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Mitochondrial dysfunction related to cell damage induced by 3-hydroxykynurenine and 3-hydroxyanthranilic acid: Non-dependent-effect of early reactive oxygen species production

J. Reyes-Ocampo [a,b,1], D. Ramírez-Ortega [a], G.I. Vázquez Cervantes [a], B. Pineda [c], Pavel Montes de Oca Balderas [d], D. González-Esquivel [a], L. Sánchez-Chapul [e], R. Lugo-Huitrón [a], D. Silva-Adaya [f], C. Ríos [a], A. Jiménez-Anguiano [b], V. Pérez-de la Cruz [a,*]

[a] Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, Insurgentes Sur 3877, México D.F. 14269, Mexico
[b] Área de Neurociencias, Departamento de Biología de la Reproducción, Universidad Autónoma Metropolitana-Iztapalapa, 09340 México, DF, Mexico
[c] Laboratorio de Neuroinmunología, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., México D.F. 14269, Mexico
[d] Unidad de Neurobiología Dinámica, Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., México D.F. 14269, Mexico
[e] Laboratorio de Bioquímica Muscular, Instituto Nacional de Rehabilitación S.S.A., México D.F. 14389, Mexico
[f] Laboratorio Experimental de Enfermedades Neurodegenerativas. Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., México D.F. 14269, Mexico

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ABSTRACT

The kynurenines 3-hydroxyanthranilic acid (3-HANA) and its precursor 3-hydroxykynurenine (3-HK) are metabolites derived from tryptophan degradation. 3-HK, has been related to diverse neurodegenerative diseases including Huntington's, Alzheimer's and Parkinson's diseases that share mitochondrial metabolic dysregulation. Nevertheless, the direct effect of these kynurenines on mitochondrial function has not been investigated despite it could be regulated by their redox properties that are controversial. A body of literature has suggested a ROS mediated cell death induced by 3-HK and 3-HANA. On the other hand, some works have supported that both kynurenines have antioxidant effects. Therefore, the aim of this study was to investigate 3-HK and 3-HANA effects on mitochondrial and cellular function in rat cultured cortical astrocytes (rCCA) and in animals intrastriatally injected with these kynurenines as well as to determinate the ROS role on these effects. First, we evaluated 3-HK and 3-HANA effect on cellular function, ROS production and mitochondrial membrane potential *in vivo* and *in vitro* in rCCA. Our results show that both kynurenines decreased MTT reduction in a concentration-dependent manner together with mitochondrial membrane potential. These observations were accompanied with increased cell death in rCCA and in circling behavior and morphological changes of injected animals. Interestingly, we found that ROS production was not increased in both *in vitro* and *in vivo* experiments, and accordingly lipid peroxidation (LP) was neither increased in striatal tissue of animals injected with both kynurenines. The lack of effect on these oxidative markers is in agreement with the $^{\bullet}OH$ and $ONOO^{-}$ scavenging capacity of both kynurenines detected by chemical combinatorial assays. Altogether, these data indicate that both kynurenines exert toxic effects through mechanisms that include impairment of cellular energy metabolism which are not related to early ROS production.

Abbreviations: ACSF, artificial cerebrospinal fluid; BSA, bovine serum albumin; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescein-diacetate; DMEM, Dulbecco's modified Eagle's medium; DTPA, diethylene triamine pentaacetic acid; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; 3-HANA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; KP, Kynurenine pathway; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbocyanine iodine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LP, lipid peroxidation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADH, Nicotinamide adenine dinucleotide reduced; NBT, nitroblue tetrazolium chloride; $O_2^{\bullet -}$, superoxide anion; $ONOO^{-}$, peroxynitrite; $OH^{\bullet}$, hydroxyl radical; PI, propidium iodine; rCCA, rat cultured cortical astrocytes; ROI's, Regions of interest; ROS, reactive oxygen species; SDH, succinate deshidrogenase; TBA, thiobarbituric acid; TBA-RS, thiobarbituric acid-reactive substances; $m\Delta\psi$, mitochondrial membrane potential.

* Corresponding author. Tel.: +52 55 5606 3822x2006.
E-mail address: veped@yahoo.com.mx (V. Pérez-de la Cruz).
[1] Programa de Doctorado en Biología Experimental, Universidad Autónoma Metropolitana-Iztapalapa.

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1. Introduction

Oxidative catabolism of tryptophan is executed by the kynurenine pathway (KP) and the metabolites produced in the KP have neuroactive and/or redox properties (Reyes Ocampo et al., 2014). The evidence suggests that kynurenine metabolism is related with physiological processes and it is known that kynurenines levels are altered in the pathophysiology of different neurological and psychiatric disorders, including Huntington's disease, Alzheimer's disease and schizophrenia (Erhardt et al., 2004; Reinhard, 2004; Schwarcz and Pellicciari, 2002; Stone and Darlington, 2002). In particular, 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HANA) have shown pro-oxidant activity inducing neuronal damage, mainly free radicals generation that causes oxidative stress (Eastman and Guilarte, 1989; Okuda et al., 1998). These kynurenines are *o*-aminophenol compounds which during their oxidation process produce reactive oxygen species (ROS). The products of 3-HK autoxidation, include hydrogen peroxide and xanthommatin, which can contribute to oxidative stress (Hiraku et al., 1995; Vazquez et al., 2000). The toxicity of 3-HANA has been explained by its conversion into quinolinic acid or, similar to 3-HK, by its autoxidation that produces ROS. Supporting the toxic effect of these kynurenines, Guidetti and Schwarcz (1999) showed that the excitotoxicity induced by quinolinic acid was potentiated by 3-HK *in vivo*. Low concentrations of 3-HK cause pronounced cell death in striatal neuronal cultures, suggesting that the elevated 3-HK levels can be indeed relevant to neurodegeneration (Okuda et al., 1998).

Nevertheless, there are evidences that show the opposing effect of these kynurenines. 3-HK and 3-HANA, similar to vitamin C and Trolox, who belong to the class of small molecules that react very fast with peroxyl radicals and are important biological antioxidants. In particular, 3-HK and 3-HANA protected B-phycoerythrin from peroxyl radical-mediated oxidative damage more effectively than equimolar amounts of either ascorbate or Trolox (Christen et al., 1990; Roberts et al., 1995).

On the other hand, disturbances of the mitochondrial function have been well documented in different neurodegenerative diseases and in the ageing process (Christen, Peterhans, 1990, Roberts, McCarthy, 1995). Related to that, the mitochondria have several important functions in the cell and its dysfunction causes abatement in ATP production, oxidative damage and cell death, events that have been involved in the pathogenesis of numerous disorders. Worth noting that in many diseases (Guidetti et al., 2004; Widner et al., 2000; Zadori et al., 2012), both kynurenines have been found altered along with mitochondrial dysfunction. However, no link has been stablished between them; and it is possible that these kynurenines alter the mitochondrial function through their redox properties. The present investigation provides experimental approaches to this question by studying the effect of 3-HK and 3-HANA on MTT reduction as a cellular functional marker and $m\Delta\psi$ in rCCA and *in vivo* after intrastriatal injection of these kynurenines; *in vitro* cell death and *in vivo* circling behavior and morphological alteration were also evaluated. In order to evidence their redox participation, we evaluated both *in vivo* and *in vitro* ROS production as well as their scavenging effect in chemical combinatorial systems. Additionally, was investigated the effect of 3-HK and 3-HANA effect on isolated mitochondrial from different brain regions through MTT reduction and succinate deshidrogenase (SDH) activity. Our results revealed that mitochondria is target of both 3-HK and 3-HANA, which can lead to cellular alteration and these effects showed to be independent to early ROS production.

2. Materials and methods

2.1. Reagents

Apomorphine, dichlorofluorescein diacetate (DCFH-DA), digitonin, D-mannitol, sucrose, K1-EGTA, bovine serum albumin (BSA; fatty-acid-free), K1-HEPES, p-iodophenyl-3-(pnitrophenyl)-5-phenyltetrazolium chloride (INT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Nicotinamide adenine dinucleotide reduced (NADH), ascorbic acid, H_2O_2, deoxyribose, diethylene triamine pentaacetic acid (DTPA), butylated hydroxytoluene in HPLC-grade, xylenol orange, thiobarbituric acid (TBA), Propidium iodide (PI), 3-HK and 3-HANA were all obtained from Sigma Chemical Company (St. Louis, MO. USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Cyclams were purchased from Mid-Century Chemicals (Posen, IL). JC-1 was purchased from Molecular Probes (Eugene, OR). All other reagents were reactive grade and obtained from known commercial suppliers. Solutions were prepared using deionized water obtained from a Milli-RQ (Millipore) purifier system.

2.2. Animals

Male Wistar rats, $n = 60$ (280–320 g) from the vivarium of the National Institute of Neurology (Mexico City) were employed throughout the study. Before they were employed for the experiments, animals were housed five per cage in acrylic cages and provided with standard commercial rat diet (Laboratory rodent diet 5001, PMI Feeds Inc., Richmond, IN, USA) and water *ad libitum*. Housing room was maintained under constant conditions of temperature (25 ± 3 °C), humidity ($50 \pm 10\%$) and lighting (12 h light/dark cycles). Tissues were collected by decapitation and immediately dissected out on ice. All procedures with animals were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the local guidelines on the ethical use of animals from the Health Ministry of Mexico. During the dissections, all efforts were made to minimize animal suffering.

2.3. Chemical combinatorial assays

2.3.1. $O_2^{\bullet-}$ scavenging assay

The ability of 3-HK and 3-HANA to scavenge $O_2^{\bullet-}$ was assessed according to previously reported methods (Fontana et al., 2001). This technique is based in the reduction of NBT (nitroblue tetrazolium chloride), where the non-enzymatic part of PMS/NADH generates $O_2^{\bullet-}$, which reduce NBT into a purple-colored formazan. The reaction mixture contained HEPES buffer (20 mM, pH 7.4), 196 μM NADH, 39.2 μM NBT, 3.92 μM PMS and increasing concentrations of both kynurenines (0–500 μM). Final mixture volume was 1.3 mL. After incubation for 5 min at room temperature, the absorbance was taken at 560 nm against an appropriate blank solution. All tests were performed six times in an independent manner.

2.3.2. $OH^{\bullet}$ scavenging assessment

$OH^{\bullet}$ scavenging was estimated throughout the Fe^{3+}-EDTA-H_2O_2-deoxyribose system (Floriano-Sanchez et al., 2006; Halliwell et al., 1987). The system contained different concentrations of 3-HK and 3-HANA (or an equivalent volume of distilled water for the control), 0.2 mM ascorbic acid, 0.2 mM $FeCl_3$, 0.208 mM EDTA, 1 mM H_2O_2, 0.56 mM deoxyribose, and 20 mM phosphate buffer (pH 7.4). $^{\bullet}OH$ was generated by incubating the mixture at 37 °C for 60 min. The iron salt ($FeCl_3$) was mixed with EDTA before addition to the reaction mixture. The deoxyribose degradation by the