STZ-induced diabetes decreases and insulin normalizes POMC mRNA in arcuate nucleus and pituitary in rats

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Kim, Eun-Mee, Martha K. Grace, Catherine C. Welch, Charles J. Billington, and Allen S. Levine. STZ-induced diabetes decreases and insulin normalizes POMC mRNA in arcuate nucleus and pituitary in rats. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1320–R1326, 1999.—Effects of streptozotocin (STZ)-induced diabetes and insulin on opioid peptide gene expression were examined in rats. In experiment 1, three groups were administered STZ (75 mg/kg ip single injection). Two groups were killed at either 2 or 4 wk. In the third group, insulin treatment (7.0 IU/kg × 1 day for 3 wk) was initiated 1 wk after STZ injection. STZ induced hyperphagia and reduced weight gain. Insulin decreased food intake and increased body weight relative to diabetes. Proopiomelanocortin (POMC) mRNA in arcuate nucleus (Arc) and pituitary decreased in diabetes and normalized after insulin treatment. Prodynorphin (proDyn) mRNA increased in diabetes and normalized in the pituitary after insulin but not in the Arc. Diabetes did not alter proenkephalin (proEnk) expression in the Arc or pituitary, nor dynorphin A1–17 or β-endorphin in paraventricular nucleus (PVN). α-Melanocyte-stimulating hormone (α-MSH) peptide levels were decreased in the PVN and normalized following insulin treatment. Diabetes increased Arc neuropeptide Y mRNA, and insulin suppressed this increase. In experiment 2, insulin (2.5 IU/kg sc) daily for 1 wk in normal rats increased Arc POMC mRNA, but not proDyn and proEnk mRNA. These results suggest that Arc POMC expression and PVN α-MSH peptide levels decrease in diabetes. Also, insulin may influence Arc and pituitary POMC activity in neurons that regulate energy metabolism.

CHANGES IN THE ENERGY status of animals following food restriction, lactation, and diabetes alter peptide levels and/or gene expression of a variety of peptides in selected brain nuclei. Such peptides include corticotropin-releasing hormone (4, 9, 31) neuropeptide Y (NPY) (4, 19, 38), opioids (17, 18), and melanocortins (22, 23). Also, peripherally circulating peptides such as leptin and insulin change in response to alterations in energy status (28, 31, 32). It is well known that diabetic animals are hyperphagic (3). Some have hypothesized that an increase in NPY and a decrease in corticotropin-releasing hormone levels contribute to diabetes-induced hyperphagia (31, 39), perhaps regulated by changes in insulin and leptin levels (16, 31, 32).

The contribution of opioid peptides to the diabetic condition is not clear. Streptozotocin (STZ)-induced diabetes has been found to increase prodynorphin (proDyn) mRNA levels in the paraventricular nucleus (PVN) (2), whereas Cheung and Tang (6) reported decreased proopiomelanocortin (POMC) mRNA levels in the pituitary, but POMC levels in whole hypothalamus did not change. Given these different findings and the lack of information relative to gene expression of POMC, proDyn, and proenkephalin (proEnk) in the hypothalamus and pituitary, the relationship between opioids and diabetic characteristics such as energy loss and hyperphagia remains to be determined. We and others have noted that gene expression of opioid peptides in the arcuate nucleus (Arc) are associated with alterations in energy status. For example, mRNA levels of all three gene families of opioid peptides (POMC, proDyn, and proEnk) decrease in the Arc of food-deprived or -restricted rats (18). Lactating rats also have lower mRNA levels of opioid peptides in the Arc (17). On the other hand, hyperphagic rats consuming a palatable high-fat-sucrose diet have higher mRNA levels of proDyn in the Arc (37). Thus one might hypothesize that hyperphagic diabetic rats, which are deprived of oxidizable fuels, might have decreased opioid gene expression in the Arc.

Measurement of mRNA levels of POMC not only reflects the synthetic potential of the opioid β-endorphin (β-End), but also reflects the synthetic potential of α-melanocortin (a-MSH). During the last few years considerable information has indicated a role for a-MSH in energy metabolism (22, 23). The obesity of the yellow mouse results from overexpression of the agouti-related protein, which competes with a-MSH for binding to the melanocortin-4-receptor (8, 24). This suggests that a-MSH plays a tonic inhibitory role in feeding and energy storage. Unlike opioids, which increase feeding by central administration (12, 13), melanocortin agonists decrease food intake and antagonists such as agouti-related protein increase feeding (11, 20). Thus it is possible that the suppression of the melanocortin system, reflected by a change in mRNA levels of POMC in the Arc, might in part be responsible for the hyperphagia observed in diabetic animals.

Other regulators known to interact with opioids and melanocortins also appear to be altered in diabetic rats. For example, mRNA levels of NPY are elevated in the Arc of STZ-induced diabetic animals (31, 38), and...
circulating levels of leptin are decreased in STZ-induced diabetic rats (16, 32). This fits with the suggestion that insulin may be a regulator of leptin and NPY synthesis and/or secretion (21, 28). In the current study we measured opioid peptide levels in the PVN and gene expression of opioids in the Arc and pituitary of STZ-induced diabetic rats and control rats, with and without insulin treatment. We also measured circulating levels of leptin, levels of α-MSH in the PVN, and mRNA levels of NPY in the Arc. To further explore the insulin-opioid interaction, we examined the effects of insulin administration on opioid peptide gene expression in the Arc.

METHODS

Subjects and treatments. Male Sprague-Dawley rats (Harlan, Madison, Wisconsin), weighing 225–250 g, were housed individually in stainless steel hanging cages with the temperature in the vivaria controlled at 22°C on a 12:12-h light-dark cycle with lights on at 0700. Subjects were given ad libitum access to water and standard laboratory diet (Rodent Chow, Teklad, IN). The experimental program was approved by the Institutional Animal Studies Committee.

Experiment 1: Effect of STZ-induced diabetes and insulin treatment on gene expression for opioid peptides. Thirty-nine subjects were randomly allotted to treatment by weight; average initial body weight 271–273 g. Four groups of subjects were employed. The control group received a saline injection. The three experimental groups were injected with STZ (Sigma, St. Louis, MO), 75 mg/kg ip, dissolved in 0.01 M sodium citrate, pH 4.5. Forty-eight hours after treatment, urine glucose concentrations were estimated using reagent strips (Diastix, Ames, Elkhart, IN) to assure the induction of a diabetic state. All saline-treated subjects showed negative reactions to reagent, whereas all STZ-treated subjects recorded urinary glucose in the 1,000–2,000 mg/dl range of reactions to reagent, whereas all STZ-treated subjects revealed positive reactions to reagent. STZ (Sigma, St. Louis, MO), 75 mg/kg ip, dissolved in 0.01 M sodium citrate, pH 4.5. Forty-eight hours after treatment, urine glucose concentrations were estimated using reagent strips (Diastix, Ames, Elkhart, IN) to assure the induction of a diabetic state. After centrifugation at 14,000 g for 15 min, the aqueous phase was precipitated with isopropanol. The RNA pellet was washed twice saturated phenol. Homogenates were mixed with 10% sarcosyl, 2 M sodium acetate, and chloroform. After centrifugation at 14,000 g for 15 min, the aqueous phase was precipitated with isopropanol overnight, resuspended in guanidine thiocyanate-β-mercaptoethanol-sarcosyl buffer for 2 h, and precipitated with isopropanol. The RNA pellet was washed twice with 75% ethanol and stored at −70°C in 100% ethanol until analysis. The RNA pellet was reconstituted in RNA storage buffer, 0.5% SDS, and sterile water, and the amount of total RNA was determined from absorbency at 260 nm. The A260/A280 ratio was used to estimate the quality of extracted RNA.

Food intake was measured daily and corrected for spillage, and body weights were measured daily. When the rats were killed, trunk blood was collected for glucose assay. Brains were rapidly excised, chilled in ice-cold saline, and sliced using a Stoepling tissue slicer. Brains were sectioned at +2.0, −2, and −5.5 mm relative to the anterior commissure, corresponding to the brain atlas of Paxinos and Watson (26). The entire PVN was removed from the 0- to −2-mm slices, and the Arc from the −2–to −5.5-mm slices. Whole pituitary was also taken. Brain tissue samples were frozen in liquid nitrogen and stored at −70°C until analyzed.

mRNA analysis. Analysis of mRNA levels in the Arc and pituitary was performed as previously described (18). In brief, total RNA was extracted by the guanidine thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (7). Tissue samples were homogenized in guanidine thiocyanate with added β-mercaptoethanol and rehomogenized in water-saturated phenol. Homogenates were mixed with 10% sarcosyl, 2 M sodium acetate, and chloroform. After centrifugation at 14,000 g for 15 min, the aqueous phase was precipitated with isopropanol overnight, resuspended in guanidine thiocyanate-β-mercaptoethanol-sarcosyl buffer for 2 h, and precipitated with isopropanol. The RNA pellet was washed twice with 75% ethanol and stored at −70°C in 100% ethanol until analysis. The RNA pellet was reconstituted in RNA storage buffer, 0.5% SDS, and sterile water, and the amount of total RNA was determined from absorbency at 260 nm. The A260/A280 ratio was used to estimate the quality of extracted RNA.

Aliquots of total RNA were dissolved in 7.4% formaldehyde and 6× saline sodium citrate (SSC) (1× SSC = 0.15 M NaCl, 0.0015 M Na citrate) and then denatured for 10 min at 68°C. Samples were blotted in duplicates of 2 µg total RNA onto nylon membranes (Zeta Probe, Bio-Rad, Richmond, CA) presoaked in 6× SSC. Control for RNA loading onto the slot-blot was verified using the ultraviolet shadowing technique, in which total unhybridized RNA is imaged (34). Membranes were prehybridized for 24 h at 42°C in 50% formamide, 5× SSC, 10× Denhardt’s solution, 0.1% SDS, and denatured salmon sperm DNA in 50 mM Na phosphate, pH 6.5. The hybridization procedure was performed using radiolabeled cDNA probe for proDyn, proEnk, or POMC and proNPY produced in our laboratory from transformed cells.
generously provided by Dr. James O. Douglass (Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences Univ., Portland, OR) and Dr. Steven L. Sabol (Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, Bethesda, MD), respectively. The cDNA utilized were 1700-, 1070-, 923-, and 511-base pair nucleotide sequences coding for the genes of rat proDyn, rat proEnk, mouse POMC, and rat proNPY, respectively. Specificity of the opioid and NPY probes was verified using Northern blot analysis. The hybridization cocktail was 50% formamide, 5× SSC, 2× Denhardt’s solution, 0.2% SDS, denatured salmon sperm DNA, and yeast tRNA in 50 mM Na phosphate, pH 6.5, with 15 × 10⁶ counts·min⁻¹·ml⁻¹ of [³²P]deoxycytidine diphosphate random primer-labeled probe. After hybridization for 48 h at 42°C, the nylon membrane was subjected to high- and low-salt washing and then exposed to X-ray film (XAR-2, Eastman Kodak, Rochester, NY) at −70°C. All RNA from a single experiment was blotted onto a single filter, hybridized in a single vessel, and autoradiographed in a single cassette to assure comparability of group treatment. Hybridization was quantified in arbitrary optical density units by scanning densitometry (Bio-Rad), and all comparisons were made relative to ad libitum control values. mRNA levels of opioid peptides and NPY in the Arc and whole pituitary were corrected with β-actin mRNA levels by calculating a ratio of opioid mRNA/β-actin mRNA and NPY mRNA/β-actin mRNA. There were no treatment effects on mRNA levels of β-actin (Arc F3,35 = 0.12, P = 0.95; pituitary F3,36 = 1.75, P = 0.17).

RIA. The concentrations of immunoreactive β-End, dynorphin A₁–₁₇ (Dyn A₁–₁₇), and α-MSH were quantitated in the PVN, using commercially available [12⁵]RIA kits [β-End and Dyn A₁–₁₇, Peninsula Laboratories, Belmont, CA; and α-MSH (acetylated α-MSH); Phoenix Pharmaceuticals]. For extraction of PVN peptides, 1 ml of 0.1 M acetic acid was added to each tissue sample, which was then transferred to a boiling water bath for 10 min. After cooling on ice, samples were homogenized in polypropylene tubes. The homogenates were centrifuged at 13,000 g for 15 min. The pellet was resuspended in 3 N NaOH and analyzed for total protein content. To assay for protein, 150 µl of supernatant were taken from each sample, and the remainder of supernatant was lyophilized and later used for β-End, Dyn A₁–₁₇, and α-MSH RIA. The RIA kit was validated before use with tissue extracts. Dose-response curves for PVN tissue extract, and increasing concentrations of the β-End, Dyn A₁–₁₇, and α-MSH standard added to a rat PVN tissue extract were parallel (P > 0.05) to the standard curve. β-End, Dyn A₁–₁₇, and α-MSH (ranging from 1 to 32 pg) added to rat PVN tissue extract was consistently recovered from 100 µl of extract (90–100%). The assay sensitivity was 4 pg/tube. The cross-reactivity test, provided by Peninsula Laboratories, indicated a cross-reactivity of <0.43% with Dyn A₁–₁₃ and 0% with Dyn A₁–₈ for Dyn A₁–₁₇ assay and 0% cross-reactivity with nonendorphin opioids for the β-End assay. The cross-reactivity test, provided by Phoenix Pharmaceuticals, indicated 0% cross-reactivity with opioid peptides, 0% cross-reactivity with β- and γ-MSH, and 0.02% cross-reactivity with ACTH for α-MSH assay. Some samples were lost during extraction, and therefore sample size for peptide assays vary from that for mRNA assays.

Serum insulin and leptin assay. The concentrations of immunoreactive serum insulin and leptin were quantitated using commercially available [12⁵] RIA kits (insulin, ICN Pharmaceutical, Costa Mesa, CA; and leptin, Linco Research, St. Louis, MO).

Glucose and protein assay. Serum glucose levels were measured using quantitative enzymatic (glucose oxidase) determination (Sigma). Total protein concentrations in PVN were measured using Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

Statistics. Data were analyzed by one-way ANOVA with post hoc testing by Fisher’s protected least-significant difference test. α-MSH peptide levels were analyzed using Bonferroni corrected t-tests. Data from mRNA levels of opioid peptides and NPY are expressed as percentage of control and presented as the means ± SE. Data of β-End, Dyn A₁–₁₇, and α-MSH peptides are expressed as absolute values and presented as the means ± SE.

RESULTS

In experiment 1, body weight gain in STZ-induced diabetic subjects was significantly lower than in control subjects (Table 1, F₃,₃₅ = 34.50, P = 0.001). Food intake increased by 74 and 88% over the 2- and 4-wk diabetes periods, respectively, compared with food intake in control subjects (Table 1, F₃,₃₅ = 39.60, P = 0.001). Daily insulin administration for 3 wk after 1 wk of STZ-diabetes induction significantly increased body weight and decreased food intake during the insulin

Fig. 1. Effects of streptozotocin (STZ)-induced diabetes and chronic insulin treatment in diabetes on mRNA levels of opioid peptides in arcuate nucleus. Values of opioid mRNA levels were corrected for β-actin mRNA levels. Values are means ± SE. a,b,c Means within mRNA type with different superscripts differ significantly (P < 0.05). POMC, proopiomelanocortin; OD, optical density.
treatment, relative to 2 and 4 wk STZ-induced diabetes untreated subjects (Table 1, P < 0.001). Serum leptin levels were significantly decreased following the induction of diabetes and were not altered following insulin treatment (Table 1, F3,36 = 82.10, P = 0.0001).

In experiment 1, STZ-induced diabetes decreased mRNA levels of POMC by 38% in the Arc of 2-wk diabetic subjects, compared with control subjects (Fig. 1, F3,34 = 3.42, P = 0.03). Diabetes also significantly suppressed gene expression for POMC in the whole pituitary (Fig. 2, F3,36 = 4.00, P = 0.02). Daily insulin administration for 3 wk normalized POMC mRNA levels in the Arc and pituitary (Figs. 1 and 2). The mRNA levels of proDyn and proEnk in the Arc were not significantly altered by STZ-induced diabetes (Fig. 1, P > 0.05). STZ-induced diabetes increased mRNA levels of proDyn in the pituitary, and insulin administration normalized proDyn mRNA levels (Fig. 2, F3,36 = 9.59, P = 0.0001), but diabetes did not alter proEnk mRNA levels in the pituitary (Fig. 2, P > 0.05). There was a tendency for PVN α-MSH peptide levels to be affected by treatment (Fig. 3, F3,34 = 2.49, P = 0.08) that was not statistically significant. Bonferroni corrected t-tests demonstrated a clear difference between PVN α-MSH levels in the diabetic rats (4 wk) and controls (P = 0.01). Peptide levels of Dyn A1–17 and β-End in the PVN were not changed by STZ-induced diabetes (Fig. 3, P > 0.05). Arc NPY mRNA levels were increased by 64 and 205% in 2 and 4-wk diabetic subjects, respectively (Fig. 4, F3,35 = 9.80, P = 0.0001), and insulin administration significantly reversed the elevation of NPY mRNA levels resulting from 4-wk STZ-induced diabetes (Fig. 4, P < 0.05).

In experiment 2, daily administration of insulin for 1 wk significantly increased 2-h food intake in subjects feeding ad libitum (Table 2, F1,18 = 29.53, P = 0.0001), whereas 24-h food intake did not change (Table 2, P > 0.05). Body weight in the insulin ad libitum fed group did not differ from the saline control group (P > 0.05). Insulin administration in subjects pair fed to control food intake decreased 24-h food intake and body weight compared with control (P < 0.05). Chronic insulin administration in both the ad libitum and pair-fed groups significantly increased POMC mRNA levels in the Arc (Fig. 5, F2,24 = 3.89, P = 0.03) but did not significantly alter proDyn and proEnk mRNA levels (Fig. 5).

DISCUSSION

As expected, we found that STZ-induced diabetic rats lost weight even though they were hyperphagic. Insulin

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**Fig. 2.** Effects of STZ-induced diabetes and chronic insulin treatment in diabetes on mRNA levels of opioid peptides in pituitary. Values of opioid mRNA levels were corrected for β-actin mRNA levels. Values are means ± SE. a,b,c Means within mRNA type with different superscripts differ significantly (P < 0.01).

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**Fig. 3.** Effects of STZ-induced diabetes and chronic insulin treatment in diabetes on β-endorphin, dynorphin A1–17, and α-melanocyte-stimulating hormone (α-MSH) peptide levels in paraventricular nucleus. Values are means ± SE. *P < 0.05 compared with control.
administration 1 wk after induction of diabetes corrected this altered energy state. Insulin-treated diabetic rats not only ate less, but they gained three times more weight compared with the nontreated diabetic animals.

We and others have noted that an energy deficit results in a decrease in gene expression of opioid peptides in the Arc of the hypothalamus (4, 18). We therefore hypothesized that diabetic rats would also have decreased levels of mRNA for the opioid peptides in the Arc. However, the current studies demonstrate that only POMC gene expression was decreased in STZ-induced insulin-dependent diabetic animals. Insulin treatment normalized the mRNA levels of POMC in the Arc without correcting long-term hyperglycemia. STZ-induced diabetes causes hypoinsulinemia, hyperphagia, and hyperglycemia, whereas food restriction results in hypoinsulinemia and hyperphagia with normoglycemia (29). This suggests that insulin deficiency, rather than hyperglycemia, may produce a signal that decreases Arc POMC gene expression in both diabetes and food restriction. We also found that insulin administration resulted in increased gene expression for Arc POMC in normal subjects supporting the idea that insulin stimulates production of POMC mRNA in the Arc. The high levels of insulin binding (36) and the presence of POMC cell bodies in the Arc (1) further support these observations.

In energy-restricted rats, mRNA levels of POMC, proDyn, and proEnk in the Arc are lower than in control animals (18). However, in the STZ-induced diabetic rats only POMC mRNA levels were lower. It is important to note that POMC is a precursor not only for the opioid β-End, but for other peptides including α-MSH (1). We found that β-End and DynA1-17 levels were not different in the PVN of diabetic and control rats. In contrast, there appeared to be a decrease in the levels of α-MSH in the Arc of diabetic rats. Central administration of α-MSH or Melanotan II (MTII), both agonists of the melanocortin-4-receptor, decreases food intake (11, 14, 20). Thus the depressed levels of α-MSH in diabetic rats may contribute to the hyperphagia associated with diabetes. This contention is supported by our observation that α-MSH levels and food intake normalized after insulin treatment.

Levels of other regulators such as NPY (31, 38) and leptin (16, 32) have been found to be altered in diabetic rats. Several studies have suggested that insulin may be a regulator of leptin and NPY synthesis and/or secretion (16, 28, 31). As noted by others, we found that Arc gene expression of NPY was elevated and that leptin serum levels were decreased in STZ-induced diabetic rats. The hypothalamic changes in NPY induced by diabetes are similar to those seen following food restriction and deprivation (25). It has been suggested that enhanced activity of NPY in the Arc-PVN pathway, in response to negative energy balance in diabetes, may induce the hyperphagia associated with diabetes and may be regulated by insulin (21, 31).

Recently, Garcia de Yebenes et al. (10) reported that intracerebroventricular injection of NPY decreased the mRNA levels of POMC in the Arc. A relationship between leptin and POMC has recently been demonstrated. For example, intracerebro-
ventricular injection of leptin increases mRNA levels of POMC in the Arc of ob/ob mice (30, 33), and the leptin receptor is colocalized with POMC-containing neurons in the Arc (5, 15). Administration of insulin increases leptin mRNA levels (28). In contrast, it has been demonstrated that STZ-induced diabetes decreases plasma leptin levels and insulin treatment in diabetic subjects reverses this trend (16, 32). Thus it is possible that insulin deficiency in STZ-induced diabetic rats resulted in the observed decrease in serum leptin levels and the decreased POMC levels in the Arc. Whereas we found that insulin treatment normalized POMC levels, leptin levels remained depressed. The lack of effect of insulin on leptin levels in our study may have been due to the dose and method of insulin administration. It is also possible that the low serum leptin levels detected in the insulin-treated diabetic animals are not reflective of value maintained during the course of insulin treatment. Serum samples were obtained 24 h after the last insulin injection when serum glucose levels were rising and serum insulin was reduced. Thus it is difficult to draw conclusions regarding the interaction of leptin, insulin, and food intake during the insulin treatment period.

We also found that POMC mRNA levels were lower in the pituitary of STZ-induced diabetic rats than in control rats. Insulin treatment returned these levels to that of control animals. In a preliminary study, Cheung and Tang (6) reported that POMC mRNA levels were decreased in both the anterior and neurointermediate lobe of the pituitary of STZ-induced diabetic rats. The pituitary is a major site of POMC synthesis (1) and contains highly specific insulin binding sites (35). Administration of insulin increases POMC gene expression in the anterior pituitary (27). In contrast to POMC gene expression we found that mRNA levels of proDyn were increased in the pituitary of diabetic rats. Bachus and Jhanwar-Uniyal (2) found that proDyn mRNA levels are increased in the PVN in diabetic rats, an area that has direct projections to the pituitary.

In conclusion, gene expression of POMC, but not proDyn or proEnk, was decreased in STZ-induced diabetic rats. This was accompanied by a decrease in α-MSH, but not in β-End or Dyn A₁₋₁₇ levels, in the PVN. The reversal of Arc POMC mRNA levels by insulin treatment in the diabetic rats and the increase in Arc POMC mRNA levels after insulin administration to normal subjects suggest that insulin may be an important regulator of POMC gene expression in diabetic rats.

Perspectives

It appears that the decrease in Arc POMC activity seen in diabetes results from the interaction of several regulators, such as NPY, insulin, and leptin. The lack of change in mRNA levels of proEnk and proDyn in the Arc as well as the lack of change in β-End peptide levels in the PVN suggest that endogenous opioids are not the major peptide family involved. Rather, it appears that α-MSH, derived from POMC, is an important player in the change in energy status of diabetic rats. Such an observation reminds us that finding a change in mRNA levels of POMC does not necessarily reflect an involvement of opioids. Posttranslational processing of POMC may yield a variety of peptides such as ACTH, β-End, and α-MSH. Our data suggest α-MSH may be an important factor in the hyperphagia associated with diabetes.

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