Increased responsiveness to glucoregulatory effect of opiates in obese-diabetic ob/ob mice

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Summary. Plasma glucose and insulin responses to opiate receptor stimulation and antagonism were determined in 12–14 week old lean and obese-diabetic Aston ob/ob mice. The opiate receptor antagonist naloxone (1 mg/kg intraperitoneally) rapidly and transiently raised glucose and suppressed insulin concentrations in lean mice, and produced qualitatively similar but more protracted responses in ob/ob mice. Selective stimulation of μ - and δ -opiate receptors using the enkephalin analogues Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg, intraperitoneally) and Tyr-D-Ala-Gly-Phe-D-Leu (10 mg/kg intraperitoneally) respectively, rapidly and tran-

siently increased glucose and insulin concentrations in lean and ob/ob mice. The ob/ob mice exhibited greater glucose and insulin responses to these analogues. The results (1) provide evidence that endogenous opiates participate in the regulation of glucose and insulin homeostasis, and (2) suggest that increased responsiveness to μ - and δ -opiate receptor stimulation may contribute to the hyperglycaemia and hyperinsulinaemia of obese-diabetic mice.

Key words: Opiates, enkephalin analogues, naloxone, obese-diabetic ob/ob mice, glucose, insulin.

Several studies have suggested that opiate peptides might contribute to the regulation of glucose homeostasis. Morphine and B-endorphin produce a hyperglycaemic effect [1–5], and stimulate insulin release from isolated pancreatic islets [6–10]. Dynorphin, met- and leu-enkephalin and enkephalin analogues with opiate agonist properties also stimulate insulin release in vitro, and this effect is inhibited by the opiate antagonist naloxone [7, 9–11]. The possible physiological relevance of these observations is brought into focus by the identification of opiate peptides within cells of the pancreatic islets and within the nervous innervation of the pancreas [12–17]. However, studies on the effects of opiates in vivo have been hampered by a lack of stable analogues with selective agonist effects at different subtypes of opiate receptors.

Using naloxone and enkephalin analogues, evidence has emerged to suggest that abnormal responses to endogenous opiates may be a factor in the development of hyperglycaemia and defective insulin secretion in human Type 2 (non-insulin-dependent) diabetes mellitus [18–20].

The obese hyperglycaemic (ob/ob) mouse has been used extensively as a model of non-insulin-dependent diabetes mellitus, being characterised by gross obesity, hyperphagia, moderate hyperglycaemia, marked hyperinsulinaemia and severe insulin resistance [21–24]. The present study was undertaken to investigate the effect of opiates on normal glucose and insulin homeostasis, and

to examine the possibility that abnormal responses to opiates contribute to the hyperglycaemia and hyperinsulinaemia of ob/ob mice. Acute changes in plasma glucose and insulin concentrations were determined in lean and ob/ob mice after administration of naloxone and stable enkephalin analogues with selective agonist properties at μ and δ -opiate receptors.

Materials and methods

Animals

Obese hyperglycaemic (ob/ob) mice and homozygous lean (+/+) mice from the colony maintained at Aston University, Birmingham, UK were used at 12–14 weeks of age. The origin and characteristics of these mice have been described previously [23, 25]. Mice were housed in an air conditioned room at 22 ± 2 °C with a lighting schedule of 12 h light (08.00–20.00 hours) and 12 h dark. A standard pellet diet (Mouse breeding diet, Heygate & Sons, Northampton, UK) and tap water were supplied ad libitum.

Chemicals

The chemicals and their sources were: naloxone hydrochloride (batch 64F-0209) from Sigma Chemical Company, Poole, UK; Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (batch 11064) and Tyr-D-Ala-Gly-Phe-D-Leu (batch 23114) from Cambridge Research Biochemicals, Cambridge, UK. Other chemicals and reagents were obtained as previously [26]. Whereas naloxone is an antagonist of all known subtypes of opiate receptors, with an especially potent effect at μ -opiate receptors, the enkephalin analogues Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH and Tyr-D-Ala-Phe-Gly-D-Leu are predominantly

Table 1. Characteristics of animals: body weights and plasma concentrations of glucose and insulin in fed and 24 h fasted lean (+/+) and obese (ob/ob) mice. Values are means $\pm$ SEM. Numbers of mice are shown in parentheses

	Body weight (g)	Fed		24-h fasted	
		Plasma glucose (mmol/l)	Plasma insulin (ng/ml)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)
Lean (+/+)	33.5 $\pm$ 0.4 (30)	7.5 $\pm$ 0.2 (10)	2.4 $\pm$ 0.5 (10)	4.2 $\pm$ 0.3 (20)	1.0 $\pm$ 0.3 (20)
Obese (ob/ob)	70.8 $\pm$ 1.6*** (30)	14.5 $\pm$ 1.2*** (12)	26.3 $\pm$ 2.3*** (12)	6.4 $\pm$ 0.7* (18)	6.2 $\pm$ 1.4** (18)

* $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$ compared with lean (+/+) mice

Fig. 1. Changes (Δ) in plasma glucose and insulin concentrations of 12–14 week old fed lean Aston mice after intraperitoneal administration of saline (5 ml/kg) and naloxone hydrochloride (1 mg/kg). Values are mean $\pm$ SEM of 5 mice. * $p < 0.05$ compared with time zero

agonists at μ - and δ -opiate receptors, respectively, and show a high degree of stability when administered in vivo [27, 28].

Experimental procedure

Test substances were dissolved in saline (0.9% NaCl) and administered by intraperitoneal (ip) injection to groups of lean and ob/ob mice. Naloxone (1 mg/kg) or saline (5 ml/kg) was administered to fed lean and ob/ob mice, and Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg), Tyr-D-Ala-Gly-Phe-D-Leu (1 and 10 mg/kg) or saline (5 ml/kg) was administered to 24 h fasted lean and ob/ob mice. Blood samples (60 μ l) for plasma glucose and insulin determination were taken from the tail tip of conscious mice immediately before and at 5, 15 and 30 min after the injection of test substances. In an additional study with 24 h fasted lean mice, ip injections of Tyr-D-Ala-MePhe-NH(CH₂)₂OH (1 mg/kg) and Tyr-D-Ala-Gly-Phe-D-Leu (10 mg/kg) were given 20 min after an ip injection of glucose (1 g/kg), and blood samples were taken immediately before and at 5 and 15 min after the injections of opiates. Food, but not water, was withheld during all tests. The mice were adapted to the manipulative procedures for 2–3 weeks before study to obviate any stress response [26]. The doses of opiates used must be regarded as pharmacological. They were selected after a preliminary study to identify the amounts required to produce measurable changes in plasma glucose and insulin after ip injection into lean mice.

Statistical analysis

Plasma glucose was measured by an automated glucose oxidase procedure, and plasma insulin was determined by double antibody

Fig. 2. Changes (Δ) in plasma glucose and insulin concentrations of 12–14 week old fed obese-diabetic Aston ob/ob mice after intraperitoneal administration of saline (5 ml/kg) and naloxone hydrochloride (1 mg/kg). Values are mean $\pm$ SEM of 6 mice. * $p < 0.05$, ** $p < 0.01$ compared with time zero

radioimmunoassay as described previously [29]. Data are expressed in the figures as the change in plasma glucose (mmol/l) or insulin (ng/ml) compared with time zero. Σ values cited in the text refer to the sum of changes in plasma glucose (mmol/l) or insulin (ng/ml) at 5, 15, and 30 min, i.e. sum of values at 5, 15 and 30 min minus 3 times the value at time zero. All values are means $\pm$ SEM of 5 lean and 6 ob/ob mice. Groups of data from the same mice were compared using Student's paired t-test, and groups of data from different mice were compared using Student's unpaired t-test. Differences were considered to be significant if $p < 0.05$.

Results

The ob/ob mice were characteristically obese, as indicated by their greater body weight, and exhibited a typically moderate hyperglycaemia and marked hyperinsulinaemia in the fed and fasted state (Table 1).

Administration of saline (5 ml/kg) did not significantly alter plasma glucose or insulin concentrations in either fed (Figs. 1a and 2a) or 24-h fasted (Figs. 3a and 4a) lean and ob/ob mice during the 30 min period of study. In fed lean mice, naloxone (1 mg/kg) produced a rapid, small and transient rise in plasma glucose ($\Sigma + 2.2 \pm 0.5$ mmol/l) accompanied by a rapid and transient fall in plasma insulin ($\Sigma - 1.8 \pm 0.6$ ng/ml) (Fig. 1b). In fed ob/ob mice naloxone produced a more protracted rise in plasma glucose ($\Sigma + 4.3 \pm 0.6$ mmol/l;

Fig. 3. Changes (Δ) in plasma glucose and insulin concentrations of 12–14 week old 24 h fasted lean Aston mice after intraperitoneal administration of saline (5 ml/kg), Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg) and Tyr-D-Ala-Gly-Phe-D-Leu (1 and 10 mg/kg). Values are mean $\pm$ SEM of 5 mice. * p < 0.05, ** p < 0.01 compared with time zero

Fig. 4. Changes (Δ) in plasma glucose and insulin concentrations of 12–14 week old 24-h fasted obese-diabetic Aston ob/ob mice after intraperitoneal injection of saline (5 ml/kg), Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg) and Tyr-D-Ala-Gly-Phe-D-Leu (1 mg/kg). Values are mean $\pm$ SEM of 6 mice. * p < 0.05, ** p < 0.01 compared with time zero

p < 0.05 compared with fed lean mice) and a more sustained fall in plasma insulin (Σ – 23.5 $\pm$ 3.5 ng/ml; p < 0.001 compared with fed lean mice) (Fig. 2b).

Fasted lean mice responded to Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg) with rapid and transient increases in plasma glucose (Σ + 2.8 $\pm$ 0.6 mmol/l) and insulin (Σ + 2.2 $\pm$ 1.1 ng/ml) (Fig. 3b). Tyr-D-Ala-Gly-Phe-D-Leu did not significantly alter either plasma glucose (Σ + 0.5 $\pm$ 0.7 mmol/l) or insulin (Σ + 1.2 $\pm$ 0.8 ng/ml) at a dose of 1 mg/kg, but a higher dose of this analogue (10 mg/kg) elevated both plasma glucose (Σ + 2.0 $\pm$ 0.4 mmol/l) and insulin (Σ + 1.45 $\pm$ 0.3 ng/ml) in a rapid and transient manner (Fig. 3c, d). In fasted ob/ob mice both Tyr-D-Ala-Gly-MePhe-

Fig. 5. Changes (Δ) in plasma glucose and insulin concentrations of 12–14 week old 24-h fasted lean Aston mice after intraperitoneal injection of glucose (1 g/kg) at time –20 min, and intraperitoneal injection of either saline (5 ml/kg), Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg) or Tyr-D-Ala-Gly-Phe-D-Leu (10 mg/kg) at time zero. Values are mean $\pm$ SEM of 5 mice. * p < 0.05 compared with time zero

NH(CH₂)₂OH (1 mg/kg) and Tyr-D-Ala-Gly-Phe-D-Leu (1 mg/kg) produced rapid transient increases in plasma glucose (Σ + 3.9 $\pm$ 1.0 and + 5.7 $\pm$ 0.5 mmol/l respectively) and insulin (Σ + 7.1 $\pm$ 1.4 and + 11.4 $\pm$ 2.5 ng/ml respectively) (Fig. 4b, c). Compared with lean mice, ob/ob mice exhibited a greater insulin response to Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (p < 0.05) and greater glucose and insulin responses to Tyr-D-Ala-Gly-Phe-D-Leu (p < 0.01 and p < 0.001 respectively).

To investigate whether the greater insulin responses to the opiate analogues in ob/ob mice were facilitated by the higher fasting glucose concentrations, the opiate analogues were administered to fasted lean mice after elevation of plasma glucose by prior administration of glucose (1 g/kg ip, 20 min previously). After glucose administration, plasma glucose in fasted lean mice (8.9 $\pm$ 0.5 mmol/l, n = 15) was higher than in untreated fasted lean mice (4.2 $\pm$ 0.3 mmol/l, n = 20, p < 0.001) and fasted ob/ob mice (6.4 $\pm$ 0.7 mmol/l, n = 18, p < 0.01). In mice treated with saline (5 ml/kg) plasma glucose con-

centrations were unchanged at 5 and 15 min, whereas plasma insulin was decreased by 0.66 ± 0.34 ng/ml ($n=5$) at 15 min (Fig. 5a). Neither Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH (1 mg/kg) nor Tyr-D-Ala-Gly-Phe-D-Leu (10 mg/kg) significantly altered plasma glucose (Fig. 5b, c). However, Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH increased insulin concentrations by 0.61 ± 0.33 ng/ml ($n=5$, $p < 0.05$ compared with saline) at 15 min, and Tyr-D-Ala-Gly-Phe-D-Leu increased insulin concentrations by 0.82 ± 0.45 ng/ml ($n=5$, $p < 0.05$ compared with saline) at 5 min. These insulin responses to the opiate analogues were considerably less than those observed in ob/ob mice (Fig. 4b, c).

Discussion

The transient hyperglycaemia and hypoinsulinaemia observed after opiate receptor antagonism with naloxone in lean mice indicate that endogenous opiate-mediated mechanisms participate in the normal control of glucose homeostasis. In particular it appears that endogenous opiates directly promote insulin secretion, as indicated by previous *in vitro* studies [6–11]. This view is substantiated herein by *in vivo* administration of the selective and stable enkephalin analogues Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH and Tyr-D-Ala-Gly-Phe-D-Leu, which induced a rapid and transient increase of insulin concentrations. Interestingly, the enkephalin analogues also produced a transient rise in glucose concentrations. This effect may involve an action on glucagon and the central nervous system [3–5], which could contribute to the accompanying hyperinsulinaemia by increasing the glycaemic stimulus for insulin release [30], and by activation of insulinotropic neuroendocrine pathways linking the central nervous system and the pancreatic islets [31]. The transient hyperglycaemia noted after opiate antagonism with naloxone may be consequent in part to the concomitant decrease of insulin concentrations. However, it would not seem unreasonable to surmise that the widespread distribution of endogenous opiates [32, 33] might be associated with additional effects on glucose homeostasis to those considered above. Indeed, it has recently been suggested that endogenous opiates influence peripheral glucose uptake by a mechanism that is independent of insulin or major counterregulatory hormones [34, 35]. The greater responses evoked in lean mice by the μ -opiate agonist (Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH) and the δ -opiate agonist (Tyr-D-Ala-Gly-Phe-D-Leu) might reflect a preponderance or a higher affinity of μ -opiate receptors on the target cells concerned. However, differences in the biological stability and reactivity of these agents could be contributory factors. The responses cannot be attributed to a stress reaction, since saline did not significantly affect glucose and insulin concentrations in this test system, as noted previously [26].

Several observations have indicated abnormalities of opiate production and action in obese-diabetic ob/

ob mice. Thus, ob/ob mice exhibit raised levels of B-enkephalin, met-enkephalin and leu-enkephalin in the pituitary, hypothalamus and pancreas [36–41]. The ob/ob mice also show heightened feeding and drinking responses to opiate agonists and antagonists, particularly those acting predominantly at K -opiate receptors [36, 40, 42]. The present study provides evidence of disturbances in the glucoregulatory effects of opiates in ob/ob mice. For example, compared with lean mice naloxone produced a more protracted hyperglycaemia and hypoinsulinaemia, and the enkephalin analogues, Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH and Tyr-D-Ala-Gly-Phe-D-Leu evoked greater responses of glucose and/or insulin. There was also no apparent difference in the glucoregulatory effectiveness of the μ -opiate agonist (Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH) and the δ -opiate agonist (Tyr-D-Ala-Gly-Phe-D-Leu) in the mutant mice. The especially marked reduction of insulin concentrations after naloxone administration, and the enhanced insulin responses to the enkephalin analogues, which do not appear to reflect the elevated glycaemia, support the view that endogenous opiates may act in addition to increased activity of the entero-insular axis [24, 43–45] to promote excessive insulin secretion in ob/ob mice. This role of opiates may reflect increased B-cell sensitivity to these insulinotropic peptides, or increased opiate biosynthesis and release by the pituitary and gastro-intestinal tract of ob/ob mice [36–42, 46]. Since ob/ob mice show a reduced insulin-releasing effect of hyperglycaemia *per se* [25, 26, 47], the greater insulin response to opiate agonists in the ob/ob mice is not attributable to the greater rise in glycaemia. The very short half life of endogenous opiates in the peripheral circulation [48] draws attention to the possible significance of recent studies demonstrating that opiate peptides are synthesised and released within the islet [12–15].

Thus, opiates appear to participate in the control of glucose homeostasis through a transient insulinotropic effect involving μ - and δ -opiate receptors. Opiates can stimulate hyperglycaemia; however, since opiate blockade can also raise glucose concentrations, it is evident that other mechanisms of glucoregulation by opiates must exist. The present study indicates that increased responsiveness to opiates may contribute to the hyperglycaemia and hyperinsulinaemia of obese-diabetic mice.

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