Title: Insulin Within the Arcuate Nucleus Has Paradoxical Effects on Nociception in Healthy and Diabetic Rats

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Abstract

Broad nervous circuits originate from the hypothalamic arcuate nucleus project to many parts of the brain which are related to pain perception. Insulin receptors have been found in the arcuate nucleus. Since nociception may be affected in type 1 diabetes, the present study aimed to investigate the intra-arcuate nucleus insulin role in pain perception in STZ-induced diabetic and healthy rats. For this purpose, regular insulin was microinjected within the arcuate nucleus and the pain tolerance was measured using the hot plate and the tail-flick apparatus in the rats. The results showed that the arcuate nucleus suppression with lidocaine could increase thermal nociception in non-diabetic animals. Also, insulin within the arcuate nucleus decreased the acute thermal pain perception in these animals. STZ-induced diabetes produced hypoalgesia which latency of these tests, progressively increased over time after induction of diabetes; also, in the same animal group, intra-arcuate injection of insulin reduced the latency of nociception. Therefore, intra-arcuate insulin has paradoxical and controversial effects in healthy and diabetic rats’ nociception. These effects seem to be due to insulin effect on releasing of pro-opio-melano-cortin and its derivatives.

Keywords: The Arcuate Nucleus; Insulin; Hot Plate; Tail-Flick; Acute Pain
1. Introduction

Pain is an undesirable feeling that is due to irritating free nerve terminals. Pain is divided into acute and chronic types. The acute pain is also called sharp pain and electrical pain. The duration of the acute pain following a painful stimulus is about 0.1 second. This pain is not felt in deep tissues. Acute pain could be measured using the hot plate apparatus [1].

Sharp or acute pain is induced by heating, mechanical and chemical irritation. This may release more chemicals from damaged tissue and along the time this process can increase acute pain intensity [2]. Acute pain projects to the brain cortex so it can be localized [3].

Pain controlling is conducted by nonsteroidal anti-inflammatory drugs and opioid anti-pain drugs. Furthermore, the pain can be modulated by endogenic opioid manipulation. Endogenic opioids consist of enkephalins, endorphins, and especially beta-endorphin can be named. Beta-endorphin is one of the polypeptides deriving from pro-opio-melano-cortin (POMC) and its level increases in response to pain [4]. The arcuate nucleus of the hypothalamus is the main part of the POMC source in the brain [5]. Beta-endorphin and related peptides derived from the POMC are in the arcuate nucleus neurons in the middle inferior part of the hypothalamus. Broad nervous circuits that originate from the hypothalamic arcuate nucleus project to many parts of the brain that have a role in pain control such as hypothalamic nuclei, limbic system, raphe nucleus, and some pons nuclei. The central POMC system has a role in the antinociceptive process [6]. Lesion of the arcuate nucleus of the hypothalamus as the main POMC source of the brain causes weakness in the antinociceptive process after anxiety and decreases the antinociceptive process
due to electrical irritating of periaqueductal gray area that exists in beta-endorphin nervous terminals. In other words, beta-endorphin neurons terminals are modulated in both the lateral ventricles and in the spinal cord that makes an analgesic process, and this function is blocked by naloxone [5, 7].

Some kinds of diseases would affect nociception. One of them is diabetes that today many people suffer from it. This is one of the most important metabolic diseases [8]. Sensory and neurosecretory nociceptor functions are sensitized in diabetes [9]. Insulin-dependent diabetes (type 1 diabetes) may display signs of hyperalgesia and allodynia [10]. Intracerebroventricular (icv) injection of insulin showed an analgesic effect in the formalin test model of pain [11]. In the tail-flick test, the nociceptive response latency was progressively elevated during chronic diabetes [12]. The pressure pain threshold during 2 weeks after STZ-induced diabetes was decreased. This hyperalgesia was independent of plasma glucose but related to low plasma insulin [13]. Diabetic rats displayed mechanical hyperalgesia by decreasing paw withdrawal thresholds to mechanical stimuli; they also indicated thermal hypoalgesia by increasing tail-flick latencies [14].

Streptozotocin (STZ) is widely used to induce experimental diabetes in animals. STZ enters the pancreatic B cells via a glucose transporter (GLUT2) and causes alkylation of DNA which leads to DNA damage. As a result of the STZ action, pancreatic beta cells are destroyed [15].

The insulin receptor (IR) exists in many brain areas including the arcuate nucleus with high density [5, 16]. Since the arcuate nucleus plays a role in nociception pathway and it possesses the insulin receptor, this study was designed to investigate the arcuate nucleus role and also insulin
effect within the arcuate nucleus in acute pain nociception in healthy rats, STZ-induced acute diabetic rats, and also STZ-induced chronic diabetic rats.

2. Materials and Methods

2.1. Animals

Male albino Wistar rats weighing 200-250 grams were used. Rats were housed under 12 hours of light and 12 hours of dark conditions. The time was set in which lighting was started at 7:00 in the morning. During the experiment, the rats had free access to enough food and water. The rats were transferred to the lab space a week before the experiment beginning to become familiar to the environment. Each rat was used just once.

2.2. Drugs

STZ (Santa Cruz biotechnology Company, Dallas, USA), was freshly prepared and dissolved in cold normal saline immediately before injection. Regular insulin (Exir pharmaceutical Company, Boroujerd, Iran), and lidocaine 2% (Aburaihan Pharmaceutical Company, Tehran, Iran), were used.

2.3. Type 1 diabetes induction

Animals were divided into 2 clusters: STZ-receiving and healthy rats. The diabetes was induced by intraperitoneal (ip) injection of 60mg/kg dose of STZ solution in cold normal saline. Six days after injection, blood samples were collected and serum glucose was measured using spectrophotometry and glucometer. Only rats with blood glucose levels higher than 250mg/dl were subjected to continuing the study as diabetic rats. Experimental diabetes also was
determined by changing the behavior of animals through polyphagia, polydipsia, polyuria and weight loss [15, 17].

2.4. Experiment design

Rats were divided into 8 groups in which each group included 6 rats (n=6). This is the final number of the rats in which led to results and participated in the statistical analysis after excluding the troubled ones. The criteria for excluding the subjects from the study were such as wrong cannulation, rats which their blood glucose was lower than 200mg/dl after STZ injection, and rats with impaired mobility.

2.4.1. The hypothalamic arcuate nucleus role in acute pain in non-diabetic rats

After a recovery period, one group that contained non-diabetic rats (healthy rats) received 0.5µl lidocaine 2% within the hypothalamic arcuate nucleus to investigate this brain structure’s role in acute pain.

2.4.2. The effect of intra-arcuate nucleus insulin microinjection on acute pain in the non-diabetic rats

After the recovery period, non-diabetic rats received 0.5µl saline as a drug vehicle (one group) and 0.5µl of three doses of Insulin (0.02, 0.1 and 0.5 IU) within the hypothalamic arcuate nucleus (in three different groups) to investigate the insulin effect within this nucleus on acute pain.

2.4.3. The effect of intra-arcuate nucleus insulin 0.5 IU on acute pain in STZ-induced diabetic rats
The diabetic rats received an intra-arcuate effective dose of insulin (0.5 IU) to investigate the insulin effect on the acute pain in these animals one, two, and three weeks after diabetes induction. This procedure was done in two different groups (one for the hot plate test and the other one for the tail-flick test).

2.4.4. The effect of insulin deficiency on acute pain during the time

The non-diabetic and insulin-dependent diabetic rats after one, two and three weeks of diabetes induction were studied by hot-plate and tail-flick tests to investigate the insulin deficiency on acute pain during the time.

2.5. Stereotaxic surgery and cannula implantation

Rats were anesthetized by ip injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). After shaving their hair, the rats were fixed into the surgical device. The scalp was cut and to reduce bleeding and local anesthesia, the lidocaine+epinephrine solution was used. To specify lambda and bregma lines, the area was cleaned using ethylic alcohol 70%. According to the atlas of Paxinos and Watson [18], arcuate nucleus coordinates are as follows: -2.28 mm to bregma with 9.4 mm depth from the surface of the skull and in the midline of the skull. The length of Cannulas (23 gauge) was considered 12 mm. The length of the needle for microinjection was also 12mm. The skull was pierced by a dental drill on the determined location. Then, the cannula guide would be placed in the brain on the surface of the nucleus and its upper part was fixed in the skull surface with dental cement. Since the arcuate nucleus is exactly in the middle line and it is small in size, the cannula was put in the midline. For more fixation of cement and cannula, small screws (glasses screw) were used which were embedded within the skull bone and dental cement. The opening of guide cannula out of the skull was blocked with a dental metal needle
and it was removed only at the time of microinjection. All instruments were sterilized to prevent infection. After cannula implantation and surgery, the rats were passed a four-day recovery period.

### 2.6. Microinjection

For microinjection in the nucleus through the cannula, a 12mm long 30 gauge needle has been joined to a thin polyethylene tube on one side. The other side of the tube was connected to the Hamilton syringe to control the microinjection volume.

### 2.7. Thermal acute pain tests

#### 2.7.1. Hot plate test

Hot plate test was implemented as follows: To adapt the animals to the environment and reducing stress, 1 day before starting the hot plate test, rats were placed on the cold (off position) surface of the hot plate device for 3 minutes to get familiar with the environment. In the test day, the hot plate temperature was set at 52°C and the animals were put on it. Reaction time or the time it takes for the animal to respond to pain stimuli was measured in seconds. Reaction time was when the animals were licking their paws or jumping from the hot plate surface and it is defined as "pain response latency" and is expressed in second. The end of this test (cut-off point) was considered 60 seconds to prevent probable animal tissue damages [19]. This test has been done for each rat 30 minutes before and about 60 minutes after microinjection. To reduce probable human errors all tests were done by one person.

#### 2.7.2. Tail flick test
The tail-flick test was run by the tail-flick apparatus (Ugo Basile, Italy). The tail-flick latency (TFL) was calculated on the average of three consecutive tail-flick tests at each time point. The heat stimulus was provided by a light source that projects infrared (IR) light. The IR light was set at an intensity that yields a TFL reaction in the range of 3–4 s (about 55% of maximal IR intensity). The cut-off point was set 10s; if the animal failed to flick its tail during 10s, the tail was removed from the coil to prevent the probable damages to the skin. The reaction time between the onset of heat stimulus and the movement of the tail was determined by an automatic sensor. The IR heat was applied in about 5cm from the caudal tip of the tail [20]. This test has been done for each rat 30 minutes before and about 60 minutes after microinjection. To reduce probable human errors all tests were done by one person.

2.8. Histological verification

At the end of the study, the rats in each group were being decapitated and to ensure and investigate the microinjection site the brain tissue sections were prepared. Rats that had a defect or a problem in cannulation were excluded from the study.

2.9. Statistical analysis

For statistical analyses, paired sample t-test and ANOVA test (continued with Newman–Keuls posthoc test) were used to compare the before-after and several group comparisons, respectively. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. The hypothalamic arcuate nucleus role in acute pain in non-diabetic rats
To show and confirm the arcuate nucleus role in the pain perception, 0.5µl lidocaine 2% was infused within this area. The arcuate nucleus neurons suppression by microinjection of lidocaine 2% caused increasing in nociception by decreasing the pain response latency in the hot plate test (fig. 1).

3.2. The effect of intra-arcuate nucleus insulin microinjection on acute pain in the non-diabetic rats

To investigate the insulin role within this nucleus on acute pain, 0.5µl of three doses of insulin (0.02, 0.1 and 0.5 IU) and saline (as the vehicle) were infused within the arcuate nucleus. Intra-arcuate nucleus insulin dose-dependently increased the pain response latency of acute thermal pain in hot plate test so that for the 0.5 IU group the t-test demonstrated that the nociception (acute pain threshold/the level of analgesia) decrease was statistically significant (P<0.05; fig. 2).

3.3. The effect of intra-arcuate nucleus insulin 0.5 IU on acute pain in STZ-induced diabetic rats

When the thermal acute pain was tested by the hot plate instrument, the t-test displayed that the microinjection of the effective dose of insulin (0.5 IU) in the hypothalamic arcuate nucleus in the first week of STZ-induced diabetes led to acute pain increase (P<0.01). In the next week, this method was performed again and pain response latency decreased in response to insulin; it means acute pain and nociception were increased (p<0.01). Similarly, in the 3rd week microinjection with the same dosage of insulin caused a reduction in pain response latency and nociception increased in insulin-dependent diabetic rats (P< 0.05; fig. 3).

In the tail-flick part, intra-arcuate insulin significantly increased the pain response latency in non-diabetic rats the same as the hot plate result (P< 0.05). Intra-arcuate nucleus injection of
0.5U insulin increased the pain response latency in the first week of STZ-induced diabetes but it was not statistically significant. In the following weeks in diabetic rats, intra-arcuate nucleus insulin injection decreased the pain response latency so that in the 3rd week, it was significantly decreased (P< 0.05; fig. 4).

3.4. The effect of insulin deficiency on acute pain during the time

When the pain response latency is compared among non-diabetic rats and different weeks after STZ-induced diabetes induction, one-way ANOVA indicated that it was increased statistically significant after diabetes induction and continued to increase in diabetic rats during the time in both hot-plate [F(3, 20)=8.179 and P<0.001; (fig. 5A)] and tail-flick [F(3, 20)=0.08911 and P<0.001; (fig. 5B)] tests.

4. Discussion

Results of the present study demonstrated that hypothalamic arcuate nucleus inhibition increased the thermal nociception. Insulin within the arcuate nucleus decreased the acute thermal pain perception, through the use of tail-flick and hot plate tests. STZ-induced diabetes made hypoalgesia in which the latency of these tests, progressively increased over time after induction of diabetes, and in the same animal group, intra-arcuate injection of insulin reduced the latency of thermal nociception, vice versa the non-diabetic rats.

Neuropathy is one of the complications of diabetic patients [21] which has a variety of manifestations including hyperalgesia, allodynia, and hypoalgesia that occurs in advanced stages of diabetic neuropathy [22]. Insulin has an analgesic effect such as modulating the analgesic system via modulating receptors of serotonin, opioids, and dopamine, as well as releasing serotonin and dopamine in the brainstem regions [23]. The arcuate nucleus has POMC
containing projections to the brainstem regions which modulate pain sensation in descending control of nociceptive, ie, serotonergic and noradrenergic neurons in the raphe nuclei and the periaqueductal gray area (PAG). Therefore, it plays an important role in controlling pain in brainstem areas [6]. The arcuate neurons' terminals release opioid peptides including beta-endorphin and adrenocorticotropic hormone which their receptors are abundant in raphe nuclei and PAG and cause inhibition of nociception [24]. In the present study, to confirm the role of the nociception inhibitory effect of the arcuate nucleus, injection of lidocaine into the arcuate nucleus caused hyperalgesia; so the inhibition of this area caused allodynia and it confirms the inhibitory role of arcuate nucleus in nociception.

So far, fewer studies have been conducted on the central effect of insulin as an analgesic hormone. Some studies showed that icv injection of insulin reduced nociception in the formalin test which was inhibited by dopamine and serotonin antagonists [11]. Also, intra-spinal IGF1 injection independently from the noradrenergic pathway, reduced hyperalgesia [25]. It seems that insulin has an analgesic effect due to the presence of high levels of receptors in the arcuate nucleus [26]. Systemic insulin administration increased the activity of the POMC neurons in the arcuate nucleus [6, 27]. Furthermore, it was demonstrated that long-term administration of insulin in healthy mice could increase the mRNA synthesis of opioid peptides in POMC neurons [27].

In the present study and a similar one [14], in the first week after diabetes induction with STZ in the rats, acute thermal hypoalgesia was observed in the tail-flick and hot plate tests, which progressively increased over time. In this regard, various studies revealed that diabetes would cause hyperalgesia due to dysfunction of serotonin and dopamine and opioid peptides as well as the sensory disorders and changes in the mechanism of pain perception in diabetes, in contrast
with the present study [11, 28-30]; while in the advanced stages of diabetes it was observed diabetic thermal or mechanical hypoalgesia [31-39]. It seems that thermal hypoalgesia in the last stages of diabetes is induced due to impairment of regeneration, neuronal degeneration, loss of sensory fibers, malfunction, and structural problems in small neurons’ fibers [14]. In fact, neuropathy in the final stages involves a variety of dysfunctions in sensory neurons including reduced conduction velocity which is related to progressing degeneration of total sensory fibers [39]. However, thermal hypoalgesia in the early stages of diabetes, which was shown in the present study as well as others [14, 34], was caused by altered nociception and a disruption in the processing of sensory neurons information. Another reason for increasing the threshold of pain perception in the early stages of diabetic neuropathy is the impairment of the epidermal nociceptors functions before the loss of peripheral terminals in sensory neurons. Abnormal status of neurotransmitters in the nociceptors is also another cause of hypoalgesia in the early stages of diabetes [14, 38].

In the present study, intra-arcuate nucleus injection of insulin increased the threshold of acute thermal pain in healthy rats but reduced thermal hypoalgesia in STZ-induced diabetic rats. In our study, single-dose insulin injection in the first, second and third weeks after diabetes induction, probably cannot influence the biosynthesis of the myelin sheath and improve the signal conduction velocity in damaged nerves [35]. Perhaps, the improvement of the insulin receptor signaling in the arcuate nucleus following the insulin injection could have altered the balance of pain and non-pain neurotransmitters.

There are many insulin receptors on both neuropeptide Y and POMC neurons in the arcuate nucleus [26]. Thus, any change in the release of β-endorphin from POMC terminals can modulate the output of serotonergic fibers of the raphe nucleus [26, 40, 41]. Some studies
showed that insulin normalizes the serotonin and norepinephrine levels in the brainstem [42]. It was demonstrated that intranasal insulin injection influenced insulin secretion in the pancreas via its receptors in the hypothalamus [43]. Fox et al. believed that changes in the content of neuronal growth factors and morphological changes in sensory neurons in diabetic models did not occur until the third week after diabetes induction [44]. In the early stages after diabetes induction, changes in response to pain tests were associated with biochemical changes. Although hyperglycemia can play a role in the development of neuropathy, it was represented that there was no correlation between the threshold of perceived thermal pain and hyperglycemia in diabetic and control animals [45]. In fact, the time of induction of biochemical and morphological changes in nociception system varies in the results and responses to pain tests, depending on different studies with different designs. Age, sex, type and race of animals, diabetes duration, use of different noxious stimulus in pain tests, duration of treatment with insulin, multiple doses of insulin treatment, method of insulin administration (peripherally or centrally), and many other things related to the design of the study would make a variety in outcomes.

In conclusion, intra-arcuate nucleus injection of insulin produced acute thermal hypoalgesia in non-diabetic rats. Insulin reduced the thermal pain threshold in diabetic rats, which was time-dependent. However further studies on the mechanism of intra-arcuate nucleus insulin effect on reducing diabetic’s hypoalgesia are needed.

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**Compliance with Ethical Standards**

**Animal Welfare**

All procedures of the study have been conducted in accordance with animal care and use guidelines approved in the ethics committee at Babol University of medical sciences (MUBABOL.HRI.REC.1397.77).

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**Conflict of Interest**

The authors declare that they had no conflict of interest.
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Figure 1. Effect of lidocaine 2% within the arcuate nucleus on pain response latency in the hot plate test. The white column displays pain response latency before and the black one displays it after lidocaine injection. Each column indicates the mean+SEM of each group. S: second.

* $P<0.05$ in t-test
Figure 2. Effect of different doses of insulin (20mU, 0.1U, and 0.5U) and the vehicle (saline) within the arcuate nucleus on pain response latency in hot plate test in different groups. The white columns display pain response latency before and the black ones display it after insulin injection. Each column indicates the mean±SEM of each group. S: second.

* \( P<0.05 \) in t-test
Figure 3. Influence of the effective dose of insulin (0.5U) and the vehicle (saline) within the arcuate nucleus on pain response latency in hot plate test during three weeks in diabetic rats. The white columns display pain response latency before and the black ones display it after insulin injection. Each column indicates the mean+SEM of each group. S: second.

* $P<0.05$, ** $P<0.01$ in t-test
Figure 4. Influence of the effective dose of insulin (0.5U) within the arcuate nucleus on pain response latency in the tail-flick test during three weeks in non-diabetic and chronic diabetic rats. The white columns display pain response latency before and the black ones display it after insulin injection. Each column indicates the mean+SEM of each group. S: second.

* $P<0.05$ in t-test
Figure 5. Influence of insulin-dependent diabetes (acute and chronic) on pain response latency via hotplate (A) and tail-flick (B) tests. The white columns display pain response latency in nondiabetic rats and the black ones display it in diabetic ones in three weeks. Each column indicates the mean+SEM of each group. S: second.

** P<0.01 and *** P<0.001 in one-way ANOVA