Effects of Streptozotocin-Induced Diabetes and Insulin Treatment on the Hypothalamic Melanocortin System and Muscle Uncoupling Protein 3 Expression in Rats

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Hypothalamic melanocortins are among several neuropeptides strongly implicated in the control of food intake. Agonists for melanocortin 4 (MC-4) receptors such as α-melanocyte–stimulating hormone (α-MSH), a product of proopiomelanocortin (POMC), reduce food intake, whereas hypothalamic agouti-related protein (AgRP) is a MC-4 receptor antagonist that increases food intake. To investigate whether reduced melanocortin signaling contributes to hyperphagia induced by uncontrolled diabetes, male Sprague-Dawley rats were studied 7 days after administration of streptozotocin (STZ) or vehicle. In addition, we wished to determine the effect of diabetes on muscle uncoupling protein 3 (UCP-3), a potential regulator of muscle energy metabolism. STZ diabetic rats were markedly hyperglycemic (31.3 ± 1.0 mmol/l; $P < 0.005$) compared with nondiabetic controls (9.3 ± 0.2 mmol/l). Insulin treatment partially corrected the hyperglycemia (18.8 ± 2.5 mol/l; $P < 0.005$). Plasma leptin was markedly reduced in STZ diabetic rats (0.4 ± 0.1 ng/ml; $P < 0.005$) compared with controls (3.0 ± 0.4 ng/ml), an effect that was also partially reversed by insulin treatment (1.8 ± 0.3 ng/ml). Untreated diabetic rats were hyperphagic, consuming 40% more food (48 ± 1 g/day; $P < 0.005$) than controls (34 ± 1 g/day). Hyperphagia was prevented by insulin treatment (32 ± 2 g/day). In untreated diabetic rats, hypothalamic POMC mRNA expression (measured by in situ hybridization) was reduced by 80% ($P < 0.005$), whereas AgRP mRNA levels were increased by 60% ($P < 0.01$), suggesting a marked decrease of hypothalamic melanocortin signaling. The change in POMC, but not in AgRP, mRNA levels was partially reversed by insulin treatment. By comparison, the effects of diabetes to increase hypothalamic neuropeptide Y (NPY) expression and to decrease corticotropin-releasing hormone (CRH) expression were normalized by insulin treatment, whereas the expression of mRNA encoding the long form of the leptin receptor in the arcuate nucleus was unaltered by diabetes or insulin treatment. UCP-3 mRNA expression in gastrocnemius muscle from diabetic rats was increased fourfold ($P < 0.005$), and the increase was prevented by insulin treatment. The effect of uncontrolled diabetes to decrease POMC, while increasing AgRP gene expression, suggests that reduced hypothalamic melanocortin signaling, along with increased NPY and decreased CRH signaling, could contribute to diabetic hyperphagia. These responses, in concert with increased muscle UCP-3 expression, may also contribute to the catabolic effects of uncontrolled diabetes on fuel metabolism in peripheral tissues. *Diabetes* 49:244–252, 2000

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AgRP, agouti-related protein; α-MSH, α-melanocyte–stimulating hormone; CNS, central nervous system; CRH, corticotropin-releasing hormone; MC-4, melanocortin 4; NEFA, nonesterified fatty acid; NPY, neuropeptide Y; Ob-Rb, Ob–receptor-b; POMC, proopiomelanocortin; PVN, paraventricular nucleus; STZ, streptozotocin; UCP-3, uncoupling protein 3.

Hyperphagia (1,2) and altered fuel metabolism (3,4) are prominent features of uncontrolled diabetes. Although the pathogenesis of diabetic hyperphagia is incompletely understood, several observations suggest a key role for reduced signaling by insulin and leptin, hormones involved in the regulation of energy balance via their effects on the central nervous system (CNS) to regulate food intake and energy expenditure (5–7). In the CNS, insulin and leptin reduce food intake, closing a negative feedback loop whereby an increase of energy intake and expansion of the adipose depot lead to a compensatory reduction of energy intake. Both hormones circulate at levels proportionate to body fat stores (8–10) and recent energy intake (10–12) in normal humans and animals, and both fall precipitously after induction of streptozotocin (STZ) diabetes in rats (13,14). Insulin regulates leptin production (12,15), and the fall of leptin in insulin-deficient diabetes is likely to be mediated by decreased insulin-mediated glucose metabolism in adipose tissue (16). Diabetic hyperphagia is suppressed by administration of low doses of insulin directly into the brain of STZ diabetic rats (17) and by infusion of leptin at a rate that prevents circulating leptin levels from decreasing after induction of STZ diabetes in rats (18). Therefore, decreased signaling by insulin and leptin is likely to have a causative role in this hyperphagic response.

A major mechanism underlying the effects of insulin and leptin to reduce food intake involves the regulation of hypothalamic neuropeptide systems (19,20). For example, central administration of either insulin or leptin inhibits the expression of the orexigenic neuropeptide, neuropeptide Y (NPY) (17,21,22), and increases the hypothalamic expression (21,22) and peptide content (23) of corticotropin-releasing hormone (CRH), a neuropeptide that inhibits food intake (24). Recently, the hypothalamic melanocortin system has emerged as a potentially important target for the effects of leptin on energy homeostasis. Central administration of melanocortin 4 (MC-4) receptor agonists causes anorexia (25), whereas antagonists have the opposite effect (25,26). Moreover, proopiomelanocortin (POMC) deficiency (27), ectopic expression of the endogenous melanocortin receptor antagonist agouti (28), or genetic deletion of MC-4 receptors (29) causes marked obesity in mice, suggesting that signaling at MC-4 receptors is critical for normal energy homeostasis. The hypothalamic melanocortin system is regulated by leptin, since conditions associated with low leptin levels, such as fasting or genetic leptin deficiency, induce both decreased hypothalamic POMC mRNA levels (30,31) and increased expression of agouti-related protein (AgRP) (32), and leptin administration blocks these responses (30,31). Increased food intake in leptin-deficient conditions may therefore arise, at least in part, from reduced melanocortin signaling. Because uncontrolled diabetes is accompanied by decreased levels of insulin and leptin, we hypothesized that hypothalamic expression of POMC mRNA should decrease, while AgRP gene expression should increase. These responses could have an important role in the pathogenesis of diabetic hyperphagia.

Uncontrolled diabetes also has marked effects on energy metabolism in peripheral tissues, and the family of uncoupling proteins has emerged as potential mediators of tissue-specific changes in fuel utilization. Uncoupling protein 3 (UCP-3) is a recently described member of this family (33,34). UCP-3 is strongly induced in skeletal muscle during fasting (35,36). Although the consequences of increased UCP-3 for muscle energy metabolism remain to be elucidated, we hypothesized that because uncontrolled diabetes shares many hormonal and metabolic responses in common with prolonged fasting (e.g., decreased insulin and leptin levels and increased levels of nonesterified fatty acids [NEFAs] and ketones), animals with uncontrolled diabetes should have increased UCP-3 expression in skeletal muscle. Therefore, in this study, we examined and compared the expression of mRNA encoding neuropeptides involved in energy homeostasis (NPY, CRH, POMC, and AgRP) as well as the long form of the leptin receptor in the hypothalamus (all by in situ hybridization) and UCP-3 expression in skeletal muscle (by Northern blot) of nondiabetic rats, rats with uncontrolled STZ diabetes, and diabetic rats treated with insulin.

RESEARCH DESIGN AND METHODS

Animals. A total of 27 adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 260–320 g were used for the studies. The animals were individually housed in hanging wire cages and fed a standard commercial diet (#557; Ralston Purina, Belmont, CA) and deionized water ad libitum. The light/dark cycle was 12 h on and 12 h off, with lights on at 0600. Experimental protocols were approved by the Institutional Animal Use and Care Committee of University of California, Davis, and were conducted in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals.

Induction of diabetes. Insulin-deficient diabetes was induced with subcutaneous injections of freshly prepared STZ (Sigma, St. Louis, MO) at a dose of 40 mg/kg in ice-cold 0.5 mol/l citrate buffer (pH 4.5). A second dose of STZ (40 mg/kg) was administered 24 h later. This regimen produces insulin-deficient diabetes (plasma glucose >450 mg/dl) in >95% of treated animals without inducing renal failure or loss of animals from hypoglycemia secondary to insulin release associated with acute B-cell destruction (37). Control animals received subcutaneous injections of citrate buffer only.

Experimental protocol. To examine changes of hypothalamic neuropeptides and UCP-3 after short-term (7 days) STZ diabetes, diabetes was induced with STZ in 18 rats as described above. Nine buffer-injected rats served as nondiabetic controls. To examine the effects of insulin treatment designed to reduce plasma glucose concentrations, at the time of the second STZ injection, nine of the diabetic animals each received a single implant impregnated with bovine insulin (Linplant, Toronto, Canada) placed subcutaneously through a 14-gauge needle under light ketamine/xylazine (30/10 mg/kg, respectively) anesthesia as previously described (13). These implants slowly release insulin (~2 U/day) for up to 40 days. The control and nine other STZ diabetic rats were sham implanted. Plasma glucose was monitored daily from a tail blood sample (~0.5 ml) collected in a heparinized tube (Sarstedt, Hayward, CA) during brief restraint (<2 min) in a polyethylene rodent restraint cone. Plasma glucose was markedly elevated (>25 mmol/l) in all untreated STZ diabetic rats.

Food intake and body weight were measured daily. After 7 days, the animals were killed by decapitation. The brains were removed immediately and placed in a cerebrum-down orientation on a glass microscope slide on a slab of dry ice and covered with powdered dry ice. This method is necessary to preserve the normal hypothalamic architecture. The liver, kidney and epididymal, retroperitoneal, and inguinal fat depots were removed and weighed. Gastrocnemius muscle was dissected, weighed, and frozen immediately in liquid nitrogen.

Assays and data analysis. Plasma glucose was measured by the glucose oxidase method with a glucose analyzer, Model 2700 (Yellow Springs Instruments, Yellow Springs, OH). Plasma NEFA concentrations were measured with a kit from Waco (Richmond, VA). Plasma insulin was measured by radioimmunoassay using rat insulin standards from Novo Nordisk (Bagsvaerd, Denmark). Plasma leptin was measured with a radioimmunoassay for rat leptin with reagents from Linco Research (St. Charles, MO) as previously described (38). Plasma corticosterone was measured by radioimmunoassay (39) in the six rats in each group that were killed during the first 8 h of the light cycle (i.e., prior to 1400), because plasma corticosterone levels are level at this time but begin to increase during the last 4 h of the light cycle (40). The intra- and interassay coefficients of variation for the assay are 4.0% and 11.2%, respectively, with a lower limit of detection of 0.2 ng/ml. The antibody used in the assay does not cross-react with insulin, proinsulin, glucagon, pancreatic polypeptide, or somatostatin. UCP-3 expression in gastrocnemius muscle was measured in six of the nine rats from each group by Northern blot analysis and as previously described (36). UCP-3 expression was normalized to the expression of β-actin.

In situ hybridization

Tissue preparation. Brains were removed immediately after decapitation between 0800–1200, frozen on dry ice, sectioned in a coronal plane at 14 μm with a cryostat, mounted on RNase-free slides, and treated with 4% paraformaldehyde, acetic anhydride, ethanol, and chloroform (22). For each probe, 3–6 slides (6–12 brain sections) that contain hypothalamus in the mid- to rostral region of the arcuate nucleus (for POMC, NPY, and AgRP mRNA) or through the paraventricular nucleus (PVN) (for CRH mRNA) were selected from each animal for hybridization. Care was taken to insure that the sections were anatomically matched among animals. For each study, all brain slices were concurrently prepared for hybridization and were used in the same hybridization assay.

Hybridization procedure. Antisense oligonucleotide probes for NPY and CRH were labeled with 33P-deoxyATP (Amersham, Arlington Heights, IL) using terminal deoxynucleotide transferase (BRL, Bethesda, MD) and purified across NENsorb columns (New England Nuclear, Boston, MA), as previously detailed (22). For hybridization to POMC or AgRP mRNA, riboprobes were transcribed from cRNA templates using [^{33}P]UTP (30). The expression of the long form of the leptin receptor (Ob–receptor-b [Ob-Rb]) in the arcuate nucleus was examined using a riboprobe specific for the rat Ob-Rb mRNA (41,42). The hybridization signal in the arcuate nucleus of each brain slice was determined from autoradiograms using an MCID image analysis system (Imaging Research, St. Catherines, Ontario), as described previously (30). Both the film density and hybridization image area were measured, the product of which was calculated as an index of mRNA levels from the mean of 6–12 sections per animal. The anatomical equivalence of hypothalamic sections among animals was obtained by selecting slides (viewed with a dark-field stereomicroscope) with the aid of a rat brain atlas before hybridization and verifying the anatomical identifications by staining sections with cresyl violet after hybridization but before computer densitometry of the film images.

Statistical analysis. Statistical analyses were performed using a software package (StatView for Macintosh; Abacus, Berkeley, CA). Comparisons of means

TABLE 1
Body weight changes and tissue weights after 7 days in nondiabetic control rats, untreated STZ diabetic rats, and diabetic rats treated with insulin implants

	Control (nondiabetic)	STZ	
		Untreated	Insulin-treated
n	9	9	9
Initial body weight (g)	294 ± 6	295 ± 4	294 ± 5
Final body weight (g)	324 ± 7	284 ± 5†	306 ± 8
Δ Body weight (g)	31 ± 3	–10 ± 3†	13 ± 3†
Liver weight (g)	13.6 ± 0.6	11.2 ± 0.3†	12.3 ± 0.7
Kidney weight (g)	2.7 ± 0.1	3.3 ± 0.1†	2.9 ± 0.1
Epididymal fat weight (g)	3.1 ± 0.2	1.8 ± 0.1†	2.6 ± 0.2
Retroperitoneal fat weight (g)	2.5 ± 0.3	1.0 ± 0.1†	2.0 ± 0.2
Inguinal fat weight (g)	4.9 ± 0.3	2.7 ± 0.1†	3.8 ± 0.3*
Total fat weight (g)	10.5 ± 0.7	5.4 ± 0.3†	8.4 ± 0.7*
Gastrocnemius muscle weight (g)	3.7 ± 0.1	3.3 ± 0.1†	3.6 ± 0.1

*P < 0.05 vs. control; †P < 0.01 vs. control.

within a group were made with a paired t test. For comparisons between groups, analysis of variance was used with a Dunnett's post test. Linear regression analysis was also performed. The null hypothesis of equal means was rejected at the P = 0.05 level of significance. Data are expressed as means ± SE.

RESULTS

Body weight and food intake. STZ diabetic rats lost 10 g of body weight by 7 days after the induction of diabetes, whereas nondiabetic animals gained 31 g during this period (Table 1). Insulin-treated diabetic rats also gained weight, but this weight gain (13 g) was less than that of the control animals (Table 1). Organ/tissue weights in the three groups of animals are provided in Table 1. The weight of three fat depots was reduced by ~50% and partially, but not completely, restored to ~80% of control by insulin treatment. The combined weight of the left and right gastrocnemius muscles was reduced by ~10% in untreated diabetic rats relative to control animals and was not decreased in diabetic rats treated with insulin implants (Table 1). Diabetic rats were hyperphagic relative to control animals, with mean daily food intake being increased by 43 ± 3% (P < 0.0001 vs. control; Fig. 1). Insulin treatment of diabetic rats on average completely prevented this hyperphagia (Fig. 1), but there was a range of food intake within the insulin-treated diabetic animals that was related to the degree of glycemic regulation (r = 0.94, P < 0.001).

Plasma hormones and metabolites. The STZ diabetic animals were markedly hyperglycemic, with mean plasma glucose levels of 31.3 ± 1.0 mmol/l (Fig. 2*A*). Treatment of diabetic rats with insulin implants reduced, but did not normalize, plasma glucose levels (Fig. 2*A*). Plasma NEFAs were nearly doubled in untreated diabetic rats compared with control animals. NEFA levels were normalized by insulin treatment in the STZ diabetic rats that received insulin implants (Fig. 2*B*). As expected, STZ-treated rats were hypoinsulinemic, with mean plasma insulin levels 85% below levels in control animals (Fig. 3*A*). In contrast, diabetic rats with insulin implants had plasma insulin levels more than twice as high as control rats (Fig. 2*A*). Plasma leptin levels were reduced by >80% in STZ diabetic rats. Although insulin-treated diabetic rats had plasma leptin concentrations four times higher than those in untreated diabetic animals, these levels remained below those in control animals (Fig. 3*B*). Within the group of insulin-treated diabetic animals, plasma leptin concentrations were correlated with plasma insulin levels (r = 0.80; P < 0.01). Plasma corticosterone was elevated fivefold in untreated STZ diabetic rats, but it was not different from control levels in insulin-implanted diabetic animals (Fig. 3*C*).

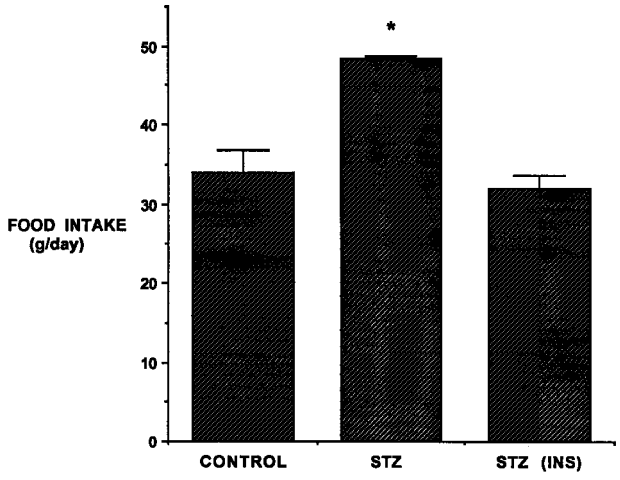

FIG. 1. Food intake 7 days after induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants (n = 9 per group). *P < 0.05 vs. control.

Hypothalamic neuropeptide, leptin receptor, and muscle UCP-3 expression. The in situ hybridization autoradiographic signal for NPY mRNA expression in the arcuate nucleus of the hypothalamus was increased by 60 ± 15% (P < 0.025) in untreated STZ diabetic rats and was not different from control levels in insulin-treated diabetic animals (Fig. 4*A*). In contrast, CRH expression was reduced by 30 ± 8% (P < 0.05) in the PVN of untreated diabetic rats and, like NPY, was normalized in insulin-treated animals (Fig. 4*B*). Hypothalamic POMC expression was markedly reduced to <20% of control levels in the arcuate nuclei of untreated diabetic rats. This reduction was partially reversed, but not normalized, by insulin treatment of diabetic animals (Fig. 5*A*). In contrast, the expression of AgRP mRNA was increased by 69 ± 21% (P < 0.01) in the hypothalamus of STZ diabetic rats, and this overexpression was unaffected by insulin treatment of diabetic animals (Fig. 5*B*). Among the nondiabetic control animals, NPY expression was significantly correlated with the expression of AgRP (r = 0.82; P < 0.025). The expression of the long form of the leptin receptor (Ob-Rb) in the arcuate nucleus was not different between nondiabetic animals and either untreated or insulin-treated diabetic rats (Fig. 5*C*).

After normalizing for β-actin, the expression of UCP-3 in gastrocnemius muscle in untreated STZ diabetic rats was nearly four times higher than in control animals. This overexpression of UCP-3 was not present in diabetic animals treated with insulin (Fig. 6). Within the nondiabetic control animals, UCP-3/β-actin was highly correlated with gastrocnemius muscle weight (r = 0.96; P < 0.003).

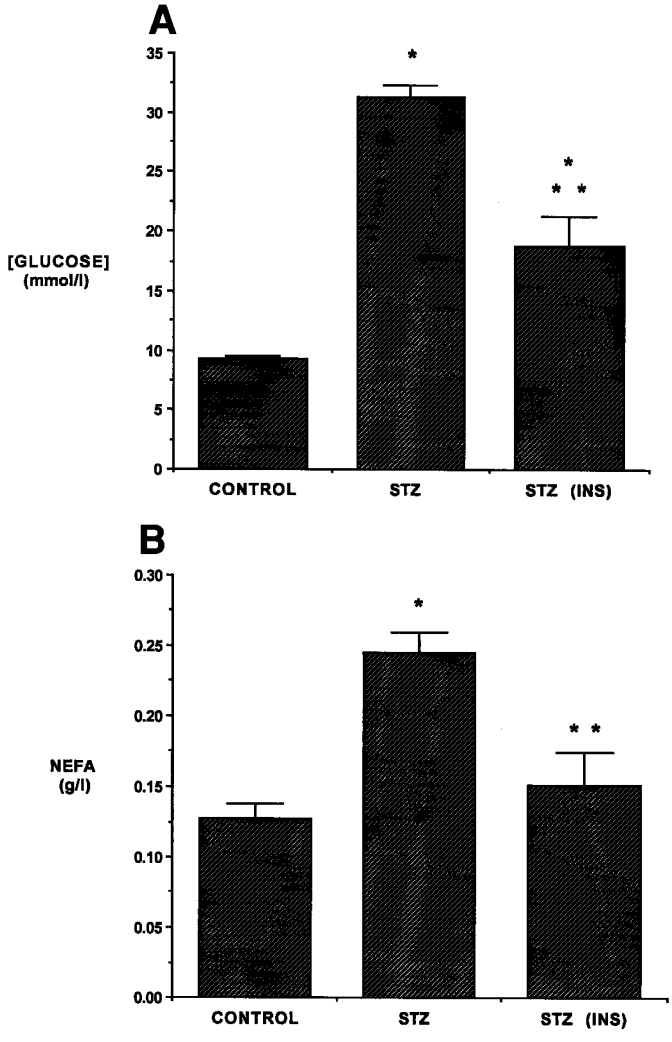

FIG. 2. Plasma glucose (*A*) and NEFA (*B*) concentrations 7 days after induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants (n = 9 per group). *P < 0.05 vs. control; **P < 0.05 vs. STZ untreated.

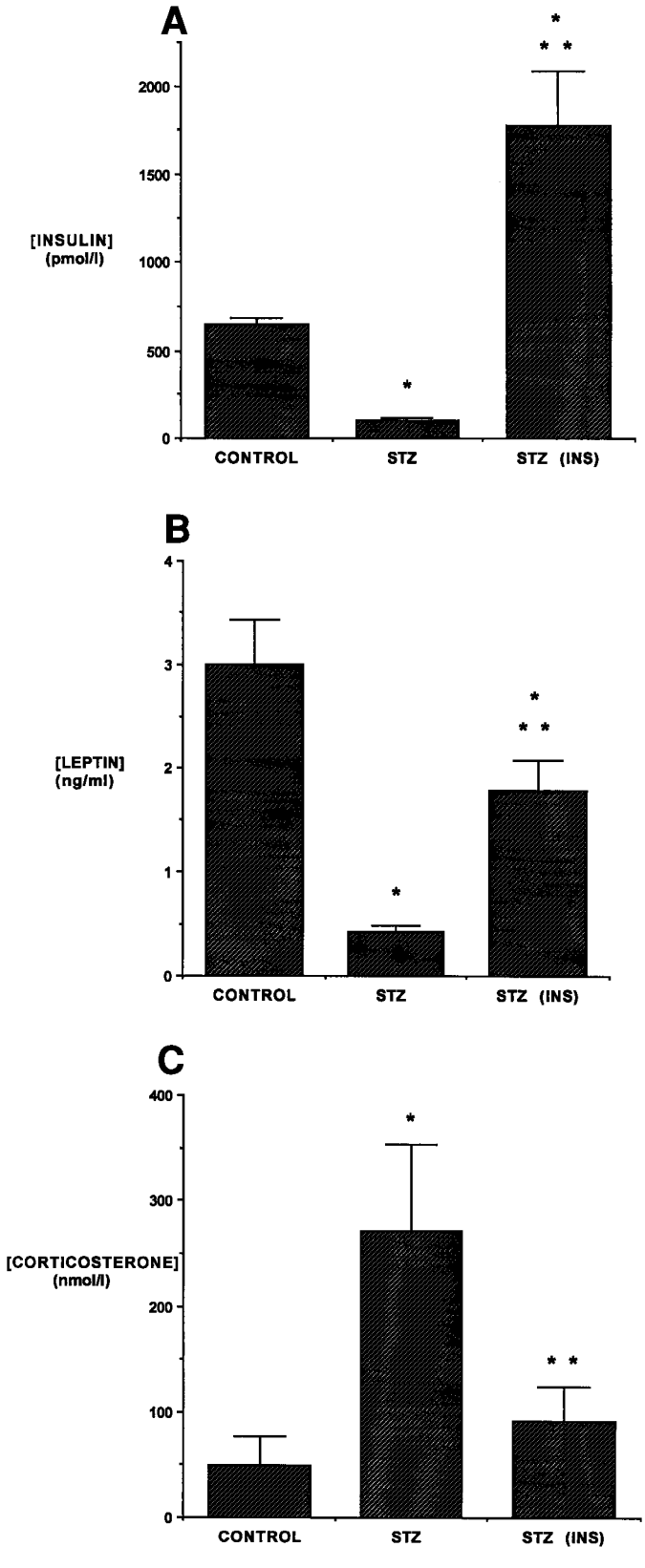

FIG. 3. Plasma insulin (*A*), leptin (*B*), and corticosterone (*C*) concentrations 7 days after induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants (n = 9 per group for insulin and leptin; n = 6 per group for corticosterone). *P < 0.05 vs. control; **P < 0.05 vs. STZ untreated.

DISCUSSION

A large body of literature supports the hypothesis that reduced signaling by insulin or leptin, as would occur in untreated diabetes, stimulates feeding behavior via changes in the activity of hypothalamic neuropeptide-containing pathways. To further investigate this hypothesis, we used in situ hybridization to measure effects of uncontrolled diabetes in the presence or absence of insulin treatment on hypothalamic expression of mRNA encoding neuropeptides implicated in the control of food intake in STZ diabetic rats. Consistent with previous reports (17,43–46), we found that uncontrolled diabetes increases NPY mRNA and decreases CRH mRNA levels in rat hypothalamus, responses that provide a potential explanation for increased food intake. A major new finding of this study is that STZ diabetes reduced hypothalamic POMC mRNA levels by 80%, while it increased AgRP gene expression by 60%. The combination of reduced synthesis of MC-4 receptor agonists, such as α-melanocyte–stimulating hormone (α-MSH) (contained within the POMC precursor), and increased signaling by the MC-4 receptor antagonist

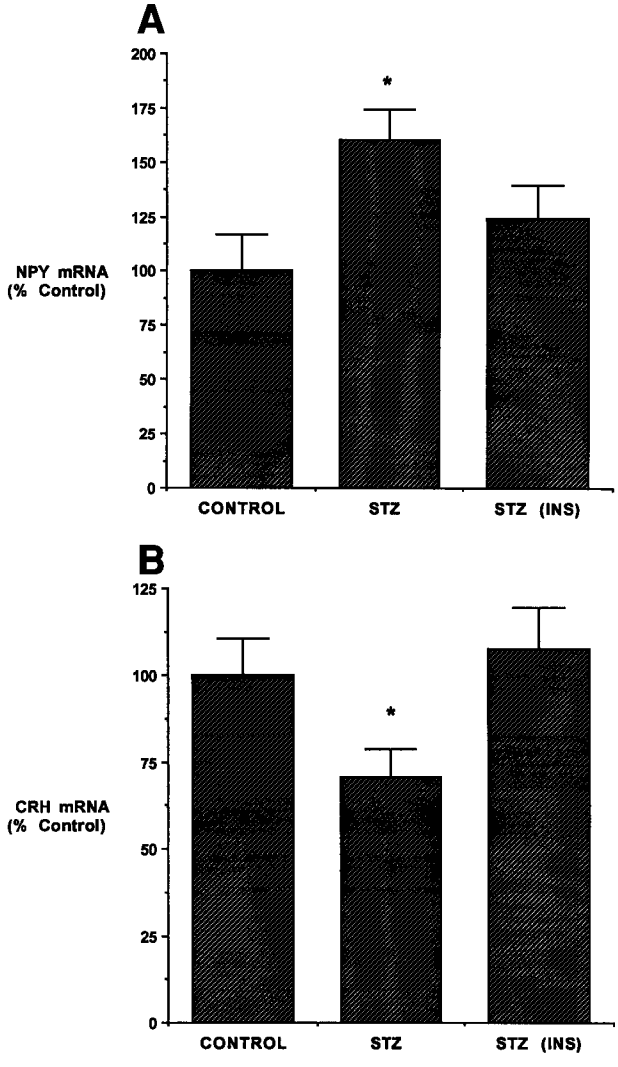

FIG. 4. Hypothalamic NPY (*A*) and CRH (*B*) expression 7 days after induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants presented as a percentage of mean control levels (n = 7–9 per group). *P < 0.05 vs. control.

AgRP suggests that a marked decrease of melanocortin signaling occurs in the hypothalamus of rats with uncontrolled diabetes. Because genetic POMC deficiency (27) and pharmacological blockade (28) or genetic deletion (29) of MC-4 receptors increase feeding, these responses could potentially play a major role in the pathogenesis of diabetic hyperphagia.

The effect of uncontrolled diabetes to increase food intake may involve reduced insulin delivery to the CNS, since central insulin administration to diabetic rats reduces the hyperphagic response (17). Uncontrolled diabetes also reduces circulating leptin levels (13,14), and this decrease is likely to contribute to diabetic hyperphagia, because replacement of basal plasma leptin levels prevents the onset of hyperphagia in STZ diabetic rats (18). The effect of lowered leptin to increase appetite is supported by reports that genetic leptin deficiency or receptor defects result in hyperphagia and obesity in animals (7) and humans (47,48), and that reductions of circulating leptin are linked to increased sensations of hunger during energy restriction in human subjects (49).

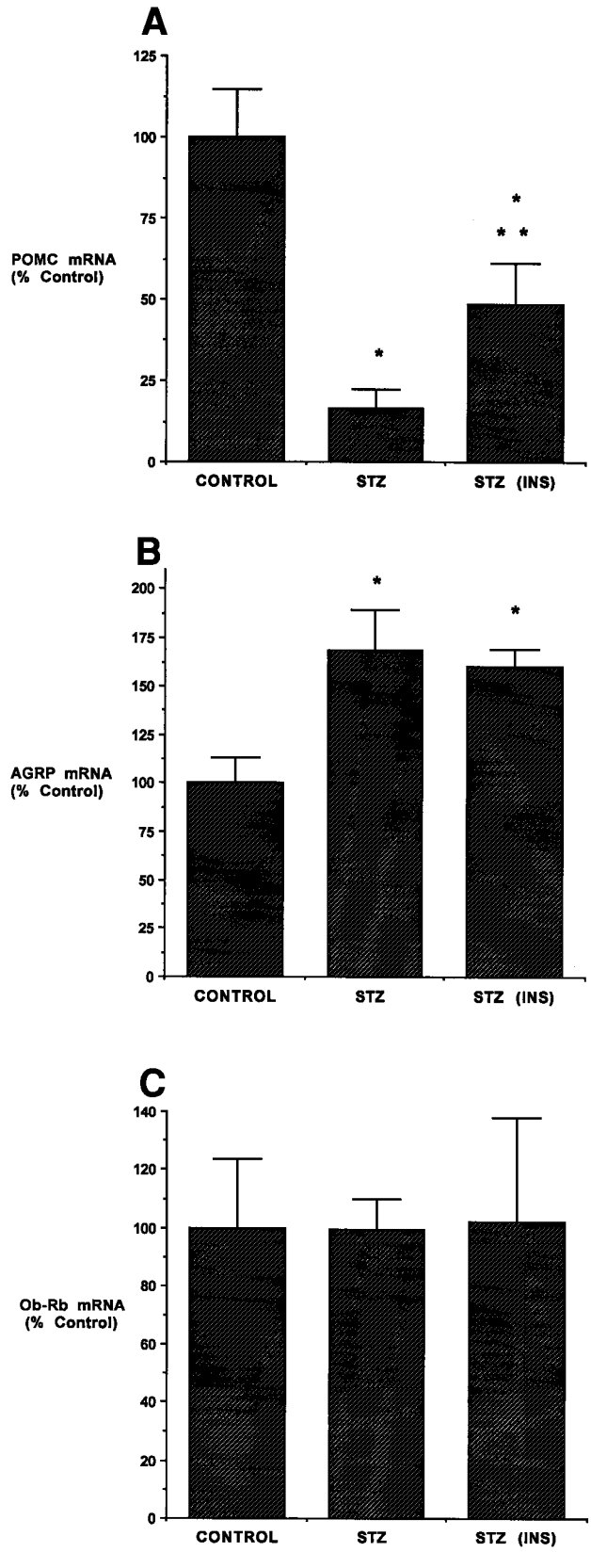

FIG. 5. Hypothalamic POMC (*A*) and AgRP (*B*) expression and expression of the long form of the leptin receptor (Ob-Rb) (*C*) 7 days after induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants presented as a percentage of mean control levels (n = 6–9 per group). *P < 0.05 vs. control; **P < 0.05 vs. STZ untreated.

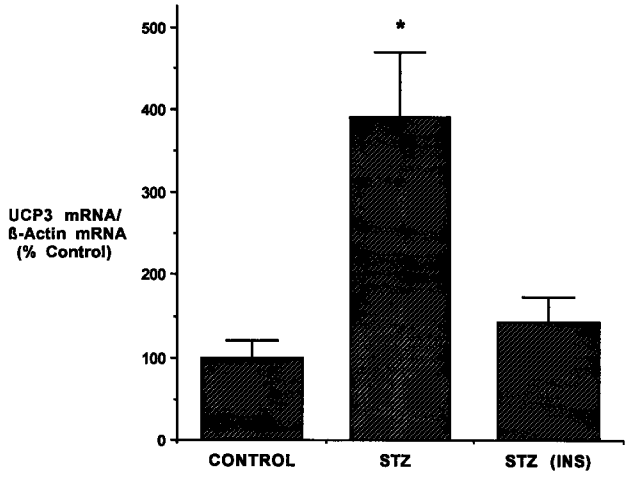

FIG. 6. Expression of UCP-3 normalized to β-actin expression (UCP-3/β-actin) in gastrocnemius muscle 7 days after the induction of diabetes in control (nondiabetic), STZ diabetic, and STZ diabetic rats treated with subcutaneous insulin (INS) implants presented as a percentage of mean control levels (n = 6 per group). *P < 0.05 vs. control.

In the current study, insulin was infused via a subcutaneously implanted pellet that slowly releases insulin into the circulation. Despite plasma insulin levels that were elevated compared with nondiabetic animals, the hyperglycemia induced by STZ was only partially normalized in the insulin-treated group. This failure to normalize plasma glucose levels is consistent with an important role for hepatic insulin delivery in glucose homeostasis, since subcutaneously administered insulin does not reproduce the preferential increase of insulin concentrations within the hepatic portal vein that occurs when insulin is secreted from the pancreas. In addition, STZ diabetes induces a state of insulin resistance in addition to insulin deficiency such that relatively high insulin levels are needed to restore normal glucose homeostasis (50).

Whatever the mechanism for the failure to normalize hyperglycemia, insulin treatment similarly failed to fully normalize the reduced adipose mass and the marked hypoleptinemia of diabetic rats. Insulin-stimulated glucose uptake and metabolism in adipose tissue, which is a major determinant of leptin synthesis and secretion (16), may therefore have been only partially restored by our insulin treatment regimen. These findings conflict with the complete reversal of diabetic hyperphagia that occurred in the insulin-treated group. One possible explanation is that circulating leptin levels must fall to values below those achieved in the insulin-treated group for hyperphagia to occur. Alternatively, the incomplete normalization of plasma leptin levels may have been counterbalanced by an effect of increased insulin signaling in the brain to reduce food intake (5), because circulating insulin levels were elevated well above control values in the insulin-treated animals.

The hypothalamic arcuate nucleus is the only area of the mammalian forebrain that expresses the gene encoding POMC, the melanocortin precursor. POMC neurons in this brain area project to adjacent hypothalamic structures that express MC-4 receptors (51,52), such as the PVN, which is a major area for the control of food intake. In the PVN, synaptic release of α-MSH, believed to be the principal endogenous ligand for neuronal melanocortin receptors, appears to reduce food intake under physiological conditions. This conclusion is based on the findings that administration of synthetic melanocortin receptor antagonists induces a hyperphagic response (25,26) and on the finding that genetic deletion of POMC (27) or MC-4 receptors leads to a severe form of hyperphagic obesity in mice (29). The potential importance of the melanocortin system to human body weight regulation was emphasized by recent reports that marked obesity occurs in families affected by mutation of genes encoding either POMC (53) or the MC-4 receptor (54,55).

The endogenous MC-4 receptor antagonist AgRP is coexpressed with NPY in neurons of the arcuate nucleus that are adjacent to, but distinct from, POMC neurons (56,57) and are activated in conditions associated with low leptin levels, such as fasting (56,58). Our finding that levels of both NPY and AgRP mRNA were elevated in diabetic animals and were highly correlated with one another within nondiabetic control animals supports the hypothesis that the expression of these two neuropeptides is regulated in parallel (56). However, insulin treatment of diabetic rats restored hypothalamic NPY expression to control levels but did not normalize AgRP expression. Therefore, it is possible that NPY and AgRP expression are differentially regulated by insulin and leptin. In this regard, it is worth noting that whereas fasting typically increases NPY mRNA levels by two- to threefold, it increases AgRP mRNA by 18-fold (56). In contrast, uncontrolled diabetes elicited comparable increases of NPY and AgRP mRNA in the arcuate nucleus, suggesting differential regulation of NPY and AgRP expression in the two conditions.

Genetic leptin deficiency in *ob*/*ob* mice is associated with reduced arcuate nucleus POMC mRNA levels (30,31) and increased AgRP mRNA levels (58), as well as with hyperphagia (a scenario similar to that observed in STZ diabetes). The hypothesis that leptin deficiency mediates these hypothalamic responses is supported by the demonstration that leptin administration to *ob*/*ob* mice increases arcuate nucleus POMC mRNA levels (30,31), that leptin receptors are expressed by POMC neurons (59), and that leptin administration to fasted animals increases hypothalamic POMC gene expression (30) while reducing AgRP mRNA levels (60). The effect of uncontrolled diabetes to lower POMC while raising AgRP mRNA levels in the hypothalamus can therefore be explained, at least in part, by reduced leptin levels. Consistent with this conclusion, we found that insulin treatment of diabetic rats caused proportionately similar increases of plasma leptin and hypothalamic POMC mRNA. However, insulin treatment did not normalize AgRP mRNA levels in diabetic rats, suggesting that factors regulating hypothalamic AgRP gene expression in rats with STZ diabetes differ from those that control expression of POMC. For example, the partial normalization of plasma leptin levels in the insulin-treated group may have been sufficient to increase POMC, but not to decrease AgRP, gene expression. Alternatively, factors in the diabetic milieu that are independent of leptin deficiency may explain the persistent increase of AgRP mRNA in the insulin-treated group.

An important role for reduced melanocortin signaling in the pathogenesis of diabetic hyperphagia is challenged by the observation that food intake was completely normalized by insulin treatment, despite persistently reduced POMC and increased AgRP mRNA levels in the arcuate nucleus. Factors other than reduced melanocortin signaling are therefore likely to contribute to diabetic hyperphagia. A number of pub-

lished reports have demonstrated that uncontrolled diabetes is associated with increased NPY mRNA and peptide levels in the arcuate nucleus (17,43–46) and increased NPY peptide levels in the PVN (44). In the current study, we confirmed previous reports that uncontrolled diabetes increases NPY mRNA levels in the arcuate nucleus and that insulin treatment normalizes both the increased NPY gene expression and the hyperphagic response. Correction of increased NPY signaling may therefore play a key role in the ability of insulin treatment to restore food intake to normal levels in diabetic animals.

Because insulin treatment normalized elevated NPY expression despite only a partial restoration of normal leptin levels, it is possible that the relatively high plasma insulin concentration in the insulin-treated group was sufficient to offset the effect of persistently low leptin levels in the regulation of NPY gene expression. In contrast, levels of mRNA encoding the anorectic neuropeptide CRH were reduced in the PVN of the untreated diabetic rats. These findings support the hypothesis that hyperphagia in animals with uncontrolled diabetes arises as a consequence of both increased signaling by hypothalamic mediators that stimulate food intake (e.g., NPY and AgRP) and reduced signaling by peptides that cause anorexia (e.g., melanocortins and CRH). Whereas reduced POMC gene expression was only partially corrected, the decline of CRH mRNA levels in the hypothalamus of diabetic rats was fully normalized by chronic insulin administration. Considering that leptin increases the expression of both POMC and CRH mRNA (22,30,31), low leptin levels may have contributed to reduced hypothalamic CRH gene expression in uncontrolled diabetes. However, uncontrolled diabetes also increases circulating glucocorticoid levels (61,62), and insulin treatment prevents this response (61). Since glucocorticoids inhibit CRH gene expression by negative feedback, the diabetes-induced rise in glucocorticoids that we observed may have lowered CRH gene expression. Indeed, STZ-induced diabetes in the rat does not alter hypothalamic CRH gene expression when adrenalectomy and glucocorticoid replacement are used to match glucocorticoid levels between diabetic and nondiabetic animals (63), suggesting a major role for increased glucocorticoids to mediate the effects of diabetes on CRH gene expression.

One mechanism that does not appear to mediate the effects of uncontrolled diabetes on food intake or hypothalamic neuropeptide expression is a change of leptin sensitivity resulting from alterations in the expression of the leptin receptor (Ob-Rb), since neither diabetes nor insulin treatment affected the expression of mRNA encoding the long form of Ob-Rb in the arcuate nucleus. This result is somewhat surprising given the observations that Ob-Rb expression is upregulated in the arcuate nucleus of *ob*/*ob* mice and in the arcuate and ventromedial nuclei of fasted rats and is decreased with exogenous leptin treatment (41). However, our recent report that STZ diabetic rats exhibit normal sensitivity to the anorexic action of exogenous leptin (18) is in agreement with the observation that CNS expression of leptin receptors is not altered in diabetic animals.

Uncontrolled diabetes exerts profound effects on patterns of fuel flux and tissue fuel utilization. For example, resting energy expenditure and muscle fatty acid oxidation (64) are increased in STZ diabetic rats. Growing interest has focused on the role of uncoupling proteins, such as UCP-3 (33,34), as potential mediators of altered metabolic responses in peripheral tissues. Although the function of UCP-3 remains to be elucidated, its expression in skeletal muscle, a major site of metabolic fuel utilization, is markedly increased by fasting in rats (35,36), an effect attributable in part to increased circulating NEFA concentrations (36). In the current experiment, we also found marked increases of UCP-3 mRNA in skeletal muscle of diabetic animals, and this effect was completely prevented by insulin treatment. Similar regulation of UCP-3 was recently reported in cardiac muscle of STZ diabetic rats (65). Because our data show that changes in plasma NEFA concentrations paralleled those of UCP-3 mRNA, it is possible that delivery of this energy substrate to muscle exerts direct effects to stimulate UCP-3 expression. Increased energy expenditure associated with partial uncoupling of oxidative phosphorylation in the large mass of skeletal muscle in the body may therefore contribute to the loss of adipose tissue that accompanies uncontrolled diabetes. Thus, the hyperphagia of uncontrolled diabetes is unable to compensate for this increase in energy expenditure. In contrast to the upregulation of UCP-3 reported here, it is worth noting that the brown adipose tissue–specific uncoupling protein UCP-1 is decreased in unregulated STZ diabetes (66,67), an effect that is prevented by insulin administration (66).

It is also possible that changes within the CNS induced by uncontrolled diabetes contribute to altered UCP-3 expression in skeletal muscle. Precedent for this hypothesis is provided by the demonstration that intrahypothalamic infusion of NPY suppresses UCP-1 expression (68), whereas central administration of CRH increases uncoupling activity in brown adipose tissue (29). NPY also modulates insulin-stimulated glucose transport in peripheral tissues, including skeletal muscle (69). Since fasting and uncontrolled diabetes are both accompanied by increased production and release of NPY and AgRP and decreased CRH and POMC expression, it is conceivable that this hypothalamic response alters autonomic outflow (70), which may in turn influence the expression of molecules such as UCP-3 (35,71). Consistent with this idea, we found that hypothalamic NPY expression in the untreated diabetic rats was positively correlated ($r = 0.99$; $P < 0.0001$), and CRH expression in insulin-treated rats was inversely correlated ($r = –0.94$; $P < 0.02$), with muscle UCP-3 expression. Further investigation of the role of UCP-3 in muscle energy metabolism and its potential link to hypothalamic neuropeptide signaling is warranted.

In conclusion, uncontrolled diabetes has major effects on the hypothalamic melanocortin system (decreased POMC and increased AgRP expression) that may contribute, in combination with increased NPY and reduced CRH signaling, to hyperphagia characteristic of the diabetic state. The findings are compatible with the hypothesis that deficiencies of insulin and leptin have potent effects on hypothalamic neuropeptide systems involved in energy homeostasis, which may in turn contribute to the marked effects of uncontrolled diabetes on fuel utilization and skeletal muscle UCP-3 gene expression.

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During the review and revision of this manuscript, a paper reporting increases of UCP-2 and UCP-3 expression in skeletal muscle in streptozotocin diabetic rats and the reversal of the overexpression with insulin administration (72) became available on the PubMed database (National Library of Medicine).

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