Increased Jejunal Absorption of Glucose in Rats Submitted to Blockade of $GABA_A$ Receptors in the Hypothalamic Paraventricular Nucleus

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Abstract: In this study we investigated the status of jejunal absorption and peripheral metabolism of glucose following disinhibition of paraventricular nucleus (PVN) induced by the $GABA_A$ antagonist bicuculline methiodide (BMI). Adult male Wistar rats (270-300g) were anesthetized to implant unilateral guide cannula targeted to PVN for later microinjection of BMI (10 pmol/100 nl, n=6) or vehicle (NaCl 0.9 % /100 nl, n=5). The jejunal loop was isolated and perfused (0.5 mL/min) with Tyrode solution containing twice the normal concentrations of glucose, sodium, and potassium. After microinjections into PVN, perfusate and blood samples were taken every 10 min over a 40 min period. In comparison with vehicle, BMI into PVN increased glucose absorption at 30 min (1.2 ± 0.1 vs. 1.8 ± 0.1 µmol/min; **P*<0.05) and at 40 min (1.3 ± 0.2 vs. 2.4 ± 0.3 µmol/min; **P*<0.05), whereas plasma insulin was significantly reduced (0.5 ± 0.04 vs. 0.3 ± 0.04 ng/ml, **P*<0.01). At the end of the experiment, samples from the liver and gastrocnemius muscle were taken to measure levels of glycogen, intermediate metabolites of the glycolytic pathway and ATP. Compared to control, muscle glucose-6-phosphate (0.380 ± 0.063 vs. 0.253 ± 0.020 µmol/g; *P*<0.05) and ATP (0.166 ± 0.065 vs. 0.038 ± 0.013 µmol/g; **P*<0.05) were reduced in the group microinjected with BMI into PVN. We conclude that PVN disinhibition changes the absorption and peripheral glucose metabolism.

Keywords: Paraventricular nucleus, hypothalamus, glucose Metabolism, $GABA_A$ receptor.

INTRODUCTION

The hypothalamus plays a key role in the organization of physiological homeostasis by regulating autonomic, neuroendocrine and intestinal functions [1, 2]. The gut and brain are closely integrated within a bi-directional autonomic pathway [2]. One of these critical pathways may be controlled by the hypothalamic paraventricular nucleus (PVN).

PVN is a complex structure that controls both neuroendocrine and autonomic functions [3]. This nucleus participates on the regulation of hypothalamus-pituitary-adrenal (HPA-axis) [4], the major axis responsible for several stress-related responses. PVN is known as a source of sympathetic activity and also has been implicated in the physiopathology of cardiovascular diseases. Anatomic studies have observed that PVN receives input from viscerosomatic afferences and from upper cortical structures [5,6]. Indeed, it sends projections to both parasympathetic and preganglionic sympathetic neurons and also to the dorsal vagal complex. Altogether, these neural pathways control visceral innervations [7, 8].

The microinjection of bicuculline methiodide (BMI) – a γ aminobutyric acid $(GABA)_A$ receptor antagonist – into the PVN increases blood pressure, heart rate [9, 10] and plasma corticosterone [11], a hallmark neuroendocrine response to stress. Recent studies have revealed that activation of the PVN neurons by blocking the inhibitory GABAergic tone results in pronounced increase in hepatic glucose production, followed by an increase in plasma glucose levels [12]. However, no attempt had yet assessed whether PVN disinhibition changes intestinal absorption of glucose and its peripheral metabolism.

The present study was undertaken to investigate the effect produced by the removal of GABAergic tonic on PVN neurons in the jejunal absorption and peripheral metabolism of glucose.

METHODS

Animals

Adult male Wistar rats from the main breeding stock of the Institute of Biological Sciences, weighing 270-300 g, were housed under standard laboratory conditions of a 12:12-h light–dark cycle and 23 ± 2°C. With water offered ad libitum, the animals were housed individually and fasted for 12 h before the experimental procedures. All experimental procedures were approved by our local Ethics Committee on Animal Experimentation (CETEA/UFMG protocol n° 21/05).

General Procedures

Under tribromoethanol anesthesia (250 mg/kg, i.p.), rats were instrumented with unilateral stainless steel guide cannulas (22 gauge, 16 mm in length) targeted to PVN. Animals were positioned on a stereotaxic frame (Stoelting, IL, USA),

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with the tooth bar fixed 3.3 mm below the level of the interaural line. The guide cannula was positioned using the bregma as reference point and following the coordinates determined by the [13] 1.8 mm posterior; 0.5 mm lateral; 7.2 mm ventral. The guide cannula was fixed with acrylic dental cement anchored by two screws placed in the skull. After the surgical procedures, animals were allowed to recover in their home cages.

After two days recovering period, the animals were again anesthetized with tribromoethanol. A small incision was made in the inguinal region and the femoral artery was exposed. A polyethylene catheter (Clay Adams, 0.0 11 I.D.) filled with heparinized saline and sealed with a stylet was inserted into the abdominal aorta through the femoral artery (~4 cm) for recording blood pressure (BP) from which were calculated mean arterial pressure (MAP) and heart rate (HR). The catheter was also routed subcutaneously to the nape of the neck, where it was exteriorized and secured. All incisions were closed with small sutures. The rats were then allowed to recover in their home cages for at least 24 h before the experiments began. All animals for which data were reported remained in conditions of good health throughout the course of surgical procedures and experimental protocol, as assessed by appearance, behavior and maintenance of body weight.

Experimental Procedures

In this study we used two groups of animals, microinjected with a $GABA_A$ antagonist bicuculline methiodide (BMI) 10 pmol/100 nl; n = 6 or vehicle (NaCl 0.9%) 100 nl; n = 5. For the duration of each experiment, HR and BP were recorded continuously (model MP100 A-CE, Biopac Systems, CA, USA). Before microinjection procedures, it was waited a minimum period of 15 min to ensure the stability of the cardiovascular parameters. Microinjections of BMI or vehicle were performed with a 30-gauge injection needle (17 mm length) connected to polyethylene tubing (Norton, 0.010 I.D.) attached to a 5-µl Hamilton syringe, as described previously [14]. HR and BP were recorded for a 15-min period after microinjections into PVN.

Jejunal Perfusion

After 15 min past microinjection into PVN, rats were anesthetized (Thiopentax, Cristalia, Brazil) (40 mg/ kg i.p.) and the abdominal cavity was opened through a median xypho-pubic laparotomy. A 20-cm segment of jejunum was isolated, preserving the nerves and vascular pedicle. Two cannulas were then introduced into both distal and proximal extremities of the jejunal loop, for perfusion and drainage respectively. The abdominal wall was closed in order to avoid tissue dehydration. Both cannulas were exteriorized through the extremities of the abdominal suture. A Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 5 mM D-glucose), at 37°C, pH 8.0 (buffered by HCO_3^-) was perfused (0.5 mL/min) for 15 min in order to equilibrate the fluids in the jejunal lumen [15]. Jejunal perfusion was then continued with a Tyrode solution containing twice the normal concentrations of glucose, sodium and potassium in a 40-min experimental period to evaluate jejunal glucose absorption. During these 40 minutes subsequent to the microinjections into PVN, the effluents from the twice concentrated tyrode were collected every 10 minutes into separated tubes and kept on ice. After the end of this 40-min jejunal perfusion period, samples from the liver and gastrocnemius muscle were collected, snap frozen in liquid nitrogen and kept frozen at –80 °C until the biochemical analysis. The rats were then euthanized by an overdose of anesthetic.

Biochemical Determinations

The glucose concentration from the effluent was determined by an enzymatic method based on glucose oxidase (Glucose God-Ana, Labtest, Brazil), using a standard glucose curve. The results were expressed by the difference between the influx and efflux of glucose (µmol/min).

Glycogen in frozen liver (1 g) or gastrocnemius muscle (1 g) was extracted by tissue homogenization in 3 mL of 6% ice-cold $HClO_4$ (w/v). The homogenate was centrifuged at 2876 x*g* for five minutes. The supernatant volume was measured and neutralized with 10% KOH (w/v). The extract was hydrolyzed by an anthrone reagent (Merck, Darmstadt, Germany) [16] and analyzed spectrophotometrically at 620 nm for free glucose. The glycogen concentration was estimated from a standard glucose curve. The metabolites glucose-6-phosphate, fructose-6-phosphate and ATP were analyzed in the supernatants of the tissue homogenates, as previously [17].

Lactate and glucose concentrations in plasma were measured using an enzymatic assay (glucose and lactate oxidase enzyme) with a Glucose Analyzer (YSI 2300-Stat Plus, Ohio, USA). Plasmatic insulin was analyzed by radioimmunoassay (Linco Research, St. Charles, MO, USA) only in the blood sample collected 15 minutes after the beginning of the perfusion.

Histology

At the end of experiments, a microinjection of 2% Alcian Blue dye (100 nl) was performed in the injection sites for subsequent histological confirmation. The brain was removed and stored in 4% paraformaldehyde for 24 h, then were placed in 20% sucrose solution for at least two days. Subsequently, coronal sections (100µm thick) in the region of the hypothalamus were cut on a freezing microtome. Sections were mounted on slides and counterstained with Neutral Red. The Atlas of Paxinos and Watson, 1986 was used as reference [13].

Data Analysis

The baseline values of mean arterial pressure (MAP) and heart rate (HR) were measured as the average values of these variables for the 5-min period immediately preceding microinjection into PVN. Changes in MAP and HR were sampled at 5-min following microinjections into PVN, calculated from the average of 1-min selected period. Comparisons between responses evoked by microinjections of BMI into PVN and control group were determined by Student's t-test. Significance was taken at $*P<0.05$. Results are reported as means ± standard error of the mean.

RESULTS

There were no differences in the basal values of MAP (vehicle 95 ± 4 vs. BMI 103 ± 5 mmHg) and HR (vehicle 372 ± 15 vs. BMI 356 ± 8 bpm) for the groups. Microinjec-

tion of BMI into the PVN (n = 6) produced significant tachycardia (ΔHR: 19 ± 4 vs. 0 ± 1 bpm; **P* < 0.05) and pressor responses (ΔMAP: 4 ± 1 vs. 0 ± 1 mmHg; **P* < 0.05), when compared with control group (n = 5). The effect evoked by microinjection of BMI into the PVN was evident just a minute after microinjection and lasted for about 15 min.

As shown in Fig. (**1**), glucose absorption throughout the jejunum at 30 and 40 min after microinjection into PVN was greater (**P* < 0.05) in the group microinjected with BMI (n = 6) when compared to control groups (n = 5).

Fig. (**2**) shows that glycogen contents in the liver (Fig. **2A**) and gastrocnemius muscle (Fig. **2B**) were not significantly altered by microinjection of BMI (n = 6) or vehicle (n = 5) into PVN.

As illustrated in Fig. (**3**) plasma glucose levels were not significantly affected by any microinjection into PVN (control, n = 5; BMI n = 6) (Fig. **3A**). However, there were significantly increases in the plasma lactate at 15 min and 40 min after microinjecting BMI (n = 6) into PVN (**P* < 0.05) when compared to control group (n = 4) (Fig. **3B**). Also, there were significant (**P* < 0.05) lower levels of insulin (Fig. **3C**) in the animals submitted to antagonism of $GABA_A$ receptors in the PVN (BMI n= 6; control, n = 5).

Fig. (**4**) displays the content of glucose-6-phosphate, fructose-6-phosphate and ATP in the liver (Fig. **4A**, **C**, **E**; respectively) and gastrocnemius muscle (Fig. **4B**, **D**, **F**; respectively) of our two experimental groups. There were no significant differences in the hepatic glucose-6-phosphate (Fig. **4A**), fructose-6-phosphate (Fig. **4C**) and ATP levels (Fig. **4E**) between groups microinjected with vehicle (n ≥ 4) or BMI (n = 6) into PVN. In the gastrocnemius muscle, the levels of glucose-6-phosphate (Fig. **4B**) and ATP (Fig. **4F**) were significantly lower (**P* < 0.05) in the group that received BMI (n = 6), when compared to control group (n ≥ 4). On the other hand, BMI into PVN did not significantly modify the muscular content of fructose-6-phosphate (Fig. **4D**).

Fig. (**5A**) displays a schematic diagram of the coronal sections of rat brains, illustrating the site of microinjection into the hypothalamic paraventricular nucleus. Figure **5B** is a photomicrograph showing a typical injection site into the PVN.

DISCUSSION

The new finding of the present study was that the $GABA_A$ antagonist BMI (10 pmol/100 nL) microinjected into PVN induced changes in MAP, HR and metabolic parameters such as glucose absorption, plasma lactate, insulin and muscle G-6-P and ATP.

Hypothalamic nuclei play a critical role in the organization of physiological responses to stress. PVN is a structure with neuroendocrine and autonomic functions [3], this nucleus is a primary controller of hypothalamo-pituitary-adrenocortical (HPA) axis [4]. PVN is directly involved in stress responses. Beside that previous studies demonstrated that the disinhibiton of this nucleus with the $GABA_A$ antagonist BMI evoked increases in blood pressure, heart rate, plasma catecholamine [9] and sympathetic nerve activity [18], which is compatible to physiological response to stress.

Previous work from Greenwood and Dimicco [19] demonstrate that disinhibition of Dorsomedial Hypothalamic Nucleus (DMH) evoked activation of both parasympathetic pathways increasing intestinal motility and sympathetic mechanism increasing MAP and HR cardiovascular function. Xavier *et al*. [20] showed that stimulation of the same nucleus evoked a decrease in mesenteric blood flow. We suggest that metabolic and cardiovascular responses under activation of PVN induced by $GABA_A$ antagonist may be modulated by connections between PVN and DMH.

Between 30-40 min after BMI injection, we observed an increase in glucose intestinal absorption. It is likely that PVN disinhibition causes changes in glucose transporter expression in the jejunum, thereby leading to an increase in glucose absorption. The absorption of jejunal glucose is directly related to specific transporters presented in jejunal membrane. The divergent results in the literature on the expression of glucose transporters following exposure to stress have been attributed to the different stress stimuli and animal strains

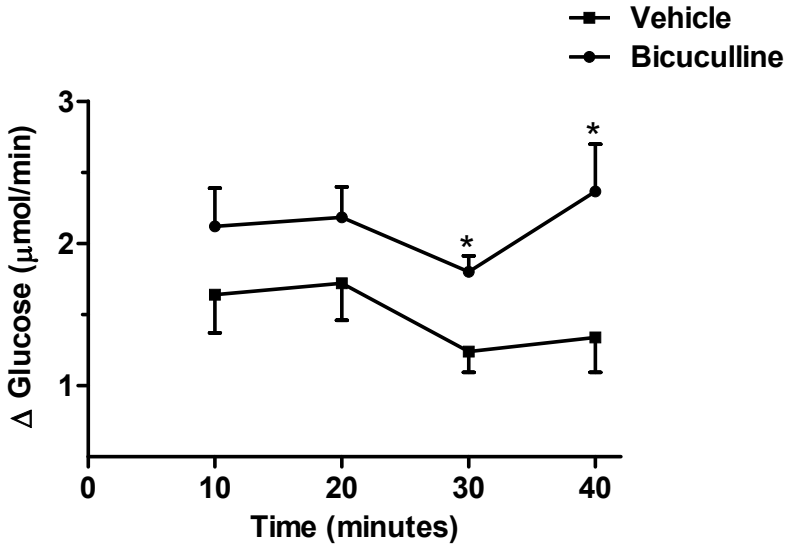

Fig. (1). Time course of glucose absorption through the isolated jejunum of control and BMI-injected rats.

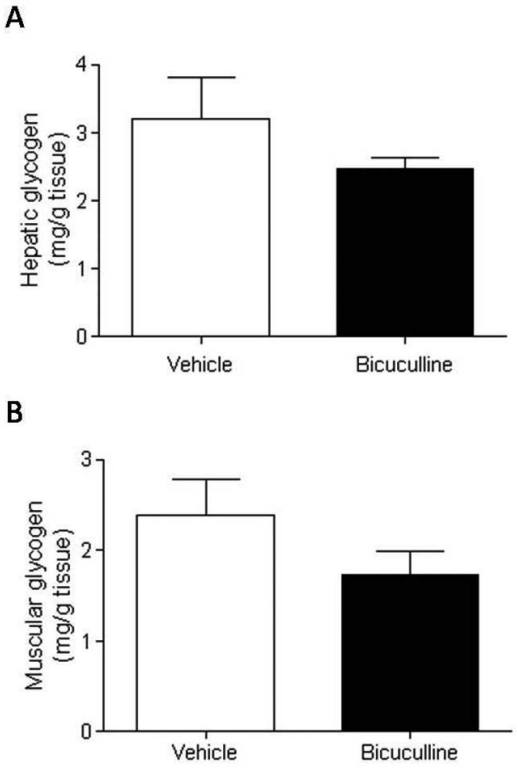

Fig. (2). Glycogen content of liver tissue (**A**) and gastrocnemius muscle (**B**) of control and BMI-injected rats.

utilized [21]. Katz *et al*. [22] demonstrated overexpression of glucose transporters in transgenic mice. Psychological stress induces no change in sodium/glucose co-transporter ($SGLT_1$) expression and an increase in $Glut_2$ expression in the brush-border membrane (BBM), leading to an increase in transporters in the BBM [23]. Shepherd *et al*. [24], describe no change in the SGLT1 component and a decrease in the apical $GLUT_2$ insertion in the apical membrane of Wistar rats. Moreover, Au *et al*. [25] used perfusion of the intestinal lumen with glucose in order to investigate the influence of glucagon-like peptide 2 and observed rapid insertion of $GLUT_2$ into the rat jejunal BBM, which is a low-affinity/high-capacity glucose entrance route. Recently, a new facilitative glucose transporter ($GLUT_7$) has been cloned and characterized in the BBM of the human small intestine [26, 27]. Innumerous possibilities would explain the increase in glucose transports in the jejunum BBM.

The increase in jejunal glucose absorption with the dose of BMI used and this effect was not accompanied by an increase in blood glucose level. Thus, these results seem to suggest that glucose was used by the enterocyte in an attempt to ensure an energy source for its own metabolism. Another possibility is that the glucose did not undergo a change in concentration due increased tissue uptake.

In the present study was observed a decrease of blood insulin levels at 15 min after perfusion. Concerning the recruitment of both sympathetic and parasympathetic autonomic components following hypothalamic stimulation, it is worth hypothesizing that PVN stimulation, by itself, may also cause direct sympathetic input to the beta pancreatic cell in order to inhibit insulin release.

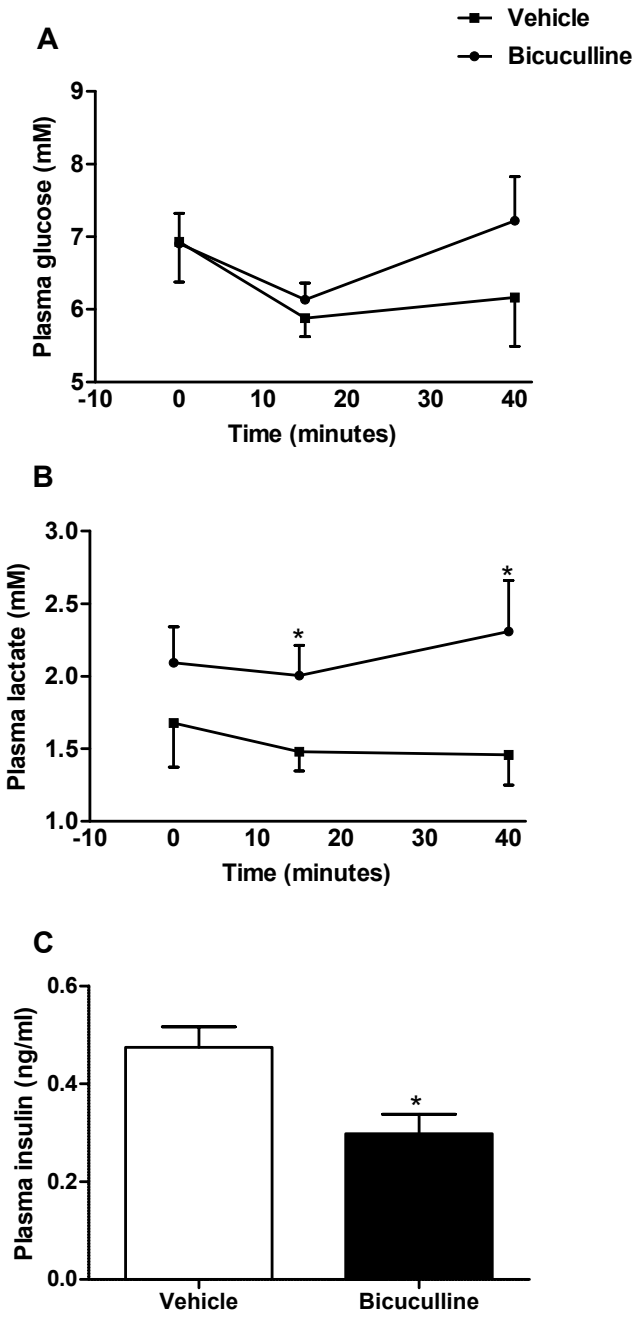

Fig. (3). Plasma glucose (**A**), plasma lactate (**B**) and insulin (**C**) of control and BMI-injected rats.

Yamada *et al*. [28] found that plasma glucose remained at the pre-stress level for 30 min, with a significant increase at 60 min during the immobilization stress. Moreover, plasma insulin concentration decreased at 15, 30 and 60 min and hepatic glycogen remained unchanged. Despite the different protocol used in the present study, our results are similar to these authors' findings.

In skeletal muscle, glycolysis is mainly concerned with the regulation of energy production and is restricted to this organ [4]. Moreover, an increase in plasma catecholamine and glucagon during immobilization stress has been described [28]. As BMI stimulus into the PVN excited a sym-

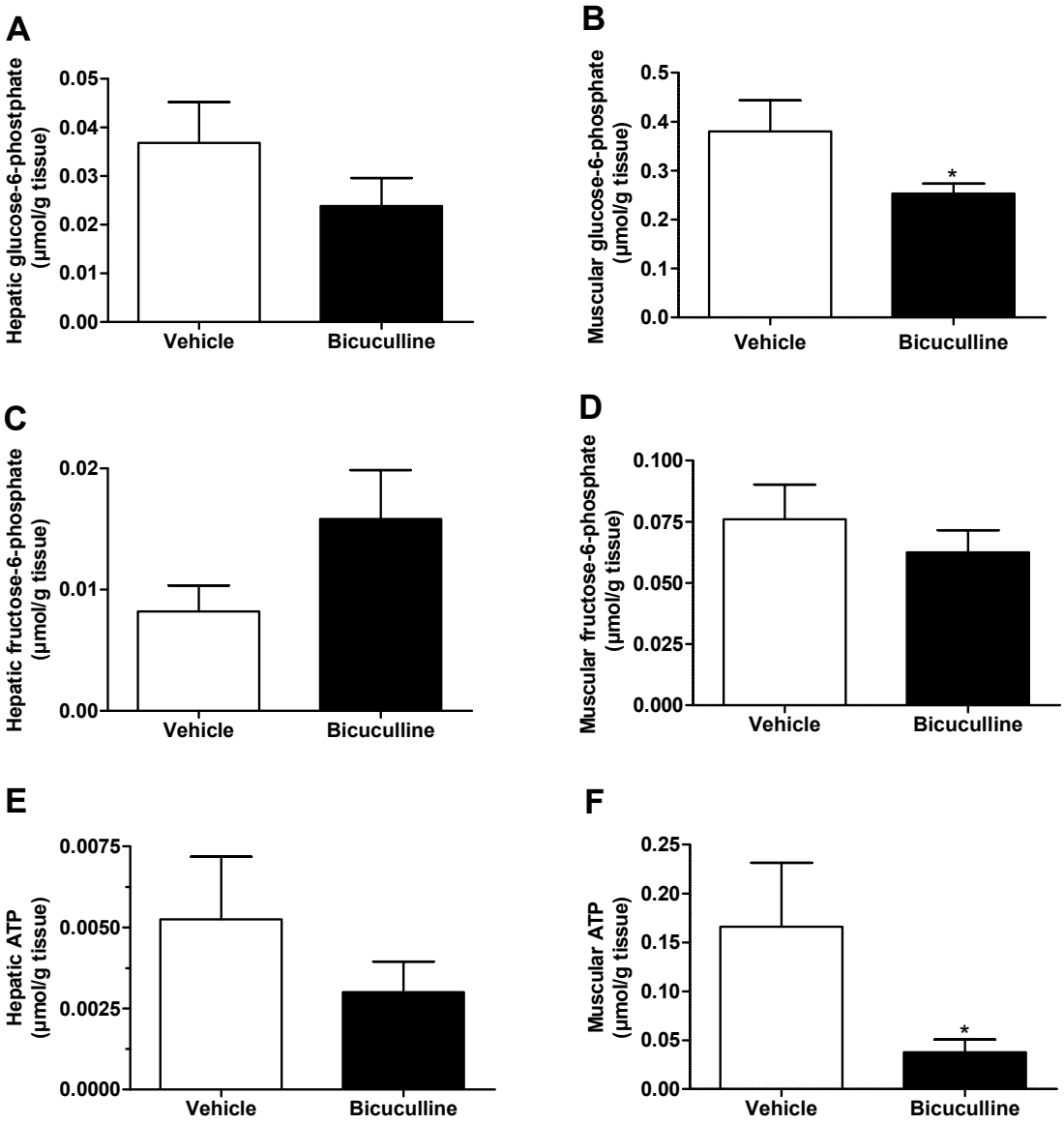

Fig. (4). Intermediate metabolites of glycolytic pathway in the liver (**A**, **C**, **E**) and gastrocnemius muscle (**B**, **D** and **F**) of control and BMI-injected rats.

pathoadrenal axis, although the content of glycogen did not change, the significant decrease in glucose-6-P and ATP in the gastrocnemius muscle in the BMI group fits better with an effect of restoring the glycogen pool, the depletion of which is a hallmark of stress stimulus. On the other hand, sympathetic activation induces an increase in plasma catecholamine and blood lactate concentration which is dependent on the adrenergic stimulation of muscle glycogenolysis [29].

In the liver, which was the main source of peripheral glucose, we did not find any change in the glycogen content. The same is true for the glycolytic and glycogenic metabolite pathways. However, the increase in blood lactate content suggests activation of the glycolitic pathway. Perhaps with a higher dose of BMI, the stimulus would be more intense and the effects on peripheral metabolic responses would be sufficiently prolonged to detect hyperglycemia, hepatic glucogenolises, glycolytic pathway activation and catabolic state expected under conditions of stress.

Previous work in our laboratory found that stress induced by epileptic seizures or the disinhibition of dorsomedial hypothalamic nucleus produced changes in metabolic parameters, also modulating the glycolytic pathways [30, 31].

The monitored rats of the present study confirm previous findings [32] that PVN disinhibition, in conscious rats, evokes an increase in MAP and HR. Thus, PVN is well established as a forebrain site at which GABAergic activity exerts a tonic inhibitory effect on the sympathoadrenal axis, thereby supporting PVN involvement in stress [4, 18]. Despite these considerations, there is evidence that the PVN region plays a role throughout the descending pathways to the gut [33]. Therefore, our data provide further evidence that the PVN can influence glucose absorption in the jejunum and its peripheral metabolism.

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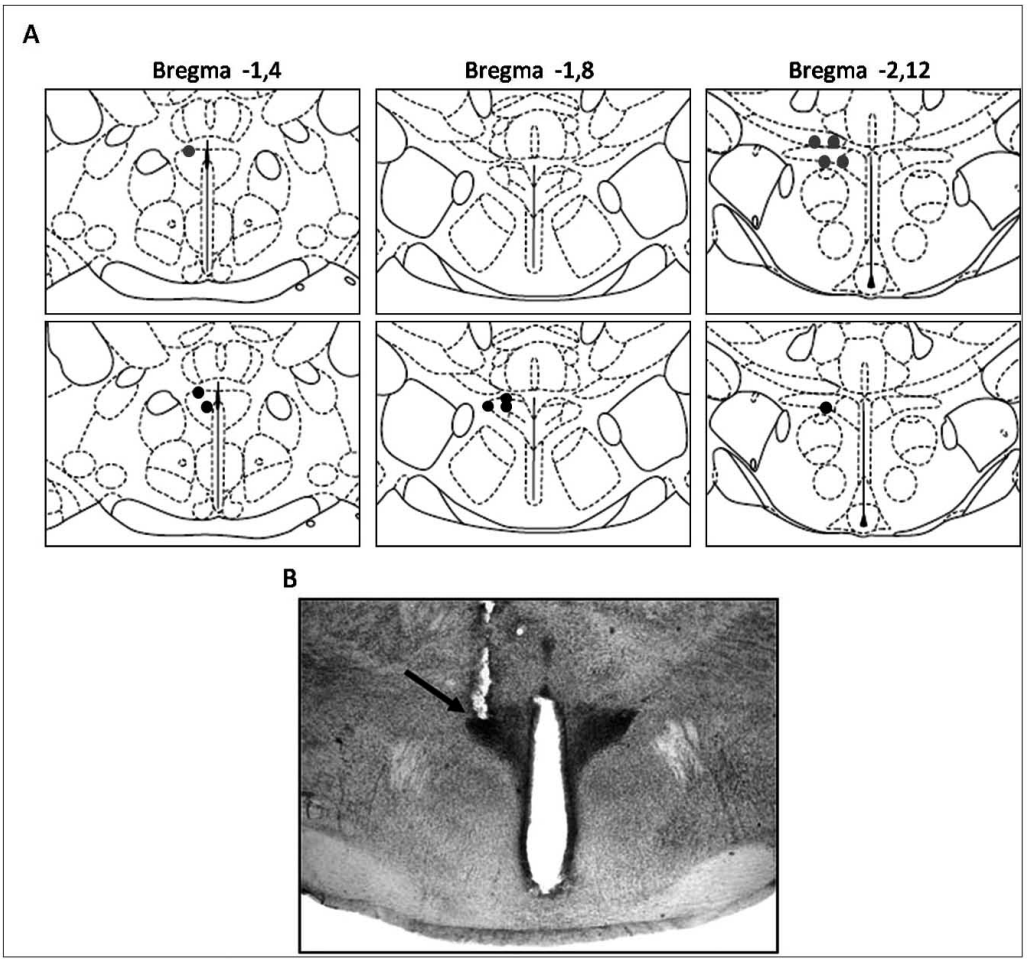

Fig. (5). (**A**) Schematic representation of coronal section showing the localization of microinjection into the paraventricular nucleus (PVN). Redrawn from the Paxinos and Watson Atlas (1986). (**B**) Photomicrograph of a cross section of the hypothalamus showing the unilateral site of microinjection in the PVN. Arrow indicates the microinjection site. Circles grey: control animals, circles black: BMI animals.

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