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# Consequences of cerebroventricular insulin injection on renal sodium handling in rats: effect of inhibition of central nitric oxide synthase

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## Abstract

In the present study, we investigated the effects of acute intracerebroventricular (*icv*) insulin administration on central mechanisms regulating urinary sodium excretion in simultaneously centrally $N^G$-nitro-L-arginine methylester (L-NAME)-injected unanesthetized rats. Male Wistar-Hannover rats were randomly assigned to one of five groups: a) *icv* 0.15 M NaCl-injected rats (control, N = 10), b) *icv* dose-response (1.26, 12.6 and 126 ng/3 µL) insulin-injected rats (N = 10), c) rats *icv* injected with 60 µg L-NAME in combination with NaCl (N = 10) or d) with insulin (N = 10), and e) subcutaneously insulin-injected rats (N = 5). Centrally administered insulin produced an increase in urinary output of sodium (NaCl: 855.6 ± 85.1 Δ%/min; 126 ng insulin: 2055 ± 310.6 Δ%/min; P = 0.005) and potassium (NaCl: 460.4 ± 100 Δ%/min; 126 ng insulin: 669.2 ± 60.8 Δ%/min; P = 0.025). The urinary sodium excretion response to *icv* 126 ng insulin microinjection was significantly attenuated by combined administration of L-NAME (126 ng insulin: 1935 ± 258.3 Δ%/min; L-NAME + 126 ng insulin: 582.3 ± 69.6 Δ%/min; P = 0.01). Insulin-induced natriuresis occurred by increasing post-proximal sodium excretion, despite an unchanged glomerular filtration rate. Although the rationale for decreased urinary sodium excretion induced by combined *icv* L-NAME and insulin administration is unknown, it is tempting to suggest that perhaps one of the efferent signals triggered by insulin in the CNS may be nitrergic in nature.

Key words: Central nervous system; Intracerebroventricular; Nitric oxide inhibition; Insulin; Natriuresis; Lithium clearance

## Introduction

Chronic elevated plasma insulin levels and resistance to the hypoglycemic effect of insulin have been associated with increased blood pressure in human and animal models of hypertension. This observation has led to speculation that insulin may play a role in the development of increased blood pressure (1,2). On the other hand, the role of the central nervous system (CNS) in the control of blood pressure and hydroelectrolyte homeostasis has been demonstrated by several studies (3-5). Further studies of insulin action on neurons have demonstrated pleiotropic effects on ion flows (6), neurotransmitter uptake and release (7), cell growth, survival, and the transcriptional regulation of genes involved with differentiation (8), as well as possible modulation of several brain functions, such as food intake regulation, reproductive function and cardiovascular function (2,9-11). The entry of insulin into the CNS has been documented in many species (12). Considerable evidence supports the concept of a specialized transport system facilitating its passage across the blood-brain barrier endothelium (12,13). In addition, we have recently provided evidence indicating a direct and positive cross-talk between insulin and leptin at the level of Janus kinase and signal transduction and activation of transcription 3 by tyrosine phosphorylation in rat hypothalamus (14). Exploration of the mechanisms by which insulin controls the CNS activity may offer insights into central mechanisms of insulin resistance and cardiovascular diseases, including hypertension. Although it has been shown that the peripheral action of insulin reduces urinary sodium excretion, suggesting an attractive reciprocal link between the renal effect of insulin, urinary sodium excre-

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tion and the development or maintenance of hypertension, studies have indicated that acute intracerebroventricular (*icv*) insulin injection significantly decreases both blood pressure and heart rate, with corresponding decreases in renal sympathetic nerve activity in anesthetized rats (15,16). Our laboratory recently showed that centrally administered insulin produced a dose-related increase in the urinary output of sodium, which was abolished by bilateral renal denervation (17) and cerebroventricular streptozotocin administration in rats (18) and that the response was significantly enhanced in long-term *icv* insulin-pretreated animals compared to control (19).

On the other hand, Shankar et al. (20) have reported that acute systemic administration of high doses of $N^G$-monomethyl-L-arginine, a competitive inhibitor of nitric oxide (NO) synthase**,** results in marked insulin resistance, hyperglycemia, defective insulin secretion, and hypertension. The latest reports on NO and neurogenesis indicate that NO participates physiologically in the control of adult neurogenesis by modulating the proliferation of the neuron progenitor cells. These effects might be partially due to a direct inhibition of growth factors by S-nitrosylation (21). Also, recent studies have demonstrated that NO modulates the synaptic activity and neuronal discharge rates in a dose-dependent manner (22,23). However, there is little information on the neural mechanisms that mediate the effects of *icv* insulin administration on renal sodium handling in rats. Thus, insulin and/or insulin-derived peptides may be thought of as neuropeptide precursors that possibly interact with the nitrergic system.

As a hypothesis, we suggest that the action of insulin in the CNS may be modulated by NO synthase activity, consequently altering urinary sodium excretion. To test this hypothesis, we investigated the effects of acute *icv* insulin administration on central mechanisms regulating urinary sodium excretion in simultaneously centrally $N^G$-nitro-L-arginine methylester (L-NAME)-injected unanesthetized rats and their appropriate control groups.

## Material and Methods

The general guidelines established by the Brazilian College of Animal Experimentation (COBEA, htpp://www.cobea.org.br/index.php) were followed throughout the study. Male Wistar-Hannover rats (250-320 g) were randomly assigned to five groups: a) *icv* 0.15 M NaCl-injected (control) rats (N = 10), b) *icv* dose-response insulin-injected rats (N = 10), c) rats injected *icv* with 60 µg L-NAME in combination with 0.15 M NaCl (N = 10) or d) with insulin (N = 10), and e) subcutaneously (*sc*) insulin-injected rats (N = 5). The animals were chronically instrumented with an *icv* guide cannula (17,19) and kept in individual metabolic cages under controlled temperature (25°C) and lighting conditions (7:00 to 19:00 h), with free access to tap water and standard laboratory rodent chow.

Briefly, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and a stainless steel cannula was stereotaxically implanted into the lateral cerebral ventricle 7 days before the experiments, using previously reported techniques and pre-established coordinates: anteroposterior, 0.2 mm from bregma; lateral, 1.5 mm from bregma, and vertical, 4.0 mm from bregma (17,19). The position of the cannula was confirmed visually by 2% blue Evans infusion through the *icv* cannula at the end of the experiment. Fourteen hours before the renal test, 60 µmol LiCl/100 g body weight was given by gavage. Systolic arterial blood pressure was estimated in additional groups of conscious rats in the morning 30 min after the *icv* administration of 0.15 M NaCl, insulin, L-NAME or L-NAME plus insulin by the tail-cuff method, using an electrosphygmomanometer (Narco Bio-System, USA). This indirect approach permits repeated measurements with a close correlation (correlation coefficient = 0.975), compared to direct intra-arterial recording (24). After an overnight fast, each animal received a load of tap water by gavage (5% of the body weight), followed by a second load of the same volume 1 h later. Thirty minutes after the second load (control period), 0.15 M NaCl (control) or insulin (100 U/mL, Eli Lilly, USA**,** 206 mOsm/kg $H_2O$) was microinjected *icv* in a volume of 3 µL at different concentrations (1.26, 12.6, and 126 ng) with a 10-µL Hamilton microsyringe and spontaneously voided urine was collected over four periods of 30 min each into a graduated centrifuge tube. In two groups, rats were centrally injected with 60 µg L-NAME in combination with 0.15 M NaCl, or with 126 ng insulin. In 5 rats, 126 ng insulin was injected *sc* in a volume of 3 µL.

At the end of the experiment, the animals were anesthetized with sodium pentobarbital, blood was drawn by cardiac puncture and urine and plasma samples were taken for analysis. Plasma and urine sodium, potassium and lithium concentrations were measured by flame photometry (Micronal, B262, Brazil), while creatinine concentration and cerebrospinal fluid (CSF) osmolarity were determined spectrophotometrically (Instruments Laboratory, Genesys V, USA) and with a wide-range osmometer (Advanced Inst. Inc., USA), respectively. Insulin levels were measured by radioimmunoassay (Diagnostic Products Corp., USA) and plasma glucose concentration by an enzymatic method (Labtest, New Zealand), glomerular filtration rate and lithium clearance ($C_{Li}$) was used to assess proximal tubule output (17,19,25). Fractional sodium excretion ($FE_{Na}$) was calculated as $C_{Na}/C_{Cr}$, where $C_{Na}$ is sodium clearance and $C_{Cr}$ is creatinine clearance. The fractional proximal ($FEP_{Na}$) and post-proximal ($FEPP_{Na}$) sodium excretion was calculated as $C_{Li}/C_{Cr}$ x 100 and $C_{Na}/C_{Li}$ x 100, respectively. Renal parameters, glycemia and insulinemia responses to *icv* microinjections were calculated as the area under the curve versus time (AUC, in Δ%/min), with all data being reported as percentage of their baseline value during the 30-min control period preceding each 30-min experimental interval.

Statistical analysis of the data was performed by ANOVA for repeated measurements. Bonferroni's *post hoc* analysis was used to determine the extent of the differences. $P \leq 0.05$ was taken to indicate statistical significance.

## Results

Figures 1 and 2 and Table 1 show the effects of *icv* and *sc* insulin, *icv* 0.15 M NaCl or combined insulin + L-NAME microinjection on renal $Na^+$ and $K^+$ handling. Glycemia and insulinemia results are reported as mean ± SEM. There were no significant differences in daily solid rat chow intake (median: 22.7 g, range: 15.3 to 31.2 g), CSF osmolarity, serum sodium, potassium, and lithium levels and systolic blood pressure (Table 1) in *icv* 0.15 M NaCl-injected rats compared with the other groups. The urinary flow rates did not differ significantly among groups during the studies of renal tubule sodium handling (Figure 1). The *icv* microinjection of insulin (1.26, 12.6, and 126 ng in a volume of 3 µL) increased $FE_{Na}$ in control (0.15 M NaCl) rats from 855.6 ± 81.1 to 1189.9 ± 308.9, 1461.6 ± 594.1, and 2055 ± 310.6 Δ%/min, respectively, and $FE_K$ in control rats from 460.4 ± 100 to 649.2 ± 100.8, 671.2 ± 175.9, and 669.2 ± 60.8 Δ%/min (Figure 1). The enhanced $FE_{Na}$ and $FE_K$ were accompanied by a significant increase in post-proximal sodium excretion compared with the rats injected *icv* with 0.15 M NaCl (Figure 1). This increase occurred despite an unchanged $FEP_{Na}$ and unaffected glomerular filtration rate estimated by $C_{Cr}$ except up to *icv* administration of 126 ng insulin (Figure 1). Intracerebroventricular injections of 126 ng insulin produced reproducible decreases in glycemia levels ($P < 0.03$; Table 1), which in turn were not modified by 126 ng *sc* insulin or *icv* saline administration. Insulinemia was not altered by *icv* insulin or 0.15 M NaCl microinjection

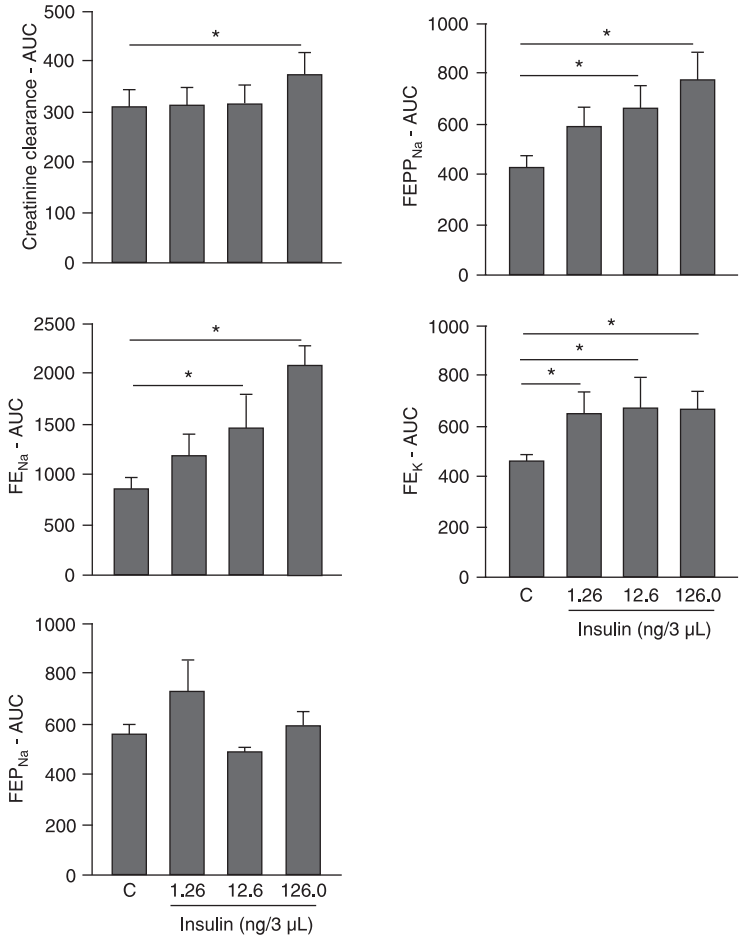


**Figure 1.** Effect of lateral intracerebroventricular microinjection of 0.15 M NaCl (C) or dose-related (1.26, 12.6 and 126.0 ng in a volume of 3 µL) insulin in Wistar-Hannover rats on creatinine clearance, fractional excretion of sodium ($FE_{Na}$), proximal ($FEP_{Na}$) and post-proximal ($FEPP_{Na}$) fractional excretion of sodium, and fractional excretion of potassium ($FE_K$). AUC = area under the curve. Data are reported as means ± SEM. *$P \leq 0.05$ as indicated by the horizontal lines (ANOVA and Bonferroni's contrast test).

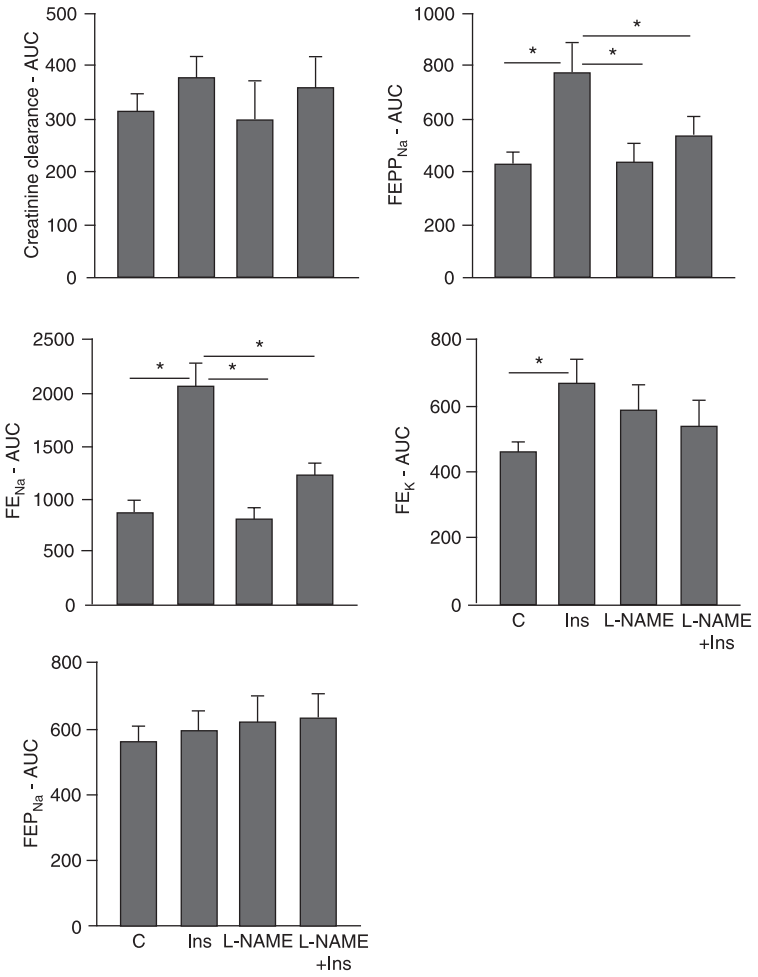


**Figure 2.** Effect of lateral intracerebroventricular microinjection of 126 ng/3 µL insulin (Ins) on creatinine clearance, fractional excretion of sodium ($FE_{Na}$), proximal ($FEP_{Na}$) and post-proximal ($FEPP_{Na}$) fractional excretion of sodium and fractional excretion of potassium ($FE_K$) compared to *icv* administration of 0.15 M NaCl (C), 126 ng/3 µL insulin + 60 µg L-NAME (Ins + L-NAME) and 60 µg L-NAME in Wistar-Hannover rats. Data are reported as means ± SEM. AUC = area under the curve. *P ≤ 0.05 as indicated by the horizontal lines (ANOVA and Bonferroni's contrast test).

**Table 1.** Effect of lateral intracerebroventricular (*icv*) or subcutaneous (*sc*) microinjection of 126 ng/3 µL insulin on cerebrospinal fluid osmolarity, serum sodium, potassium and lithium levels, and insulinemia, glycemia and systolic blood pressure (SBP) compared to *icv* administration of 0.15 M NaCl (control), 126 ng/3 µL insulin + 60 µg L-NAME and 60 µg L-NAME administration in Wistar-Hannover rats.

| Groups | $Na^+$ (mM) | $K^+$ (mM) | $Li^+$ (µM) | Insulinemia (AUC) | Glycemia (AUC) | CSF (mOsm/kg $H_2O$) | SBP (mmHg) |
|---|---|---|---|---|---|---|---|
| NaCl (*icv*, N = 10) | 144 ± 2.3 | 4.2 ± 0.3 | 87 ± 16 | 14.91 ± 2.78[b] | 436 ± 21[c] | 306 ± 2.0[a] | 132 ± 11.0 |
| Insulin (*icv,* N = 10) | 143 ± 2.1 | 4.1 ± 0.2 | 69 ± 19 | 18.48 ± 2.02[d] | 298 ± 27[c,*] | 301 ± 3.0[a] | 127 ± 9.5 |
| Insulin + L-NAME (*icv,* N = 10) | 145 ± 3.4 | 4.2 ± 0.5 | 91 ± 32 | 17.72 ± 3.71[d] | 378 ± 58[c] | 307 ± 2.8[a] | 131 ± 9.0 |
| L-NAME (*icv,* N = 10) | 142 ± 2.7 | 3.9 ± 0.7 | 82 ± 28 | - | - | - | 137 ± 10.2 |
| Insulin (*sc,* N = 5) | 143 ± 3.5 | 3.5 ± 0.2 | 100 ± 10 | 14.03 ± 3.12 | 398 ± 33 | 301 ± 4.1[a] | 132 ± 8.2 |

Data are reported as means ± SEM. AUC = area under the curve (in Δ%/min); CSF = cerebrospinal fluid osmolarity**;** N = number of animals; [a]N = 3; [b]N = 5; [c]N = 7; [d]N = 6.*P ≤ 0.05 NaCl *vs* all groups (ANOVA and Bonferroni's contrast test).

(Table 1). The urinary sodium excretion response to *icv* 126 ng insulin injection was blunted and significantly reduced by simultaneous *icv* administration of 60 µg L-NAME, from 0.15 M NaCl + 126 ng insulin: 2054.9 ± 211.5 Δ%/min to L-NAME + 126 ng insulin: 1267.4 ± 134.1 Δ%/min (see Figure 2). This attenuated urinary ion excretion was associated with a significant increase in post-proximal sodium reabsorption (Figure 2). The renal natriuretic responses, confirming previous studies, were not altered by centrally 0.15 M NaCl or isolated 60 µg L-NAME administration (Figure 2). Likewise, $C_{Cr}$, natriuresis and kaliuresis were unaffected by 126 ng insulin administered *sc*.

## Discussion

In the current study, we confirmed that centrally administered insulin produced a substantial increase in the urinary output of $Na^+$ and $K^+$, and tested the hypothesis that the centrally insulin-induced renal ion excretion is, at least in part, related to changes in CNS NO-dependent neural pathways since the insulin response was significantly attenuated by simultaneous *icv* L-NAME administration. In addition, we showed that blunted insulin-induced natriuresis occurred by increasing post-proximal tubule $Na^+$ reabsorption, despite an unchanged $C_{Cr}$ (Figure 1) and was proportional to the filtered $Na^+$ load.

Several investigators have shown that insulin infused into the cerebroventricular space can reach neuronal loci through ependymal cells or glial processes and enter the interstices of the underlying cerebral neuropil (12,26,27). Injection of labeled insulin into the lateral cerebral ventricles of rats produced heavy staining in regions closer to the third ventricle (14,28). We and other authors have carried out immunohistochemical analysis of the rat hypothalamus using an insulin receptor-specific antibody and the results showed a high concentration of this receptor in the arcuate nucleus and, to a lesser extent, in some periventricular neuronal bodies (11,12,14,27).

We have shown that acute *icv* insulin microinjection in rats promotes a dose-dependent increase in sodium excretion, followed by a post-proximal sodium excretion (17,18,29). Conversely, intravenous hyperinsulinemic euglycemic clamp and oral glucose test in humans and rats lead to antinatriuresis (30,31). The action of insulin in the CNS produces sympathetic nervous system activation although the neuronal intracellular mechanisms that mediate this are unknown. Muntzel et al. (10), using concentrations (0.42 to 42 µg/µL) close to ours, showed that administration of insulin into the third cerebral ventricle produces regionally nonuniform increases in sympathetic neural outflow. In this study, *icv* insulin administration failed to significantly increase adrenal or renal nerve activity. On the other hand, studies have shown that insulin injection in the periventricular area significantly reduces the efferent firing rate of peripheral sympathetic nerves and that this hypothalamic effect is abolished when neurons are destroyed by injection of kainic acid (15). We, as well as others (3,4), have shown that carbachol and norepinephrine injection into the septal area, anterior lateral hypothalamus, and subfornical organ as well as the anterior portion of the third ventricle induces a dose-related natriuresis accompanied by a lesser degree of kaliuresis. All of these findings have led us to suggest that the natriuresis observed in the present study may result from either a significant and transient renal sympathetic inhibition or indirectly from a contribution of sympathetic and parasympathetic nervous system activation.

Studies have demonstrated that NO modulates synaptic activity and neuronal discharge rates in a dose-dependent manner. This response was more prominent in stimulus-on than in stimulus-off neurons and the inhibitory effect is partly mediated via purinergic or metabotropic glutamate receptors (22,23,32). Although the rationale for attenuated urinary sodium excretion observed after *icv* insulin administration in animals simultaneously treated with L-NAME remains unknown, speculatively, it is tempting to suggest that perhaps one of the efferent signals triggered by insulin in the CNS may be nitrergic in nature. Recently, several investigators have demonstrated that NO secretion regulates sympathetic neuronal activity in central cardiovascular control nuclei (33). Thus, it is possible that neurons modulated by insulin and containing nitrergic fibers (34) project from the CNS to peripheral organs, including the kidney.

It has been shown that many brain-specific natriuretic factors are located in periventricular structures related to water and salt balance control (3-5), demonstrating a possible link between insulin and natriuresis. Alternatively, we also cannot rule out the possibility that central NO-dependent neural pathways may control cholinergic, adrenergic or non-adrenergic non-cholinergic neurons and/or the hypothalamic/pituitary release of hormones, which in turn acutely modulates the action of insulin in the brain.

A possible indirect mechanism underlying the increase in renal sodium excretion includes insulin-induced changes in CNS glucose metabolism. However, experiments using relatively large doses of *icv* insulin or cultured neurons labeled with radioactive 2-deoxy-D-glucose support the traditional view that the brain is not responsive to insulin with respect to glucose uptake and metabolism (35). Furthermore, in a recent study, relatively large doses of *icv* insulin did not change the measured CSF glucose levels, supporting our conclusion that the insulin effect in the present study was not mediated by glucose deprivation (36,37). Under our experimental conditions, we showed that central insulin (126 ng) by itself decreased blood glucose levels, with no change in insulinemia. Because our experiments were not specifically designed to distinguish the mechanisms involved in this result we cannot rule out, at least in part, that decreased plasma glucose levels are associated with a significantly reduced efferent firing rate of peripheral sympathetic nerves induced by central insulin administra-

tion. Taking into account the data from the present study, we suggest that the peripheral effect of insulin on urinary sodium retention may be physiologically counterbalanced by an acute central insulin action.

The remarkable findings of the present study suggest a novel concept, i.e., that central NO-dependent pathways may control the central action of insulin on renal function and that this system might be related to alterations of the brain insulin circuit.

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