Insulin Attenuates Formalin-Induced Nociceptive Response in Mice through a Mechanism that Is Deranged by Diabetes Mellitus

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Accepted for publication December 13, 1996

ABSTRACT
Although hypoglycemic doses of insulin (0.24-7.5 U/kg s.c.) did not significantly change acetic acid-induced writhing in mice, they dose-dependently attenuated formalin-induced nociceptive responses, and their effects were more potent on the second phase (ID50 = .62 U/kg) than on the first (ID50 > 7.5 U/kg). Intracerebroventricular doses of insulin (250-1000 μU/animal) mimicked the effects of the s.c. dose, but caused little change in blood glucose levels. The antinociceptive activity of insulin (0.75 U/kg, s.c.) in the formalin test was significantly inhibited by naloxone (10 mg/kg i.p., an opiate receptor antagonist), sulpiride (10 mg/kg i.p., a dopamine 2 receptor antagonist), pindolol (1 mg/kg i.p., a 5-hydroxytryptamine 1 receptor antagonist) and ketanserin (1 mg/kg i.p., a 5-hydroxytryptamine 2 receptor antagonist), but not by 3-tropanyl-indole-3-carboxylate (1 mg/kg i.p., a 5-hydroxytryptamine 3 receptor antagonist). Insulin also exerted antinociception in streptozotocin-induced diabetic mice and genetically diabetic db/db mice which, however, were less sensitive (ID50s around 7.5 U/kg) to the of insulin effect than normal mice. The results suggest that insulin attenuates chronic rather than acute pains through a mechanism mediated by dopamine, 5-hydroxytryptamine and opioids. The antinociceptive pathway appears to be deranged by diabetes mellitus.

Davis et al. (1956) were the first to report that insulin-induced hypoglycemia potentiated the antinociceptive action of morphine in the rat tail-flick test. This was confirmed by later studies demonstrating that insulin potentiates the antinociceptive effects of sodium salicylates (Wisniewski and Zarebski, 1968) and morphine (Singh et al., 1983), while Simon and Dewey (1981) and Simon et al. (1981) reported that streptozotocin-induced diabetic mice and rats as well as genetically diabetic db/db mice are significantly less sensitive to the antinociceptive effect of morphine in the tail-flick test. This evidence indicated that blood glucose levels affect pain perception mechanisms, and has given a theoretical basis for the hyperalgesia in diabetic patients.

The abovementioned experiments measure transient pain induced by brief exposure to physical stresses, although the most frequently encountered complaints in patients are of continuous pain, usually of pathological origin. The formalin test was originally described in rats and cats by Dubuisson and Dennis (1977), and the s.c. injection of formalin produced a biphasic pain response in rats. Several lines of evidence have indicated that the first phase represents a phasic pain response to direct stimulation of the nerve endings, and the second phase represents a tonic pain response to subsequent inflammation (Dubuisson and Dennis, 1977; Shibata et al., 1989). However, only a few drugs have been studied in the formalin test, especially for their effects in diabetic animals (Acton et al., 1992; Calcutt et al., 1994; Takeshita et al., 1995; Takeshita and Yamaguchi, 1995).

Using the formalin test, we recently found that the antinociceptive activity of morphine was significantly reduced in STZ-induced diabetic mice, but was not changed in the genetically diabetic db/db mice compared with their controls (Takeshita et al., unpublished observation). Although hyperglycemia is common in both STZ-induced diabetic mice and db/db mice, the latter are hyperinsulinemic, and the former are hypoinsulinemic (Coleman and Hummel, 1967; Kodama et al., 1994). This in combination with the evidence cited above led us to hypothesize that hyperinsulinemia in the db/db mice might have potentiated the analgesic effect of morphine and compensated for the attenuated pain perception mechanism due to hyperglycemia. During the course of a study on the synergism between morphine and insulin, we found that insulin injection per se induces antinociception in the formalin test.

We report on the mechanism of action of insulin in normal

ABBREVIATIONS: STZ, streptozotocin; ICS205-930, 3-tropanyl-indole-3-carboxylate; i.c.v., intracerebroventricular; 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine.
mice, and compare the antinociceptive effect of insulin in STZ-induced diabetic and genetically diabetic db/db mice.

Methods

Materials

Bovine pancreas insulin, (±)-pindolol hydrochloride, naloxone hydrochloride and streptozotocin (STZ) were obtained from Sigma Chemical Co. (St. Louis, MO). Ketanserin tartrate was from Research Biochemical Inc. (Natick, MA). Formalin and acetic acid were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sulpiride and 3-tropanyl-indole-3-carboxylate hydrochloride (ICS205-930) were synthesized in our laboratory. Insulin, ketanserin, naloxone and ICS205-930 were dissolved in saline. Pindolol and sulpiride were dissolved in 1% tartaric acid, adjusted to pH 7 with 1N NaOH and diluted in saline. STZ was dissolved in 2 mM citrate buffer at pH 4.5. Formalin and acetic acid were diluted in saline. Sulpiride, naloxone, pindolol, ketanserin and ICS205-930 were administered i.p. 30 min before the injection of insulin.

Animals

All mice were housed at 22 ± 1°C and 55 ± 5% humidity under a 12 hr light/12 hr dark cycle and given free access to water and food ad libitum.

Normal mice. Male ddY strain mice (5 wk of age, SLIC, Shizuoka, Japan) were purchased and used for the formalin test and the acetic acid writhing test at the age of 7 wk (body weight, around 35 g).

STZ-induced diabetic mice. Two hundred mg/kg of STZ were injected i.p. to the 5-wk-old male ddY mice. Ten days later blood was obtained from the orbital sinus and plasma glucose levels were determined by a commercial kit (Glucose B-test Wako, Wako Pure Chemical Industries, Ltd. Osaka, Japan). We used mice with blood glucose levels of >400 mg/dl. The formalin-test was performed 2 wk after administration of STZ. The body weight of the mice was around 27 g.

Genetically diabetic mice. Female C57BL/KsJ-db/db mice (6 wk of age, The Jackson Laboratory, Bar Harbor, ME) were purchased. The formalin test was performed with 9- to 10-week-old mice (body weight around 44 g) because hyperglycemia became steady at 7 to 8 wk of age. Blood was obtained from the orbital sinus and plasma glucose levels were determined by the commercial kit described above. We used db/db mice with blood glucose levels of >400 mg/dl.

Formalin Test

We used the formalin test previously published by Hunskaa et al. (1985) with slight modifications (Takeshita et al., 1995). Each mouse was placed in an observation chamber 5 min before the injection of diluted formalin to allow acclimation to the new environment. Ten μl of 1% formaldehyde in saline were administered into the left hindpaw with an Ito microsyringe (Shizuoka, Japan). Each animal was then returned to the observation chamber and nociceptive response was recorded for a period of 30 min. The summation of time (sec) spent in licking and biting of the paw that received injections during each 5 min block was measured as an indicator of the pain response. The duration of responses in the first 10 min and that from 10 to 30 min represent first and second phases, respectively. Insulin was injected s.c. and i.c.v. 20 and 10 min before injection of formalin, respectively. This test was performed in a temperature- and humidity-controlled (22 ± 1°C, 55 ± 5%) room.

Acetic Acid Writhing Test

Writhing was induced by an i.p. injection of 120 mg/kg of acetic acid (0.6% in saline solution) 20 min after s.c. injection of insulin. The mice were placed individually in observation chambers immediately after the acetic acid injection, and 3 min later the number of writhes produced by each mouse was counted for 10 min.

Procedure for i.c.v. Injections

Intracerebroventricular injection essentially followed a previously published method (Haley and McCormick, 1957). Insulin was delivered in a volume of 5 μl using a Hamilton microsyringe (Hamilton Company, Reno, NV). To minimize leakage of the solution, we injected the volume over a period of 15 sec and left the needle in place for a further 15 sec. After some practice using a dye, we found little difficulty in reproducibly localizing drugs intracerebroventricularly.

Time Course Studies of Blood Glucose after Insulin Injection

Insulin (s.c.: 0.75 U/kg; i.c.v.: 1000 μU) was injected to mice and blood was taken from the orbital sinus s.c. at pre, 20, 30 and 50 min, and i.c.v. at pre, 10, 20 and 40 min. Plasma was obtained by centrifuging the blood. Plasma glucose was determined with the commercially available kit described above.

Statistical Analysis

The results are presented as the mean ± S.E., and statistical significance of differences between groups was analyzed by means of analysis of variance followed by Dunnett’s t test or by the unpaired t test where indicated. P < 0.05 were considered significant. The ID₅₀ values (i.e., the dose of drugs that reduced formalin-induced nociceptive response by 50% relative to control values) were estimated from individual experiments by using the linear regression methods in a computer program produced in our laboratory.

Results

Effects of insulin on nociceptive responses induced by formalin and acetic acid in normal mice. Although insulin tended to attenuate the acetic acid-induced writhing in normal mice in a dose-dependent fashion (fig. 1), even the largest dose (7.5 U/kg) gave only 19% inhibition, and the overall dose effect of the drug was not statistically significant (F₄,₃₅ = 0.68, P > .05). The activity of insulin was more potent on the latter than on the former; 7.5 U/kg caused only...

![Fig. 1. Effects of insulin on acetic acid-induced writhing responses in normal ddY mice.](image-url)
Antinociceptive Effect of Insulin

Effects of i.c.v. insulin on formalin-induced nociceptive response. An i.c.v. injection of insulin did not change the biphasic pattern of formalin-induced nociception, but attenuated the response time at each 5-min period (fig. 4). The total response time are shown in figure 5; the overall dose effect of insulin was statistically significant for the second phase (F3,28 = 4.72, P < .01). The maximal and statistically significant inhibition (57%) was obtained at 1000 μU. The overall dose effect of insulin on the first phase was not statistically significant (F3,28 = 0.13, P > .05).

Blood glucose levels after the treatment with insulin. Twenty minutes after a s.c. injection of insulin (0.75 U/kg) plasma glucose levels were significantly lowered by 29% compared with the saline treated group (fig. 6a). There was also a statistically significant difference between the two at 30 min but not at 50 min.

No significant changes in plasma glucose levels were obtained by i.c.v. injection of insulin (1000 μU), and there were no statistically significant differences between the vehicle- and insulin-treated groups at any time points (fig. 6b).

Effects of naloxone and sulpiride on the antinociceptive activity of insulin in normal mice. Reproducing the abovementioned results, a s.c. injection of insulin (0.75 U/kg) significantly inhibited the second phase of the formalin test in normal mice (compare saline-saline with insulin-saline in figure 7).

Treatment with naloxone (10 mg/kg i.p., an opiate receptor antagonist) per se did not significantly change the nociceptive response to the formalin injection (compare saline-saline with saline-naloxone in fig. 7a), but it greatly reduced the antinociceptive activity of insulin; insulin inhibited the formalin-induced nociceptive response by 74% in the saline-treated mice and by 26% in the naloxone-treated ones. The latter change (saline-naloxone vs. insulin-naloxone) was not statistically significant.

Similarly, treatment with sulpiride (10 mg/kg i.p., a DA2 receptor antagonist) per se did not significantly change the nociceptive response to the formalin injection, but it greatly reduced the antinociceptive activity of insulin (fig. 7b). The
difference between the saline-sulpiride and insulin-sulpiride groups was not statistically significant.

**Effects of pindolol, ketanserin and ICS205-930 on the antinociceptive activity of insulin in normal mice.** In the three sets of experiments in normal mice, a s.c. injection of insulin (0.75 U/kg) induced comparable and statistically significant inhibition of the second phase of the formalin test (fig. 8).

Treatment with pindolol (1 mg/kg i.p., a 5-HT1 receptor antagonist) per se did not significantly change the nociceptive response to the formalin injection (compare saline-vehicle with saline-pindolol in fig. 8a), but it reduced the antinociceptive activity of insulin; insulin inhibited the formalin-induced nociceptive response by 56% in the vehicle-treated mice and by 26% in the pindolol-treated ones, and the latter change (saline-pindolol vs. insulin-pindolol) was not statistically significant.

Treatment with ketanserin (1 mg/kg i.p., a 5-HT2 receptor antagonist) per se did not significantly change the nociceptive response to the formalin injection (compare saline-saline with saline-ketanserin in fig. 8b), but it greatly reduced the antinociceptive activity of insulin; insulin inhibited the response by 66% in the saline-treated mice (saline-saline vs. insulin-saline) and 47% in the ketanserin-treated ones (saline-ketanserin vs. insulin-ketanserin). Both of the changes were statistically significant.

Treatment with ICS205-930 (1 mg/kg i.p., a 5-HT3 receptor antagonist) per se affected neither the nociceptive response to the formalin injection (compare saline-saline with saline-ICS205-930 in fig. 8c), nor the antinociceptive activity of insulin. Insulin inhibited the response by 66% in the saline-treated mice (saline-saline vs. insulin-saline) and 47% in the ICS205-930-treated ones (saline-ICS205-930 vs. insulin-ICS205-930). Both of the changes were statistically significant.

**Effects of insulin on the formalin-induced nociceptive response in STZ-induced diabetic mice.** The biphasic pattern of formalin-induced nociception was not changed in STZ-induced diabetic mice compared with normal ddY mice, although the response time of the second phase was attenuated in the former (compare fig. 9 with fig. 2). Insulin attenuated the response time of the second phase with minimal effects on that of the first phase (fig. 9). The total response times are shown in figure 10; the overall dose effect of insulin was statistically significant for the second phase.

**Fig. 6.** Time course changes in blood glucose levels after injection of insulin. Each value represents mean ± S.E. (n = 6-8). a: s.c. injection, b: i.c.v. injection. Open symbols: Injection of vehicle; closed symbols: injection of insulin. Dunnett’s t test for multiple comparisons subsequent to a repeated measurements analysis of variance was used for evaluation. Statistical significance at **P < .01, ***P < .001.

**Fig. 7.** Effects of pretreatment with naloxone and sulpiride on insulin (0.75 U/kg, s.c.) analgesia in formalin-induced tonic pain responses. a; Naloxone, b; sulpiride. Naloxone (10 mg/kg) and sulpiride (10 mg/kg) were injected i.p. 30 min before administration of insulin. The nociceptive responses represent the summation of biting and licking responses during 10 to 30 min after injection of formalin. Each value represents mean ± S.E. (n = 10-13). *P < .05, **P < .001; Student’s t test.

**Fig. 8.** Effects of pretreatment with pindolol, ketanserin and ICS205-930 on insulin (0.75 U/kg, s.c.) analgesia in formalin-induced tonic pain responses. a: Pindolol, b: ketanserin, c: ICS205-930. These compounds (1 mg/kg) were injected i.p. 30 min before administration of insulin. The nociceptive responses represent the summation of biting and licking responses during 10 to 30 min after injection of formalin. Each value represents mean ± S.E. (n = 10-16). *P < .05, **P < .001; Student’s t test.
The biphasic pattern of formalin-induced nociception was not changed in genetically diabetic db/db mice compared with normal ddY mice, although the nociceptive responses were attenuated in the former (compare fig. 11 with fig. 2). Insulin attenuated the second phase with minimal effects on the first phase (fig. 11). The total response times are shown in figure 12; the overall dose effect of insulin was statistically significant for the second phase (F4,45 = 4.37, P < .01) but not for the first phase (F4,45 = 0.97, P > .05). The ID_{50} for the former was around 7.5 U/kg.

**Discussion**

It is a novel and interesting finding that insulin strongly attenuated the second phase of the formalin-induced nociceptive response in our study. Although the experimental data demonstrating that insulin per se has analgesic effects are still scanty, this may possibly be ascribed to the nature of methods used. Most behavioral studies concerning pain transmission use tests determining acute or phasic pain induced by thermal, mechanical or chemical stimuli. The formalin test was originally described in rats and cats by Dubuisson and Dennis (1977), and a s.c. injection of formalin produced a biphasic pain response in rats. Several lines of evidence have indicated that the first phase represents a phasic pain response to direct stimulation of the nerve endings, and the second phase represents a tonic pain response to subsequent inflammation (Dubuisson and Dennis, 1977; Shibata et al., 1989). We thus speculate that insulin, which resembles mild analgesics such as paracetamol and acetylsalicylic acid (Hunskaar et al., 1985), induces specific modulation of tonic pain compared with phasic pain. The assumption is supported, in part, by the present finding that insulin had a less potent effect on the first phase of the formalin test and on the acetic acid-induced writhing test than on the second phase of the formalin test. However, there is clinical evidence that normal subjects with higher insulin levels...
show an elevated threshold for thermal nociceptive stimuli (Delaney et al., 1994) and that insulin effectively blocks diabetic pain (Samanta and Burden, 1985). In addition to the effects of insulin on sensory nerve function (Delaney et al., 1994) and on glucose metabolism (Samanta and Burden, 1985), the mild analgesic action of insulin may also be responsible for its clinical effects. In this respect, it is interesting to note that amitriptyline and tiapride, which are clinically effective for painful diabetic neuropathy, specifically attenuated the second phase of the formalin test (Acton et al., 1992, Takeshita et al., 1995). Further, Calcutt et al. (1994) have suggested that animal studies using the formalin test might be useful in elucidating the etiology of painful diabetic neuropathy.

An i.c.v. injection of insulin dose-dependently attenuated only the second phase of the formalin test, but it did not significantly lower the blood glucose levels even at the largest dose. The degree of the change by 1000 μU/animal i.c.v. was comparable to that by 0.75 U/kg s.c., i.e., the i.c.v. dose was calculated to be about 20 times more potent than the s.c. dose because the body weight of the mice was around 35 g. However, it has been reported that insulin receptors are widely distributed in the central nervous system (Havrankova et al., 1978; Pacold and Blackard, 1979), and that peripherally administered insulin crosses the blood-brain barrier to reach the central nervous system (Wallum et al., 1987; Steffens et al., 1988). This evidence, taken together, favours the view that the central nervous system is the site of the antinociceptive action of insulin. However, as there is still a possibility that s.c. and i.c.v. insulin activate different mechanisms, further studies are needed to clarify the point.

Another important finding of our study is that the antinociceptive effect of a systemic dose of insulin was significantly inhibited by pretreatment with naloxone (10 mg/kg i.p., an opiate receptor antagonist), sulpiride (10 mg/kg i.p., a DA2 receptor antagonist), pindolol (1 mg/kg i.p., a 5-HT1 receptor antagonist) and ketanserin (1 mg/kg i.p., a 5-HT2 receptor antagonist), but not by that with ICS205-930 (1 mg/kg i.p., a 5-HT3 receptor antagonist). Pindolol and ketanserin effectively blocked the antinociceptive effect of FR64822 (a dopaminergic enhancer) in the acetic acid writhing test (Ohkubo et al., 1995), although sulpiride blocked the antinociceptive effect of meta-chlorophenylpiperazine (Takeshita and Yamaguchi, 1991). In line with this speculation, there have been papers demonstrating that insulin injection releases brain amines including DA and 5-HT (Gordon and Meldrum, 1970; Gupta et al., 1992).

The antinociceptive effects of insulin were less potent in db/db mice and STZ-treated mice than in normal ddY mice. The difference in insulin effects was too large (about 10-fold) to be ascribed to that in body weight (less than 2-fold) between the diabetic mice and the normal ddY mice. However, we have recently observed that the antinociceptive effects of FR64822 (a dopaminergic enhancer, Ohkubo et al., 1991) are also reduced in both of these diabetic models (Takeshita et al., unpublished observation), whereas the effects of serotoninergic agents such as tiapride (Takeshita et al., 1995) and meta-chlorophenylpiperazine (Takeshita and Yamaguchi, 1995) are not changed in mice compared with normal ddY mice. Therefore derangement of the dopaminergic antinociceptive pathway would appear to be responsible for the reduced activity of insulin in the diabetic mice. In line with this assumption, it has been shown that the brain DA synthesis rate is decreased in STZ-induced diabetic rats (Trulson and Himmel, 1983). Alternately, the following evidence argues against the possibility that derangement of insulin- or opiate-receptor mechanisms is responsible for the change in the effect of insulin. Although it is widely accepted that insulin receptors in the periphery undergo up- and down-regulation, a balance of evidence indicates that insulin receptors in the central nervous system do not undergo a similar regulation (Schwartz et al., 1992). Insulin binding to brain homogenates was not affected by hyperinsulinemia or STZ-treatment (Havrankova et al., 1979; Pacold and Blackard, 1979). Also, naloxone binding in brain membranes was not affected by the induction of diabetes via STZ or in db/db mice compared with the nondiabetic control (Brase et al., 1987).

In conclusion, insulin attenuated specifically the second phase of formalin-induced nociception. An activation of central dopaminergic, serotonergic and opioidergic pathways rather than systemic hypoglycemia appears to be the mechanism of action. Some of the antinociceptive pathways initiated by insulin appear to be attenuated in both the STZ-induced diabetic mice and the genetically diabetic db/db mice.

Acknowledgments

The authors thank Dr. T. Ohashi for helpful advice during the preparation of this manuscript.

References


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