Effects of Glucose and Diabetes on Binding of Naloxone and Dihydromorphine to Opiate Receptors in Mouse Brain

DAVID A. BRASE, YI-HONG HAN, AND WILLIAM L. DEWEY

SUMMARY

The effects of glucose and diabetes on the high-affinity lofentanil-displaceable opiate-receptor binding in mouse brain membranes were studied to determine if the attenuation of opiate actions by hyperglycemia previously observed in our laboratory was due to a modification of receptor affinity or number. With membranes from normal ICR mice, glucose (100–400 mg/dl) caused small but significant concentration-dependent decreases in receptor affinities for [^{3}H]naloxone and [^{3}H]dihydromorphine, both in the absence and presence of 20 mM NaCl, without changing the maximum number of binding sites. Fructose and the nonmetabolizable sugar 3-O-methylglucose had intermediate effects on naloxone affinity in the presence of NaCl that were not significantly different from control or from the effect of glucose. Similar results were obtained with brain membranes from streptozocin-induced diabetic mice. The binding affinity for [^{3}H]naloxone in the presence of NaCl was not affected by the induction of diabetes in ICR mice via streptozocin or in spontaneously diabetic (*db*/*db*) C57BL/KsJ mice compared with their nondiabetic (m^+/m^+) littermates. These results indicate that the previously observed attenuation of opiate effects by glucose may be partly due to a glucose-induced decrease in opiate-receptor affinity. However, the much greater attenuation of morphine by fructose in vivo cannot be explained by this mechanism. *Diabetes* 36:1173–77, 1987

As recently reviewed, studies in our laboratory have demonstrated antagonistic effects of glucose on opiate actions in vivo and in vitro (1). The antinociceptive potency of morphine was decreased in several rodent models of hyperglycemia, including a spontaneously diabetic strain of mice, streptozocin-induced diabetes (STZ-D), and acute glucose loading (2). Conversely, the induction of hypoglycemia in mice by fasting plus insulin treatment increased the potency of morphine in the tail-flick test (2). The induction of physical dependence on morphine was also significantly decreased in STZ-D rats and genetically diabetic mice (3). Increasing concentrations of glucose in vitro were found to decrease the potency of normorphine in the electrically stimulated guinea pig ileum and mouse vas deferens preparations (4). Furthermore, glucose attenuated the development of acute dependence in vitro in the guinea pig ileum (4). In a clinical study, Morley et al. (5) showed a significantly decreased pain tolerance in diabetic patients and in normal subjects loaded with glucose compared with normal fasted subjects and suggested that the painful neuropathy experienced by some diabetic patients might involve an interaction of glucose with the action of endogenous opioid peptides.

The sensitivity of animals to the opioid antagonist naloxone has also been reported to be changed by hyperglycemia (6,7). Levine et al. (6) reported that both STZ-D mice and genetically diabetic C57BL/KsJ mice were markedly more sensitive to the naloxone-induced suppression of food intake than nondiabetic controls. This observation was later confirmed in STZ-D rats when they were tested in a novel cage but not when tested in their home cages, indicating a significant influence of environment on sensitivity to this effect of naloxone (7).

Among the possible mechanisms for these observations could be a glucose-induced alteration of opiate-receptor affinity or number. Lending support to the former hypothesis was the report that glucose at concentrations of 100 and 300 mg/dl caused a concentration-dependent decrease in mouse brain opiate-receptor affinity for [^{3}H]naloxone in the presence of NaCl (8). In the same experiments, however, glucose also caused a concentration-dependent increase in

From the Central Nervous System Division, Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.
Address correspondence and reprint requests to Dr. William L. Dewey, Box 613, MCV Station, Richmond, VA 23298.
Received for publication 22 September 1986 and accepted in revised form 13 March 1987.

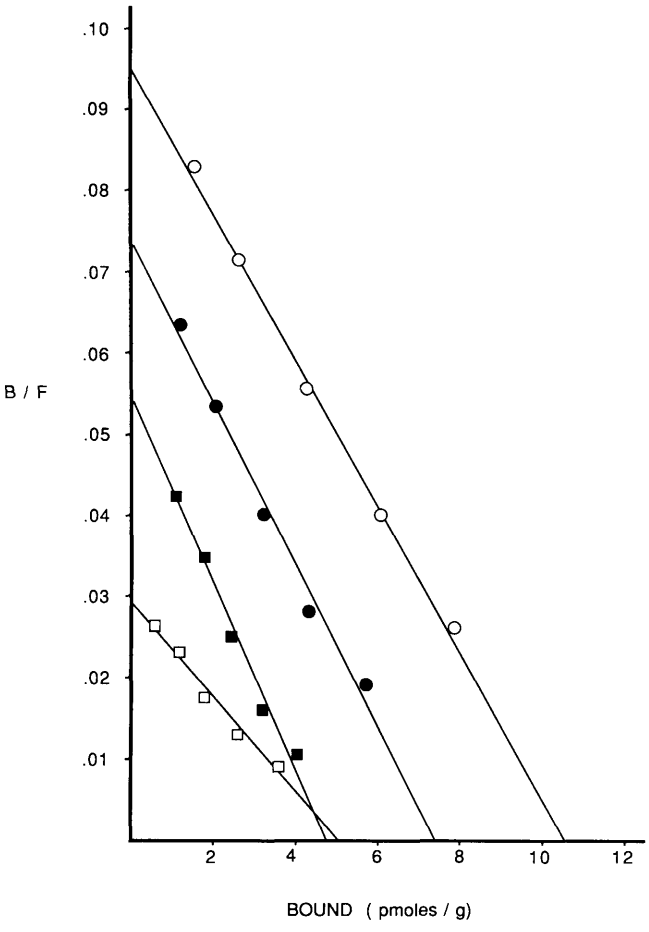

FIG. 1. Scatchard plots of lofentanil-displaceable binding of [^{3}H]naloxone (*circles*) and [^{3}H]dihydromorphine (DHM) (*squares*) to particulate fraction from ICR mouse brains in the absence (*closed symbols*) and presence (*open symbols*) of 20 mM NaCl at 25°C. *Points* on each *line* represent means of 4 separate experiments. B/F, bound-to-free ratio.

the number of binding sites for [^{3}H]naloxone (8). It was of interest to reinvestigate the effects of glucose on opiate-receptor binding, because the 5–100 nM range of naloxone concentrations used in that study would not be expected to yield binding parameters for the high-affinity opiate binding site in isolated brain membranes, which has a dissociation constant (K_d) for naloxone ≤1.6 nM under usual binding-assay conditions with rat membranes (9–11). Recently, a K_d of 1.04 ± 0.20 nM was reported for the binding of [^{3}H]naloxone to hypothalamic slices from mouse brain (12). It is a high-affinity binding site that is thought to be primarily involved in the analgesic effect of morphinelike agonists (13). Because naloxone is an opiate antagonist, it was also of interest to study the effects of glucose on the binding of an opiate agonist, because other studies have shown that the binding of agonists and antagonists are not always modulated (e.g., by Na^+) in the same direction (9). In addition, the effects of fructose and the nonmetabolizable sugar 3-*O*-methylglucose on high-affinity naloxone binding were compared with the effects of glucose, because previous studies in our laboratory demonstrated a greater attenuation of morphine in vivo by fructose than by glucose, whereas 3-*O*-methylglucose failed to significantly alter the effects of morphine (2).

Finally, we compared the effects of spontaneous and STZ-D on the high-affinity binding of naloxone, because it was reported that brain membranes from genetically diabetic C57BL/KsJ mice had a 43% higher K_d for naloxone than membranes from nondiabetic control (8).

MATERIALS AND METHODS

Animals. Male ICR mice weighing 25–30 g were obtained from Dominion Animal Supplies (Dublin, VA). Some of these mice were rendered diabetic by a single injection of STZ (200 mg/kg i.v; Sigma, St. Louis, MO), and controls received an equivalent volume of the 0.01 M sodium citrate vehicle (pH 4.0), as described previously (2). Mice were housed in groups of six per cage and allowed free access to food and water. Induction of diabetes was verified in the laboratory on the day of use (6–7 days after STZ) by testing blood from the retroorbital sinus with Dextrostix reagent strips (Ames, Miles, Elkhart, IN), as described previously (3). Genetically diabetic (*db/db*) C57BL/KsJ male mice and their nondiabetic littermates (m^+/m^+) were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in groups of four per cage for 6–7 days before measurement of blood glucose levels just before death at the age of 6 wk.

Binding assay. After decapitation the brains were removed and weighed. The whole brains were homogenized in pairs in 10 vol (1 g/10 ml) of ice-cold 50 mM Tris-HCl buffer (pH

TABLE 1
Effect of glucose on lofentanil-displaceable [^{3}H]naloxone and [^{3}H]dihydromorphine binding to mouse brain membranes in the absence and presence of NaCl

		K_d (nM)		B_{max} (pmol/g)	
^{3}H-labeled ligand	Glucose (mg/dl)	Minus NaCl	Plus 20 mM NaCl	Minus NaCl	Plus 20 mM NaCl
Naloxone*	0	1.06 ± 0.06	1.10 ± 0.02	7.58 ± 0.17	10.6 ± 0.2
	100	1.13 ± 0.08	1.16 ± 0.03	7.59 ± 0.19	10.7 ± 0.3
	200	1.21 ± 0.02	1.22 ± 0.05	7.66 ± 0.24	10.7 ± 0.4
	400	1.35 ± 0.06†	1.36 ± 0.05†	7.75 ± 0.28	10.9 ± 0.2
Dihydromorphine	0	0.85 ± 0.06	1.64 ± 0.14	4.67 ± 0.37	4.80 ± 0.41
	100	0.94 ± 0.07	1.70 ± 0.11	4.65 ± 0.30	4.82 ± 0.44
	200	0.99 ± 0.04	1.93 ± 0.12	4.56 ± 0.24	5.04 ± 0.22
	400	1.16 ± 0.07†	2.00 ± 0.16	4.68 ± 0.26	4.86 ± 0.33

B_{max}, maximum binding.
*Sp act = 60 Ci/mmol (batch 27).
†Significantly different from control at 99% confidence level (Dunnett's test).

TABLE 2
Comparison of effects of glucose, 3-*O*-methylglucose, and fructose on specific [^{3}H]naloxone* binding to normal ICR mouse brain membranes in presence of 20 mM NaCl

Sugar (mg/dl)	K_d (nM)	B_{max} (pmol/g)
None	1.23 ± 0.03	10.9 ± 0.3
Glucose (400)	1.39 ± 0.05†	11.1 ± 0.2
3-*O*-methylglucose (431)	1.33 ± 0.04	11.4 ± 0.4
Fructose (400)	1.30 ± 0.04	11.4 ± 0.3

B_{max}, maximum binding.
*Sp act = 58 Ci/mmol (batch 30).
†Significantly different from control at 95% confidence level (Dunnett's test).

7.4 at room temperature) containing 2 mM dithiothreitol (DTT), with a motor-driven Teflon/glass homogenizer. After centrifugation at 40,000 × *g* for 15 min, the supernatant fraction was discarded and the pellet was rehomogenized in another 10 vol of buffer plus DTT and recentrifuged. The final pellet was resuspended in another 10 vol of buffer, equivalent to ~100 mg brain/ml. To an aliquot of this particulate fraction of brain was added 25 pmol/ml (final incubation concentration 2.5 nM) of the extremely high-affinity opiate-receptor agonist lofentanil (14), for the determination of nonspecific binding. Incubations were carried out at 25°C for 30 min in a total volume of 2.0 ml, containing 50 mM Tris-HCl (pH 7.4) plus 2 mM DTT, 0.2 ml of the particulate fraction, and the appropriate concentrations of [^{3}H]naloxone (sp act 58 or 60 Ci/mmol; Amersham, Arlington Heights, IL) or [^{3}H]dihydromorphine (sp act 83.3 Ci/mmol; New England Nuclear, Boston, MA). Incubations were terminated by filtration through 25-mm glass-fiber filters (#32; Schleicher and Schuell, Keene, NH) and rapid washing with two 5-ml aliquots of 50 mM Tris-HCl buffer without DTT (pH 7.4), under reduced pressure (−250 mmHg) with a 40-place filtration manifold. Each wet filter was allowed to stand overnight in 10 ml of ACS scintillation fluid (Amersham) and mixed before determination of radioactivity by liquid scintillation spectrometry. Each incubation was carried out in duplicate, and binding parameters were calculated from 4 or 5 different concentrations of [^{3}H]naloxone (0.2–3.2 nM) or [^{3}H]dihydromorphine (0.25–4.0 nM), with a computer program for the Scatchard method (15).

Statistics. Comparisons of multiple treatment groups with a control group were carried out with a computer program for Dunnett's test (15), with *t* tables for one-sided comparisons (16). Significant differences between two groups were determined with a *t* test for grouped data (15). All data are expressed as means ± SE.

RESULTS

Effects of NaCl on binding parameters. The effects of 20 mM NaCl on the lofentanil-displaceable binding of [^{3}H]-naloxone and [^{3}H]dihydromorphine to the particulate fraction of mouse brain are illustrated in the Scatchard plots in Fig. 1. NaCl increased the number of binding sites for [^{3}H]naloxone without significantly affecting its affinity. On the other hand, NaCl decreased the affinity for [^{3}H]dihydromorphine without affecting the maximum number of binding sites (B_{max}). As reported previously for rat brain (17), there were fewer high-affinity sites for [^{3}H]dihydromorphine than for [^{3}H]naloxone binding in both the absence and presence of NaCl.

Effects of sugars on binding parameters. The effects of glucose on the binding parameters for the lofentanil-displaceable binding of [^{3}H]naloxone and [^{3}H]dihydromorphine to the particulate fraction from brains of normal ICR mice in the absence and presence of 20 mM NaCl are summarized in Table 1. Glucose caused concentration-dependent increases in the K_d values for both ^{3}H-labeled ligands in the absence and presence of NaCl but did not significantly affect the maximum number of binding sites. The effects of glucose and NaCl appeared to be independent of each other.

Because previous in vivo studies indicated a greater effect of fructose loading than glucose loading but no significant effect of 3-*O*-methylglucose loading on the ED_{50} for morphine in the mouse tail-flick test (2), it was of interest to compare the effects of these three sugars on opiate-receptor binding in vitro (Table 2). Although somewhat higher control values for the naloxone K_d and B_{max} were obtained in this series of experiments, glucose (400 mg/dl) again caused a significant increase in the K_d for [^{3}H]naloxone. Intermediate values for the naloxone K_d were obtained in incubations with equimolar concentrations of 3-*O*-methylglucose and fructose, which were not significantly different from either control incubations or incubations in the presence of glucose.

Effects of diabetes on naloxone binding. At death, the STZ-D ICR mice all had blood glucose levels >400 mg/dl, the maximum concentration measurable by the Glucometer. The control ICR mice pretreated with vehicle had a mean (±SE) blood glucose level of 180 ± 9 mg/dl (n = 8). As indicated in Table 3, the induction of diabetes with STZ had no significant effect on the binding parameters for [^{3}H]naloxone in the presence of 20 mM NaCl.

The diabetic (*db/db*) C57BL/KsJ mice had a mean (±SE) blood glucose concentration of 318 ± 25 mg/dl (n = 8), compared to a concentration of only 83 ± 5 mg/dl (n = 8) for their nondiabetic (m^+/m^+) littermates. There was no difference between these two groups in opiate-receptor affinity for [^{3}H]naloxone in the presence of 20 mM NaCl (Table 3). The diabetic mice had a mean of 14.4% more binding sites per gram of brain than the nondiabetic mice (Table 3). However, the brains of the diabetic mice weighed a mean of 8.6% less ($P < .01$, n = 4 pairs) than the brains of their nondiabetic littermates, so the number of binding sites per brain was probably the same for both groups.

Although these results indicated that the diabetic state did not affect the affinity of opiate receptors for naloxone, it was

TABLE 3
Effects of spontaneous and streptozocin-induced diabetes on binding of [^{3}H]naloxone* to brain membranes in presence of 20 mM NaCl

Strain	Treatment or condition	K_d (nM)	B_{max} (pmol/g)
ICR	Vehicle	1.13 ± 0.07	9.51 ± 0.60
ICR	Streptozocin	1.21 ± 0.10	9.93 ± 0.54
C57BL/KsJ	Nondiabetic (m^+/m^+)	1.09 ± 0.15	9.12 ± 0.91
C57BL/KsJ	Diabetic (*db/db*)	1.11 ± 0.14	10.43 ± 1.16

Values are means ± SE of 4 experiments, each with membranes prepared from 2 brains. B_{max}, maximum binding.
*Sp act = 58 Ci/mmol (batch 30).

TABLE 4
Comparison of effects of glucose, 3-*O*-methylglucose, and fructose on specific [^{3}H]naloxone* binding to brain membranes from streptozocin-induced diabetic ICR mice

Sugar (mg/dl)	K_d (nM)	B_{max} (pmol/g)
None	0.97 ± 0.05	8.21 ± 0.52
Glucose (400)	1.16 ± 0.05†	8.56 ± 0.50
3-*O*-methylglucose (431)	1.04 ± 0.06	8.52 ± 0.54
Fructose (400)	1.09 ± 0.04	8.21 ± 0.47

B_{max}, maximum binding.
*Sp act = 60 Ci/mmol (batch 32).
†Significantly different from control at 95% confidence level (Dunnett's test).

of interest to determine whether the membrane receptors from diabetic mice displayed a change in sensitivity to the addition of sugars to the incubation medium. However, the effects of glucose, fructose, and 3-*O*-methylglucose on naloxone binding to membranes from STZ-D mice (Table 4) were not substantially different from their effects on the binding parameters for naloxone in membranes from normal ICR mice (Table 2).

DISCUSSION

Opiate-receptor–binding studies have shown the presence of both high- and low-affinity binding sites for [^{3}H]naloxone and [^{3}H]dihydromorphine in brain membranes (17,18). The in vivo administration of naloxazone was reported to selectively inhibit binding to the high-affinity sites and to markedly decrease the antinociceptive potency of morphine (19). Thus, it appeared that the high-affinity binding sites were primarily involved in mediating the analgesic effects of morphine. Because diabetes or the acute administration of glucose or fructose have also been shown to decrease the antinociceptive potency of morphine (2), it was of interest to determine whether diabetes or the direct addition of various sugars to the binding assay would affect binding parameters for the high-affinity opiate binding sites. In similar studies of the low-affinity binding site for [^{3}H]naloxone, concentration-dependent increases in the K_d and B_{max} caused by the addition of glucose to incubations containing Na^+ were found (8).

In the study of the low-affinity sites, Na^+ itself increased the affinity for [^{3}H]naloxone without affecting the maximum number of binding sites (8). Opposite effects of Na^+ were observed in this study of the high-affinity sites, in which sodium increased the B_{max} without affecting the K_d for naloxone. A similar effect of Na^+ (25 mM) on the high-affinity binding of naloxone to rat brain membranes at 25°C has been reported (10). Thus, the high-affinity sites for naloxone in mouse brain membranes appear to respond to Na^+ similarly to those in rat brain membranes, but differently from the lower-affinity binding sites in mouse brain membranes (8).

Like the low-affinity binding sites for [^{3}H]naloxone (8), the high-affinity sites displayed concentration-dependent decreases in affinity with increasing concentrations of glucose in vitro. However, glucose did not significantly affect the maximum number of high-affinity binding sites. Similar results were obtained with the high-affinity binding of the agonist [^{3}H]dihydromorphine, and the effects of glucose on the binding of both ligands appeared to be independent of the effects of Na^+.

Although it was reported that brain membranes from diabetic (*db/db*) mice had a lower affinity for naloxone than membranes from the corresponding controls (8), no difference in the high-affinity binding of naloxone was observed between these two groups in this study. STZ-D also did not affect the high-affinity binding of naloxone or significantly modify the effects of glucose, 3-O-methylglucose, or fructose on that binding. Consequently, the previously reported decreased potency of morphine in diabetic animals does not appear to be due to an alteration of opiate receptors by the diabetic state (2). The hyperglycemia associated with the diabetic state may contribute to the decreased potency of morphine observed in vivo (2). The glucose-induced decreases in opiate-receptor affinity were moderate, however, compared with the marked decrease in the antinociceptive potency of morphine previously observed in genetically diabetic mice (2). To account for the difference in magnitude between the effects of glucose in vitro and in vivo, the modest effect of glucose on binding in vitro may be due solely to some physicochemical mechanism, whereas the more marked effect of hyperglycemia in vivo may also involve the metabolism of glucose, an interaction of glucose with ion transport, or an interaction of glucose with endogenous opioid peptides.

The finding by Simon and Dewey (2), that the nonmetabolizable 3-*O*-methylglucose did not significantly affect the potency of morphine in vivo lends support to the possibility that the significant effect of glucose in vivo may involve its metabolism. Recent evidence that hyperglycemia significantly affects the transport of Na^+ into the central nervous system (20) indicates the possibility that the effect of glucose in vivo may be secondary to changes in the disposition of this ion. Changes in intracellular Na^+ concentrations appear to regulate opiate agonist binding to cultured cells (21). In addition, STZ-D has recently been reported to decrease pain tolerance and β-endorphin levels in rats (22), and it has been postulated that the attenuation of the analgesic effect of morphine in diabetic rats may be related to reduced hypothalamic levels of β-endorphin (23). Diabetic C57BL/KsJ mice were reported to exhibit neuronal degeneration in the arcuate nucleus of the hypothalamus (24), an area rich in β-endorphin–containing neurons (25). In view of the hypothesis that at least part of the analgesic action of morphine may be mediated by the release of endogenous opioid peptides, including β-endorphin (26), it is possible that part of the decreased antinociceptive response in diabetic animals could involve changes in β-endorphin. Other studies, however, have not found significant changes in hypothalamic β-endorphin levels in STZ-D rats (27) or diabetic C57BL/KsJ mice (28). In addition, studies in isolated tissues have demonstrated a direct inhibitory effect of hyperglycemia on opiate potency (4).

In contrast to glucose, fructose and 3-O-methylglucose failed to have a significant effect on high-affinity naloxone binding, although previous studies in mice indicated that the intraperitoneal administration of fructose produced a considerably greater attenuation of morphine-induced antinociception than glucose (2,29). Therefore, it is not likely that the fructose-induced decrease in opiate potency previously

observed in our laboratory can be explained by a direct physicochemical effect of fructose on opiate-receptor affinity or number.

We conclude that, although glucose decreases the affinity of the high-affinity opiate receptors in brain, the magnitude of this decrease is not sufficient to account totally for the reduction of opiate potency observed in vivo in various models of hyperglycemia. In addition, the opiate antagonism by fructose in vivo does not appear to be mediated through an alteration of opiate-receptor–binding parameters.

ACKNOWLEDGMENTS

We thank Dr. Fred Lux for the blood glucose measurements.

This research was supported in part by USPHS Grants DA-01647 and T32-DA-07027.

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