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Hyperglycemia enhances the inhibitory effect of mitochondrial toxins and D,L-homocysteine on the brain production of kynurenic acid

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Abstract:
We have evaluated the effect of diabetes-mimicking conditions on the inhibition of kynurenic acid (KYNA) production exerted by mitochondrial toxins: 3-nitropropionic acid (3-NPA) and aminooxyacetic acid (AOAA), by endogenous agonists of glutamate receptors: L-glutamate and L-cysteine sulfinate, and by a risk factor of atherosclerosis, D,L-homocysteine. Hyperglycemia (30 mM; 2 h) itself did not influence KYNA synthesis in brain cortical slices. However, it significantly enhanced the inhibitory effects of 3-NPA, AOAA and D,L-homocysteine, but not of L-glutamate and L-cysteine sulfinate, on KYNA production. Their IC_{50} values were lowered from 5.8 (4.5–7.4) to 3.7 (3.1–4.5) mM ($p < 0.01$), from 11.6 (8.6–15.5) to 7.1 (4.9–10.3) μM ($p < 0.05$), and from 4.5 (3.5–5.8) to 2.4 (1.8–3.2) mM ($p < 0.01$), respectively. The obtained data suggest that during hyperglycemia, the mitochondrial impairment and high levels of D,L-homocysteine evoke stronger inhibition of KYNA synthesis what may further exacerbate brain dysfunction and play a role in central complications of diabetes.

Key words:
rat brain, kynurenic acid, hyperglycemia, mitochondrial toxins, *in vitro*

Abbreviations: 3-NPA – 3-nitropropionic acid, AOAA – aminooxyacetic acid, HPLC – high pressure liquid chromatography, KAT – kynurenine aminotransferase, KYNA – kynurenic acid

Introduction

Numerous data indicate that the excessive activation of glutamate receptors may contribute to various pathologies, such as neurodegenerative disorders, epilepsy or psychiatric illnesses [26]. Kynurenic acid (KYNA) is the only known endogenous compound able to block all ionotropic glutamate receptors [19]. At low concentration, KYNA inhibits the strychnine-insensitive glycine site of N-methyl-D-aspartate (NMDA) receptor and α7 nicotinic receptors [7]. KYNA is formed by an irreversible transamination of L-kynurenine catalyzed in the brain mainly within glial cells, by kynurenine aminotrans-

ferases (KAT) I and II [6]. KYNA acts as neuroprotectant and anticonvulsant under various experimental paradigms and disturbances in its level have been implicated in neurodegeneration and other neuropathologies [19].

Brain synthesis of KYNA may be controlled by several distinct mechanisms. Changed availability of a bioprecursor, altered activity of biosynthetic enzymes or modified mitochondrial respiration may influence KYNA production [12]. Endogenous agonists of glutamate receptors, e.g. L-glutamate, L-aspartate or endogenous sulfur-containing amino acids were demonstrated to inhibit KYNA formation [8, 22, 27], whereas D,L-homocysteine, a risk factor of atherosclerosis was shown to act biphasically [11].

3-Nitropropionic acid (3-NPA) and aminooxyacetic acid (AOAA) are mitochondrial toxins interfering with mitochondrial respiratory chain [23, 24]. 3-NPA irreversibly inhibits mitochondrial complex II and, at millimolar concentrations affects the activity of KATs [2, 12, 13]. Chronic experimental administration of 3-NPA evokes neuropathological and behavioral changes resembling Huntington's disease in humans [2, 13]. Acute application of 3-NPA induces seizures in rodents [25]. AOAA is also a mitochondrial toxin interfering with oxidative phosphorylation that, in contrast to 3-NPA, potently inhibits KYNA biosynthetic enzymes with IC_{50} values in micromolar range [20, 21, 23, 24].

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia. Despite proper therapeutic regimens, a high proportion of patients suffer from diabetic complications. Their severity usually correlates with the serum level of glucose [3, 4]. In the brain, hyperglycemia affects not only neuronal but also glial cell function [16]. Chronic hyperglycemia is associated with excessive formation of reactive oxygen species, increased production of advanced glycation end-products, reduced synthesis of growth factors and others [3, 17]. Moreover, accumulation of extracellular glutamate in the course of ischemia is potentiated by hyperglycemic milieu [9].

In this study, the influence of hyperglycemia on the inhibitory effects exerted by mitochondrial inhibitors, endogenous agonists of excitatory amino acids and by D,L-homocysteine on the cortical synthesis of KYNA was evaluated *in vitro*.

Materials and Methods

Animals

Experiments were performed on male Wistar rats (220–250 g). Animals were housed under standard laboratory conditions, at 20°C ambient temperature, with food and water available *ad libitum*. Experimental procedures have been approved by the Local Ethical Committee in Lublin and are in agreement with European Communities Council Directive on the use of animals in experimental studies.

Substances

L-kynurenine sulfate salt, kynurenic acid, D,L-homocysteine, L-glutamate, 3-NPA, AOAA, L-cysteine sulfinate were obtained from Sigma-Aldrich (St. Louis, USA). All the high pressure liquid chromatography (HPLC) reagents were purchased from J.T. Baker Laboratory Chemicals (Holland). All other reagents were obtained from POCH (Gliwice, Poland).

KYNA synthesis in cortical slices

Synthesis of KYNA in rat cortical slices was examined as described before [27]. Briefly, rats were killed by decapitation, and their brains were rapidly removed from the skull. Brain cortical slices were prepared with McIlwain tissue chopper, placed at random into the culture wells (10 slices per well), and incubated in the oxygenated, standard or high-glucose Krebs-Ringer buffer (final volume of 1 ml). Standard Krebs-Ringer buffer, pH 7.4, contained 118.5 mM NaCl, 4.75 mM KCl, 1.77 mM $CaCl_2$, 1.18 mM $MgSO_4$, 12.9 mM NaH_2PO_4, 3 mM Na_2HPO_4, and 5 mM glucose. High-glucose Krebs-Ringer buffer was composed as above, except for glucose used at 30 mM concentration. KYNA synthesis was tested in normoglycemic (5 mM) and hyperglycemic (30 mM) Krebs-Ringer solutions in parallel. After the preincubation period (10 min), the tested substances were added in a volume of 50 µl. The incubation (37°C, 2 h) was carried out in the presence of 10 µM L-kynurenine. At least six wells were used for each concentration of the studied substances. Blanks contained all components of incubation buffer except for the brain tissue. Following incubation period, the media were rapidly separated from the tissue and centrifuged

with 0.1 ml of 1 M HCl and 14 μl of 50% trichloroacetic acid. Supernatants were applied to the cation-exchange columns (Dowex 50 W, hydrogen form), which were prewashed with 1 ml of water and 1 ml of 0.1 M HCl. Columns were subsequently washed with 1 ml of 0.1 M HCl and 1 ml of water. KYNA was eluted with 2.5 ml of water.

Quantification of KYNA

Eluted KYNA was subjected to the HPLC and quantified fluorimetrically (Varian HPLC system; ESA catecholamine HR-80, 3 μm, C_{18} reverse-phase column), as previously described [27]. For HPLC detection, a mobile phase pH 6.2 containing 250 mM zinc acetate, 50 mM sodium acetate and 4% acetonitrile, was used. Retention time of KYNA was 4.5–5.0 min with 1 ml/min mobile phase flow. Each chromatographic assay was preceded by the measurements of standardized concentrations of KYNA (0.2, 0.4, 0.6, 0.8 and 1.0 pmol of KYNA) in order to obtain calibration curve. The mean control production of KYNA in the presence of 10 μM L-kynurenine was 6.37 ± 1.02 pmol/1 h/well. Each experiment was repeated at least twice.

Statistical analyses

The concentration of a compound necessary to induce 50% inhibition of KYNA synthesis (IC_{50}), with 95% confidence limits, was calculated using the computerized linear regression analysis of quantal log dose-probit function. Data are presented as a percentage of control values ± SD. The statistical analyses were performed using ANOVA, with the adjustment of p value by the Bonferroni method.

Results

KYNA synthesis was not changed during incubation of cortical slices in solutions containing up to 30 mM glucose (94.0 ± 10.5, 96.0 ± 8.5 and 95.4 ± 4.6% of control in 15, 25 and 30 mM glucose, respectively).

L-cysteine sulfinate inhibited KYNA synthesis reaching the IC_{50} value of 49.4 (37.0–65.9) μM in normoglycemia and 49.4 (33.2–73.5) μM in hyperglycemia. Inhibitory effects of L-glutamate were also not changed and the IC_{50} value reached 0.4 (0.3–0.5) mM (normoglycemia) *vs.* 0.4 (0.3–0.6) mM (hyperglycemia).

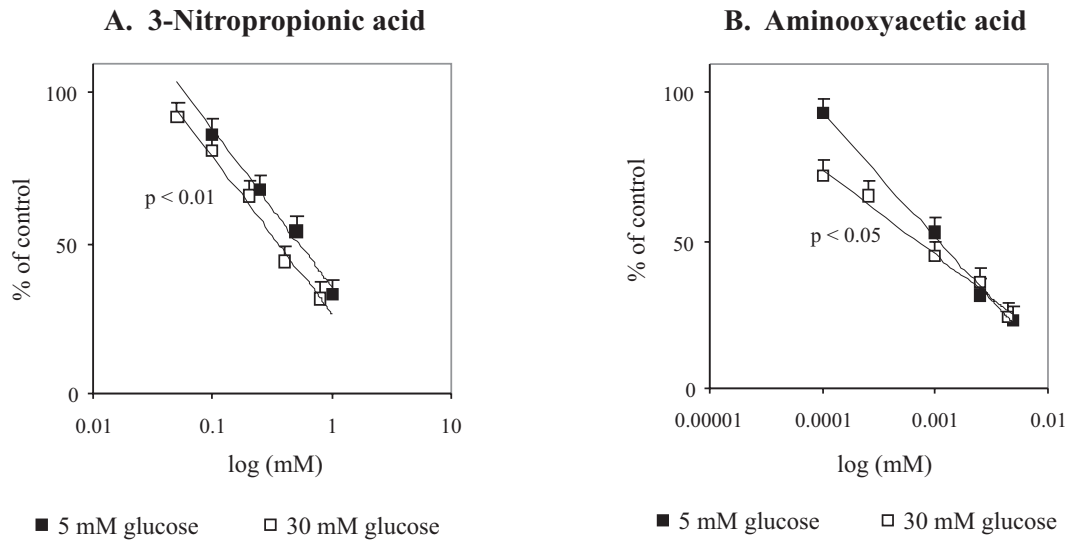

Fig. 1. The effect of hyperglycemia on the inhibitory effect of **(A)** 3-nitropropionic acid (3-NPA) and **(B)** aminooxyacetic acid (AOAA) on the production of kynurenic acid (KYNA). Data are the mean values ± SD (N = 12) and are expressed as % of control values. The concentration of a compound necessary to induce 50% inhibition of kynurenic acid synthesis (IC_{50}), with 95% confidence limits, was calculated using the computerized linear regression analysis of quantal log dose-probit function. **(A)** 5 mM glucose: y = –22.836Ln(x) + 35.358, r = 0.9892; 30 mM glucose: y = –22.65Ln(x) + 26.546, r = 0.9875 **(B)** 5 mM glucose: y = –18.415Ln(x) –76.181, r = 0.969; 30 mM glucose: y = –12.326Ln(x) –39.616, r = 0.9902

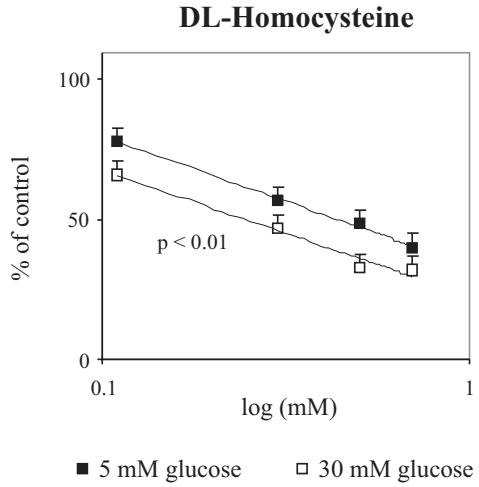

Fig. 2. The effect of hyperglycemia on the inhibitory effect of D,L-homocysteine at millimolar concentrations on the production of kynurenic acid (KYNA). Data are the mean values ± SD (N = 12) and are expressed as % of control values. The concentration of a compound necessary to induce 50% inhibition of kynurenic acid synthesis (IC_{50}), with 95% confidence limits, was calculated using the computerized linear regression analysis of quantal log dose-probit function. 5 mM glucose: y = −19.042Ln(x) + 34.309, r = 0.9955; 30 mM glucose: y = −18.482Ln(x) + 23.448, r = 0.9787

Hyperglycemia enhanced the 3-NPA-evoked inhibition of KYNA synthesis, as revealed by the IC_{50} value lowered from 5.8 (4.5–7.4) mM (normoglycemia) to 3.7 (3.1–4.5) mM (hyperglycemia) with $p < 0.01$ (Fig. 1A).

AOAA-induced reduction of KYNA synthesis was enhanced by hyperglycemia and the IC_{50} value reached 7.1 (4.9–10.3) μM *vs.* 11.6 (8.6–15.5) μM ($p < 0.05$). (Fig. 1B).

The inhibitory effects of D,L-homocysteine on KYNA synthesis were increased in hyperglycemia, as revealed by the IC_{50} value decreased from 4.5 (3.5–5.8) mM to 2.4 (1.8–3.2) mM ($p < 0.01$) (Fig. 2).

Discussion

High glucose concentrations (up to 30 mM) did not influence the synthesis of KYNA in rat cortical slices *per se*. However, hyperglycemia intensified the inhibitory effects of D,L-homocysteine and mitochondrial toxins, 3-NPA and AOAA, but not of endogenous glutamate receptor agonists, L-glutamate and L-cysteine sulfinate, on the synthesis of KYNA *in vitro*.

Increased level of D,L-homocysteine is considered an independent risk factor of cerebro- and cardiovascular disease [4, 5]. Congenital hyperhomocysteinemia is associated with neurological abnormalities, such as cerebral atrophy or mental retardation [5]. Yet, it is still not fully recognized why homocysteine exerts harmful effects. In the brain, the compound acts as a partial antagonist of the glycine site and as an agonist at the glutamate site of NMDA receptor [10]. Moreover, it was recently demonstrated that neurotoxic effects of D,L-homocysteine were also associated with an activation of type II metabotropic glutamate receptors [28, 29]. At high millimolar concentrations, D,L-homocysteine may also affect glial viability in cultures [14]. We have previously shown that D,L-homocysteine can alter the synthesis of KYNA. In cortical slices and aortic rings, D,L-homocysteine stimulates KYNA production at micromolar concentrations and inhibits it when used at millimolar concentrations [11, 18]. In this study, we demonstrated that hyperglycemia enhanced the action of D,L-homocysteine on KYNA synthesis. The IC_{50} value for D,L-homocysteine was lowered almost two-fold under hyperglycemic conditions. Our data suggest that harmful effects of an elevated homocysteine level on the availability of KYNA may be further increased by hyperglycemia. This could be one of the factors possibly contributing to the development of central complications of diabetes.

The obtained results indicated also that hyperglycemia enhanced the decline in synthesis of KYNA caused by mitochondrial toxins, AOAA and 3-NPA. Both compounds are known to act within mitochondrial respiratory chain and to cause neuronal injuries in the brain [15, 23, 24]. *In vitro*, 3-NPA enhances the L-glutamate-induced reduction of KYNA synthesis [12]. Herein, hyperglycemia enhanced the inhibitory effect of 3-NPA and AOAA on KYNA production. Possibly, during diabetes-associated hyperglycemia, the impairment of mitochondrial metabolism may exert stronger decline in KYNA synthesis and, therefore, render the central nervous system more susceptible to the neurodegeneration.

Neurons have a very low potential to store energetic substrates and they need constant delivery of ATP. The oxidation of main energetic substrate, glucose, requires proper supply of NADPH, the level of which is paradoxically reduced by hyperglycemia [3].

Moreover, an excess of glucose stimulates the formation of reactive oxygen species which can impair mitochondrial oxidative phosphorylation [3, 17]. Thus, it was proposed that hyperglycemia actually disturbs mitochondrial function [3, 17]. High concentration of reactive oxygen species and sorbitol, non-enzymatic intracellular protein glycation, caspases activation and intracellular ionic and energetic disturbances may all contribute to neurotoxicity and gliotoxicity [3, 17]. Hyperglycemia, *via* similar effects, may be equally harmful for glial cells [3, 16]. This view seems to be supported by our data considering the fact that glial cells are the major source of KYNA in the brain. Furthermore, our results are in line with the data suggesting that hyperglycemia increases the vulnerability of brain mitochondria to damaging factors [1].

As demonstrated, L-glutamate and L-cysteine sulfinate, in contrast to D,L-homocysteine and mitochondrial toxins, inhibited KYNA production with the same potency in normoglycemic and hyperglycemic conditions. These data are in line with the observation indicating certain discrepancy between L-glutamate- and D,L-homocysteine-induced neurotoxicity [28]. In cerebellar granule cells, these compounds evoke a qualitatively different mitochondrial dysfunction, which in case of D,L-homocysteine is much less susceptible to the protective action of cyclosporine A [28]. Our previous studies have shown that L-glutamate and L-cysteine sulfinate diminish KYNA synthesis most probably due to the direct interference with KAT I or II [8, 27]. L-cysteine sulfinate is an extremely potent inhibitor of KYNA synthesis acting at low micromolar concentrations. Inhibitory effects of L-glutamate are about 10 times weaker [8]. L-cysteine sulfinate selectively inhibits KAT II, the enzyme responsible for about 75% of brain KYNA synthesis, whereas L-glutamate modifies action of both enzymes, KAT I and KAT II, but with a lower efficacy [8]. In view of the data presented here, hyperglycemia does not seem to influence the action of compounds which alter KYNA synthesis solely at the level of its biosynthetic enzymes.

In conclusion, the data obtained *in vitro* suggest that short hyperglycemia may enhance the inhibition of KYNA synthesis evoked by the mitochondrial impairment and high levels of D,L-homocysteine. This mechanism may additionally aggravate brain dysfunction and contribute to the central complications of diabetes. The investigations presented herein will be continued by conducting the *in vivo* studies utilizing an animal model of diabetes.

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