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CARACTERIZACION DE LOS PROCESOS GENERADORES DE ESTRES OXIDATIVO INDUCIDOS POR LA ACTIVACION DE LOS RECEPTORES PARA N-METIL-D-ASPARTATO EN EL SISTEMA NERVIOSO CENTRAL DE LA RATA

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P R E S E N T A :

ABEL SANTAMARIA DEL ANGEL

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ABREVIATURAS

QUIN	-	ácido quinolínico
NMDA	-	N-metil-D-aspartato
Glu	-	ácido glutámico
Asp	-	ácido aspártico
Fe^{2+}	-	fierro
Cu^{+}	-	cobre
$FeSO_4$	-	sulfato ferroso
$CuSO_4$	-	sulfato de cobre
MK-801	-	dizocilpina
APV	-	ácido 2-amino-5-fosfonovalérico
GSH	-	glutatión reducido
GSSG	-	glutatión oxidado
CNS	-	Sistema Nervioso Central
EAA	-	aminoácidos excitadores
KYN	-	kinurenina
KYNA	-	ácido kinurénico
GABA	-	ácido γ-aminobutírico
GAD	-	glutamato descarboxilasa
HD	-	corea de Huntington
Ach	-	acetilcolina
CAT	-	colina acetil transferasa
AMPA	-	ácido alfa-amino-3-hidroxi-5-metil-4-isoxasolil propiónico
LP	-	peroxidación de lípidos
LFP	-	productos lipídicos fluorescentes
TBA	-	ácido tiobarbitúrico
TBARS	-	sustancias reactivas al ácido tiobarbitúrico
MDA	-	malondialdehído
ROS	-	sustancias reactivas del oxígeno
MAO	-	monoamino oxidasa

RESUMEN

La peroxidación de lípidos (LP) es un proceso citotóxico particularmente activo en el sistema nervioso central (CNS) que ocurre por la generación de especies reactivas del oxígeno (ROS), entre las que se encuentran moléculas tóxicas altamente reactivas, los radicales libres. Como una expresión del estrés oxidativo, la LP se caracteriza por el ataque de dichos radicales a los diferentes sustratos biológicos que son fuente de lípidos insaturados, los cuales predominan en las membranas celulares. Aunque el estrés oxidativo es inherente a todas las biomoléculas (peroxidación de lípidos, proteólisis peroxidativa y daño por radicales a ácidos nucléicos), es particularmente evidente alterando estructural y funcionalmente a los componentes lipídicos. El estrés oxidante ocurre cuando ciertos procesos del metabolismo celular se ven alterados por factores fisiopatológicos, conduciendo así a la actividad redox a un desbalance orientado a la formación de más especies reactivas del oxígeno. Destaca entre dichos factores la excesiva activación de receptores para aminoácidos excitadores, la cual constituye la señal para la producción de radicales libres y la consecuente LP. En este trabajo se evaluaron algunos de los procesos generadores de estrés oxidativo que ocurren en el CNS como consecuencia de la sobre-excitación de los receptores glutamatérgicos para N-metil-D-aspartato (NMDA). El estrés oxidativo fue caracterizado tanto en condiciones *in vitro* como *in vivo*, en diferentes preparaciones biológicas y regiones cerebrales de la rata, en términos de los niveles de LP. La activación selectiva de los receptores para NMDA se realizó mediante la administración tópica de un potente agonista endógeno del NMDA, el ácido quinolínico (QUIN), un metabolito del L-triptofano con acción neurotóxica, considerado por sus efectos como un modelo experimental de la corea de Huntington, así como una excitotoxina involucrada en la patogénesis de enfermedades neurodegenerativas infecciosas e inflamatorias. Los factores generadores de estrés oxidativo caracterizados en este trabajo por métodos de análisis bioquímico fueron: a) La susceptibilidad diferencial de las distintas regiones cerebrales con alta actividad redox (corteza entorrinal, cuerpo estriado e hipocampo) a la peroxidación de lípidos inducida por el QUIN; b) La identificación de algunas de las estructuras celulares preferencialmente generadoras de daño oxidativo que sean sensibles a la acción tóxica del QUIN (sinaptosomas y capilares); c) La participación del óxido nítrico en la inducción y potenciación de la LP debida al QUIN, así como la evaluación del efecto de otras moléculas con acción antioxidante (glutatión) o antagonística sobre el receptor para NMDA (ácido 2-amino-5-fosfonovalérico) sobre la potencia pro-oxidante del QUIN; y d) el papel de algunos metales de transición (fierro, cobre y manganeso) en el daño oxidativo generado por la acción tóxica del QUIN. Los resultados obtenidos del análisis de estos factores sugieren una participación activa de todos ellos en la modulación del estrés redox generado por la activación de receptores para NMDA, a la vez que propone una explicación multifactorial para la neurotoxicidad del QUIN, enfatizando la relevancia de los radicales libres y sus efectos como mediadores de toxicidad en el sistema nervioso.

ABSTRACT

Lipid peroxidation (LP) is a well-known cytotoxic process mainly active in the central nervous system (CNS). LP is related to the generation of reactive oxygen species (ROS), including some highly reactive molecules known as free radicals. As a suitable index of oxidative stress, LP is characterized by free radicals attack to the several biological sources of unsaturated fatty acids located into the cell membranes. Although oxidative injury can be observed in all biomolecules (lipid peroxidation, peroxidative proteolisis and damage to nucleic acid by free radicals), it is particularly evident modifiying structurally and functionally the lipidic components. Oxidative stress occurs when some metabolic process of the cells are altered by physiopatological factors, leading the basal redox activity to a disbalance oriented to the formation of ROS. Among these factors, excesive activation of excitatory aminoacid receptors (EAA) is one of the key signals for the production of free radicals as well as for initiation of LP. In this work, we evaluated some of those events related to the generation of oxidative stress occuring in the CNS as a consequence of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors overstimulation. Oxidative damage, expressed as LP, was evaluated both under *in vitro* and *in vivo* conditions, in several biological preparations as well as in different rat brain regions. Selective overactivation of NMDA receptors was assessed by topic administration of a potent endogenous NMDA agonist, quinolinic acid (QUIN), an L-tryptophan metabolite showing neurotoxic activity and considered by its effects as the experimental model of Huntington's chorea, as well as an excitotoxin involved in the pathogenesis of inflammatory and infectious neurodegenerative diseases. The factors related to the generation of oxidative stress which were characterized in this work by biochemical methods were: a) The differential susceptibility of every rat brain region showing high redox activity (enthorhinal cortex, corpus striatum and hippocampus) to the LP induced by QUIN; b) The identification of some of the cellular structures potentially related to the generation of oxidative stress which are also sensitive to the toxic action of QUIN (synaptosomes and microvessels); c) The participation of nitric oxide on the induction and potentiation of QUIN-induced LP, as well as the evaluation of the effect of some other molecules presenting either antioxidant (as glutathione) or antagonic (as 2-amine-5-phosphonovaleric acid) actions on the NMDA receptors overactivation; d) The role of some trace metals (iron, copper and manganese) on the oxidative injury produced by QUIN. Results obtained from the analysis of all these factors suggest an active role of them in the modulation of redox stress after NMDA receptor stimulation , and let us to propose a multifactorial explanation for the neurotoxicity of QUIN, pointing out a role of free radicals and their effects as mediators of toxicity in the CNS.

INTRODUCCION

Durante la década pasada, múltiples hallazgos vinculados a la caracterización de los efectos neurotóxicos de los aminoácidos excitadores (EAA), glutamato y aspartato, y su importancia como posibles factores etiológicos en enfermedades neurodegenerativas, llevaron a ciertos grupos de investigación a profundizar en el estudio de aquellos factores involucrados en la toxicidad mediada por la activación selectiva de receptores para N-metil-D-aspartato (NMDA) por el ácido quinolínico (QUIN), un metabolito endógeno del triptofano, como una alternativa para explicar el origen de la enfermedad de Huntington. Previamente surgió la hipótesis de la "excitotoxicidad" a partir de las observaciones de Coyle y colaboradores, la cual proporcionaba una explicación simple e integrativa de la serie de eventos tóxicos que ocurrían como resultado de la permanencia persistente y prolongada de agentes excitadores en el espacio sináptico con disponibilidad suficiente para saturar los receptores para EAA. En el caso de los receptores para NMDA, la mayor parte de los eventos tóxicos observados por este proceso fueron adjudicados al exacerbado incremento intracelular de calcio resultante de la apertura de canales iónicos asociados a dichos receptores. Sin embargo, una fracción de este patrón de neurotoxicidad quedaba sin completa explicación desde el punto de vista de la evidente carencia de correlación entre la potencia excitotóxica del QUIN y el incremento proporcionalmente menor en los niveles intracelulares de calcio por acción de este metabolito en el sistema nervioso. Paralelamente, surge en la literatura la noción de que el estrés oxidativo puede estar involucrado en el patrón de toxicidad inherente a la acción del glutamato, entendiéndose como estrés oxidante el daño producido a cualquier biomolécula por la acción tóxica de los radicales libres, los cuales son generados por un desbalance en la actividad redox en los sistemas biológicos.

Aún bajo el conocimiento de la existencia de agonistas tipo NMDA aún más potentes que el mismo QUIN, los estudios experimentales y clínicos con este metabolito tienen hoy día gran relevancia debido a tres aspectos fundamentales: a) su naturaleza endógena, la cual, a diferencia de otros agonistas NMDA, le confiere la potencialidad de ser considerado un posible factor iniciador de patologías neurológicas; b) su alta especificidad como agonista NMDA, que a diferencia del mismo glutamato, produce un patrón de neurotoxicidad muy selectivo; y c) la evidencia reciente que lo involucra directamente en enfermedades inflamatorias e infecciosas con componentes neurológicos, tales como la encefalopatía hepática y el complejo SIDA-demencia.

Es bajo este esquema que a principios de esta década surge la necesidad de caracterizar el patrón de toxicidad producido por el QUIN en el Sistema Nervioso. Debido a la ya existente evidencia experimental de que la activación prolongada de receptores para NMDA puede generar estrés oxidante como un factor vinculado a la muerte neuronal, surge la necesidad de evaluar si el QUIN, con potencial clínico, es capaz de producir dicho efecto y si este último está vinculado a la excitación de receptores para NMDA.

En el presente trabajo se describen algunos de los posibles factores involucrados en la generación de estrés oxidativo mediado por la activación de receptores para NMDA por el QUIN, en relación a previas evidencias de nuestro grupo que ha demostrado el efecto de dicha toxina en la peroxidación de lípidos, un parámetro de daño oxidativo sobre los lípidos de las membranas celulares. La relevancia de este estudio radica fundamentalmente en su posible impacto sobre el conocimiento de los mecanismos tóxicos involucrados en la manifestación de algunas enfermedades neurodegenerativas y representa un esfuerzo culminante por dar continuidad a una línea de investigación que hemos desarrollado exitosamente durante los últimos siete años.

El ácido quinolínico y la peroxidación de lípidos *in vitro*, en cerebro de rata

Una primera aproximación a la caracterización de la potencia tóxica del QUIN en términos de su habilidad para generar eventos oxidativos capaces de producir daño celular debió ser inicialmente probada bajo condiciones controladas y a la vez generales. El diseño experimental realizado para tal efecto consistió en la evaluación de los niveles de productos derivados de la peroxidación de lípidos generados por esta toxina en homogenados de cerebro de rata, una preparación rica en sustratos biológicos y que preserva la integridad funcional de los receptores glutamatérgicos y de las membranas que los contienen. Para estos propósitos fue necesario el empleo de dos técnicas experimentales para garantizar la ocurrencia de estrés oxidante, la evaluación de sustancias reactivas al ácido tiobarbitúrico (TBARS) y la generación de productos lipídicos fluorescentes de la peroxidación (LFP).

Adicional a la evaluación del posible efecto pro-oxidante del QUIN, debió obtenerse mayor información acerca del mecanismo involucrado en su toxicidad. En consideración al hecho de que el QUIN fue inicialmente caracterizado en la literatura como un agonista selectivo de los receptores para NMDA, nuestra hipótesis inicial sugirió que el mecanismo tóxico involucrado en la producción de daño oxidativo en el CNS mediado por el QUIN tendría un evidente componente de excesiva activación de dichos receptores, seguido por la apertura de los canales de calcio asociados. Dicho planteamiento permitiría postular que un importante factor generador de estrés oxidante dentro del perfil neurotóxico del QUIN bien podría ser atribuido al incremento en los niveles intracelulares de calcio, siendo éste el responsable de la activación de proteasas y lipasas, así como también de un incremento exacerbado en el metabolismo celular y de la generación de radicales libres. De ser así, la adición de fármacos con acción directa sobre la actividad del receptor a los medios incubados conteniendo los homogenados

tendría un efecto directo sobre la peroxidación registrada en presencia del QUIN. Entre éstos destaca el empleo del ácido kinurénico (KYNA), un antagonista de los receptores para NMDA, y de su precursor, la kinurenina (KYN). Los efectos de otro agonista glutamatérgico, el ácido kaínico (KA), así como del glutamato y el aspartato también fueron probados.

A continuación se presentan los resultados correspondientes a dichos experimentos en el respectivo reporte original.

ANEXO I

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Quinolinic Acid is a Potent Lipid Peroxidant in Rat Brain Homogenates

Camilo Rios[1] and Abel Santamaria[1]

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In this study, we describe the lipoperoxidative effect of quinolinic acid (QUIN) in vitro. The formation of thiobarbituric acid reactive products (TBA-RP), an index of lipid peroxidation, was measured in rat brain homogenates after incubation at 37°C for 30 min in the presence of QUIN and some structurally and metabolically related compounds such as Kynurenine, Kynurenic acid, Glutamate, Aspartate and Kainate. Concentrations of QUIN in the range of 20 to 80 μM increased lipid peroxidation in a concentration-dependent manner from about 15% to about 50%. Kynurenic acid, a compound metabollically related to QUIN that can block its neurotoxic actions in vivo, also inhibited completely the QUIN-induced TBA-RP formation in our system. Lipid fluorescent material, another index of lipid peroxidation was also found increased by 49% after incubation with 40 μM QUIN. It is concluded that lipid peroxidation may be a damaging process involved in the neurotoxicity of QUIN.

KEY WORDS: Quinolinic acid; lipid peroxidation; N-methyl-D-aspartate receptors; Neurotoxicity; Huntington's disease.

INTRODUCTION

The tryptothan metabolite 2,3-pyridine dicarboxylic acid, known as quinolinic acid (QUIN), has been postulated as a candidate to explain the etiology of some neurodegenerative diseases of humans as Huntington's disease (HD) and epilepsy (1,2,3). When applied topically into the rat corpus striatum, QUIN produce axon-sparing lesions similar to those observed in HD. The lesions result in a depletion of GABA and substance-P, while other neurotransmitters are unaffected (4). QUIN also acts as epileptogenic when administered systemically or intracerebrally (5,6). There is also some evidence that relates QUIN actions with the neural damage produced by hepatic encephalopathy (7). This broad spectrum of actions led us to investigate the molecular mechanism by which QUIN exerts its toxic effects. It has been proposed that QUIN is the endogenous ligand of the excitatory aminoacid N-methyl-D-Aspartate (NMDA) receptor (8) suggesting that its neurotoxic effects may be related to the "excitotoxic" properties of the compound, inducing massive calcium entry into the neuron (9); however, Schwarcz and coworkers have shown that the neurotoxic characteristics of QUIN cannot be explained by its action on NMDA receptors (10), indicating that perhaps an additional mechanism exists which wholly explains the QUIN-induced damage.

Lipid peroxidation, on the other hand, is a deleterous process that may participate in the induction of various pathologies of man (11). Lipid peroxidation has been shown to occur in brain tissue in vitro (12) and in vivo (13,14), and it has been associated with neuronal damage after trauma (15) and increased membrane permeability (16). Membranal functions, as GABA uptake, are altered by lipid peroxidation (17), and lipoperoxidative agents as Fe^{2+} can induce persisting

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epileptiform discharges in the rat after intracerebral administration (18).

This work describes for the first time, the lipoperoxidant effect of QUIN and some structurally and metabolically related compounds in vitro in an effort to further characterize their neurotoxic actions in brain tissue.

EXPERIMENTAL PROCEDURE

Materials. Quinolinic acid, Kynurenine (KYN), Kynurenic (KYNA), Kainic (KA), Thiobarbituric (TBA), Aspartic (ASP) and Glutamic (GLU) acids were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were obtained from Merck (Mexico). Deionized water (Milli R/Q System, Millipore) was used for preparation of all reagents and solutions.

Animals. Male Wistar rats (250–300 g) were used throughout the experiments. Animals were sacrificed by decapitation, brains isolated (including cerebellum) and homogenized (1:20 g/mL) in ice cold 0.05 M phosphate buffer (pH = 7.0) containing 0.015 M NaCl and 0.145 M KCl.

Assay of Thiobarbituric Acid Reactive Products (TBA-RP). One mL aliquots of the homogenate were incubated under air at 37°C in a shaking water bath for 30 min (unless specified). Drugs were added as aqueous solutions in micromolar concentrations at the beginning of the incubation period. After this, lipid peroxidation was measured by the Thiobarbituric acid test (19). Two ml of the TBA reagent (0.375 g of TBA + 15 g of Trichloroacetic acid + 2.5 ml of concentrated HCl in 100 ml of water) were added to the homogenate and the solution was heated in a boiling water bath for 20 min. After 5 min of ice-cooling, centrifugation was done at 3000 g for 15 min. The absorbance of the supernatant was read in a DU-6 Beckman Spectrophotometer at 532 nm. TBA-RP concentrations as nmol of Malondialdehide in the samples were obtained by interpolation in a standard curve constructed by periodic oxidation of known concentrations of 2-deoxy-D-ribose as described by Waravdekar, et al. (20). Protein was measured according to Lowry et al. (21). Zero time peroxidation was subtracted in all cases. Results were expressed as nmoles of TBA-RP formed per mg of protein or as percent of change against the control incubated in parallel. All samples were done in duplicate.

Assay of Lipid Fluorescent Products (LFP). The formation of lipid-soluble fluorescence was monitored using the technique described by Triggs and Willmore (22) modified by us for in vitro analysis of LFP. One mL of the homogenate was incubated as previously described. After incubation, 4.0 ml of chloroform-methanol 2:1 mixture were added. The tubes were capped, gently mixed and placed on ice for 20 min to permit phase separation. Aqueous phase was discarded and 1 mL of the chloroformic layer was transferred into a quartz cuvette, and 0.1 mL of methanol was added. Fluorescence was measured in a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer at 370 nm of excitation and 430 nm of emission. Sensitivity of the spectrophotometer was adjusted to 160 fluorescence units with a 0.1 μg/ml quinine standard prepared in 0.05 M aqueous Sulfuric acid solution, prior to the measurement of the samples. Results were expressed as fluorescence units per gram of wet tissue per mL of extraction read.

In order to assess the results of LFP monitoring, an additional mililiter of the brain homogenate was incubated in each experiment with 0.4 μM of ferrous sulphate, a well-known in vitro promoter of lipid peroxidation (23).

Statistics. Results were statistically analyzed by one-way analysis of variance followed by Dunnet's test for comparisons against the control (24). Paired *t*-test was also used for some data. Values of $p<0.05$ and $p<0.01$ were considered significant.

RESULTS

Incubation of the homogenates in the presence of 40 μM of QUIN resulted in a significant increase of TBA-RP at every time point examined when compared with the values obtained with controls (Figure 1). The percent of increase induced by QUIN varied at different incubation times: 15% at 15 min, 24% at 30 min and 5% at 1 hr. Based on this finding, we fixed the time at 30 min in subsequent experiments.

The enhancement of TBA-RP formation induced by QUIN was concentration-related (Figure 2). In this experiment we also tested the effect of the addition of Kynurenic acid (KYNA) and Kynurenine (KYN), two compounds metabollically related to QUIN. Kynurenine exerts a strong inhibitory action on TBA-RP formation (Figure 2) which seems to be not concentration-dependent at the range examined. KYNA did not change sig-

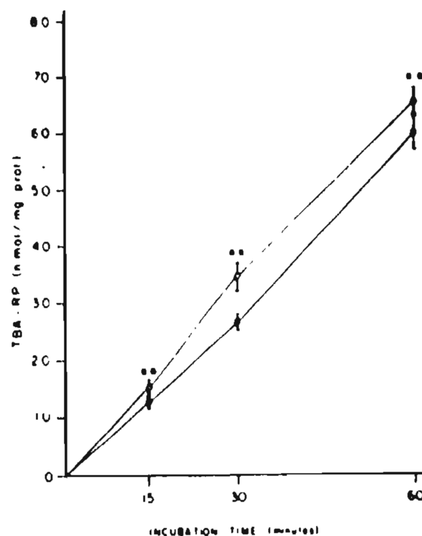

Fig. 1. Time course of TBA-RP formation of brain homogenates incubated in the presence of 40 μM of QUIN. Mean values of n = 7 independent experiments ± SEM. Black dots are the control homogenates.** $p<0.01$ paired *t*-test

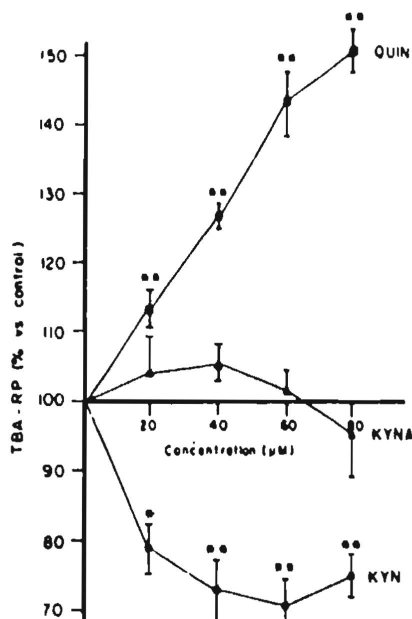

Fig. 2. Effect of increasing concentrations of QUIN, KYNA and KYN on TBA-RP formation. Mean values of n = 5-7 independent experiments ± SEM. The absolute control peroxidation value, which was similar to that of Figure 1, was considered as 100 %. * $p<0.05$, ** $p<0.01$ Dunnet's test.

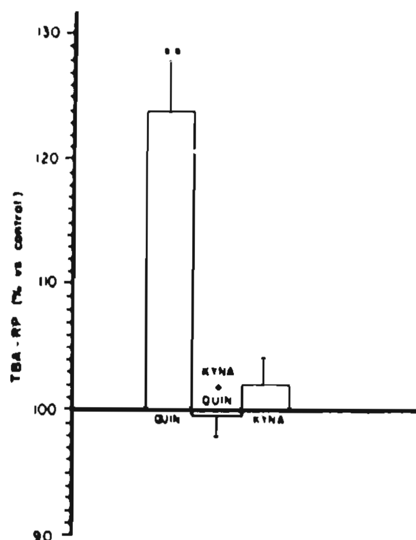

Fig. 3. Effect of simultaneous addition of QUIN (40 μM) and KYNA (40 μM) on TBA-RP formation. Mean values of n = 5-7 independent experiments ± SEM. ** $p<0.01$ Dunnet's test.

nificantly the formation of TBA-RP. However, when added together with QUIN, KYNA was able to completely block the QUIN-induced lipid peroxidation at a molar ratio of 3:1 (see Figure 3).

To further characterize this antagonic effect, we increased the concentration of QUIN in the presence of 20 μM of KYNA or KYN (see Figure 4). Inhibition of the QUIN-induced lipid peroxidation by KYNA was again demonstrated at the entire range of QUIN concentrations used, simultaneously, KYN displayed a similar inhibitory effect. In Figure 5, the TBA-RP promotion effect of QUIN was compared with that of other dicarboxilic compounds added in equimolar concentrations, such as Aspartic, Kainic and Glutamic acids. None of the latter induced significative changes at the concentration tested.

Table 1 shows the level of fluorescence in the homogenates after 30 min of incubation with 40 μM QUIN, 0.4 μM Fe^{2+}, and the control. Significant accumulation of LFP occurred both in the Fe^{2+}-incubated and the QUIN-incubated homogenates as compared with the control. These results indicate that lipid peroxidation measured both as LFP or as TBA-RP formations is enhanced in the presence of QUIN.

DISCUSSION

In this study we found an increased formation of TBA-RP and LFP, two indexes of lipid peroxidation, in the presence of QUIN in rat brain homogenates. In this system, lipid peroxidation has been found to be associated to a decrease in the content of polyunsaturated fatty acids of three or more double bounds (25), leading to several physico-chemical changes in the cell membranes (26). It is interesting to note that micromolar concentrations of QUIN, as those used in this work, have been demonstrated to produce neuronal damage in rat cerebellar slices (27) and in organotypic cultures (28), pointing out the importance of the lipoperoxidative effect of QUIN.

Increased in vivo lipid peroxidation after kainic acid-

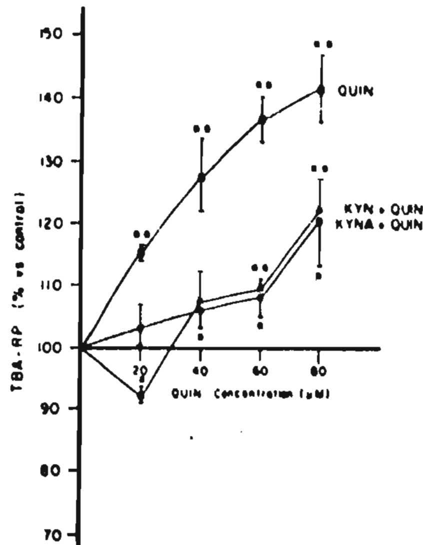

Fig. 4. TBA-RP formation in the presence of variable concentrations of QUIN plus KYN or KYNA at fixed concentration (20 μM). Mean values of n = 6–7 independent experiments ± SEM. * $p<0.01$ Dunnet's test.

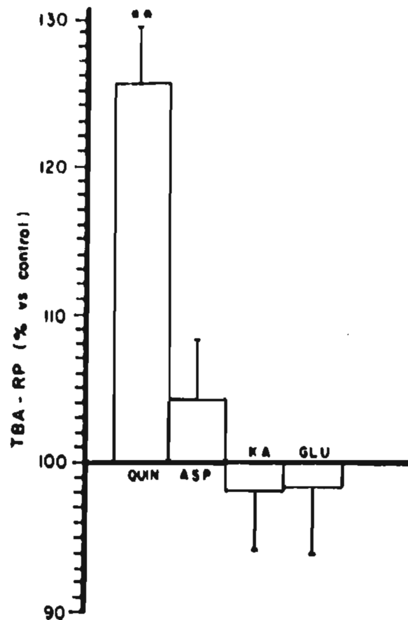

Fig. 5. Comparison of the TBA-RP formation effects of QUIN, Glutamic, Aspartic and Kainic acids, all at 40 μM concentration. Mean values of n = 7 independent experiments ± SEM. ** $p<0.01$ Dunnet's test.

induced seizures has been reported (29). However, this may be a consequence of the seizure activity itself and not the KA direct action over neuronal tissue, since KA was not able to induce TBA-RP formation in our in vitro system.

The meaning of QUIN-induced lipid peroxidation for its neurotoxicity in vivo remain to be studied. However, it is interesting to note that KYNA blocked the formation of QUIN-induced TBA-RP, as in vivo KYNA can also prevent QUIN neurotoxicity and seizure induction (30), perhaps through NMDA receptor antagonism (31,32). This finding suggests that the lipoperoxidative properties of QUIN might be related to NMDA receptor activation, however, our in vitro preparation is not adequate to conclude about NMDA involvement; this hypothesis needs to be tested in a system of preservated cells (as tissue culture or brain slices).

Lipid peroxidation enhancement after Ca^{2+} entry to cells has been reported (33), and this may be a mechanism by which QUIN may produce the increase in lipid peroxidation observed in this work, since NMDA receptor activation by QUIN induces Ca^{2+} influx and delayed Ca^{2+} neurotoxicity (9,34).

Table I. Formation of Lipid-Soluble Fluorescence

Solution	Fluorescence (Units/g wet wt/ml) ($\bar{x}$ ± S.E.M.)	Percent vs Control
Control	6.3 ± 0.5	.....
QUIN (40 μM)	9.4 ± 0.3 *	49.1
Fe^{2+} (0.4 μM)	11.9 ± 0.8 **	89.4

Fluorescence was measured in chloroform-methanol extracts from 1 ml of brain homogenates. Values are means ± SEM from 5 rats. * $p<0.05$, ** $p<0.01$ Dunnet's test.

QUIN-induced lipid peroxidation could be the result of enzymatic or non enzymatic processes such as: overproduction of oxygen-derived free radicals, inactivation of protective enzymes (catalase, superoxide dis-

mutase, etc.) or excessive uptake of calcium (35). Whether or not this effects may be related to NMDA receptor activation by QUIN remain to be proved. We are currently investigating some of those mechanisms to further characterize the action of QUIN over the formation of lipid peroxides.

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El ácido quinolínico y la peroxidación de lípidos *in vivo*, en cuerpo estriado de rata

En toxicología, así como en otras múltiples disciplinas, los estudios *in vivo* tienen gran importancia debido a que permiten la evaluación de procesos generales en sistemas que preservan su integridad estructural y funcional. Por ello, los efectos de los fármacos que son probados bajo condiciones *in vivo* son considerados más reales al estar sometidos a las condiciones naturales de los sistemas.

A partir del conocimiento de que el QUIN es capaz de promover la peroxidación de lípidos *in vitro* en el cerebro de la rata, surgió la necesidad de obtener más información sobre dicho efecto, evaluando su acción *in vivo* sobre la peroxidación de lípidos en una región del cerebro de la rata que es conocida por ser especialmente susceptible tanto a los efectos tóxicos del QUIN así como al ataque por radicales libres, el cuerpo estriado. Más aún, era necesario comprobar que, de acuerdo a los hallazgos *in vitro*, la peroxidación de lípidos inducida por el QUIN era dependiente de la activación de los receptores para NMDA y no de un efecto secundario inherente a la naturaleza química de la molécula. Por lo anterior, se emprendió una serie de experimentos orientados a caracterizar los niveles de daño oxidativo en cuerpo estriado de animales lesionados a diferentes tiempos después de la administración intraestriatal de la toxina, probando adicionalmente el efecto de la administración sistémica de un potente antagonista de los receptores para NMDA, el MK-801. La peroxidación de lípidos fue analizada por dos técnicas: sustancias reactivas al ácido tiobarbitúrico y productos lipídicos fluorescentes de la peroxidación. Los resultados de esta evaluación, mostrados en la publicación anexa, confirman la potencia pro-oxidante del QUIN en el sistema nervioso, asi como la correlación que guarda dicho efecto con la activación específica de receptores tipo NMDA, evidenciada por el efecto protector del uso de antagonistas de dichos receptores.

A partir de estos hallazgos, nuestro interés se centró en la caracterización de aquellos posibles factores involucrados en la generación del daño oxidativo, motivo de las siguientes investigaciones.

ANEXO II

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MK-801, an N-methyl-D-aspartate receptor antagonist, blocks quinolinic acid-induced lipid peroxidation in rat corpus striatum

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Key words: Quinolinic acid; Lipid peroxidation; NMDA receptor; Huntington's chorea; Excitotoxicity; Brain injury; Corpus striatum; MK-801

In this study, we evaluate the possible participation of lipid peroxidation (LP) in the neurotoxic events that follow after quinolinic acid (QUIN) microinjection into the rat corpus striatum. Two hours after QUIN (240 nmol/μl) intrastriatal administration, lipid peroxidation was found increased by 32% vs. control as measured by thiobarbituric acid-reactive substances (TBARS). At the same time tested, the enhancement in LP was of 55% vs. control as measured by lipid flourescent products (LFP) formation (a second index of lipid peroxidation employed). The increase of QUIN-induced lipid peroxidation was completely abolished by pretreatment of rats with an N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 (10 mg/kg, i.p.), 60 min before QUIN microinjection. Results suggest an NMDA receptor involvement in the QUIN-induced oxidative processes.

Quinolinic acid (QUIN) has been postulated as an endogenous ligand of the N-methyl-D-aspartate (NMDA) receptor in the rat brain [11]. QUIN is a tryptophan metabolite of the kynurenine pathway [9]. When administered topically into the rat corpus striatum, QUIN is able to produce neuronal damage that leads to a characteristic pattern of cell loss similar to that found in Huntington's chorea [1]. Some authors have proposed that QUIN may produce the neuronal damage observed by a sustained activation of the NMDA receptor [4], through the process called excitotoxicity, which refers to the events that follow after receptor-mediated excessive excitation, such as high Ca^{2+} cytosolic concentrations, ATP exhaustion, etc. and the toxic processes thereafter [13]. A possible consequence of excitotoxicity is a calcium-mediated free radicals overproduction [2, 5]. This lead us to hypothesize the following series of events after QUIN microinjection into the corpus striatum: (a) NMDA receptor over-activation by QUIN which produces a selective opening of cation channels; (b) increased intracellular Ca^{2+} concentration; (c) Ca^{2+}-dependent free radicals overproduction; (d) cells destruction by free radicals-mediated mechanisms, such as lipid peroxidation.

Recently, we reported that in vitro lipid peroxidation is highly increased in the presence of micromolar concentrations of QUIN [12], given support to the possible relationship between excitotoxic events and oxidative processes after QUIN administration.

In order to further substantiate this hypothesis, we evaluate the ability of QUIN to produce in vivo lipid peroxidation and the NMDA receptor involvement in this relationship.

Male Wistar rats (150–170 g) were used for all experiments. QUIN, thiobarbituric acid, butylated hydroxytoluene and desferrioxamine were purchased from Sigma Chemical CO. (St. Louis, MO). MK-801 was obtained from RBI Biochemicals (Natick, MA). All other reagents were from E. Merck (México).

Rats (n = 19) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then received a stereotaxic microinjection with QUIN at three different doses: 120, 240 and 480 nmol/μl into their right corpora striata. Stereotaxic coordinates were 0.5 mm anterior to bregma, 2.6 mm lateral to bregma, and 4.5 mm ventral to the dura, according to the rat brain atlas of Paxinos and Watson [10]. Twelve control animals were similarly microinjected with phosphate buffered-saline solution (PBS, pH = 7.4).

In order to test the possibility that cellular death itself could be responsible for the lipoperoxidant effect of QUIN, an additional group of 5 animals was intrastriatally injected with kainic acid (10 nmol/μl), a neurotoxin which produces extensive cellular death when adminis-

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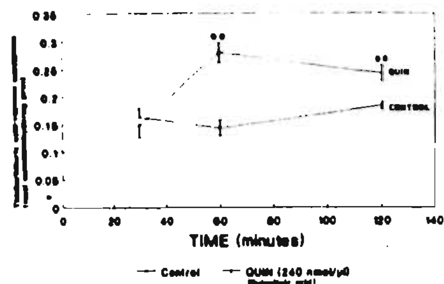

Fig. 1. Time course of lipid peroxidation after quinolinic acid intrastriatal microinjection to rats. Animals were administered with either phosphate buffered-saline solution (PBS, pH = 7.4) as control, or quinolinic acid (QUIN, 240 nmol/μl) and sacrificed at different times after injection. Lipid peroxidation was measured as thibarbituric acid-reactive substances (TBARS) production. Mean values of n = 5–7 independent experiments ± 1 S.E.M. $^{**}P < 0.01$, t-test.

tered topically into the striatum, similar to the one produced by QUIN [1, 17].

To investigate the possible participation of the NMDA receptor activation on QUIN-induced lipid peroxidation, an additional group of animals was injected with an aqueous solution of MK-801 (10 mg/kg, i.p.), a non-competitive NMDA receptor antagonist able to prevent QUIN neurotoxicity [6]. One hour after MK-801 administration, animals were microinjected with QUIN, as described above. Additional control groups, administered with MK-801 (10 mg/kg) 1 h before intrastriatal injection of 1 μl of PBS, were tested.

After injection, animals from all groups were sacrificed at different times (30, 60 and 120 min) and their striata dissected out to measure lipid peroxidation using the techniques described below.

We used two different techniques to estimate lipid peroxidation: (1) thiobarbituric acid-reactive substances (TBARS) content and (2) lipid fluorescent products (LFP) formation.

To measure TBARS, the striatal tissue was homogenized in 1 ml of saline solution (0.9% NaCl). An aliquot of 0.25 ml of this homogenate was added to 0.5 ml of the thiobarbituric acid (TBA) reagent (0.5% w/v of thiobarbituric acid + 16% w/v of trichloroacetic acid + 2.5% v/v of hydrochloric acid). To this mixture, 10 μl of aqueous desferrioxamine (1.5 mM) and 10 μl of butylated hydroxytoluene (3.75% w/v) in methanol-water (50% v/v) were added in order to prevent artifactual increase of lipid peroxidation due to the presence of ferrous ions and to the in situ overproduction of TBARS by the boiling step of the technique [3], respectively. The mixture was ubmerged in a boiling water bath for 20 min and centrifuged (3000 × g, 15 min). The absorbance of the supernatant was measured at 532 nm in a Beckman DU-6 UV-visible spectrophotometer. A standard curve for TBARS formation was constructed using a periodic acid oxidation rate of 2-deoxy-D-ribose [18]. Results were expressed as nmol of TBARS/mg of protein. Protein was measured in the homogenate according to Lowry et al. [8].

To measure LFP, we used the technique previously described by Triggs and Willmore [16]. The striata of animals were homogenized in 3 ml of saline solution (0.9% NaCl). One-ml aliquots of the homogenate were added to 4 ml of a chloroform-methanol mixture (2:1 v/v). After stirring, the mixture was ice-cool for 30 min to permit phase separation and the fluorescence of the chloroformic layer was measured in a Perkin-Elmer MPF-44A fluorescence spectrophotometer at 370 nm of excitation and 430 nm of emission wavelenghts. The sensitivity of the spectrophotometer was adjusted to 140 fluorescence units with a quinine standard solution (0.1 μg/ml). Results were expressed as fluorescence units per gram of tissue. In order to test the reliability of the LFP formation measurement of lipid peroxidation, a group of 5 rats was intrastriatally administered with $FeSO_4$, a well-known lipid peroxidant [16]. 120 min after ferrous sulfate microinjection, lipid peroxidation was measured in corpus striatum as LFP formation.

The neurotoxicity of QUIN on striatal neurons was confirmed by challenging a group of QUIN-lesioned rats with apomorphine (1 mg/kg, s.c.) 6 days after QUIN microinjection (240 nmol/μl). Apomorphine challenge produces circling behavior to the QUIN-lesioned side [14].

Fig. 1 shows the TBARS production at different times after QUIN microinjection. At 30 min, TBARS production in the QUIN-injected striatum does not differ significantly from those values of the control group. One hour after QUIN microinjection an overproduction of TBARS was observed (95% vs. control values) that reaches statistical significance (see Fig. 1). This enhancement of lipid peroxidation persisted at least until 120 min after microinjection (32% vs. control values). TBARS production at 60 min was completely blocked by pretreatment of rats with MK-801 (0.14 ± 0.011 (MK-801 + QUIN) vs. 0.28 ± 0.016 (QUIN), $P < 0.01$, Dunnett's test). MK-801 administered to control rats does not modify TBARS production (data not shown).

Table 1 shows the effect of QUIN microinjection on LFP formation. In this case, lipid peroxidation was measured at 120 min after QUIN injection. LFP was increased by 0.06, 55 and 58% at the 120, 240 and 480 nmol/μl of QUIN doses, respectively. In the same Table 1, we present the antagonic effect of MK-801 on QUIN-induced lipid peroxidation. MK-801 pretreatment decreases this process to their basal levels (0.11% vs. con-

TABLE I

FORMATION OF LIPID-SOLUBLE FLUORESCENCE IN RAT CORPUS STRIATUM

Fluorescence was measured in chloroform-methanol extracts from 1 ml of striatum homogenates 2 h after a single intrastriatal administration of phosphate buffered-saline solution (PBS), quinolinic acid (QUIN), kainic acid (KA) or ferrous sulfate ($FeSO_4$). Values are means ± one S.E.M. of 5–12 independent experiments. n.s., no significant; *$P < 0.05$, **$P < 0.01$, Dunnett's test.

Solution	Lipid fluorescent products (LFP) (Units/g wet wt/ml) ($\bar{X}$ ± S.E.M.)
Control (PBS)	198.6 ± 13.5
QUIN (120 nmol/µl)	198.7 ± 8.0 n.s.
QUIN (240 nmol/µl)	309.3 ± 22.6**
QUIN (480 nmol/µl)	314.8 ± 13.1**
QUIN (240 nmol/µl) + MK-801 (10 mg/kg)	198.8 ± 14.5 n.s.
PBS + MK-801 (10 mg/kg)	184.5 ± 6.9 n.s.
KA (10 nmol/µl)	190.0 ± 6.2 n.s.
$FeSO_4$ (100 nmol/µl)	358.3 ± 40.4**

trol animals). MK-801 alone does not change significantly lipid fluorescent products formation as compared to the control group, as presented in Table I.

Rats ($n = 7$) lesioned with QUIN (240 nmol/µl) exhibited circling behavior to the lesioned side when apomorphine (1 mg/kg, s.c.) was challenged 6 days after QUIN microinjection (212.1 ± 28.6, mean ± S.E.M.). QUIN-induced circling behavior in rats ($n = 6$) was blocked by MK-801 pretreatment (10 mg/kg, i.p.) (9 ± 3.5, mean ± S.E.M.).

Kainic acid (KA) administration, at a dose reported to produce neuronal damage [1], was unable to promote lipid peroxidation (see again Table I), suggesting that QUIN-induced peroxidative stress is not the result of non-specific cell destruction.

Animals microinjected with the $FeSO_4$ (100 nmol/µl) solution presented an increased LFP formation of 80% vs. control, as shown in Table I. This result indicates that LFP formation is a sensitive index of lipid peroxidation.

Results of this work show a significant increase in lipid peroxidation after administration of quinolinic acid into the rat corpus striatum. This effect was observed by two different methods of measuring lipid peroxidation : (a) TBARS production and (b) LFP formation, assessing the reliability of the results. Complete inhibition of QUIN-induced lipid peroxidation by MK-801 pretreatment suggests the participation of the NMDA receptor in the oxidative processes induced by QUIN, as MK-801 is a specific antagonist of this receptor [17]. KA was not able to induce lipid peroxidation at the nanomolar concentration (10 nmol/µl) which has been reported to produce neuropathological changes in the rat corpus striatum [1], suggesting that neuronal death itself does not enhance lipid peroxidation at the time examined. All these results lead us to propose a specific role of NMDA receptor in the oxidant events induced after in vivo QUIN administration to rats. The physiological significance of this finding is remarked by the recent report about the existence of an NMDA receptor redox modulatory site which can block NMDA responses in cultured cells [15]. This putative modulatory site is activated by increased concentrations of oxidized glutathione (GSSG) which in turn is produced by cells submitted to oxidative stress [7]. In accord with these findings, results of the present work suggest a novel role for NMDA receptor as a transducer of oxidative events in corpus striatum with a putative GSSG-mediated negative feedback control.

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RESULTADOS

A continuación se describen los principales hallazgos obtenidos de los diferentes diseños experimentales orientados a la caracterización de los factores generadores de estrés oxidativo mediado por el QUIN.

Se consideró que la mejor forma de describir la metodología correspondiente a cada estudio, así como sus principales conclusiones, debía ser directamente mediante la presentación anexa del artículo publicado o, en su caso, del manuscrito enviado a publicación.

Todos los experimentos descritos en estos estudios se llevaron a cabo en el Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S., con los recursos y la infraestructura de esta Institución.

El efecto del ácido quinolínico sobre el contenido cerebral de cobre y manganeso en la rata

La importancia de los metales de transición en la modulación de la actividad redox en el cerebro está fundamentada en la capacidad de éstos para intervenir en reacciones de óxido-reducción mediante su cualidad de modificar su valencia y su capacidad para reaccionar con múltiples moléculas. Durante este proceso, estos elementos pueden ganar o perder electrones, transitando entre sus estados reducidos y oxidados, representando así tanto sustratos potenciales para la generación de especies reactivas del oxígeno como posibles agentes inactivadores de radicales libres. Es justamente esta dualidad de efectos la que confiere cierta dificultad para establecer con precisión el papel que juegan los metales de transición en los eventos que conducen al estrés oxidativo en el CNS. Más aún, bajo condiciones tan específicas de alteración en la homeostasis celular como lo son la activación selectiva y prolongada de grupos neuronales que poseen receptores para NMDA activados por QUIN, el posible papel de metales de transición como el cobre y el manganeso, ya sea en la promoción o en la inhibición del daño oxidativo producido por esta toxina se antoja, al menos en principio, impredecible. Es por ello que un paso lógico en la caracterización de los posibles efectos de dichos elementos en el modelo de excitotoxicidad producido por el QUIN es la evaluación de la homeostasis de dichos metales en el cerebro con la finalidad de evidenciar si la excesiva activación de los receptores para NMDA podría eventualmente conducir a alteraciones en los niveles de cobre y manganeso en distintas regiones cerebrales de la rata. Con esta primera aproximación al problema de los metales en el cerebro, es posible correlacionar ciertos cambios en sus niveles con probables alteraciones en la actividad redox cerebral, pero aún sin esclarecer su participación específica en el daño oxidativo. Por lo anterior, esta primera parte describe los cambios a mediano plazo en los niveles cerebrales de ambos metales 7 días después de la

administración intraestriatal de QUIN, plazo para el cuál la neurotoxicidad de este metabolito ya se ha manifestado en términos de que induce la pérdida selectiva de células GABAérgicas y colinérgicas, proliferación glial, alteración de los perfiles neuroquímicos y metabólicos de ciertos transmisores y conducta estereotipada en los animales tratados, de acuerdo a las observaciones de Schwarcz y colaboradores (1984). A continuación se presentan los dos reportes que describen los resultados de dicho análisis.

ANEXOS III Y IV

ELSEVIER

Toxicology Letters 87 (1996) 113-119

Toxicology Letters

Quinolinic acid neurotoxicity: In vivo increased copper and manganese content in rat corpus striatum after quinolinate intrastriatal injection

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Abstract

Copper and manganese, two essential metals involved in physiological and physiopathological processes in the brain, were measured in corpora striata of rats 7 days after intrastriatal injection of quinolinic acid (QUIN, 240 nmol/1 μl), an N-methyl-D-aspartate (NMDA) receptor agonist with toxic activity. Seven days after QUIN administration, copper and manganese contents were assessed by graphite furnace atomic absorption spectrophotometry. Total copper content was increased by 152% in QUIN-treated rats (18.74 ± 2.05 μg/g) as compared to control animals (7.44 ± 1.15 μg/g), whereas manganese striatal levels were enhanced by 35% (0.30 ± 0.02 μg/g) vs. control values (0.22 ± 0.02 μg/g). Quinolinate-induced striatal increase in copper and manganese levels were prevented by 23% (9.18 ± 1.43 μg/g) and −0.45% (0.22 ± 0.03 μg/g) vs. control values, respectively, in rats pretreated with an NMDA receptor antagonist, dizocilpine (MK-801, 10 mg/kg, i.p.), 60 min before QUIN administration. As an index of QUIN neurotoxicity, striatal GABA levels were also measured 7 days after QUIN injection. GABA content was decreased by − 55% in QUIN-lesioned rats (96.37 ± 8.92 μg/g), whereas MK-801 was able to block QUIN-induced GABA depletion by 2% (219.37 ± 10.60) vs. control values (214.2 ± 21.88 μg/g). These findings suggest that increased concentrations of transition metals can be mediated by selective overactivation of NMDA receptors and might be a consequence of neural loss as well as glial response to damage.

Keywords: Quinolinic acid; Copper; Manganese; Excitotoxicity; N-methyl-D-aspartate receptors; Corpus striatum

1. Introduction

The neurotoxic tryptophan metabolite, quinolinic acid (QUIN), an endogenous agonist of the N-methyl-D-aspartate (NMDA) glutamate receptor subtype, is able to reproduce the pathological features of Huntington's disease (HD), such as

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Gamma-aminobutyric (GABA) depletion and striatal spiny cell loss, when administered topically into the rat corpus striatum [1]. QUIN also seems to play an important role in neurodegenerative inflammatory and infectious diseases [2]. The mechanism by which QUIN exerts its neurotoxic effects has been ascribed to its ability to induce excessive activation of NMDA receptors, calcium channels opening and consequent massive calcium entry into the cells [3]. In addition to these mechanisms, we have previously described the involvement of lipid peroxidation and oxidative stress in QUIN-induced lesions [4,5].

Essential transition metals, such as copper and manganese, seem to play a complex role in the central nervous system: they are implicated in regulatory events when they are present as components of metalloproteins [6]; whereas, under physiopathological conditions, they are involved in cytotoxic processes such as free radical generation and oxidative stress [7–9].

Shoham et al. [10] have shown that after single unilateral injections of quinolinate and kainate into the rat ventral-striatal region, iron is accumulated in high concentrations in basal ganglia areas such as globus pallidus and substantia nigra pars reticulata. Interestingly, Dexter et al. [11] reported increased concentrations of copper and iron in caudate nucleus, putamen, substantia nigra and cerebral cortex of post-mortem HD human brains. This pattern of increased copper in HD patients is not shared by other neurodegenerative diseases of the basal ganglia, such as Parkinson's disease (PD) and progressive supranuclear palsy, suggesting that specific changes in transition metals are linked to the different neuronal populations affected. In the same report, however, no difference in manganese content was found in HD brain regions as compared against control brains.

In order to investigate whether the NMDA receptor overstimulation by QUIN may produce a striatal copper and manganese accumulation, similar to that found in HD brains, we conducted experiments to evaluate possible changes in the content of these transition metals as a consequence of QUIN intrastriatal injection to rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (6–9 per group), bred-in-house strain, weighing 250–300 g, were used throughout the study. Animals were housed five per cage in acrylic box cages and provided with Rodent Chow (Purina, St. Louis, MO) and water *ad libitum*. Animals were maintained under conditions of constant temperature (25 ± 3°C), humidity (50 ± 10%) and lighting (12:12 light:dark cycle).

2.2. Chemicals

Deionized water (Milli R/Q water purifier) was used for preparation of all reagents and solutions. For graphite furnace atomic absorption spectrophotometry (GFAAS) measurements, water was further purified using a chelating resin column (Chelex, Sigma). Quinolinic acid, Triton X-100, *o*-phthaldehyde (OPA), 3-mercaptopropionic acid, 2-mercaptoethanol and GABA were all purchased from Sigma (St. Louis, MO). Suprapur nitric acid, copper and manganese atomic absorption standards and sodium nitrite were purchased from E. Merck (México). Dizocilpine (MK-801) was obtained from RBI Biochemicals (Natick, MA).

2.3. Surgical lesion technique and treatments

Rats (5–9 per group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). QUIN was dissolved in 0.1 M phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 0.1 N NaOH. Single unilateral 1 μl-injections were made with a 10 μl Hamilton syringe into the right striatum at the stereotaxic coordinates 0.5 mm anterior to bregma, 2.6 mm lateral to bregma and 4.5 mm ventral to the dura, according to the brain Atlas of Paxinos and Watson [12]. QUIN (240 nmol) was injected over 2 min. The needle was left in place for another 2 min and then slowly withdrawn. Control animals were similarly injected with PBS alone. In order to test the possible participation of the NMDA receptor on QUIN-

induced copper and manganese changes, a group of rats was pretreated with the non-competitive NMDA receptor antagonist, dizocilpine (10 mg/kg, i.p.), 1 h before QUIN injection.

2.4. Determination of copper and manganese concentrations in striatal tissue

Copper and manganese contents in rat corpus striatum were both analyzed as previously described [13]. Seven days after QUIN or PBS striatal injections, rats were sacrificed by decapitation, their brains removed and the lesioned striata were dissected out on ice. Then, tissue samples placed in polypropylene tubes were digested in 1 ml of concentrated trace metals-free HNO_3 in a shaking water bath at 60°C for 30 min. After digestion, a 100-μl aliquot was taken from the clear solution and diluted (1:20, v:v) with an aqueous solution containing 0.1% w/v Triton X-100 and 1% diammonium hydrogen phosphate. Aliquots of the solution were then injected into the graphite furnace for copper and manganese analysis. Calibration curves for metals were constructed for each element by adding known amounts of the respective Titrisol (E. Merck) atomic absorption spectrophotometric standard solution to brain tissue digested and diluted as described above.

Analysis of diluted samples was performed using a Perkin-Elmer 360 Atomic Absorption Spectrophotometer with an HGA-2200 graphite furnace. For analysis of both samples and standards, a volume of 20 μl of each final diluted solution was injected into the furnace. The furnace program for each element was optimized by measuring the standards' signal height at several temperature settings, for both char and atomization stages, according to the procedure of Welz [14].

2.5. Analysis of striatal GABA levels

Seven days after QUIN injection, a group of animals treated under the previously described experimental conditions was injected with an inhibitor of glutamate decarboxilase (GAD) activity, 3-mercaptopropionic acid (1.2 mmol/kg, i.v.). in order to prevent artifactual GABA post mortem increase [15]. Two minutes later, rats were sacrificed by decapitation and their brains removed. Right striata of the lesioned animals were dissected out on ice and homogenized in 15 volumes of methanol-water (85% v/v). Samples were then centrifuged (3000 *g* for 15 min) and aliquots of the supernatant were stored at −5°C until high performance liquid chromatography (HPLC) analysis. Striatal GABA levels were analyzed by HPLC with fluorometric detection as previously described [16–18].

For precolumn derivatization procedure, 100 μl of the OPA reagent (containing 5 mg of OPA dissolved in 625 μl of methanol + 5.6 ml of borate buffer 0.4 M, pH 9.5 + 25 μl of 2-mercaptoethanol) were added to 100 μl of the tissue supernatants and after stirring and standing for exactly 1 min, 20 μl of the mixture were injected with a 25 μl Hamilton syringe into a Perkin-Elmer Series 3B liquid chromatograph. A 100 × 4.8 mm Alltech Adsorbosphere OPA-HS column with a particle size of 3 μm was used. Mobile phase consisted in: (A) 50 mM sodium acetate buffer solution (pH 5.9) containing 1.5% tetrahydrofuran and (B) HPLC-grade methanol as solvent. Linear gradient programming was made in two stages: increase of solvent (B) from 10% to 65% in 15 min, and then from 65% to 99% in 5 min, returning to 10% in 5 min. Fluorescence was measured using a Beckman 157 fluorescence detector with OPA cuttoff filters. Peak areas were obtained with a Perkin-Elmer Sigma 10 Data Station. Calibration curves for GABA were constructed by injecting known concentrations of OPA-derivatized GABA standards into the 20 μl loop of the chromatograph. GABA concentrations were obtained by interpolation in the standard curve.

2.6. Statistical analysis

Metals content and GABA levels data were both analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test [19]. Values of $P < 0.05$ were considered of statistical significance.

3. Results

3.1. Effect of quinolinic acid injection on striatal copper content

Striatal copper content in control rats was 7.44 ± 1.15 μg/g of tissue (Fig. 1). Copper concentration in QUIN-lesioned striata (18.74 ± 2.05 μg/g) was remarkably increased by 152% as compared to control values, while dizocilpine pretreatment (9.18 ± 1.43 μg/g) prevented QUIN-induced copper acummulation by −51% as compared to QUIN-treatment (Fig. 1).

3.2. Effect of quinolinic acid on striatal manganese content

Striatal manganese content in control animals was 0.22 ± 0.03 μg/g (Fig. 2). Manganese concentration was increased in QUIN-treated rats (0.30 ± 0.02 μg/g) by 35% vs. control values (Fig. 2). Accumulation of manganese induced by QUIN injection was also prevented in dizocilpine-pretreated rats (0.22 ± 0.03 μg/g) by −27% as compared to QUIN alone (Fig. 2).

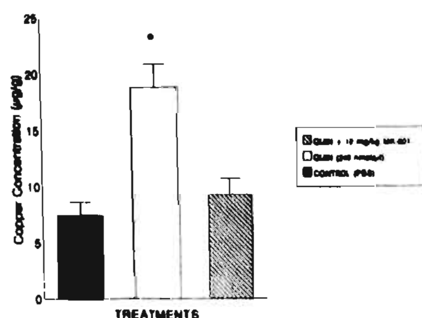


Fig. 1. Effect of quinolinic acid (QUIN) on striatal copper content. Rats were administered i.p. with saline or dizocilpine (MK-801, 10 mg/kg) 1 h before a phosphate buffered saline (PBS, pH 7.4) or QUIN (240 nmol/μl) intrastriatal injection. Seven days after lesion, copper content was measured by graphite furnace atomic absorption spectrophotometry in the lesioned striata. Mean values of $n = 6-9$ independent experiments ± S.E.M. are shown. $^*P < 0.05$, different from control; one-way ANOVA followed by Tukey's test.

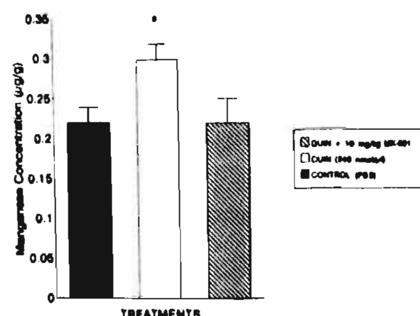


Fig. 2. Effect of quinolinic acid (QUIN) on striatal manganese content. Rats were administered i.p. with saline or dizocilpine (MK-801, 10 mg/kg) 1 h before phosphate buffered saline (PBS, pH 7.4) or QUIN (240 nmol/μl) intrastriatal injection. Seven days after lesion, manganese content was measured by graphite furnace atomic absorption spectrophotometry in lesioned striata. Mean values of $n = 6-9$ independent experiments ± S.E.M. are shown. $^*P < 0.05$, different from control; one-way ANOVA followed by Tukey's test.

3.3. Effect of quinolinic acid on striatal GABA levels

As shown in Fig. 3, a significant decrease in GABA content (−55% vs control) in striatal tissue of QUIN-treated rats (96.37 ± 8.92 μg/g) was found, as compared with control contents (214.20 ± 21.88 μg/g). In this case, preadministration of the 10 mg/kg dose of dizocilpine (219.37 ± 10.60 μg/g) was completely effective to prevent QUIN-induced GABA depletion by 128% as compared to QUIN-treated group (Fig. 3).

4. Discussion

The main finding of this work was the significant increase in striatal copper content 7 days after topical injection of quinolinic acid and the slight increase in manganese induced by the excitotoxin. Excessive copper accumulation in putamen and substantia nigra in post-mortem brains of HD subjects has been described, as compared to normal brain levels [11]. The similarity between QUIN-mediated cell damage and the selective neuronal destruction observed in Huntington's

disease suggests that this parameter can be considered as a suitable tool to reproduce some of the physiopathological consequences of HD in the experimental model induced by QUIN. Accumulation of transition metals, such as copper, iron and manganese, has been associated with degenerative changes in neural tissue. Wilson's disease, for example, is a genetic alteration that produces an accumulation of free copper in brain and liver [20,21]. The severity of the neurological signs in Wilson's disease relates to the extent of copper accumulation in the brain [22,23]. Recently, an alteration in the human chromosome 13 q14.3. has been described to be responsible for the excessive uptake of copper, associated with Wilson's disease [24,25]. Free copper, normally at very low concentrations in the brain and blood plasma [26], can alter the redox balance of living systems, leading to oxidative damage to membranes. Also, free copper is as active as iron in stimulating the decomposition of hydrogen peroxide and hydroperoxide [27], causing DNA damage [28], protein and peptide modification [29], hemolysis, formation of fluorescent lipid complexes and oxidation of low density proteins [30].

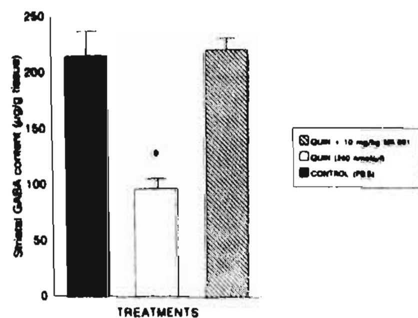
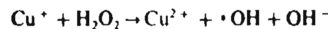


Fig. 3. Effect of quinolinic acid (QUIN) on striatal GABA content. Rats were administered i.p. with saline or dizocilpine (MK-801, 10 mg/kg) 1 h before phosphate buffered saline (PBS, pH 7.4) or QUIN (240 nmol/µl) intrastriatal injection. Seven days after, GABA concentration was measured by OPA-derivatization/high performance liquid chromatography in lesioned striata. Mean values of $n = 6$–9 independent experiments ± S.E.M. are shown. $^{*}P < 0.05$, different from control; one-way ANOVA followed by Tukey's test.

Copper ions can produce increased DNA strand breaks and crosslinks, a damaging process which is thought to be the result of free radicals' attack to this biomolecule [31]. Moreover, it has been proposed that high levels of copper and iron may generate hydroxyl radicals in brain tissue through the Fenton's reaction [32]:



$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + \cdot OH + OH^{-}$$

On the other hand, manganese, mainly Mn^{3+} species, is suspected to generate free radicals in those brain regions in which the redox activity is high, such as basal ganglia, via an increased autoxidation of dopamine [33].

Manganese neurotoxicity, probably mediated by Mn(III), involves alterations in calcium homeostasis [34], autooxidation of dopamine [35], formation of reactive oxygen species [36], production of 6-hydroxydopamine or other toxic catecholamines and quinones [37], and decreased brain catalase and GSH-peroxidase levels [38]. All these factors may account for the redox stress that leads to cell damage [39].

Although the evidence presented in this work might initially suggest that QUIN-induced lipid peroxidation found in previous reports [4,5] could be partially attributed to an accumulation of copper and manganese after QUIN administration [7–9], other evidence suggests that copper can also be partially neuroprotective. For example, a deficiency of copper can cause neuronal degeneration [40].

A possible explanation of the observed alterations in metals content is that, as a consecuence of neurodegeneration, changes could be the result of a glial response to damage, as it has been proposed in the case of increased iron after QUIN-induced striatal lesion [10]. Nevertheless, a role of metal ions as toxic agents contributing to cell damage after NMDA receptor overstimulation can not be discarded and remains to be elucidated.

The ability of dizocilpine to completely prevent QUIN-induced metals accumulation suggests that increased amounts of copper and manganese in the striatum is exclusively dependent on NMDA-receptor activation, since dizocilpine was completely effective to prevent QUIN-induced GABA depletion, verifying its antagonistic potency on NMDA receptor [18].

In conclusion, increased copper and manganese striatal content may be the result of several processes activated by QUIN: (a) increased uptake of metals by glial or neural elements, (b) decreased release of transition metals from intracellular depots, and (c) increased expression of metal-binding proteins in brain.

All these possibilities or the combination of them may provide interesting working hypotheses to explain NMDA-mediated neurotoxicity.

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Original Article

Changes in Transition Metal Contents in Rat Brain Regions After *In Vivo* Quinolinate Intrastriatal Administration[1]

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Abstract

Total copper and manganese contents were measured in five rat brain regions 7 days after a unilateral striatal injection of quinolinic acid (QUIN, 240 nmol/1 µl), an endogenous N-methyl-D-aspartate (NMDA) receptor agonist. Concentrations of both transition metals were evaluated in tissue of brain cortex, hippocampus, corpus striatum, midbrain and cerebellum of saline- and QUIN-treated rats using graphite furnace atomic absorption spectrophotometry. Increases in copper content were observed after QUIN striatal injection in cerebellum, hippocampus, midbrain and corpus striatum (37, 55, 71 and 152% as compared against control values, respectively) but not in brain cortex. Manganese levels were found enhanced only in corpus striatum of QUIN-treated rats by 35% vs. control values, but not in all other brain regions analyzed. QUIN-induced increases in regional copper content were partially prevented in hippocampus, midbrain and striatum (17, 57, and 23% vs. control, respectively) by pretreatment of rats with an NMDA receptor antagonist, dizocilpine (MK-801, 10 mg/kg, i.p.), administered 60 min before QUIN microinjection. The same protective effect of dizocilpine was observed against QUIN-induced enhancement of striatal manganese content (-0.45% vs. control). These findings resemble those changes observed in post-mortem Huntington's disease brains and suggest that alterations in regional content of copper, but not in manganese, may be a consequence of the spreading of QUIN-induced neurotoxic events into the striatal tissue to the neighboring regions of the brain, by action of QUIN on NMDA receptors. (*Arch Med Res* 1996; 27:449)

KEY WORDS: Quinolinate; NMDA receptor; Copper; Manganese; Brain regions; MK-801; Excitotoxicity

Introduction

Quinolinic acid (QUIN), a neurotoxic tryptophan metabolite, is also an endogenous agonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. QUIN is able to reproduce some neurochemical and histopathological features of Huntington's disease (HD), such as gamma-aminobutyric (GABA) depletion and striatal spiny cell loss, if administered topically into the rat corpus striatum (1). QUIN has been described to be involved in neurodegenerative inflammatory and infectious diseases (2). QUIN-induced neurotoxicity can be explained by its ability to overstimulate NMDA

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receptors and massive calcium entry into the cells (3). An involvement of oxidative stress in QUIN-mediated excitotoxicity has also been described (4,5).

On the other hand, some transition metals are involved in oxidative events such as lipid peroxidation. High amounts of copper and iron are commonly associated with generation of hydroxyl radicals in brain tissue through the Fenton reaction (6).

It has been demonstrated that after a single unilateral injection of quinolinate into the rat ventral striatal region, increased iron is accumulated in high amounts in basal ganglia areas such as globus pallidus and substantia nigra pars reticulata (7). In addition, there is some evidence of increased concentrations of copper and iron in caudate nucleus, putamen, substantia nigra and cerebral cortex of post-mortem HD human brains (8). Furthermore, such a pattern of increased copper concentration in HD patients is not similar to those observed for other neurodegenerative diseases of the basal ganglia, such as Parkinson's disease (PD) and progressive supranuclear palsy, suggesting that specific changes in transition metals are linked to HD due to the different neuronal populations affected in such a disease (8).

In the case of manganese, Mn(III) neurotoxicity, mainly mediated by oxidative stress and free radicals generation in brain regions with high redox activity seems to be related to the production of superoxide and hydroxyl radical (9) and decreased levels of brain catalase and GSH-peroxidase (10).

In order to investigate the possible link between QUIN-induced neurotoxicity, NMDA receptor-mediated excitotoxicity and copper and manganese accumulation in rat brain regions, we conducted experiments to detect possible changes in brain regional content of these metals as a consequence of QUIN intrastriatal injection to rats.

Materials and Methods

Male Wistar rats (250 - 300 g) were used throughout the study. Animals were provided with Purina Rodent Chow and water *ad libitum*, and maintained under standard conditions of temperature, humidity and lighting.

For preparation of all solutions, deionized water (Milli R/Q water purifier) was used. Water was further purified for graphite furnace atomic absorption spectrophotometry (GFAAS) using a chelating resin column (Chelex, Sigma Chemical Co., St. Louis, MO, USA).

Rats (5 - 9 per group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). QUIN was dissolved in 0.1 M phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 0.1 N NaOH. Single unilateral 1 µl injections were made into the right striata of rats at the stereotaxic coordinates 0.5 mm anterior to bregma, 2.6 mm lateral to bregma and 4.5 mm ventral to the dura, according to the brain Atlas of Paxinos and Watson (11). Then, 240 nmol of QUIN was injected over a 2-min period. Control animals were similarly administered with PBS alone. In order to test a possible participation of NMDA receptor on QUIN-induced copper and manganese changes, a group of rats was previously treated with the NMDA receptor antagonist, dizocilpine (10 mg/kg, i.p.), 1 h before QUIN injection.

Copper and manganese contents in rat brain regions were analyzed as previously described (12) by GFAAS. Seven days after the striatal injection of QUIN or PBS, rats were sacrificed by decapitation, their brains removed and tissue samples from cerebral cortex, hippocampus, corpus striatum, midbrain and cerebellum were dissected out on ice, according to Glowinski and Iversen (13). Then, samples were digested with 1 ml of concentrated Suprapur HNO_3 (E. Merck) in a shaking water bath at 60°C for 30 min. After digestion, a 100 µl aliquot was taken from the clear solution and diluted (1:20, v:v) with an aqueous solution containing 0.1% w/v Triton X-100 and 1% diammonium hydrogen phosphate. Aliquots of the solution were then injected into the graphite furnace for copper and manganese analysis. Calibration curves were constructed for each element by adding known amounts of the respective Titrisol atomic absorption spectrophotometric standard solution to brain tissue digested samples, as described above.

Analysis of the diluted samples was performed using a Perkin-Elmer 360 Atomic Absorption Spectrophotometer with an HGA-2200 graphite furnace. For analysis of both samples and standards, a volume of 20 µl of each final diluted solution was injected into the furnace. Furnace program for metals detection was optimized by measuring the standards signal height at several temperature settings, for both char and atomization stages, according to the procedure of Welz (14).

Brain regional copper and manganese concentrations, expressed as mg/g of wet tissue, were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test (15). Values of $p < 0.05$ or $p < 0.01$ were considered of statistical significance.

Results

Values of brain regional copper content after striatal administration of QUIN and pretreatment of rats with dizocilpine are shown in Figure 1. Copper concentrations in cerebellum, hippocampus, midbrain and corpus striatum (but not brain cortex) after QUIN injection were increased by 37, 55, 71 and 152% as compared against control values, respectively (see Figure 1); whereas dizocilpine pretreatment to rats was effective in preventing these effects (17, 57 and 23% vs. control in hippocampus, midbrain and striatum, respectively) (see again Figure 1).

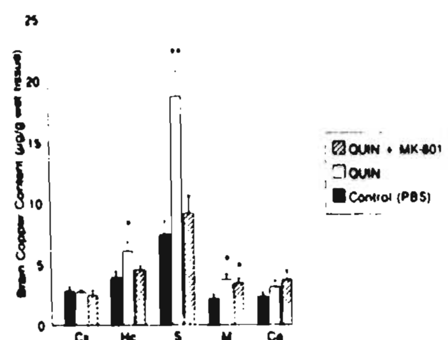

Figure 1. Effect of quinolinic acid (QUIN) intrastnatal injection on rat brain regional copper content. Rats were i.p. injected with saline or dizocilpine (MK-801, 10 mg/kg) 1 h before a phosphate buffered saline (PBS, pH 7.4) or QUIN (240 nmol/µl) intrastriatal administration. Seven days later, copper levels were measured by graphite furnace atomic absorption spectrophotometry in brain cortex (Cx), hippocampus (Hc), corpus striatum (S), midbrain (M) and cerebellum (Ce). Mean values of six to nine independent experiments ± SEM are shown. *p <0.05, **p <0.01, differences against control values; Dunnett's test.

Figure 2 shows manganese levels in five rat brain regions after QUIN intrastriatal injection. Manganese concentrations were significantly increased only in corpus striatum of QUIN-treated rats by 35% against control values, but not in all other brain regions analyzed (see Figure 2). Accumulation of manganese induced by QUIN injection was fully prevented by pretreatment to rats with dizocilpine by -0.45% vs. control (see again Figure 2).

Discussion

Data presented in this work show a remarkable increase in copper content mainly in hippocampus, midbrain and corpus striatum after intrastriatal injection of quinolinic acid. We also observed a significant increase in manganese content in corpus striatum, but not in all other rat brain regions analyzed.

Accumulation of transition metals, such as copper, iron and manganese, has been associated with neurodegenerative processes. Wilson's disease is an example of a genetic alteration produced by excessive accumulation of copper in brain and liver (16). It seems that the severity of the neurological signs in Wilson's disease relates to the extent of copper accumulation in the brain (17).

Free copper is normally found at low concentrations in the brain and blood plasma (18). However, at high concentrations, free copper seems to be a potent free radical generator (19), causing hemolysis, formation of fluorescent lipid complexes and oxidation of low density proteins (20), and such effects are related to oxidative stress induced by this metal.

Excessive accumulation of copper in putamen and substantia nigra in post-mortem brains of HD subjects has also been reported (8), pointing out a similarity between QUIN-mediated cell damage and the selective neuronal destruction observed in Huntington's disease. On the other hand, manganese neurotoxicity involves changes in calcium homeostasis (21) among other toxic events. In regard to this issue, it is well known that the neurotoxicity induced by QUIN is mediated by alterations in calcium levels in the intracellular space (22). Therefore, manganese could contribute to such an effect of QUIN by modifying calcium movements in the nervous system. Manganese is also responsible for production of toxic metabolites such as hydroxydopamine, oxidized catecholamines and quinones (23). All these factors can account for the manganese-mediated redox stress that leads to cell damage (24).

The evidence presented in this work could be partially taken to hypothesize that QUIN-induced enhanced lipid peroxidation found in our previous reports (4,5) could be

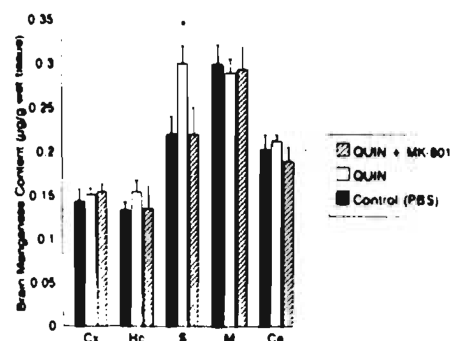

Figure 2. Effect of quinolinic acid (QUIN) intrastriatal administration on rat brain regional manganese content. Rats were i.p. injected with saline or dizocilpine (MK-801, 10 mg/kg) 1 h before a phosphate buffered saline (PBS, pH 7.4) or QUIN (240 nmol/µl) intrastriatal administration. Seven days later, manganese levels were measured by graphite furnace atomic absorption spectrophotometry in brain cortex (Cx), hippocampus (Hc) corpus striatum (S), midbrain (M) and cerebellum (Ce). Mean values of six to nine independent experiments ± SEM are shown. *p <0.05, differences against control values, Dunnett's test.

partially attributed to a high copper and manganese accumulation in corpus striatum, and copper increase in other brain areas, as such metals are known to induce oxidative stress, via the production of free radicals and lipid peroxidation, when it is present in high concentrations in brain tissue (25,26).

Dizocilpine was partially effective in preventing the QUIN-induced increase of these transition metals, suggesting that the accumulation of copper and manganese ions is partially dependent on the NMDA-receptor activation by QUIN. Metals accumulation after QUIN striatal lesion is not likely to be just a consequence of neural loss mediated by NMDA receptor overactivation, but it also could be the result of a glial response to damage, as has been demonstrated in the case of increased iron content after QUIN-induced striatal lesion (7).

Regional changes in copper content after QUIN intrastriatal injection could also be related to the spreading of QUIN-induced toxic effects, leading to an extensive cell damage mediated by the mobilization of metals to the neighboring regions, such as hippocampus, or even in those regions which are distant to the lesioned site, such as midbrain and cerebellum.

In summary, increased brain content of copper and manganese may be the result of several processes activated by QUIN such as increased uptake of metals by glial or neuronal components, decreased release of transition metals from intracellular depots or increased expression of metal-binding proteins in brain. Combinations of all these possibilities, which in turn could be mediated by alterations in calcium homeostasis induced by QUIN, may provide working hypotheses to explain NMDA receptor-mediated neurotoxicity. How such an increased regional contents of transition metals may contribute to QUIN neurotoxicity will be investigated in further studies.

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El papel del cobre en la peroxidación de lípidos inducida por el ácido quinolínico en cuerpo estriado de rata

La evidencia obtenida a partir de la evaluación de los niveles cerebrales de cobre y manganeso después de la aplicación *in vivo* del QUIN, que muestra cambios significativos de estos metales sobre todo en la región lesionada, el cuerpo estriado, conducen a la interrogante de cuál es el efecto específico de estos metales en el daño oxidativo generado por el QUIN en el cerebro. En particular, el cobre es uno de los metales más abundantes en el CNS y se encuentra involucrado en el control de múltiples procesos metabólicos. La mayor proporción de dicho metal se encuentra unido a proteínas reguladoras de actividad redox, tales como la ceruloplasmina y la metalotioneina, modulando la homeostasis del fierro y de otros metales. Por su parte, se ha mostrado recientemente que el patrón de daño oxidativo producido por el QUIN en el cerebro depende en gran medida del ión fierro, un promotor de radicales libres. Sin embargo, aquella porción de cobre que se encuentra en el cerebro en forma libre ha sido relacionada frecuentemente al daño oxidativo mediante procesos tales como la reacción de Fenton:

$$Cu^{+} + H_2O_2 \dashrightarrow Cu^{2+} + \cdot OH + OH^{-}$$

Más aún, una considerable cantidad de evidencias referidas en los artículos previos, apuntan a que la carencia de cobre en el cerebro puede producir neurotoxicidad relacionada a la imposibilidad de regular una serie de procesos fisiológicos dependientes de cobre y también que la administración de cobre bien puede estar relacionada a la inhibición selectiva de receptores para NMDA, así como a la inducción de ceruloplasmina. Todos estos eventos controversiales descritos en el siguiente reporte, establecen las bases para la evaluación del efecto del cobre a corto plazo sobre la peroxidación de lípidos inducida 2 horas después de la

inyección del QUIN *in vivo* en el cuerpo estriado de ratas lesionadas. Dicho informe conserva el formato en el que será enviado para su publicación.

ANEXO V

SYSTEMIC ADMINISTRATION OF COPPER SULFATE BLOCKS QUINOLINIC ACID-INDUCED STRIATAL LIPID PEROXIDATION

Running head: **COPPER AND NMDA RECEPTOR-MEDIATED OXIDATIVE INJURY**

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors are highly modulated glutamate entities involved in the control of several metabolic responses. Inhibition of their activity during excitotoxic conditions would represent an alternative for treatment of neurological diseases, since excessive activation of NMDA receptors is thought to be involved in neurodegenerative processes. In this work, increasing doses of copper (II), as sulfate, (2.5, 5.0, 7.5 and 10.0 mg/kg), administered i.p. to rats, were used to investigate whether or not this essential metal is able to modify the lipoperoxidant action of quinolinic acid (QUIN), an NMDA receptor agonist. Copper doses of 2.5, 5.0 and 7.5 mg/kg antagonized quinolinic acid-induced striatal lipid peroxidation (LP), as measured 2 hours after a single unilateral intrastriatal 1 µl-injection of quinolinate (240 nmol/µl). Dose-dependency was found since partial protection was observed with the 2.5 mg/kg dose of copper, whereas complete prevention of LP was observed at doses of 5.0 and 7.5 mg/kg. The dose of 10 mg/kg of copper was lethal to animals. The administration of ascorbate (500 mg/kg, i.p.), either alone or in combination with copper (5 mg/kg), QUIN or copper plus QUIN, did not significantly modify any of the effects of these drugs when administered separately. We conclude that copper ions prevent QUIN-induced LP by a protective mechanism perhaps involving blockade of NMDA receptors.

Key words: Excitotoxicity, Copper, NMDA receptor, Quinolinate neurotoxicity, Lipid peroxidation, Corpus striatum.

INTRODUCTION

Blockade of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors by NMDA antagonists represents a potential therapeutic strategy to prevent the neurotoxic events observed after hypoxia, ischemia, trauma, hypoglycemia and several other neurodegenerative conditions.[1] However, most synthetic NMDA antagonists can not be still considered the most appropiate therapeutic agents against excitotoxic events since they have been shown to produce neuronal damage.[2] Therefore, it is highly desirable to find alternative agents, preferentially endogenous molecules, capables of modulating NMDA receptor activity. So far, some endogenous NMDA modulators, such as glutathione[3] and kynurenic acid,[4] have been described to be effective in preventing NMDA-mediated neurotoxicity.

We have previously shown a significant increse in the striatal content of copper in rats topically lesioned with quinolinic acid (QUIN),[5] a well-known NMDA receptor agonist. On the basis of other previous findings, this change could be related to a tissular response after QUIN-induced oxidative stress.[6] Recently, a modulatory effect of copper ions on NMDA receptor responses has been reported in hippocampal neurons of rodents,[7] also suggesting that this trace metal can inhibit the activity of those receptors.

In order to elucidate the precise role of copper ions on NMDA receptor-mediated oxidative damage, in this work we tested the ability of this metal to prevent QUIN-induced *in vivo* lipid peroxidation, a toxic process observed after overactivation of NMDA receptors.[6] Our findings show for the first time an antagonistic action of copper on NMDA receptor-mediated oxidative damage which may be related to a putative

inhibition of NMDA receptor activation, suggesting that copper ions could be acting as NMDA antagonists.

MATERIALS AND METHODS

Groups of 5-11 male Wistar rats (250-300 g) were injected i.p. with copper (II) sulfate (2.5, 5.0, 7.5 or 10 mg Cu^{2+}/kg)[8] and/or ascorbic acid (500 mg/kg) in 1 ml of saline. Animals were then immediately anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and 30 min later, infused for two min with a single intrastriatal injection of quinolinate (240 nmol/µl)[6] dissolved in 1 µl of saline at the following stereotaxic coordinates: 0.5 mm anterior to bregma, 2.6 mm lateral to bregma and 4.5 mm ventral to the dura.[9] Control animals were similarly administered, intrastriatally or intraperitoneally, with saline (pH 7.4). Two hours after microinjection, animals from all groups were killed by decapitation and their striata dissected out on ice and briefly stored at -5°C until their biochemical analysis. The measurement of lipid peroxidation in the rat brain by the assay of lipid fluorescent products used by us in this study has been previously described.[6,10] Briefly, the striatal tissue samples were homogenized in 3 ml of saline. One-ml aliquots were then added to 4 ml of a chloroform-methanol mixture (2:1, v/v). After vortexed during 10 seconds, mixture was ice-cooled for 30 min to permit phase separation and the fluorescence of the chloroformic layer was measured in a Perkin-Elmer LS50B luminescence spectrometer at 370 nm of excitation and 430 nm of emission wavelenghts. The sensitivity of the equipment was adjusted to a fluorescent signal of 130-140 fluorescence units with a quinine standard solution (0.1 µg/ml). Results were expressed as fluorescence units per mg of protein. Protein was measured in the homogenates according to Lowry's assay.[11] All data were analyzed by a one-way ANOVA followed by Tukey's test.[12]

RESULTS

Figure 1 shows the effect of increasing i.p. doses of copper (II) sulfate alone on lipid peroxidation from corpora striata of rats intrastriatally administered with 1 μl saline. Copper had no significant effect on basal lipid peroxidation (100 %) at any dose tested (135 %, 119 % and 112 % as compared to control values at 2.5, 5.0 and 7.5 mg/kg, respectively). At a dose of 7.5 mg/kg, copper produced about 40 % of mortality, whereas the 10 mg/kg dose showed 100 % of mortality before measurement of lipid peroxidation.

LOCATE FIGURE 1 HERE

Figure 2 presents the effect of increasing doses of copper on the quinolinate-induced striatal lipid peroxidation. QUIN alone significantly increased lipid peroxidation by 184 %, as compared to control rats (100 %). The combination of QUIN plus copper resulted in a dose-dependent protective effect, with partial prevention of QUIN-induced lipid peroxidation observed at the 2.5 mg/kg dose of copper (133 %), as compared to control values (133 %). Complete protection of QUIN-induced lipid peroxidation was observed at copper doses of 5.0 and 7.5 mg/kg, being not statistically different from control values (77 % and 84 % of control, respectively).

LOCATE FIGURE 2 HERE

The i.p. administration of ascorbate (500 mg/kg) to rats intrastriatally treated either with saline or QUIN previously treated i.p. with $CuSO_4$ (5.0 mg/kg) had not effect on the action of any of these drugs when administered (data not shown).

DISCUSSION

Lipid peroxidation is a deletereous process affecting both the structure and function of cell membranes. Lipoperoxidation currently occurs in the mammalian brain due to the high amount of fatty acids contained in neural tissue. Some pathologic factors in the nervous system, such as excessive activation of NMDA receptors[6], have been related to the generation of free radicals, leading to lipid peroxidation as an index of oxidative stress.

On the other hand, copper is an important component of the brain; most of this trace element remains bound to some transport and storage proteins, such as ceruloplasmin. Copper is involved in several metabolic processes and is also required as a cofactor for the activity of some enzymes. However, due to its high reactivity, copper can also be associated to cell damage if it remains free in brain tissue[13], because some reactions involving the generation of free radicals, such as Fenton's reaction, are dependent on the presence of free copper or iron.

The findings of the present study indicate that copper is able to prevent the NMDA receptor-mediated *in vivo* QUIN-induced lipid peroxidation in rat corpus striatum. Copper ions, when administered alone, did not produced any significant effect on the striatal lipid peroxidation at doses of 2.5 and 7.5 mg/kg, whereas 5.0 mg/kg resulted in a moderately enhanced peroxidation. The protective action produced by this metal on quinolinate oxidative injury was partially observed at dose of 2.5 mg/kg, while complete protection was found at 5.0 and 7.5 mg/kg, suggesting that some dose-dependency would be found between the first two doses tested. The dual action of copper described above, either as a modulatory element or as a neurotoxic metal, as well as its balance

between free and bound amounts, might eventually serve to explain both its pro-oxidant effect observed when injected alone at the dose of 5.0 mg/kg on basal lipid peroxidation and its protective effect at the same dose when administered against quinolinate injury. Although copper resulted protective in our experiments, the precise mechanism(s) by which this trace metal is acting to attenuate such an index of neurotoxicity still remain unknown.

In a previous report[5] showing a significant increase in the total amount of copper after the intrastriatal injection of quinolinate to rats, we suggested that some of the neurotoxic effects of quinolinate in the nervous system, such as oxidative stress and neurodegeneration, would be partially related to a disruption in the balance of copper into the brain; however, considering the findings of the present study, it is likely that copper could be eventually more related to modulatory and neuroprotective actions than to neurotoxicity. Such an idea is reinforced by recent evidences demonstrating that deficiency of copper can produce neuronal degeneration in rats[14], resembling those findings showing that some neurological alterations, such as Menkes syndrome, as well as their respective experimental models, are related to a defect in copper metabolism. Moreover, it has been shown that free copper is capable to antagonize the responses mediated by NMDA receptors in a voltage-independent inhibition[7], also suggesting that Cu^{2+} may act on an extracellular binding site of the NMDA receptor-channel complex. Considering these evidences together, it is likely that copper is acting as a potent inhibitor of NMDA receptor-mediated responses either under *in vitro* or *in vivo* conditions.

Thus, what is the copper species involved in its antioxidant effect? It has been reported that intraperitoneal administration of increasing doses of copper (similar to those

employed in this study) to rats reached peak concentrations of this metal in hypothalamus when measured 30 minutes after its injection[8]. In the same report, ascorbate (500 mg/kg) decreased the levels of hypothalamic copper, also reducing some of its toxic effects, such as increased dopamine and decreased noradrenaline levels. Therefore, a protective action of ascorbate was suggested on copper-induced changes in brain catecholamines on the basis of vitamin-induced changes in copper ion status: ascorbate is able to decrease the brain copper content by limiting the rate of its absorption when Cu^{2+} is reduced to Cu^{+}. In contrast, in our experiments ascorbate had no effect on copper inhibition of quinolinate-induced lipid peroxidation when coadministered. From these results one would initially say that under our experimental conditions, considering the lack of effect of ascorbate on copper antioxidant action, Cu^{+} and Cu^{2+} are both equally responsible for such an effect. However, it is well known that Cu^{+}, which have to be predominant in the presence of ascorbate, is related to oxidative events when is active during Fenton's reaction. One possible explanation for this is that both species of copper could be forming inactive complexes with other small molecules, such as glutamate[7].

Further explanations for the observed protective effect of copper against the quinolinate-induced lipid peroxidation have been recently reported: a) Since it is known that quinolinate-induced lipid peroxidation in the brain is dependent on the presence of iron[15], and also that copper is an essential component of many metalloproteins which serve regulatory purposes[14], it is probable that elevated concentrations of copper might enhance the activity of some cuproenzimes having ferritin-like or ferroxidase activity such as ceruloplasmin and metallothionein[16], modulating and preventing the oxidative action of iron; b) some interesting interactions between copper and iron have been also reported[13], pointing out a possible synergistical relationship in which overload of one of

them might result in modified absorption of the other by changing the mechanisms of brain transport, which would eventually serve to explain changes in the distribution of both of these metals in the brain and the consequent modification of the basal oxidative activity; c) copper has been also related to the inhibition of nitric oxide synthesis in murine macrophages[17], probably involving a mechanism affecting the expression of nitric oxide synthase, while NMDA receptor-mediated cell damage is known to be related to the production of toxic nitric oxide and other oxygen-derived reactive species[18]. If quinolinate exert its toxic effects via an NMDA receptor-mediated production of nitric oxide which will lead to a further generation of free radicals, it is likely that copper could be acting by inhibiting of nitric oxide.

Regarding the evidences discussed here, there are some questions emerging from the finding that copper may reduce the activity of NMDA receptors[7] in direct relationship to our previous report[5]: it is possible that the increased copper concentrations observed after the striatal injection of quinolinate to rats could be partially a response oriented to reduce the activity of NMDA receptors or it was merely the result of copper removal from some storages after cell death?, what is the precise mechanism of action of copper in the CNS?, is the action of copper depending on its bound or on its free status?, if so, its action also depends on its mono- or divalent species? Whatever the answer to all these questions, copper seems to offer an interesting alternative to understand some modulatory mechanisms in the brain as well as it represent a promising therapeutic tool for treatment of neurological alterations.

CONCLUSION

This work shows that copper is capable to prevent the NMDA receptor-mediated lipid peroxidation after its excessive activation by quinolinic acid. Whatever the precise

mechanism of copper against quinolinate-induced lipid peroxidation found in our system, the relevance of these results arise from the facts that they were observed under *in vivo* conditions and that copper is a trace metal present in physiololgical concentrations in the brain, supporting a role of copper as an endogenous modulator of NMDA receptors.

ACKNOWLEDGEMENTS

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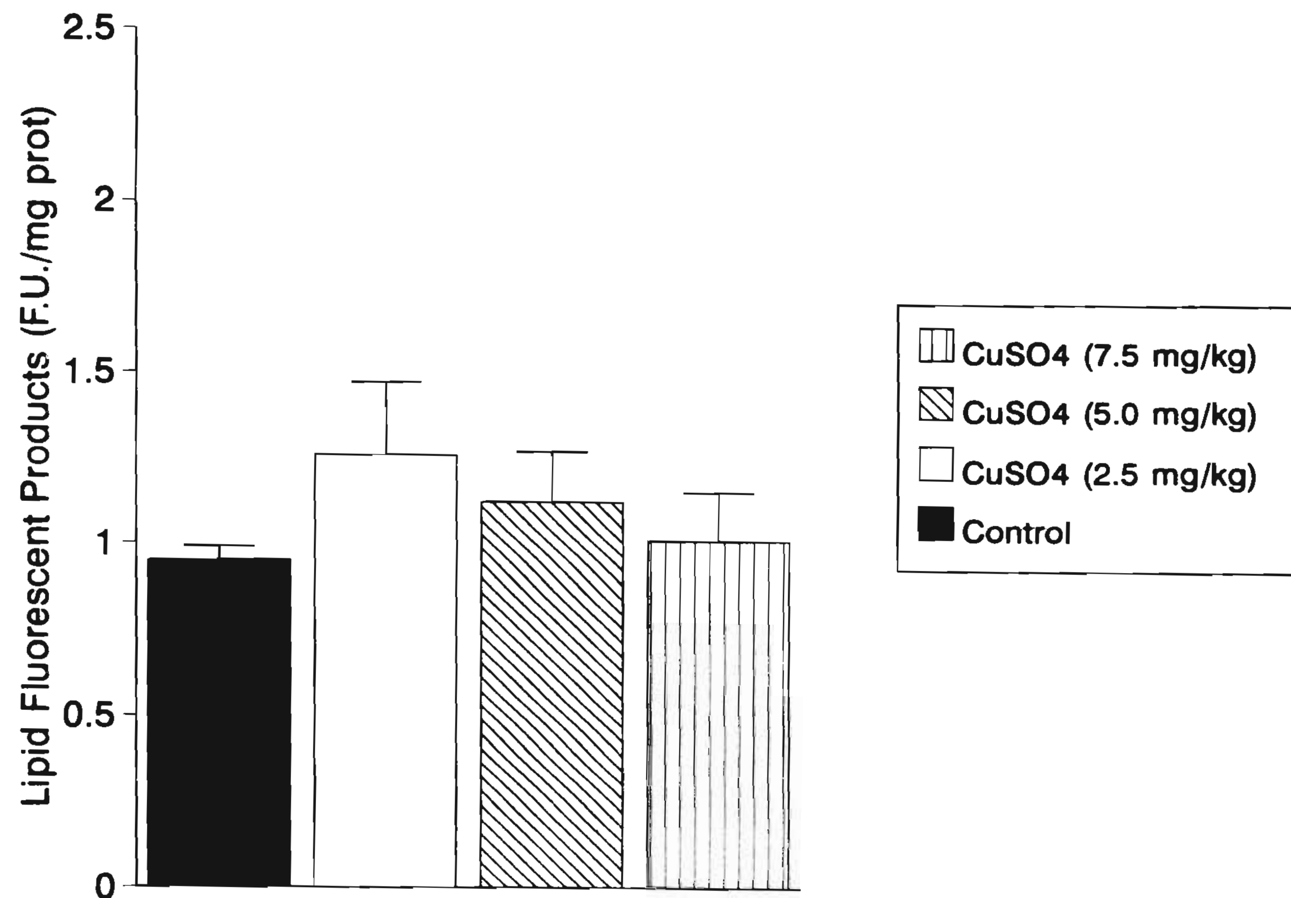
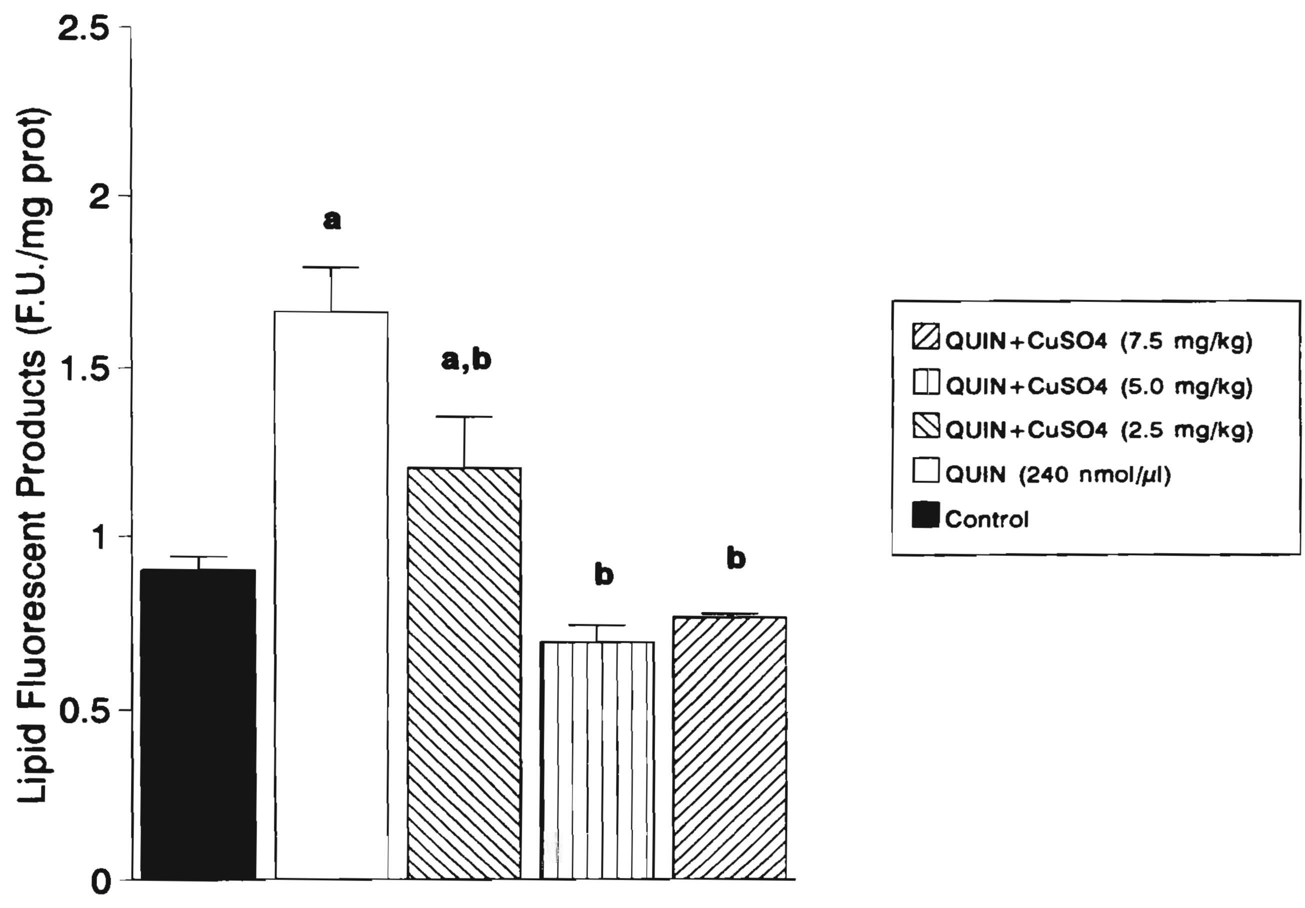
FIGURE LEGENDS

FIG. 1. Effect of systemic copper (II) sulfate on basal lipid peroxidation from rat corpus striatum. Male Wistar adult rats were administered i.p. either with saline or copper as sulfate (2.5, 5.0 and 7.5 mg/kg) 30 minutes before a single intrastriatal injection of 1 µl saline (pH 7.4). Two hours after the lesion, lipid peroxidation was measured in the injected striata by the assay of lipid fluorescent products. Averages of n=5-11 independent experiments ± S.D. are shown. Statistical analysis was performed using one-way ANOVA followed by Tukey's test.

FIG. 2. Effect of systemic copper (II) sulfate on quinolinate-induced striatal lipid peroxidation in rats. Animals were administered i.p. with copper as sulfate (2.5, 5.0 and 7.5 mg/kg) 30 minutes before a single intrastriatal injection of 1 µl quinolinic acid (240 nmol/µl). Control animals received saline (pH 7.4) i.p. or intrastriatally. Two hours after the lesion, lipid fluorescent products were assessed as an index of lipid peroxidation. Mean values of n=5-11 independent experiments ± S.D. are shown. [a] $p<0.05$, differences against control values. [b] $p<0.05$, differences against QUIN treatment; one-way ANOVA followed by Tukey's test.


Lipid Fluorescent Products (F.U./mg prot)
2.5
2
1.5
1
0.5
0
CuSO4 (7.5 mg/kg)
CuSO4 (5.0 mg/kg)
CuSO4 (2.5 mg/kg)
Control


Lipid Fluorescent Products (F.U./mg prot)
2.5
2
1.5
1
0.5
0
a
a,b
b
b
QUIN+CuSO4 (7.5 mg/kg)
QUIN+CuSO4 (5.0 mg/kg)
QUIN+CuSO4 (2.5 mg/kg)
QUIN (240 nmol/μl)
Control

El papel del fierro y del glutatión en la peroxidación de lípidos inducida por el ácido quinolínico en sinaptosomas y capilares de cerebro de rata

El siguiente paso en la caracterización de factores generadores de estrés oxidativo inducido por el QUIN corresponde a la evaluación del efecto del fierro endógeno en el CNS como posible factor potenciador de la peroxidación de lípidos dentro del patrón de toxicidad probado en este estudio. El fierro, un metal esencial que normalmente modula la actividad de algunas proteínas reguladoras del metabolismo celular y que en su mayor proporción se encuentra en el cerebro unido a dichas proteínas, es también un potente agente pro-oxidante cuando se presenta en su forma libre. Como podría contribuir este metal al daño oxidativo inducido por el QUIN? Se sabe que la acción oxidante del fierro tiene que ver con su participación en reacciones que generan radicales libres, empleando para ello sustratos endógenos tales como el peróxido de hidrógeno (reacción de Fenton). Por su parte, una de las características en la toxicidad del QUIN es incrementar la actividad metabólica celular, lo cual genera múltiples sustratos de radicales libres disponibles para reaccionar con el fierro, mismo que también se incrementa en su forma libre como resultado de la acción del QUIN. Más aún, se ha demostrado recientemente (Goda y col., 1996) la capacidad del QUIN de formar un complejo con el fierro libre que resulta ser más tóxico y pro-oxidante que cuando se considera la actividad de cada una de estas moléculas por separado.

En este contexto surge la iniciativa de evaluar el efecto de otra molécula endógena, el glutatión (GSH-GSSG), como posible inhibidor del daño oxidativo producido por el QUIN. El GSH, o especie reducida del glutatión es conocido por intervenir en reacciones que generan radicales libres inclusive por acción del fierro u otros metales de transición, inhibiendo así este

proceso. Por su parte, la forma oxidada del glutatión o GSSG presenta una interesante actividad inhibiendo la actividad de los receptores para NMDA por su unión selectiva a éstos, y bloqueando así la posible toxicidad generada por al excesiva activación de dichos receptores. Desde esta perspectiva es de gran importancia analizar el efecto que esta molécula pueda expresar en este modelo.

A continuación se presenta el correspondiente artículo conteniendo los resultados de estas evaluaciones, considerando no solo los efectos de estas dos importantes moléculas, sino también mostrando un estudio comparativo que contempla el empleo de dos preparaciones biológicas muy específicas (sinaptosomas y capilares cerebrales) de tres diferentes regiones cerebrales (hipocampo, cuerpo estriado y corteza entorrinal) especialmente susceptibles al ataque por radicales libres y a la toxicidad del QUIN. Se presentan datos comparativos también de la posible influencia de la edad (ratas jóvenes y adultas) en la generación de eventos oxidativos mediados por el QUIN; y por último, se evalua el efecto de otro antagonista NMDA, el ácido 2-amino-5-fosfonovalérico (APV), sobre la peroxidación de lípidos.

Este trabajo se realizó en colaboración con el Dr. Frantisek Stastny del Departamento de Neurobiología Molecular, Instituto de Fisiología, Academia de Ciencias de la República Checa.

ANEXO VI

Lipid peroxidation in synaptosomes and microvessels isolated from rat brain: Involvement of age, iron and quinolinate

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Abstract

Lipid peroxidation is particularly active in synaptosomes and brain microvessel endothelial cells which are very susceptible to pathological alterations during development. Therefore, the measurement of lipid fluorescent products (LFPs) in brain synaptosomes and capillaries was used as an indicator of their free radical damage. We found that the LFP formation was approximately 6 - 10fold higher in isolated brain capillaries than in synaptosomes from the entorhinal cortex, hippocampus and striatum of 50-day-old rats. In 12-day-old animals the differences were less pronounced owing to regularly lower LFP levels in brain microvessels. The lipid peroxidation was significantly inhibited in the brain microvessels by iron-chelation (deferoxamine; 10 μM), but in the case of synaptosomes, only in those from the entorhinal cortex of adult rats. In contrast, the addition of Fe^{2+} (2 μM $FeSO_4$/250 μM ascorbate) increased the formation of LFPs in synaptosomes to or even above their levels in capillaries from rats of both age groups. Quinolinic acid (QUIN; 100μM), a neuroactive metabolite of tryptophan acting at N-methyl-D-aspartate (NMDA)-type of glutamate receptor, was much less potent peroxidant than exogenous iron. However, the lipid peroxidation was increased significant only in those brain regions in which QUIN potently displaced the binding of L-[^{3}H]glutamate to cell membranes, i. e., in the hippocampal formation and striatum, but not in the entorhinal cortex. The addition of 2-amino-5-phosphonovaleric acid (APV; 250 μM), an inhibitor of the NMDA-glutamate receptor, and reduced glutathione (GSH; 50 μM), a scavenger of free radicals with affinity to the NMDA-receptor, inhibited the increased production of LFPs induced by QUIN with regular efficiency in adult rats.

The results show that the spontaneous formation of lipid peroxides in brain capillary endothelium is more intensive and exhibits high dependence on endogenous iron than in

nerve endings in which, however, a higher activity of nitric oxide synthase (NOS) and exogenous iron can induce more intensive oxidative stress. The excitotoxic action of QUIN includes stimulation of lipoperoxidation in both these cellular-like structures and can be diminished by substances exhibiting an antagonist action at the NMDA-receptor/ion channel complex.

Key words: Lipid peroxidation; Synaptosomes; Brain capillaries; Iron; Quinolinic acid; [^{3}H]glutamate binding; Nitric oxide synthase; 2-amino-5-phosphonovaleric acid; Glutathion

1. Introduction

Oxidative stress, the result of stimulated oxidative metabolic events leading to the formation of O_2-derived free radicals, represents in nerve cells one of the main risk factors in some acute (ischemia, stroke trauma) and chronic (neurodegenerative) disorders of the brain (Braughler and Hall, 1989, Jesberger 1991, Traystman et al., 1991). The O_2-derived free radical species ($O_2^{\cdot-}$, $OH^{\cdot}$) and hydrogen peroxide (H_2O_2) can induce peroxidative damage of membrane lipids and proteins and fragmentation of DNA strands, leading to disruption of nerve cell function and integrity (LeBel and Bondy, 1991).

Lipid peroxidation induced by oxidative stress, is particularly active in isolated nerve terminals that behave as metabolically autonomous minicells and in endothelial cells of brain microvessels, membranes of which are rich in polyunsaturated, highly peroxidable fatty acids (Bénistant et al., 1995). Free radical-induced lipid peroxidation which disturbs the barrier function of endothelial cells (Patel et al., 1995), can causes a failure in the brain capillary transport of iron (Broadwell et al., 1996) and later leads to its accumulation in some brain regions (Shoham et al., 1992; Kondo et al., 1995). The iron derived from blood and/or from storage proteins is, at least partially, chelated to low molecular weight compounds (e.g., ADP, citrate, histidin) which can further potently catalyze the lipid peroxidation by ascorbate-dependent mechanism (Braughler et al., 1986, Miller and Aust, 1989). From this is evident that iron may contribute to initiation and catalytic propagation of nerve and endothelial cell damage which can be attenuated by antagonists of N-methyl-D-aspartate (NMDA)-type of excitatory glutamate (GLU) receptor (Zhang et al., 1993).

Neurons and brain endothelial cells, which possess the NMDA receptors in their membrane surfaces (Monahan and Michel, 1987; Koenig et al., 1992), are particularly vulnerable to the excitotoxic/free radical damage, induced by excitatory amino acids

(glutamate/aspartate) and their agonists (quinolinic acid). Activation of NMDA receptors results in sustained elevation of $(Ca^{2+})_i$ with subsequent overactivation of some Ca^{2+}-dependent enzymes (e.g., calpain I and II, phospholipase A_2, constitutive nitric oxide synthase) and irreversible conversion of xanthine dehydrogenases to xanthine oxidase (Choi, 1988). The xanthine oxidase activity, which is highly enriched in brain endothelial cells (Betz, 1985), is responsible for an increased formation of O_2^- and H_2O_2 and for a further disturbances in Ca^{2+} homeostasis. The resultant increased production of nitric oxide (NO) and its rapid conversion to more stable peroxinitrite (ONOO) in synaptosomes and/or in brain microvessels can be the responsible for NMDA-induced neuronal death (Lafon-Cazal et al., 1993). Resulting brain damage comprises a dysfunction of the endothelial cell barrier (Dietrich et al., 1992; Nag, 1992) which can be accompanied by additional deposits of transient metals in some brain regions (Santamaria et al., 1996).

The concept of oxidative stress-mediated brain cell damage induced by excitatory amino acids is characterized by two main mechanisms: (1) receptor mediated alteration of Ca^{2+} homeostasis, resulting in the formation of O_2-derived free radicals and (2) the iron-catalyzed free radical generation, followed by enhanced lipid peroxidation, leading to changes in neuronal and endothelial cell membrane fluidity, receptor function and ion permeability (Coyle and Puttfarcken, 1993). Also QUIN, a neuroactive metabolite of L-tryptophan with binding affinity at some subpopulations of NMDA receptors (Prado de Carvalho et al., 1996), can act as a potent lipid peroxidant in the rat brain (Rios and Santamaria, 1991; Santamaria and Rios, 1993) by an iron-dependent mechanism (Štipek et al., 1997). Because neuronal susceptibility to QUIN toxicity depends on age (Keilhoff et al., 1991) and brain region vulnerability (Lisý et al., 1994), we decided to bring some new data concerning the mechanism of excitotoxic action of QUIN in those rat brain regions which exhibit the highest sensitive to oxidative stress. Therefore, we studied the formation of lipid fluorescent product (LFP) in enriched fractions of synaptosomes and brain

capillaries isolated from the entorhinal cortex, hippocampal formation and corpus striatum (a) in dependence on age (spontaneous lipid peroxidation); (b) after the addition of Fe^{2+} and/or QUIN (stimulated lipid peroxidation) and (3) to test neuroprotectivity of some endogenous (reduced glutathione; GSH) and exogenous (desferoxamine; DFA) substances, including the NMDA-receptor antagonist (D,L-2-amino-5-phosphonovaleric acid; APV), in relation to the QUIN-stimulated, iron-dependent lipid peroxidation. Our results show that the spontaneous LFP formation in synaptosomes was markedly lower than in freshly isolated brain microvessels in which this process was regularly inhibited by chelation of endogenous iron. In contrast, in nerve endings this process was more sensitive to the addition of exogenous iron than in brain microvessels. In addition, we suggest that the inhibitory action of APV and GSH at NMDA-receptor complex exhibit some age-dependent differences in relation to the QUIN-induced lipid peroxidation.

2. MATERIAL AND METHODS

2. 1. Chemicals

Quinolinic acid (QUIN), ferrous sulfate ($FeSO_4$) hephahydrate, desferoxamine mesylate, D,L-2-amino-5-phosphonovaleric acid (APV), (HEPES) (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic]), chelating resin (iminodiacetic acid), quinine, sucrose and SIGMA 7-9 (Tris [hydroxymethyl]-aminomethane) were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). Sodium, potassium and calcium as chlorides of analytical grade as well as chloroform and methanol were obtained from Merck-Mexico. Ether was from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) and Anestesal (Na-pentobarbital) was from SmithKline Beeham. All solutions were prepared in deionized water made in MILLI-R/q water purifier (Millipore). For rat body perfusions we used isotonic solution of sodium chloride from Abbott-Mexico. Heparin 1000 (Helberina) was from Helber-Mexico. Prior to incubation of tissue samples, the isotonic NaCl solution was

passed through a chelating resin column to remove possible trace amounts of transient metals. Chemicals for γ-glutamyl transpeptidase (GGT) determination (L-γ-glutamyl-p-nitroanilide, glycylglycine, acetic acid, Trizma (Sigma 7-9), and Triton X-100) were from Sigma-Aldrich, Prague, Czech Republic. Also reagents for nitric oxide synthase (NOS) determination, i.e., solution for homogenization (EDTA, EGTA, 2-merkaptoethanol) containing protease inhibitors (aprotinin, leupeptin hydrochloride, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, NP-40) and for reaction mixture (L-arginine, (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, calcium chloride, β-nicotinamide adenine dinucleotide phosphate (NADPH) as tetrapotassium salt, calmodulin) and Dowex-50W-X8-200 mesh were from Sigma-Aldrich. [^{3}H]Arginine (L-[2,3,4,5 - ^{3}H]arginine monohydrochloride, 63 Ci/mmol) and [^{3}H]glutamate (L-G-[^{3}H]glutamic acid, 21 Ci/mmol) were from Amersham, England.

2. 2. *Experimental protocols*

Male Wistar rats at the age of 12 days (18 - 28 g of body weight) or 50 days (200 - 250 g of body weight) were used. Caged animals had free access to food (Purina, Ralston-Nitrocubos, México) and water. The housing room was maintained under conditions of constant temperature (25±3 ^{0}C) and humidity (50±10 %). To standardize diurnal oscillations in rat brain lipid peroxidation (Diaz-Muñoz et al., 1985), all experiments were carried out during morning period (0900-1100 h).

In the first set of experiments, adult rats under light ether anaesthesia were perfused with 100 ml of cold saline solution through the left ventricle of heart, with widely cut right auricle, to eliminate blood from the brain vascular tree. When lipid peroxidation was measured in incubated homogenates or isolated brain microvessels, the perfused animals were decapitated under light ether anaesthesia, the brains were rapidly removed and rinsed in ice cold saline. After removal of meninges, the entorhinal cortices (both entorhinal

cortical areas), the hippocampal formations (hippocampus proper plus dentate gyrus) and corpora striata (nucleus caudatus and putamen) from the left and right half of rat brains were dissected on a cooled plate and transferred into ice cold Ringer's solution (plus 10 mM HEPES, adjusted to pH 7.4). Pooled samples were homogenized and used directly for the determination of LFP in incubated samples or, first, brain microvessels were isolated from the homogenates and the spontaneous LFP generation was assayed in them.

In the second set of experiments, young or adult rats were decapitated in light ether anesthesia and their brains were removed in cold without previous body perfusion. Tissue samples of individual brain regions were prepared from 10-11 rat pups on postnatal day 12 or from 5-6 rats (lipid peroxidation) and/or from 10-16 animals (NOS activity and [^{3}H]glutamate binding) on postnatal day 50. Then, the pooled samples were homogenized in buffered Ringer's solution and used for the preparation of enriched synaptosomal P_2 and capillary fractions (GGT and NOS activity, lipoperoxidation) or in 0.32 sucrose for the isolation of crude synaptosomal membranes ([^{3}H]glutamate binding).

2. 3. Isolation of synaptosomes and brain capillaries

Synaptosomal P_2 and capillary fractions from the entorhinal cortex, hippocampal formation and corpus striatum were isolated according to procedures described by Cotman and Matthews (1971) and Mršulja et al. (1976), respectively. Pooled tissue samples obtained from young and adult rat brains were homogenized in 20 volumes of cold Ringer's solution with 10 mM HEPES (pH 7.4), but without bovine serum albumin. The homogenates were centrifuge (3-times) at 1500 x g for 15 min and decanted supernatants were pooled for isolation of crude synaptosomes (P_2 fraction). Then, synaptosomal sediments were obtained by centrifugation at 10,000 x g for 20 min. The sedimented P_2 material was resuspended in isotonic saline solution, the pH of which was carefully adjusted to the value of 7.0 just prior to use. The third low-speed-sediments obtained at

1500 x g were resuspended in 0.25 M sucrose (pH 7.0), layered on a discontinuous sucrose gradient (1.0 and 1.5 M sucrose, each 12 ml) and centrifuge at 58,000 x g using a Sorvall AH-627 rotor in Sorvall OTD 55b Ultracentrifuge for 30 min. The packed capillary sediments were resuspended in 6 ml of isotonic saline (pH 7.0) and immediately used for incubation and following determination of LFP or frozen and stored for the GGT assay.

2. 4. Assay of GGT activity

The capillary origin of sedimented microvessels was evaluated by the examination of γ-glutamyl transpeptidase (GGT) activity, used as a unique biochemical marker of cerebral endothelium, distinguishing endothelium of small capillaries from that in larger vessels and from synaptosomes of P_2 fraction (Šťastný et al., 1988; Šťastný et al., 1992). For this purpose the sediments of brain microvessels and crude synaptosomes (and/or washed homogenates) were resuspended in 0.9 % NaCl and GGT activity was assayed using γ-glutamyl-p-nitroanilide (2.5 mM) as a donor and glycylglycine (20 mM) as an acceptor of γ-glutamyl residue.

2. 5. Assay of NOS activity

The activity of NOS was measured by means of conversion of [^{3}H]arginine to [^{3}H]citrulline as previously described by Bredt and Snyder (1989; 1990). Freshly isolated synaptosomes and capillaries were resuspended in the homogenization buffer solution (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA and 0.1% 2-mercaptoethanol; pH 7.5) containing protease inhibitors (2 μM/ml aprotinin, 100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor) and 0.1% NP-40. Samples were frozen in liquid nitrogen until use. Thawed samples were rehomogenized and 500 μg of synaptosomal protein or 250 μg of capillary protein was added to the reaction mixture containing 10 μM L-arginine, 2.5 mM $CaCl_2$, 10 μM calmodulin, 30 μM

tetrahydrobiopterin, 1 mM NADPH, 0.2 μCi [^{3}H]arginine and the volume was adjusted to 200 μl by the addition of homogenization solution. After 15 min incubation in a shaking-bath at 37 °C, the samples were transferred into ice and the reaction was terminated by the addition of 1 ml of ice-cold stop-solution (20 mM HEPES, 2 mM EDTA, 2 mM EGTA; pH 5.5). Total volumes of the mixture were passed over the columns of 1 ml Dowex-50W-X8 (Na^+-form) equilibrated with stop-solution. Test tubes were washed with 1 ml of distilled water and remaining trace amounts of radioactivity were applied onto the columns. Solutions were collected into vials to which 5 ml of Bray's scintillation liquid were added.

2. 6. Assay of lipid fluorescent products

The determination of lipid fluorescent products is a complex, but very sensitive method for the measurement of later stages in the lipid peroxidation process (Triggs and Willmore, 1984) which can overcome some problems of the thiobarbituric acid (TBA) test in the correct estimation of lipid peroxidation in the brain development (Krištofiková et al., 1995). The initial method used by Triggs and Willmore (1984) was slightly modified for our *in vitro* determination of LFP. In brief, aliquots of 970 μl of synaptosomal and capillary pellets (or washed homogenates), resuspended in 6 ml of chelating resin-purified isotonic NaCl solution (pH 7.0), were incubated at 37 °C (in a Lab-line Dubnoff incu-shaker) for 60 min in the absence or in the presence in deionized water dissolved DFA (final conc. 10 μM) and/or iron (final conc. 2 μM $FeSO_4$/250 μM ascorbate) as well as after the addition of QUIN (final conc. 100 μM) alone or QUIN plus APV (final conc. 250 μM), QUIN plus GSH (final conc. 50 μM). Final volumes of the mixtures were adjusted to 1000 μl. In some measurements we also tested samples from young animals for a possible additional effect of APV (250 μM) together with GSH (50 μM) on the QUIN-stimulated lipid peroxidation.

The incubation was stopped by the transfer of samples to an ice bath with a subsequent addition of 3 ml of cold chloroform-methanol (2:1) mixture. The mixtures were intensively vortexed for 30 s and stored in a refringerator for 30 min to permit phase separation. After suction of water phase and tissue material off, the fluorescence of the chloroformic layer was measured (0.1 ml of methanol was added to 0.8 ml of extracts) in a Perkin-Elmer LS 50 luminiscence spectrometer at 370 nm of excitation and 430 nm of emission wavelengths. Freshly prepared quinine sulfate, containing 0.1 μg quinine per ml of 0.05 M H_2SO_4, was used as a standard. All values of LFP were expressed in lipid fluorescence units per mg of protein per minute.

2. 7. Membrane preparation and [^{3}H]glutamate binding

Pooled samples of entorhinal cortex, hippocampal formation and corpus striatum obtained from adult rat brains were homogenized in 0.32 M sucrose using a glass homogenizer equipped with a motor driven Teflon pestle. The homogenates were centrifuged at 1000g for 10 min and the pellets were discarded. The supernatants were centrifuged at 48000g for 20 min and the pellets were suspended in 40 vol of cold distilled water. Further steps of membrane preparation were carried out as described (Ragsdale et al., 1989; Folbergrová et al. 1997). The resultant pellets were suspended in 50 mM Tris-acetate buffer (pH 7.4). Aliquots (100 μl) of membraneous suspension (100-300 μg protein) were incubated at 35 °C for 30 min with 10 nM (^{3}H)glutamate and 200 μM quinolinate in a final volume of 1 ml of 50 mM Tris-acetate (pH 7.4). Incubation was terminated by the addition of 4 ml of ice-cold buffer with subsequent filtration through Whatman GF/C glass microfibre filters under a constant vacuum. After repeated washing, the radioactivity trapped on the filters was measured in a modified Triton-toluene scintillant using a Beckman 980 Scintillation Counter (Yoneda and Ogita, 1986). The radioactivity found in the presence of 1 mM nonradioactive glutamate was subtracted from

each experimental value as nonspecific binding to obtain the specitic binding of (^{3}H)glutamate.

All the preparations except of binding incubation were performed at 2 ^{0}C. Buffers were sterilized before use by filtration through a nitrocellulose membrane filter with a pore size of 450 nm to avoid possible microbial contamination (Yoneda and Ogita, 1989).

2.8. Protein determination

Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. In the case of NOS activity determination the Bradford reagent (Sigma) for protein determination was used.

2. 9. Stastistical analysis

Data were analysed by means of one-way analysis of variance followed by Turke's test for multiple comparisons (Steel and Torrie, 1980) and by the Pharmacologic System (t-test I; version 4.0). The values of $p< 0.05$ and $p< 0.01$ were calculated to be significant.

3. RESULTS

3. 1. Brain synaptosomes and microvessels

We used changes in GGT activity as an enzymatic marker of the enrichment of synaptosomes in P_2 fractions and of capillary endothelium in brain microvessel sediments. The specific GGT activity in homogenates was low with relative order of hippocampus>striatum>entorhinal cortex (Table I). Only the difference between the GGT activity in hippocampal and cortical homogenates was significant. Comparing GGT activity in homogenates to that in enriched synaptosomal fraction isolated from the adult brain regions, the differences found initially in homogenates were also preserved in synaptosomal P_2 fractions, but at the higher activity levels than in the initial preparations.

Microvessels isolated from the low-speed sediments were mostly of capillary origin as they showed a 8-9-fold increase in the activity of GGT comparing to brain homogenates.

Incubation of rat brain homogenates in the air atmosphere resulted in spontaneous accumulation of lipid peroxides in the incubated samples as determined at zero time and following 60 min of incubation at 37 °C (Zaleska and Floyd, 1985). In our samples the spontaneous accumulation of LFP elevated minimally a 6-fold (in cortical synaptosomes) and a 10-fold (in striatal capillaries) the endogenous („basal") levels of these products found in freshly isolated synaptosomes or capillaries at zero time of incubation (data not shown). To know if iron contained in biologically inactive forms bound to plasma transferrin and/or in hemoglobin and ferritin may be released by tissue homogenization and perhaps by proteolysis of iron proteins during the isolation of brain microvesels, we measured the amounts of the LFP in homogenates and isolated capillaries from regions of perfused or non-perfused brains of adult rats (Table 2). We found regularly lower levels of LFP present in homogenates prepared from the perfused rat brains in comparison to non-perfused animals. Interestingly, cortical microvessels isolated from these homogenates did not exhibit any differences in the formation of LFP and even increased generation of LFP was determined in the hippocampal and striatal capillary fractions isolated from homogenates prepared from the perfused brains. Taking all the above into considerations, we decided to use enriched fractions of synaptosomes and brain capillaries isolated from the non-perfused rat brains for the further determination of LFP levels.

3. 2. Age-dependent and regional differences in the formation of LFP in synaptosomes and brain capillaries

Studying the age-dependent changes in brain lipid peroxidation, we measured the production of LFP in synaptosome- and capillary-enriched fractions isolated from the most vulnerable brain regions in 12-day-old (young) and 50-day-old (adult) rats (Table 3). We

found that the non-stimulated LFP formation in cortical synaptosomes isolated from the brain of young rats was significantly lower than their levels in synaptosomes isolated from the hippocampi and striata of rats of the same age. In contrast, the „adult" spontaneous levels of LFP in hippocampal and striatal synaptosomes were a 2-fold lower than their levels determined in the cortical synaptosomes. Therefore, a developmental increase in the formation of LFP was demonstrated only in synaptosomes isolated from the entorhinal cortex (+69% *vs* young rats) whereas the generation of LFP decreased significantly in hippocampal and striatal synaptosomes during the same developmental period.

In young rat brains the formation of LFP in capillaries from the entorhinal cortex was a 2-fold higher than that in corresponding preparations from the hippocampus and striatum (Table 3). However, no further developmental increase in the formation of the products was observed in the cortical capillaries whereas in hippocampal and striatal microvessels an approximately twice higher levels of LFP were found in capillary sediments prepared from the adult rat brains.

Comparing the higher spontaneous LFP formation in brain capillaries to that in synaptosomes from young rats, the values in cortical microvessels prevailed a 9-times those in cortical synaptosomes whereas in the hippocampus and striatum the capillary values of these products were about 3-times higher only (Table 3). In adult rat brain, the cortical capillary/synaptosome ratio of the generated LFP reached only a value of 6 which was due to, mainly, their almost unchanged spontaneous generation in microvessels and their significantly higher formation in synaptosomes. In contrast, the hippocampal and striatal ratios in adult animals were 10 and 12, respectively, mainly, owing to higher developmental increases in the spontaneous LFP formation in capillaries isolated from these brain regions, but also owing to their significantly lower formation in the brain synaptosomes.

3. 3. Iron-dependent generation of LFP in synaptosomes and capillaries from young and adult rat brain

To demonstrate a possible dependence of the formation of LFP on the presence of endogenous iron, we incubated the sediments enriched in synaptosomes and capillaries from the adult rat brain in the presence of 10 μM DFA which inhibited the process of lipid peroxidation in freshly prepared homogenates from the rat prosencephalon by chelation of endogenous iron (Štipek et al., 1997). This chelator was able to decrease significantly the spontaneous LFP formation only in cortical synaptosomes to levels found in synaptosomal sediments isolated from the adult hippocampi and striata (Fig.1). On the contrary, the decrease in the spontaneous production of LFP in brain capillaries was significant in all brain regions studied, but the "inhibited" levels still remained above the levels found in the enriched fractions of synaptosomes.

Therefore, we tried to stimulated the LFP production by the addition of exogenous iron at the cerebrospinal fluid concentration of 2 μM (Halliwell and Gutteridge, 1984), to document possible differences in the metal-dependent production of LFP in both isolated fractions enriched in synaptosomes and microvessels from 12- and 50-day-old rat brains. The addition of 2 μM Fe^{2+} together with 250 μM ascorbate, the concentration of which present in the cerebrospinal fluid (Grünewald, 1993), increased the formation of LFP in synaptosomes isolated from all the vulnerable brain regions of young animals with high significancy (Fig. 2A). The stimulatory action was expressed preferentially in cortical structures as it was documented by a 22-fold elevation of the synaptosomal formation of LFP above the non-stimulated value. In hippocampal and striatal synaptosomes the increase was comparable to that in the entorhinal cortex, but owing to higher spontaneous LFP levels, the increases were a 16- and 13-fold, respectively, of corresponding non-stimulated values.

In brain capillaries from 12-day-old rats the situation was comparable to that in brain synaptosomes, exhibiting a 4-fold increase of the amount of LFP in cortical microvessels in which the the stimulatory action of 2 μM Fe^{2+}/250 μM was most pronounced (Fig. 2A). In contrast to the entorhinal cortex, the stimulatory action of this transient metal was significantly lower in hippocampal and striatal capillaries and, therefore, the increases were only 3-fold of their spontaneous LFP generation.

The stimulatory action of exogenous iron was less pronounced in synaptosomes from the investigated brain regions in the adult rat (Fig. 2B). Thus, only a 14-fold stimulation of the LFP formation was shown in cortical synaptosomes whereas in striatal synaptosomes the increase remained almost at the same level and even decreased in hippocampal synaptosomes to 13-fold of the spontaneous LFP generation. Nevertheless, all differences in the values of the transient metal-stimulated lipid peroxidation, in comparison to spontaneous LFP generation in nerve endings, remained at levels of high statistical significancy.

In brain microvessels isolated from adults rats the stimulatory effect of Fe^{2+} was comparable to findings in young rats, with the exception of the hippocampus, in which the value increased significantly above the value in young rats, reaching almost a 4-fold of spontaneous lipoperoxidation in this brain region (Fig. 2B). In contrast, in the brain microvessels from the entorhinal cortex and striatum the rises did not reach of a 2-fold of the spontaneous peroxidation in these structures.

3. 4. QUIN-stimulated generation of LFP in synaptosomes and capillaries from young and adult rat brain

From Figs. 3A and 3B is evident that QUIN is much less effective lipid peroxidant than iron, but at the selected concentration of 100 μM this excitotoxin regularly increased the levels of LFP in brain synaptosomes and capillaries from young rats. QUIN increased

the generation of LFP in synaptosomes isolated from all vulnerable regions of young brains with high significancy and their highest levels were found in the striatum (Fig. 3A). Comparable findings were received with microvessels from young rats, but arising from the presence of higher levels of the spontaneously formed products than in synaptosomes, the increases were percentually lower, but with significancy in all studied brain region (Fig. 3A).

In adult rats, the QUIN-stimulated formation of LFP in cortical synaptosome reached the highest level, but owing to the increased level of spontaneously formed products in this fraction, the rise was not statistically significant (Fig. 3B). On the other hand, this excitotoxin increased significantly the levels of LFP in hippocampal and striatal synaptosomes. In cortical capillaries the QUIN-induced rise was lower and the non-stimulated level LFP was slightly higher than that in young animals and, therefore, the difference did not reach of statistical significancy. In contrast, the differences between QUIN-stimulated and spontaneous (control) levels of LFP in hippocampal and striatal capillaries were statistical significant.

3. 5. Effect of D,L-2-amino-5-phosphonovaleric acid and reduced glutathione on QUIN-stimulated generation of LFP in synaptosomes and capillaries from young and adult rat brain

To determine if the QUIN-stimulated generation of LFP also involves an interaction of this excitotoxin with NMDA-type of GLU receptor in synaptosomal and endothelial plasma membranes, we incubated P_2 synaptosomes in the absence or presence of 250 μM APV. This concentration of NMDA-receptor antagonist inhibited QUIN-induced damage in cultured hippocampal neurons (Khaspekov et al., 1990). The action of APV was compared to the effect of 50 μM reduced GSH, a concentration of which corresponds to that found in the rat cerebrospinal fluid (Rehncrona et al., 1980). We assume that this

concentration of GSH can be very similar to those which are present in the brain extracellular fluid.

The concentration of 250 μM APV did not inhibit the QUIN-stimulated formation of LFP in synaptosomes isolated from the entorhinal cortex and corpus striatum and in the hippocampal synaptosomes even significantly potentiated the stimulated generation of LFP by this excitotoxin (Fig. 4A). In contrast, GSH in 50 μM concentration was a more potent inhibitor of the QUIN-induced generation of LFP in brain synaptosomes than APV and, in the case of striatal synaptosomes, this tripeptide significantly inhibited the stimulated formation of LFP. Incubation of the capillaries in the presence of APV *plus* GSH did not potentiate their action when used separately (data not shown).

In capillaries isolated from young rats APV regularly inhibited the QUIN-induced generation of LFP and the decreases in hippocampal and striatal microvessels reached statistical significancy (Fig. 4A). As in synaptosomes, the GSH-induced suppression of QUIN-elevated LFP was more pronounced in brain capillaries and the diminutions were statistically significant in all three brain regions. Also in this case, APV and GSH did no exhibited a mutual potentiation of their individual inhibitory actions (data not shown).

In adult animals, the inhibitory action of both these substances, probably meadiated by NMDA-type of GLU receptor, was more expressed than in 12-day-old rats (Fig. 4B). In contradiction to young animals, APV significantly inhibited the stimulated formation of LFP in synaptosomes from the adult rat hippocampi. The higher potency of GSH to inhibit the QUIN-stimulated formation of LFP was expressed in significant diminution of LFP levels not only in hippocampal, but also in striatal synaptosomes. However, in cortical synaptosomes, neither GSH nor APV influenced the LFP levels determined in the presence of QUIN.

In contrast to synaptosomes, in brain microvessels from the adult rats, APV as well as GSH significantly inhibited the QUIN-stimulated formation of LFP (Fig. 4B).

However, in contrast to young rats, the substances also diminished the spontaneous levels of LFP in cortical capillaries.

3.6. Effect of QUIN on (^{3}H)glutamate binding into synaptic membranes derived from adult rat brain.

In order to reveal if the effect of QUIN on lipid peroxidation can be realized through its direct interaction with glutamate receptor we decided to test QUIN as a displacer in binding of (^{3}H)glutamate in membranes separated from some regions of adult rat brain. As it is shown in Table 4, the binding of (^{3}H)glutamate into membranes from hippocampal formation and corpus striatum was significantly dacreased (by 15 %) in the presence of QUIN. But any displacement of binding could not be observed in the case of entorhinal cortex. These results indicate that at least in some brain regions, the interaction of QUIN with glutamate receptor (NMDA-receptor/ion channel complex) is quite indisputable.

4. DISCUSSION

4. 1. Regional and structural characterization of LFP generation in brain homogenates and in enriched fractions

The aim of this paper was to bring some additional data enlarging our previous findings concerning the mechanism(s) of excitotoxic action of QUIN (Rios and Santamaria, 1991; Santamaria and Rios, 1993; Štipek et al., 1997). Because there are some doubts about the exactness of the "TBA test" to estimate real initial rates of lipid peroxidation (Krištofiková et al., 1995), we have investigated the regional and age-dependent changes in the spontaneous and stimulated formation of LFP in brain synaptosomes and capillaries to show that the generation exhibits structurally different

dependance on iron and that the QUIN-stimulated generation can be influenced by some exogenous or endogenous antagonists acting at the NMDA-receptor recognition site of the GLU receptor. For this purpose we isolated synaptosomal and capillary fractions from the brain homogenates in which the enrichment in nerve endings and endothelial cells is closely related to the activity of membrane-bound GGT (Šťastný et al., 1988; Šťastný et al., 1997). The specific activity of capillary GGT was ~10-times higher than that in brain homogenates, corresponding to the activity in freshly isolated rat brain microvessels (Hilgier et al., 1991) and to endothelial cells in cocultures with astrocytes (Dehouck et al., 1990). The enrichment in GGT was accepted to be sufficient to reveal possible differences in the LFP formation in isolated synaptosomes and brain capillaries

The intrinsic feature of lipid peroxidation in brain tissue is its dependence on the presence of endogenous or exogenous iron (Triggs and Willmore, 1984; Zaleska and Floyd, 1985; Braughler et al., 1986; Cini et al, 1994; Štípek et al, 1997). It could explain the fact that we found small amounts of extractable products of lipid peroxidation in the non-incubated brain preparations formed mainly during the isolation procedure of enriched fractions. In fresh brain homogenates the endogenous levels of LFP were just above the sensitivity of this method whereas in freshly isolated capillaries at zero time of incubation they represented 4-10 % of the levels formed spontaneously over the period of 60 min (data were not shown). To evaluate a possible role of iron which could dissociate from iron-containing proteins (mainly from transferrin and ferritin), we compared the levels of LFP in perfused and non-perfused rat brain. However, our results showed that the effect of trace amounts of iron bound to low molecular weight components (ADP, citrate, histidin) in blood plasma or released from erythrocytes and/or nerve cells, mainly from glia (Francois et al., 1981), is evident only in homogenates, but is negligible, if any, in isolated enriched fractions of brain microvessels. In dependance on the conditions of determination, the ultrafiltrable, biologically active iron in brain homogenates represents 2.6 or 5.7% of the

total iron content of the tissue (Bralet et al., 1992), but during the isolation procedure of synaptosomes and capillaries it is diluted to concentrations which are ineffective to induce initiation of lipid peroxidation. In contrast to this, during saline perfusion of rat brains, resulting hypoxia can activate the xanthine-xanthine oxidase system or liberate some iron in brain microvessels and in this way it can lead to some elevations of LFP in perfused capillaries (and synaptosomes). Therefore, in further experiments with capillaries and synaptosomes we prepared pooled samples of the brain regions from non-perfused rat brains to shorten the the time of their isolation and to minimalize the spontaneously formed LFP as in them.

We found that the non-stimulated levels of LFP were higher in hippocampal homogenates than in the preparations from the adult corpus striatum. It is in a reciprocal relationship to the topographical localization of iron (Francois et al., 1981) and to its concentratins found in these brain regions by Zaleska and Floyd (1985). Also in neuronal cultures from the embryonic rat brain, the nigro-mesencephalic neurons exhibit lower sensitivity to Fe^{2+} (Michel et al., 1992) in comparison to hippocampal neurons for which concentrations of 100 µM $FeSO_4$ or lower (together with ascorbate) were toxic (Zhang et al., 1993). However, both these brain regions are highly sensitive to submicromolar amounts of the intraregionally injected $FeCl_2$ which caused an excessive accumulation of Ca^{2+}, followed by a neuronal loss in adult rat brain (Sloot er al., 1994). This is in agreement with previous findings demonstrating that micromolar or lower concentrations of Fe^{2+} are able to initiate lipid peroxidation in rat brain homogenates (Štípek et al., 1997). Non-parallel relationship between the iron content and lipid peroxidation also exists in the cerebral cortex. In this brain region we found the highest spontaneous generation of LFP in homogenates (and in synaptosomes), but the content of endogenous iron is lower than in previous two brain regions (Zaleska and Floyd, 1985). It suggests that, besides of iron,

some other pro-oxidant or anti-oxidant factor(s) or their mutual dysbalance may be involved in the initiation of lipid peroxidation in these brain preparations.

The endothelial cells of brain capillaries are potential targets of reactive O_2 radicals (Mak et al., 1992) and their subsequent damage may represent an early event in brain damage, mainly during the excitotoxic (Dietrich et al., 1992) or ischemia/reperfusion injury (Plateel et al., 1995; Siesjö et al., 1995). The involvement of brain endothelial cells in a receptor-mediated transport of iron (Broadwell et al., 1996), the high activity of xanthine oxidase in them (Betz, 1985) and the accumulation of highly oxidizable polyunsaturated fatty acids in their membranes (Bénistant et al., 1995), form from the brain endothelium a very susceptible structure to lipid peroxidation. Therefore, the brain endothelial cells require more protection against peroxidative damage than other brain cells (Tayarami et al., 1987). The protection is represented not only by the presence of peroxide-detoxifying enzymes (Tayarami et al., 1987; Plateel et al., 1995), but also by a relatively high content (~ 3.5 mM) of GSH (Plateel et al., 1995) which is similar to that in astroglia (Thorburne and Juurlick et al., 1996), but higher than in neurones (Sagara J.-i. et al., 1993). This very labile balance between pro- and anti-peroxidative processes in brain capillaries may explain our findings in which we demonstrated several times higher spontaneous formation of LFP in brain capillaries that in brain homogenates (or synaptosomes). Similarly to homogenates, the high formation of LFP was found in cortical synaptosomes (and capillaries) suggesting for high vulnerability of this brain region to oxidative stress in adulthood.

4. 2. Age-dependent changes in the formation of LFP in enriched fractions of rat brain synaptosomes and capillaries

From our experiments is also evident that in immature (12-day-old) rats the spontaneous formation of LFP is regularly higher in synaptosomes from the subcortical

brain regions than in young adult (50-day-old) animals. These results (Fig.3) are in agreement with the observation of Koudelová and Mourek (1992) who demonstrated higher levels of TBA-reactive substances in homogenates prepared from the subcortical brain formations of 5- and 21-day-old rats than in adults. The decrease of TBA-reactive substances is not final and gets on with age so that their levels are lower in the hippocampus than in the striatum of 28-month-old animals (Krištofiková et al., 1995). In contrast to the subcortical structures, in cortical preparations, a gradual postnatal increase in the endogenous levels of these substances was observed (Koudelová and Mourek, 1992), with a peak in 35-day-old rats. Then their levels decreased again (Koudelová and Mourek, 1992), but remaind unchanged betwoen 3- and 28 postnatal months, being significantly higher than in the hippocampus and striatum of old rats (Krištofiková et al., 1995). The results suggest that the adult cerebral cortex as a ontogenetically younger brain region, can be more susceptible to different pathological insults, in contrast to the immature brain in which subcortical brain regions seem to be transiently more susceptible to oxidative stress than in adulthood.

However, comparable data concerning postnatal changes in capillary formation of lipid peroxides are absent. Qualitative and quantitative ultrastructural changes in the brain microvessel wall of 3-month-old and aging rats suggest for the developmental reduction of mean thickness of capillary endothelium and of relative volume of mitochondria in endothelial cells and pericytes (Heinsen and Heinsen, 1983) and for a subsequent blood-brain-barrier dysfunction. These findings may suggest for a difference between the brain aging and the process of postnatal brain maturation.

4. 3. Dependence of the LFP generation on endogenous and exogenous iron in enriched fractions of synaptosomes and capillaries

In the rat brain, the main bulk of iron is deposited intracellularly (mainly in oligodendroctes and microglia) in the storage protein ferritin or bound to the transport protein transferrin (Bmkovic et al., 1993) whereas only a small fraction of iron is extracellularly in a form of low molecular weight components (Braughler et al. 1986). However, the regional and cellular distribution of iron deposits (Francois et al., 1981; Hill and Switzer, 1984) do not overlap in the distribution of iron-binding sites (Barkai et al., 1991) and transferrin receptors (Hill et al., 1985). It was suggested (Hill et al., 1985) that iron-rich brain areas are efferent to areas of high transferrin receptor density and that these sites receive iron through neuronal transport. Our results with desferoxamine (see Fig. 1) show that the nerve terminal accumulation of transported iron could play a role in the initiation of lipid peroxidation only in synaptosomes from the entorhinal cortex in which the density of a transferrin-like protein is relatively high (Hill et al. 1985).

Iron is taken up by nerve cells after transferrin binds to specific surface receptors and then the complex is internalized. Transferrin receptors have been localized on the luminal endothelial surfaces of brain capillaries suggesting a route through which transferrin can transport iron from plasma to the cells of the brain (Jefferies et al., 1984). Mainly rat brain capillaries in cortical areas and in basal ganglia exhibited high density of transferrin receptor. This is in parallel with our findings demonstrating higher spontaneous formation of LFP in the brain capillaries isolated from the entorhinal cortex and striatum than in those from the hippocampus. But a potent inhibition of the spontaneous generation of LFP by desferoxamine in all these brain regions supports the observation of Broadwell et al. (1996) demonstrating that the internalized, iron-containing tranferrrin may leave its iron within the endothelium. This lability of transferrin-bound iron, together with the high activity of xanthine oxidase in the brain endothelial cells (Betz, 1985), could also be responsible for much higher spontaneous formation of LFP in brain capillaries (comparing to synaptosomes) which was regularly inibited by the addition of desferoxamine.

The addition of increasing concentrations of exogenous iron resulted in a linear accumulation of peroxidative products in brain homogenates of about 50-day-old rats (Cini et al., 1994). Also exposure of brain synaptosomes to the Fe^{2+}/ascorbate (5 μM/100 μM) oxidizing system increased their peroxidative activity in a time dependent manner (Bondy et al., 1990) which was connected with a significant decrease in the content of polyunsaturated free and phospholipid fatty acids in synaptosomal membranes (Viani et al., 1991) and with enhanced basal release of excitatory amino acids from the synaptosomal compartment (Gilman et al., 1994). We found that exogenous iron (2 μM) in the presence of ascorbate (250 μM) enhances the generation of LFP in synaptosomes from different regions of adult rat brain in comparable rate which parallells the similar capacity of the cortical, hipocampal and striatal tissue to bind exogenous iron in rats of comparable age (Barkai et al., 1991). But the higher potency of the exogenous iron/ascorbate system to enhance the generation of LFP in brain synaptosomes from 12-day-old rats than in adult animals remains unclear, mainly, if the brain amounts of unsaturated fatty acids, the substrates for peroxidative processes, are lower in young than in adult rats (Šmídová et al., 1984). In contrast, lipid composition of brain capillary membranes (Tayarani et al., 1987; Bénistant et al., 1995) and the receptor-mediated transport of iron in the brain endothelium (Hill et al., 1985; Broadwell et al., 1996) make from the brain microvessels an important target for the iron-induced oxidative stress.

4. 4. Involvement of NMDA-type of glutamate receptor in the generation of LFP in enriched fractions of synaptosomes and capillaries

Glutamate (Krištofikova et al., 1995) and its agonists, kainic acid (Bose et al., 1992), seem to be a less potent inducers of oxidative stress than QUIN (Ríos and Santamaría, 1991; Santamaría and Ríos, 1993). QUIN, an endogenous agonist of NMDA-type of GLU receptor, stimulates the rat brain lipid peroxidation by an iron-

dependent mechanism (Štípek et al., 1997). Excessive activation of this type of GLU receptor by QUIN can lead to neuronal death (Foster et al., 1988; Keilhoff et al., 1991), implicating free radical formation in this process (Fagni et al., 1994). We demonstrated that QUIN stimulates the formation of LFP in all synaptosomal preparations from immature rats more potently than in young adult rats (see Fig. 3A an 3B) and, in the case of the adult entorhinal cortex, this excitotoxin was even ineffective. This can be related to the transient overexpression of NMDA-glutamate receptors in some rat brain regions between the second and forth postnatal week (Tremblay et al., 1988; Insel et al., 1990; Folbergrová et al., 1997) and to their unequal distribution in adult rat brain (Monaghan and Cotman, 1985). The NMDA-binding sites are well-characterized on membranes of rat cerebral synaptosomes (Monahan and Michel, 1987) and are also detectable on rat cerebral capillary membranes (Koenig et al., 1992). However, in contrast to NMDA-binding sites on developing synaptosomes (Chaudieu et al., 1991), any developmental data are absent in the case of brain capillaries and, therefore, any correlation to changes in QUIN-induced formation of LFP in brain microvessels cannot be carried out.

In relation to the QUIN-induced, NMDA-receptor mediated generation of LFP in synaptosomes and capillaries, we tested the inhibitory action of exogenous (APV) and endogenous (GSH) antagonists acting at the NMDA-type of GLU receptors. The CSF concentration of total GSH (50 μM), reflecting the presence of low levels of GSH in the brain interstitial fluid (Rehncrona et al., 1980), is so low than its inhibitory action on QUIN-stimulated formation of LFP, observed regularly in synaptosomes from the adult rat brain and irregularly in young rats (see Fig. 4A and 4B), is more probably mediated by GSH-induced inhibition of an agonist binding to glutamate receptors in the rat synaptosomal membranes (Ogita et al., 1986) than by its action as a scavenger of free radicals. In this study we show that GSH at 50 μM concentration is also capable to attenuate the QUIN-induced lipid peroxidation in brain microvessels despite of the fact that

it is a good substrate for GGT, the activity of which is high in their surfaces (see Table I). The inhibitory action of GSH is more regular than that of 250 μM APV. However, in contrast to the micromolar concentrations of GSH which inhibit the synaptosomal and capillary formation of LFP, millimolar concentrations of GSH or GSSG are necessary to protect neuronal cells against the NMDA-receptor mediated neurotoxicity (Levy et al., 1991). It can suggests that lipid peroxidation is only one of the NMDA receptor-mediated processes which lead to a neuronal cell death (Choi, 1988). But the finding that APV (and partially GSH) can further stimulate the QUIN-induced formation of LFP in hippocampal synaptosomes of 12-day-old rats is not easy to explain and can be, perhaps, related to the differential expression of NMDA-recognition sites with respect to glycine and phencyclidine modulatory sites of the receptor/channel complex observed in the rat hippocampal formation between postnatal days 7 and 21 (McDonald et al., 1990)

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TABLE 1. Specific activity of GGT in synaptosomal and capillary fractions isolated from homogenates of entorhinal cortex, hippocampal formation and striatal complex in adult rat brain

Fraction	Entorhinal cortex		Hippocampal formation		Corpus striatum	
	GGT	NOS	GGT	NOS	GGT	NOS
Homogenate	3.9 ± 0.6	n.d.	5.4 ± 0.5	n.d.	4.6 ± 0.5	n.d.
Synaptosomes	4.7 ± 0.5	5.56+	7.9 ± 1.0	4.77+	7.2 ± 0.9	3.35+
Microvessels	32.5 ± 4.0	1.74+	50.7 ± 3.4	1.60+	42.1 ± 6.1	1.88+

Values are mean of 3 determinations (in duplicate or triplicate) ± S.D.

TABLE 2. Comparison of the non-stimulated formation of lipid fluorescent products in homogenates and capillaries prepared from entorhinal cortex, hippocampal formation and corpus striatum of non-perfused and saline-perfused adult rat brain

Fraction	Lipid fluorescent product formation expressed as U/mg protein per min		
	Entorhinal cortex	**Hippocampal formation**	**Corpus striatum**
Homogenate (non-perfused brain)	2.730 (100%)	1.411 (100%)	1.272 (100%)
Homogenate (perfused brain)	2.294 (84%)	1.197 (85%)	1.032 (81%)
Microvessels (non-perfused brain)	13.955 (100%)	6.509 (100%)	11.164 (100%)
Microvessels (perfused brain)	13.951 (100%)	7.105 (109%)	13.375 (120%)

Values are mean of two experiments (in duplicates).

TABLE 3. **Developmental and regional differences in the formation of lipid fluorescent products in synaptosomes and capillaries isolated from young and adult rat brain**

Age in days	Fraction	Lipid fluorescent products expressed as U.F./mg protein/min.		
		Entorhinal cortex	Hippocampal formation	Corpus striatum
12	Synaptosomes	1.568 ± 0.194	2.277 ± 0.280	2.584 ± 0.441
12	Capillaries	14.680 ± 2.600^{b}	7.426 ± 1.157^{a}	7.554 ± 2.117^{a}
50	Synaptosomes	2.656 ± 0.833^{c}	1.159 ± 0.376	1.437 ± 0.388
50	Capillaries	16.075 ± 3.365^{b}	11.839 ± 2.433^{c}	13.310 ± 4.746^{c}

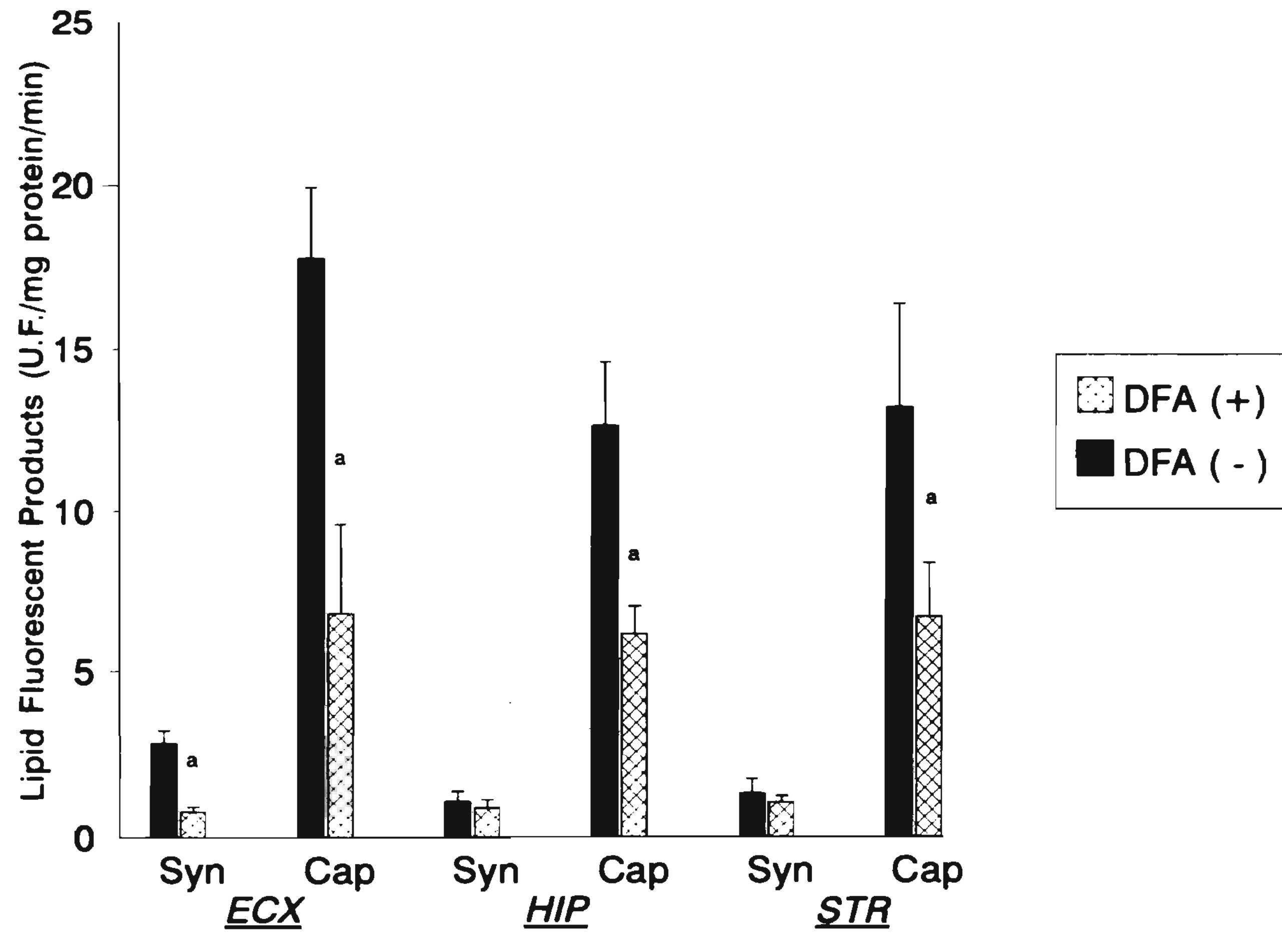
1

Values are mean ± S.D. of 8 - 12 individual experiments. The age-dependent changes are expressed in percentages of corresponding values from 12-day-old rats. $^{a}p < 0.05$ vs entorhinal cortex, respecting the same fraction and age of rats. $^{b}p < 0.01$ vs hippocampal formation, respecting the same fraction and age of rats, $^{c}p < 0.01$ vs 12-day-old rats, respecting brain region and fraction.

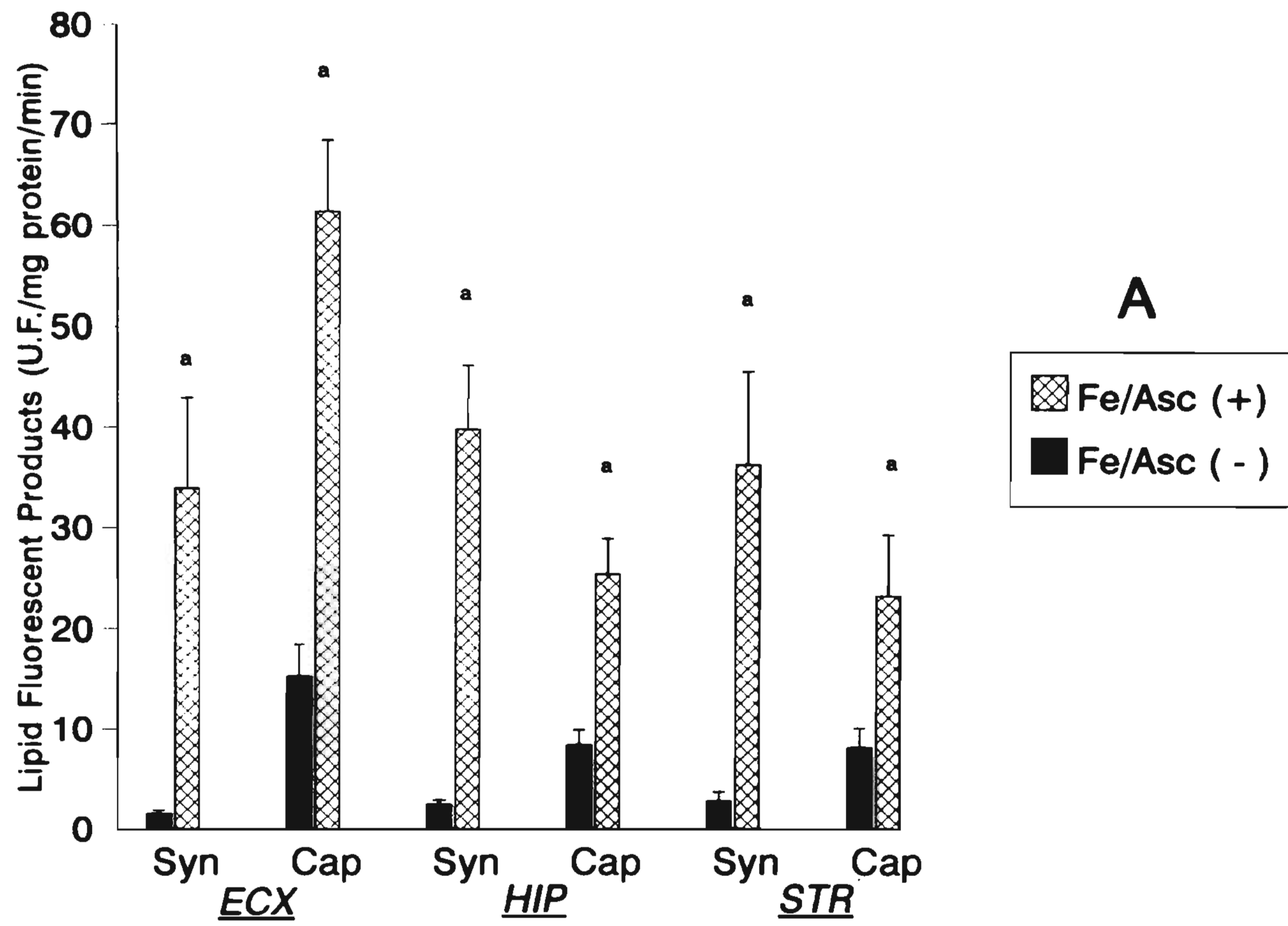
TABLE 4. Effect of quinolinic acid (QUIN) on specific receptor binding of [^{3}H]glutamate in crude synaptic membranes isolated from the entorhinal cortex, hippocampal formation and corpus striatum of adult rat brain

	(^{3}H)glutamate binding (fmol/mg protein)		
	Entorhinal cortex	Hippocampal formation	Corpus striatus
Control	1419 ± 350	1172 ± 312	546 ± 130
QUIN	1364 ± 155	777 ± 187^b	413 ± 183^a

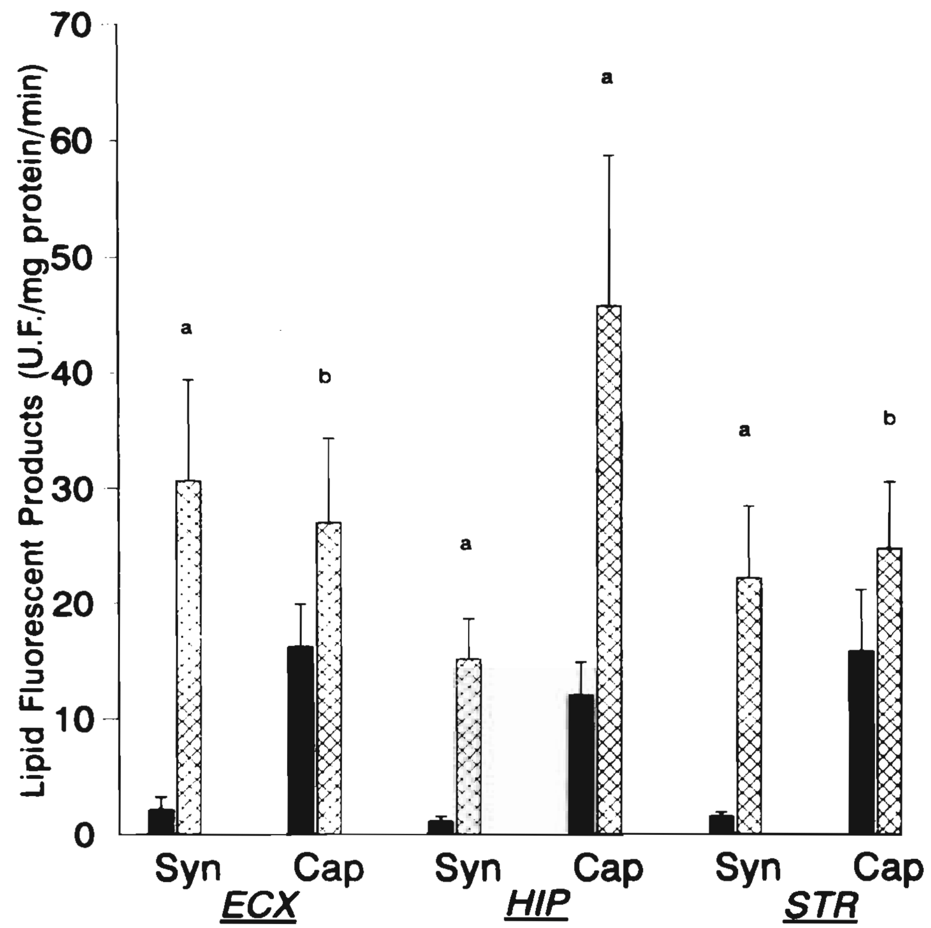
Values are mean ± S.D. of 12-15 measurements representing membrane preparations from 4 individual experiments. $^a p < 0.05$ and $^b p < 0.01$ vs corresponding control value.



Lipid Fluorescent Products (U.F./mg protein/min)
25
20
15
10
5
0
a
a
a
a
Syn
Cap
Syn
Cap
Syn
Cap
ECX
HIP
STR
DFA (+)
DFA (-)



Lipid Fluorescent Products (U.F./mg protein/min)
80
70
60
50
40
30
20
10
0
a
a
a
a
a
a
Syn
Cap
Syn
Cap
Syn
Cap
ECX
HIP
STR
A
Fe/Asc (+)
Fe/Asc (-)



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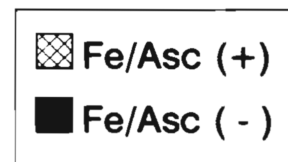
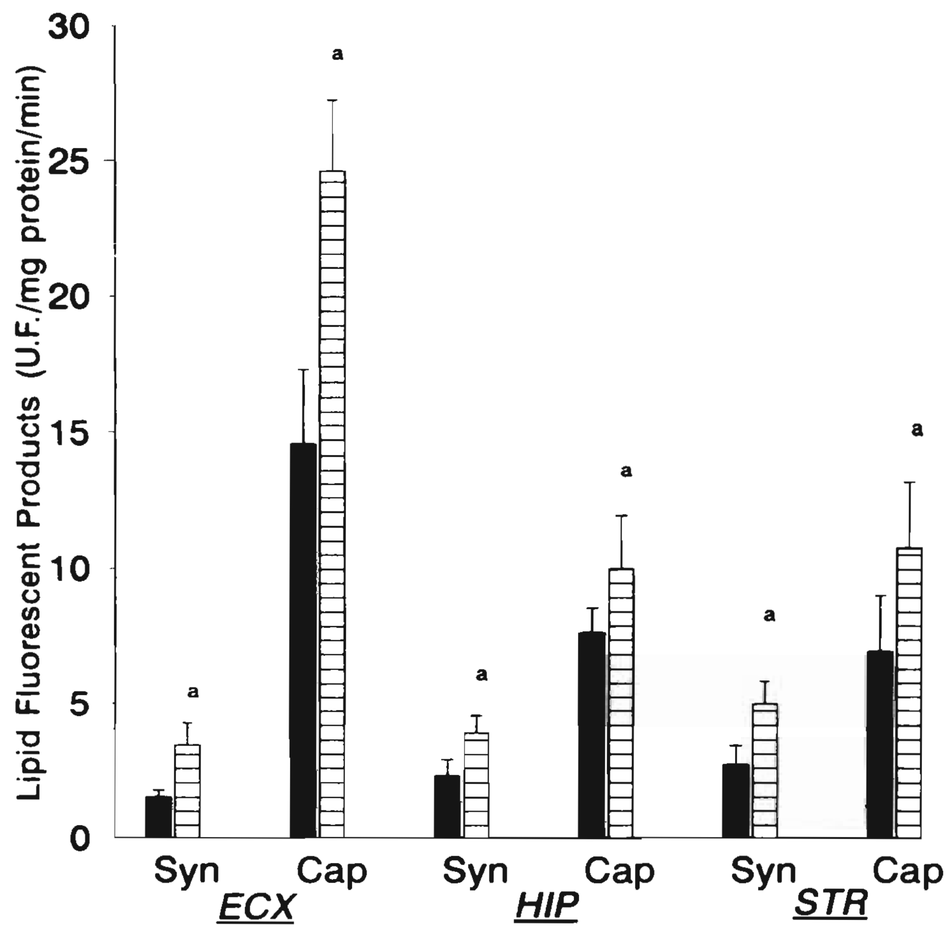
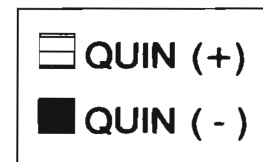
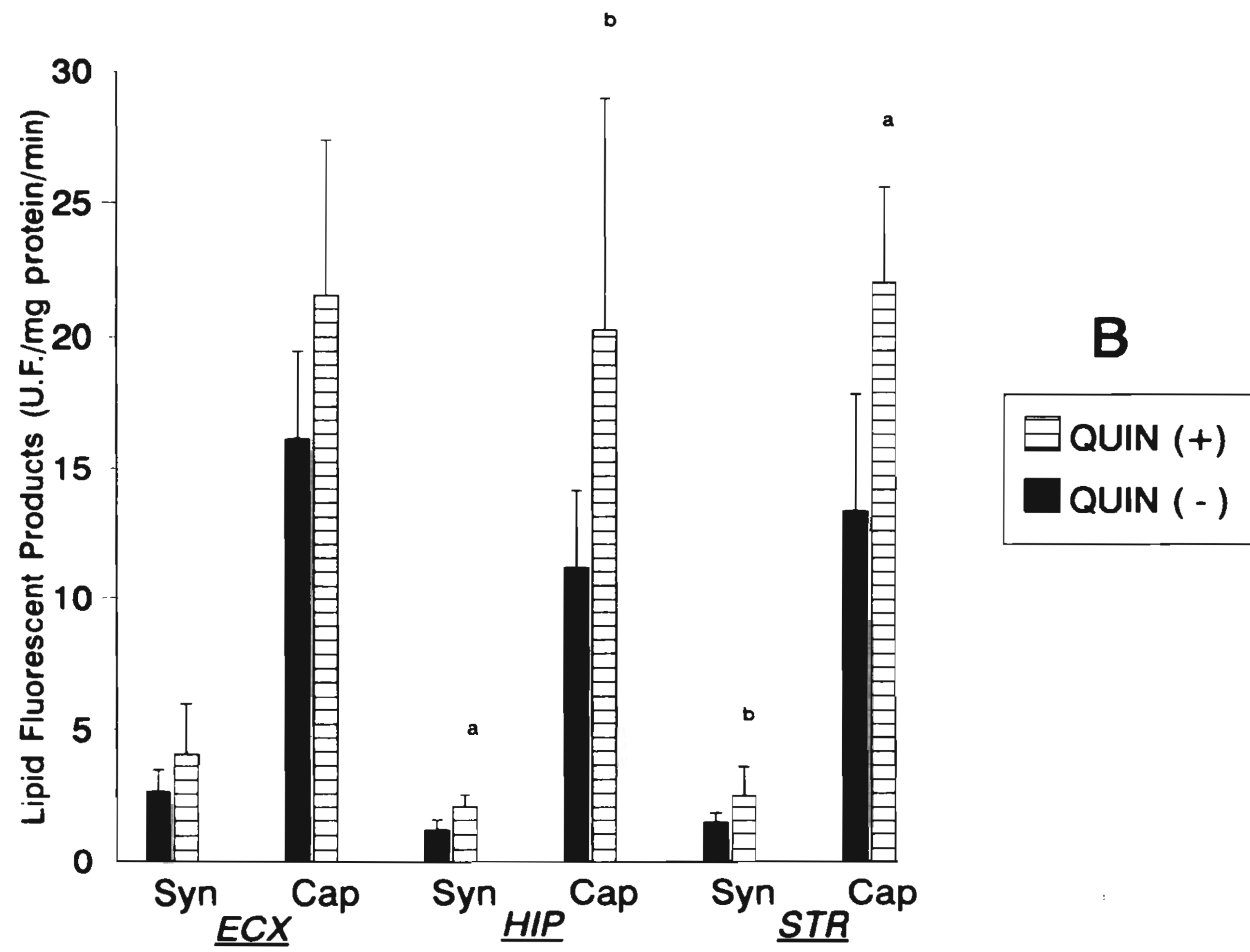




A




Lipid Fluorescent Products (U.F./mg protein/min)
30
25
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ECX
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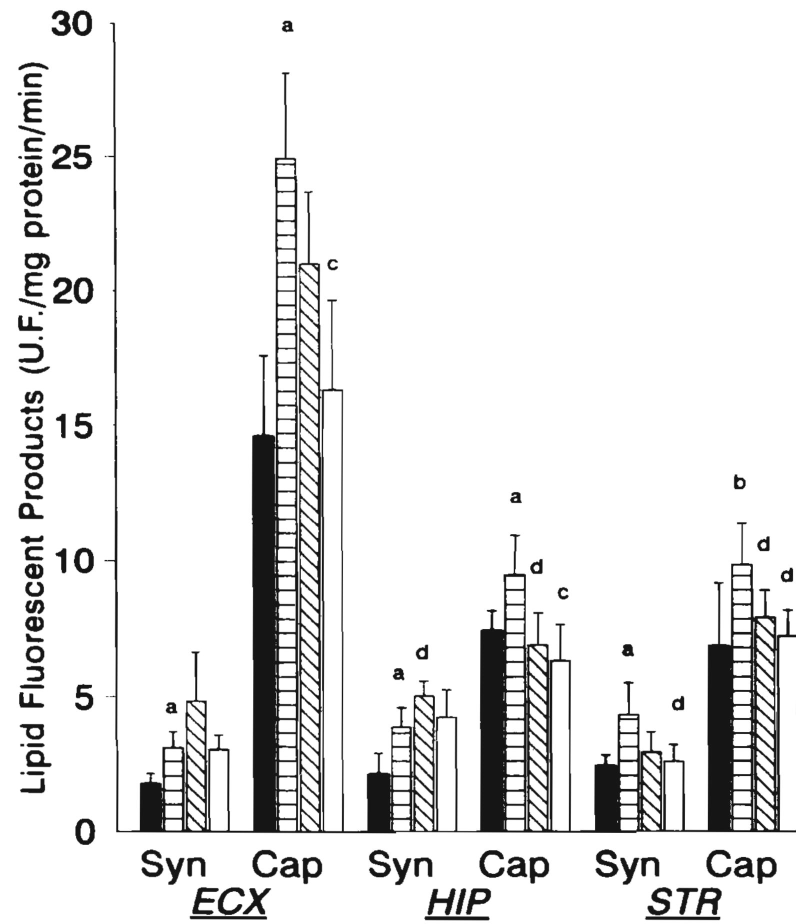
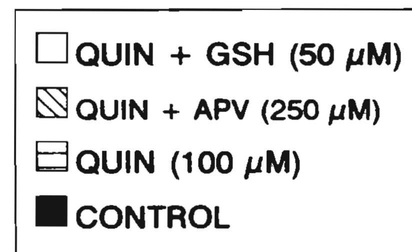
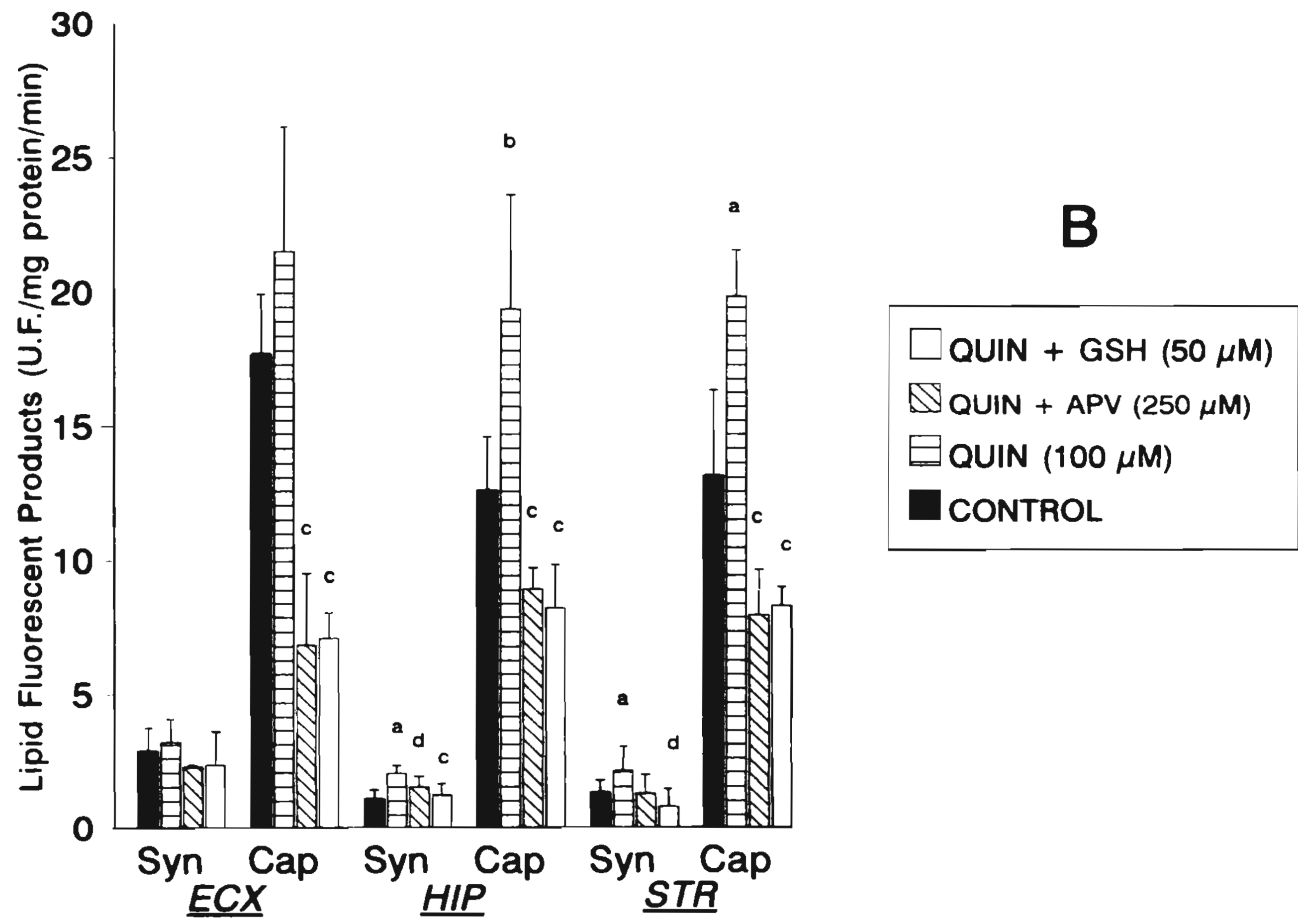
QUIN (+)
QUIN (-)



A





B
Lipid Fluorescent Products (U.F./mg protein/min)
30
25
20
15
10
5
0
Syn
Cap
Syn
Cap
Syn
Cap
ECX
HIP
STR
QUIN + GSH (50 μM)
QUIN + APV (250 μM)
QUIN (100 μM)
CONTROL

El óxido nítrico y la peroxidación de lípidos inducida por el ácido quinolínico en sinaptosomas de cerebro de rata

Existe para el óxido nítrico (NO), como lo existe también para otras pequeñas moléculas tales como los metales de transición, un papel dual sobre el SNC ampliamente demostrado en la literatura. En el caso de este gas, hay reportes que lo proponen como un neuromodulador de la transmisión glutamatérgica involucrado en el control y la regulación de procesos metabólicos dependientes de calcio y que incluso puede resultar potencialmente neuroprotector en condiciones muy específicas de daño neuronal. Sin embargo, también se ha reportado que el NO puede ser un importante mediador de daño neuronal y de estrés oxidativo bajo condiciones de excitotoxicidad. Su naturaleza gaseosa le confiere además la capacidad de alcanzar sustratos remotos en los sistemas biológicos y manifestar así sus efectos sobre múltiples biomoléculas blanco, sean éstos tóxicos o fisiológicos. Parece ser que dicha dualidad del NO obedece a dos factores fundamentales: a) su concentración en el SNC y b) su especie molecular predominante.

En vista de la considerable cantidad de reportes en la literatura que demuestran que la activación de receptores para NMDA produce un incremento en los niveles de NO y que dicho proceso puede estar relacionado ya sea a la generación de procesos tóxicos que eventualmente conduzcan a la muerte neuronal, o bien al reestablecimiento de la homeostasis celular, se evaluó el efecto tanto de precursores del NO, como de inhibidores de la actividad de la sintasa del NO, la NOS, sobre la peroxidación de lípidos inducida por el QUIN en sinaptosomas de cerebro de rata, bajo la hipótesis de que dichas manipulaciones farmacológicas permitirían eventualmente sugerir un papel pro-oxidante del NO en condiciones de excitotoxicidad mediadas por el QUIN, constituyéndose en un factor potencialmente vinculado al patrón de daño oxidativo inducido por esta neurotoxina.

Entre los fármacos probados en este estudio se encuentran la L-arginina (el precursor endógeno del NO), así como diferentes dosis de Nω-nitro-L-arginina (L-NARG), un potente inhibidor de la NOS. A continuación se anexan los respectivos resultados de dicho trabajo en la forma en que fueron enviados para su publicación.

Este trabajo se realizó en colaboración con el Dr. Mauricio Díaz del Departamento de Neurociencias del Instituto de Fisiología, U.N.A.M.

ANEXO VII

TOXICOLOGY LETTERS

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Effects of Nω-nitro-L-arginine and L-arginine on quinolinic acid-induced lipid peroxidation

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Keywords: Oxidative stress, Nitric oxide, NMDA receptor, Quinolinate neurotoxicity, Nω-nitro-L-arginine, L-arginine, Synaptosomal fractions.

Abstract

The effects of a nitric oxide synthase inhibitor, Nω-nitro-L-arginine (L-NARG), and a nitric oxide precursor, L-arginine (L-ARG), on the lipid peroxidation induced by quinolinic acid (QUIN, an NMDA receptor agonist), were both tested in synaptosomal fractions from whole rat brain. Baseline of lipid peroxidation was found at 2.43 ± 0.24 fluorescence units/mg protein or 14.27 ± 1.24 nmoles of TBARS/mg protein (100 %). QUIN (100 μM)-induced lipid peroxidation in synaptosomes (256 % and 166 % vs control, as measured by lipid fluorescent products and thiobarbituric acid-reactive substances, respectively) was inhibited by concentrations of 10, 40, 100, 200 and 400 μM of L-NARG (74 %, 58 %, 56 %, 48 % and 48 % vs quinolinate value, respectively). Coincubation of synaptosomes with QUIN plus L-ARG (100 μM), which alone resulted a potent pro-oxidant (277 % vs control), increased the lipoperoxidative effect induced by QUIN alone in 120 % (290 % vs control). Synaptosomes simultaneously exposed to QUIN (100 μM) plus L-ARG (100 μM) plus L-NARG (200 μM) showed levels of lipid peroxidation similar to those of quinolinate alone. These findings suggest that nitric oxide may contribute to the oxidative damage induced *in vitro* by QUIN.

1. Introduction

Quinolinic acid (2,3-pyridine dicarboxylic acid, QUIN), an endogenous tryptophan metabolite from the kynurenine pathway, is also a potent neurotoxin. Its excitotoxic action is mediated by overactivation of glutamate receptors, as QUIN is a selective NMDA subtype of glutamate receptor agonist [1]. QUIN has been commonly employed to reproduce some of the pathological consequences of Huntington's disease [2], and more recently, Heyes and coworkers [3] described an involvement of QUIN in inflammatory and infectious human brain disorders as AIDS-dementia complex. Enhanced cytosolic Ca^{2+} concentrations, ATP exhaustion, circling behavior and GABA depletion [2,4,5] are all important features observed as a result of QUIN-induced neurotoxicity, as well as increased oxidative stress and lipid peroxidation [6,7,8].

On the other hand, nitric oxide (NO), a novel neuronal modulator, has been postulated to play a role as a retrograde messenger in the central nervous system, showing both regulatory and neurotoxic effects, depending on its redox status [9,10] and on its concentrations [11]. NO is involved in several physiological processes such as vascular relaxation, long-term potentiation, learning and memory [12]; while, under pathological conditions, NO may promote apoptosis, oxidative stress and cell damage [13].

An increased production of NO mediated by a Ca^{2+}-calmodulin-dependent activation of the constitutive nitric oxide synthase (cNOS) has been demonstrated after stimulation of NMDA receptors [14,15]. In order to elucidate whether nitric oxide is able to modify the quinolinate-induced oxidative stress *in vitro*, a NOS inhibitor, Nω-nitro-L-arginine, and the NO precursor, L-arginine, were both employed to evaluate the generation of lipid peroxidation in QUIN-exposed

rat brain synaptosomes, as measured by two current indexes of oxidative stress, the assay of lipid fluorescent products (LFP) and the assay of thiobarbituric acid-reactive substances (TBARS).

2. Materials and Methods

2.1. Animals

Male Wistar bred-in-house rats (250-300 g) were used throughout the experiments. Animals were housed in acrylic box cages and provided with Rodent Chow (Purina Chow) and water *ad libitum*. Animals were mantained under conditions of constant temperature (25 ± 3° C), humidity (50 ± 10 %) and lighting (12:12 light:dark cycle). Rats were killed by decapitation and their brains were then removed for tissue homogenization.

2.2. Chemicals

Deionized water (Milli R/Q System, Millipore) was used for preparation of all solutions. QUIN, 2-amino-5-phosphonovaleric acid (APV), Folin & Ciocalteu's phenol reagent, quinine, 2-thiobarbituric acid (TBA), ferrous sulfate ($FeSO_4$), copper sulfate ($CuSO_4$) and sucrose were purchased from Sigma Chemical Co. (St Louis, MO). L-arginine (L-ARG) and Nω-nitro-L-arginine (L-NARG) were purchased from RBI. All other chemicals were obtained from E. Merck (Mexico).

2.3. Isolation of synaptosomal-enriched fractions

Synaptosomal fractions were obtained according to a modification [16] of the procedure reported by Löscher and coworkers [17]. Animals were killed by decapitation and their brains rapidly removed and briefly stored at -5° C in isotonic saline solution (0.9 %, pH 7.4). Pooled brains (without cerebellum) from 2 animals were gently homogenized in a 0.32 M sucrose solution with a Teflon pestle-glass homogenizer (8 up-and-down strokes). Homogenates were then centrifuged at 1,500 g for 10 min and the supernatants centrifuged again at 11,000 g for 20

min. The final supernatants were discarded and the pellets resuspended in 5 ml of sucrose (0.32 M). Resuspended preparations were gently layered onto 20 ml of a 0.8 M sucrose solution in polycarbonate tubes and centrifuged at 10,800 g for 27 min at 4° C in a swinging bucket rotor. After removal of the first layer, the interface was recovered and diluted in 30 ml of a sucrose solution (0.32 M). A final centrifugation was performed at 24,000 g for 15 min. Pellets obtained at the end of this procedure were finally resuspended in 9 ml of a non-calcium-free isotonic saline solution and represented the synaptosomal fraction.

2.4. Treatment of brain synaptosomes

Volumes of 970 µl of the resuspended synaptosomal aliquots (final volume adjusted to 1 ml with deionized water) were exposed to equal volumes of 10 µl of either isotonic saline (pH 7.4) to reach final concentrations of QUIN (100 µM), APV (250 µM), L-ARG (100 µM), L-NARG (10, 40, 100, 200 and 400 µM) or some combinations of them. The concentrations of L-ARG and L-NARG used here were selected according to previous reports [15,18,19]. Aliquots were then gently vortexed, incubated in a shaking water bath at 37° C during 1 hour, and assayed either for the content of lipid fluorescent products or the thiobarbituric acid-reactive substances, as described below.

2.5. Assay of lipid fluorescent products (LFP)

Formation of lipid-soluble fluorescent products was measured by the technique described by Triggs and Willmore [20] and modified for *in vitro* studies [6]. After incubation, synaptosomal-enriched 1 ml-aliquots were added with 3 ml of a chloroform-methanol mixture (2:1, v:v). Tubes were capped, gently vortexed for 15 seconds and placed on ice during 30 min. The aqueous phase was discarded and 800 µl of the chloroform layer were transfered into a quartz cuvette and mixed with 100 µl of methanol. The fluorescent signals of the samples were measured at 370 nm and

430 nm of excitation and emission wavelengths, respectively. The sensitivity of the spectrophotometer was adjusted just before measurement of samples, to 140 fluorescence units, with a quinine standard solution (0.1 μg/ml) prepared in 0.05 M of sulfuric acid. Final results were expressed as fluorescence units per mg of protein or as a percent of lipid peroxidation vs control values.

In order to assess the quality of the results of LFP monitoring, additional aliquots containing the synaptosomes were incubated under the same conditions in the presence of a well-known peroxidant, ferrous sulfate (2 μM).

2.6. Assay of thiobarbituric acid-reactive substances (TBARS)

Production of thiobarbituric acid-reactive substances was measured according to the technique previously described for *in vitro* studies [6]. One ml-aliquots containing the incubated synaptosomes were added with two ml of the TBA reagent (0.375 g of TBA + 15 g of trichloroacetic acid + 2.5 ml of concentrated HCl in 100 ml of water) and the final solution (3 ml total volume) was heated in a boiling water bath for 30 min. After ice-cooled, samples were centrifugated at 3000 g for 15 min. The absorbance was measured in the respective supernatants by spectrophotometry at 532 nm. Concentrations of TBARS were calculated by interpolation in a standard curve of periodic oxidation of 2-deoxy-D-ribose. Final results were expressed as nmoles of TBARS per mg of protein.

2.7. Protein measurement

Content of protein in tissue samples from brain synaptosomes was measured by the Folin and Ciocalteu's phenol reagent [21]. Results of lipid peroxidation were corrected by protein content in each sample.

2.8. Statistical analysis

All data were analyzed employing a one-way ANOVA, followed by Tukey's test for multiple comparisons [22]. Values of $p<0.05$ were considered of statistical significance.

3. Results

3.1. Effect of iron on synaptosomal lipid peroxidation

Measurement of lipid peroxidation (LP) was first assessed by exposure of synaptosomal fractions to 2 µM of ferrous sulfate ($FeSO_4$), a well-known lipid peroxidant [20]. LP induced by iron was enhanced by 581 % ($p<0.05$), as compared to control values (data not shown).

3.2. Effect of APV on quinolinate-induced lipid peroxidation

Figure 1 shows the effect of APV on QUIN-induced LP. APV, a well-known competitive NMDA receptor antagonist, was first employed in our system in order to assess an involvement of NMDA receptors activation on LP mediated by QUIN, as previously reported [8,9]. Basal values of LP (100 %) were found to be 2.43 ± 0.24 F.U./mg prot (n=8, Fig. 1) or 14.27 ± 1.24 nmol TBARS/mg prot (n=4, Table 1). Incubation of brain synaptosomes in the presence of QUIN resulted in a significant increase of LP, as compared to control values (256 % as fluorescent products in Fig. 1 or 166 % as TBA-reactive substances in Table 1). APV alone had no effect on basal LP (110 % vs control), whereas co-incubation of synaptosomes with QUIN plus APV completely inhibited the QUIN-induced LP (109 % vs control).

3.3. Effect of L-NARG on quinolinate-induced lipid peroxidation

Figure 2 shows the effect of increasing concentrations of L-NARG, a NOS inhibitor, on QUIN-induced LP, as measured by the assay of LFP. Co-administration of QUIN plus L-NARG at concentrations of 10, 40, 100, 200 and 400 µM, decreased the lipoperoxidative effect produced by QUIN alone (74 %, 58 %, 56 %, 48 % and 48 % vs QUIN, respectively), showing concentration-dependency mainly among the lower concentrations (10 and 40 µM). The same

concentrations of L-NARG, administered alone, did not produced significant changes on LP, as compared to control values (data not shown).

The effect of L-NARG (200 μM) on QUIN-induced LP was also tested by the assay of TBARS (Table 1). L-NARG alone had no effect on basal LP in the incubated synaptosomes, whereas coadministered with QUIN, L-NARG inhibited the oxidative action produced by QUIN (76 % as compared to QUIN values).

3.4. Effect of L-ARG on quinolinate-induced lipid peroxidation

Figure 3 presents data (as percentage vs control values) of the effect of L-ARG, the precursor of NO, as well as L-NARG at a concentration of 200 μM, on QUIN-induced LP. QUIN, coadministered with 100 μM L-ARG, slightly enhanced the pro-oxidant effect of QUIN alone by 120 % (290 % vs control values). L-ARG alone significantly promoted the basal LP (277 % vs control). As shown in Figure 2, QUIN, coadministered with L-NARG, significantly prevented the QUIN-induced LP, whereas L-NARG alone had not effect (see Figure 3). Synaptosomes coincubated with L-ARG (100 μM) + L-NARG (200 μM) showed no significant increase in LP values as compared to control (103 %, data not shown in Fig. 3). The effect of the same combined treatment (L-ARG + L-NARG) resulted to be significantly lower as compared to L-ARG alone (23 % vs L-ARG, $p<0.05$). Finally, when synaptosomes were incubated in the presence of QUIN + L-ARG + L-NARG, lipid peroxidation was significantly increased by 233 % as compared to control values, but below the level of QUIN alone (91 % vs QUIN).

4. Discussion

In accordance with previous reports [6,7], we observed that quinolinic acid augmented the oxidative damage produced *in vitro* by the enhancement of lipid peroxidation, as measured both by LPS and TBARS assays. Such an effect is thought to be mediated by the selective activation of

NMDA receptors, since an NMDA antagonist, APV, was able to completely abolish the quinolinate-mediated lipid peroxidation in rat brain synaptosomes. The same protective effect of NMDA receptor antagonists, such as kynurenic acid and MK-801, on quinolinate-induced lipid peroxidation, has been reported [6,7]. Stípek and coworkers [8] have recently proposed that quinolinate lipid peroxidation in the rat brain is also dependent on iron concentrations, pointing out a non-receptor-mediated mechanism of QUIN neurotoxicity which seems to be partially related to the ability of quinolinate to form complexes with iron. It has been also reported that nitric oxide can cause a release of iron from ferritin, producing cell death [23], suggesting that nitric oxide might contribute to the quinolinate-induced neurotoxicity by this mechanism.

In this work, we also found that the inhibition of nitric oxide synthase by L-NARG was able to inhibit lipid peroxidation after incubation of synaptosomes with quinolinate, suggesting that blockade of NO synthesis constitutes an important factor to prevent the production of reactive oxygen species and the further oxidative cell damage. In addition, L-ARG, the substrate of NOS which forms citrulline and NO, significantly increased the basal levels of lipid peroxidation, but it did not significantly increase the QUIN-induced peroxidative effect. The lack of summation of the peroxidative effects of L-ARG and QUIN suggests that both are acting through the same mechanism: enhanced formation of NO by NOS activation. However, this consideration remains to be elucidated. Interestingly, the coadministration of L-ARG plus L-NARG to synaptosomes, completely prevented the peroxidative effect induced by L-ARG alone, suggesting that the peroxidative effect of L-ARG is dependent on the production of NO by NOS. From these findings, it is not surprising to observe a partial protective effect of L-NARG on lipid peroxidation produced by QUIN + L-ARG, since L-NARG, in the ratio (2L-NARG:1L-ARG) employed for

this purpose, has been shown to supress the toxic action of L-ARG [24], reducing the peroxidation to the same level of QUIN alone, as observed in this study.

Although several reports suggested both modulatory and neuroprotective effects of NO in the CNS, such as regulation of mitochondrial respiration [25], reduction of brain damage in focal ischemia [26], antioxidant actions [27], blocked calcium influx in NADPH diaphorase-containing cortical neurons [28] and inactivation of the NMDA receptors by reaction with sulfhydryl groups at the redox modulatory site by formation of S-nitrosothiols and the consequent generation of disulfide bonds [29]; other groups have described alternative neurotoxic events mediated by NO, involving excessive stimulation of neurons by glutamate, besides of calcium influx and Ca^{2+}-calmoduline dependent NOS activation, protection against NMDA-induced neurotoxicity by inhibition of NOS and the participation of superoxide anions in the neurotoxicity elicited by NMDA [18]. Moreover, it has been demonstrated that increased levels of hydroxyl radicals are associated to NOS activation after NMDA receptors overstimulation [30,31].

Some other authors have shown that the stimulation of NMDA receptors by glutamate resulted in the activation of NOS, and a further production of NO [15]. Nakamura and coworkers [24] tested the effect of different drugs either related to the inhibition or the activation of NOS, on quinolinate-induced convulsions in mice. They found that L-ARG, either alone or in combination with 5,6,7,8-tetrahydrobiopterine (a cofactor of NOS), potentiated the clonic and tonic convulsions induced by QUIN, whereas N^G-monomethyl-L-arginine, a competitive NOS inhibitor, decreased the toxic action of QUIN.

Further studies testing other nitric oxide donors, as well as other NOS inhibitors, are needed to clarify the nature of nitric oxide-derived species involved in this pattern of toxicity. The relevance of this work can be also related to their implications on those neurotoxic mechanisms involved in

the action of quinolinic acid as an endogenous metabolite in the CNS, which has been demonstrated to play a role in human neurological disorders [3].

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Table 1. Lipid peroxidation in quinolinate- and L-NARG-treated synaptosomes

	nmol TBARS/mg prot (mean ± S.E.M.)	% vs Control
Control	14.27 ± 1.24	100
QUIN (100 μM)	23.65 ± 1.30*	166
L-NARG (200 μM)	12.01 ± 0.70	84
QUIN + L-NARG	18.09 ± 0.87	127

Synaptosomal fractions were incubated during one hour at 37°C in the presence of the different treatments; n=4 experiments per group; * $p<0.05$, significantly different from control value.

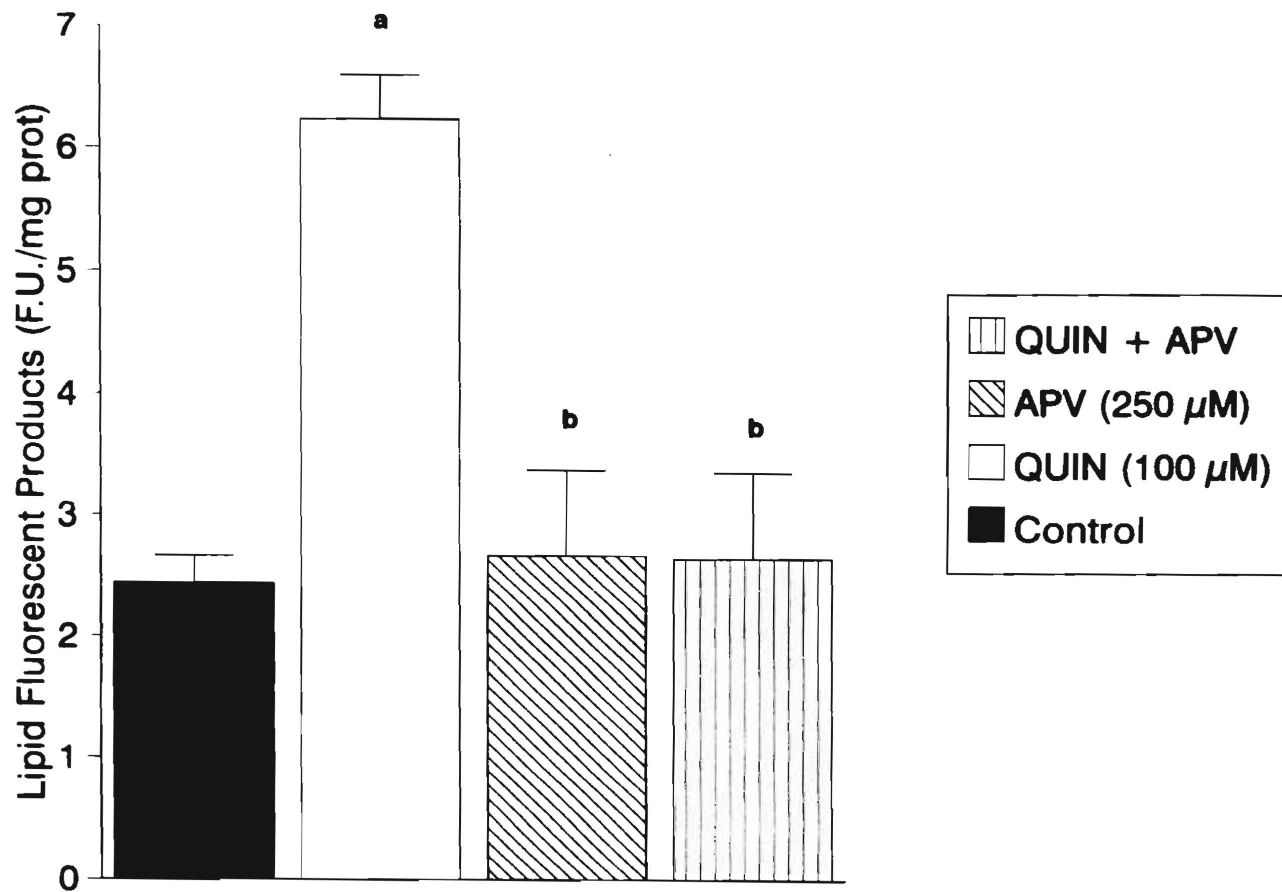
Figure Legends

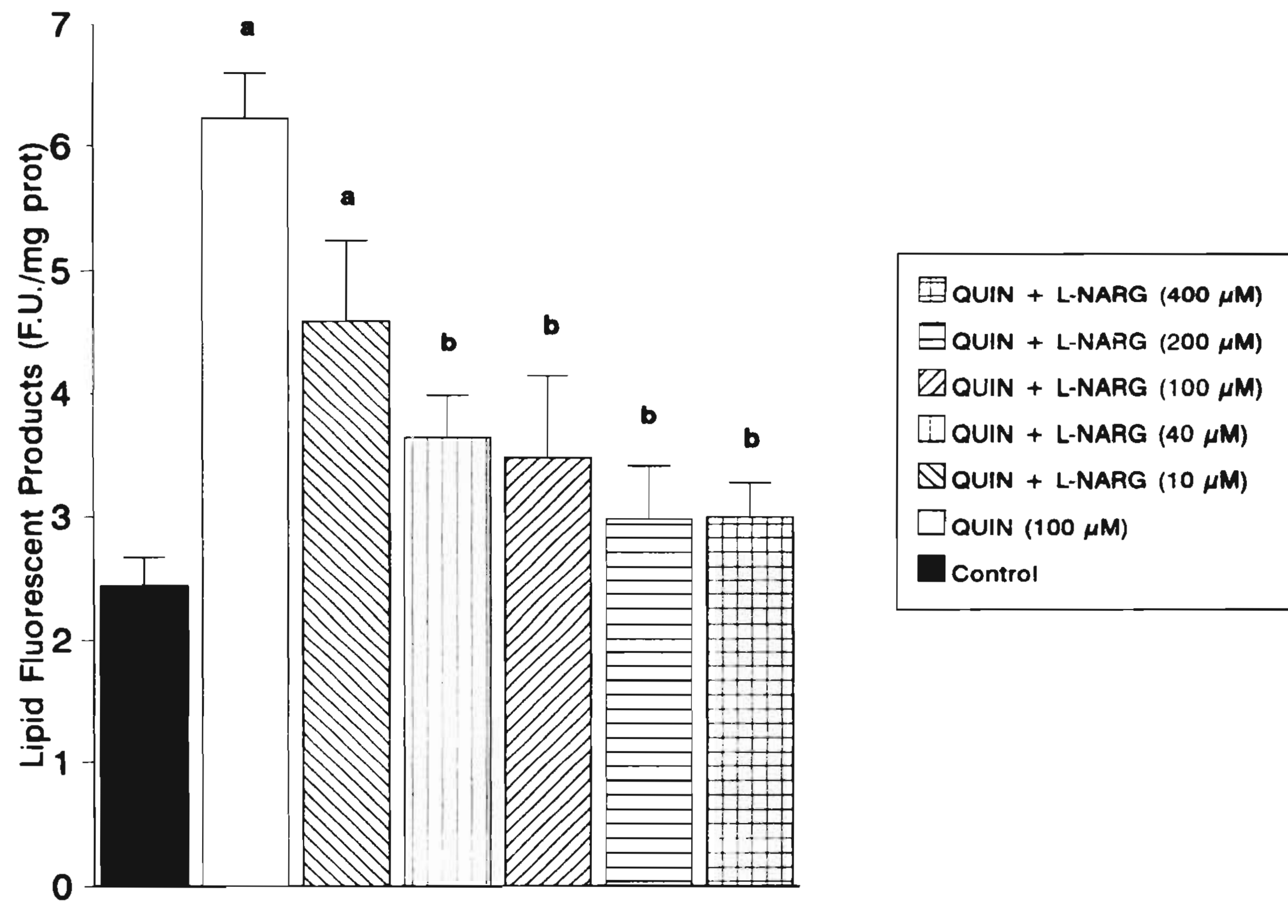
Fig. 1. Effect of 2-amino-5-phosphonovaleric acid (APV) on quinolinic acid (QUIN)-induced lipid peroxidation in rat brain synaptosomes. Synaptosomal fractions (1 ml of final volume) were incubated in isotonic saline solution in a shaking water bath during 1 hour at 37° C in the presence of QUIN and/or APV. Lipid peroxidation, expressed as fluorescence units per mg of protein, was measured in synaptosomes by detection of lipid fluorescent products. Mean values (± one S.E.M.) of n=6-12 experiments are shown. *a* $p<0.05$, treatments different to control values; *b* $p<0.05$, different from QUIN treatment; one-way ANOVA followed by Tukey's test.

Fig. 2. Effect of increasing concentrations of Nω-nitro-L-arginine (L-NARG) on quinolinic acid (QUIN)-induced lipid peroxidation in rat brain synaptosomes. Synaptosomal fractions (1 ml of final volume) were incubated in isotonic saline solution in a shaking water bath during 1 hour at 37° C in the presence of QUIN and/or L-NARG. Lipid peroxidation, expressed as fluorescence units per mg of protein, was measured in synaptosomes by detection of lipid fluorescent products. Mean values (± one S.E.M.) of n=6-11 experiments are shown. *a* $p<0.05$, treatments different to control values; *b* $p<0.05$, different from QUIN treatment; one-way ANOVA followed by Tukey's test.

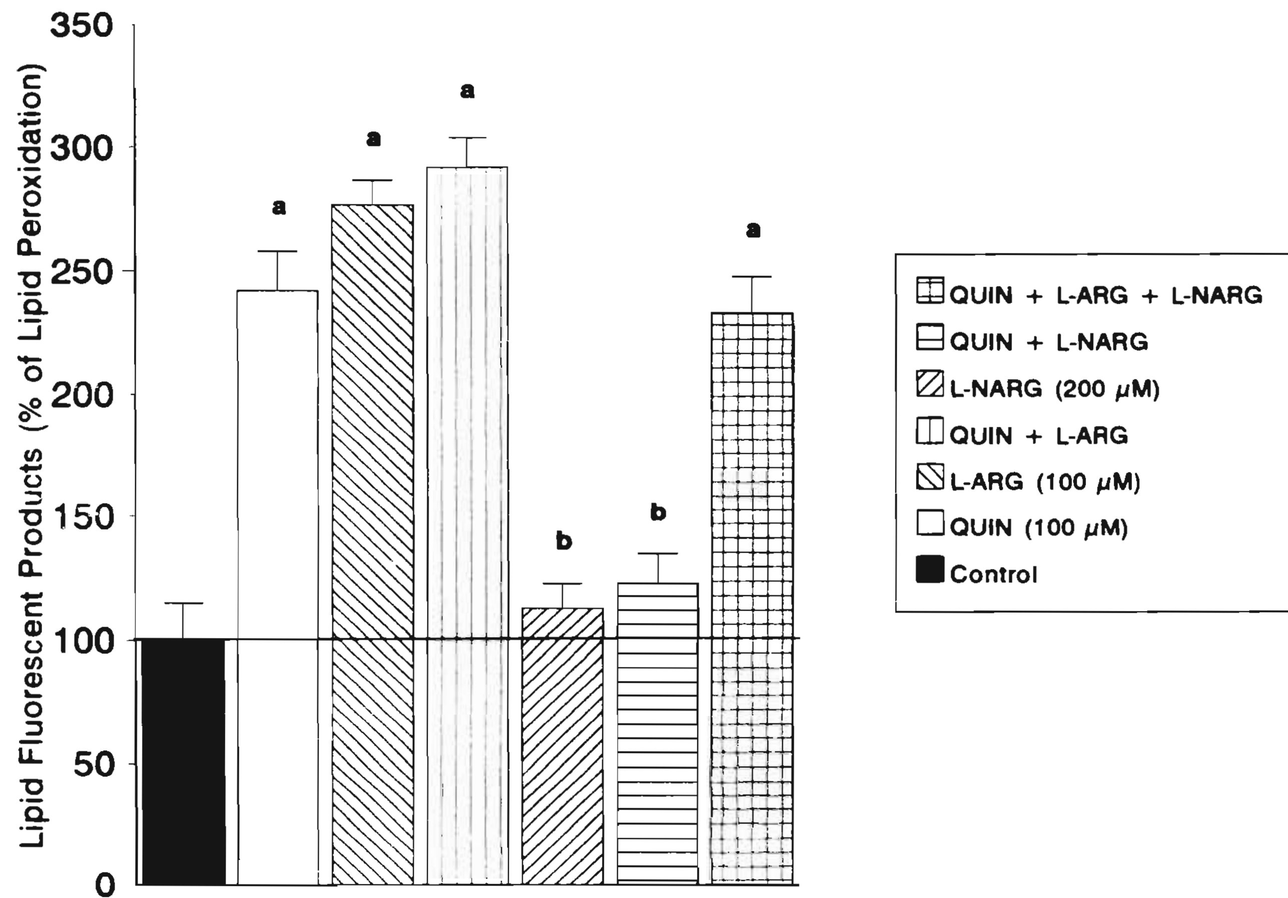
Fig. 3. Effect of L-arginine (L-ARG) and Nω-nitro-L-arginine (L-NARG) on quinolinic acid (QUIN)-induced lipid peroxidation. Synaptosomal fractions (1 ml of final volume) were incubated in isotonic saline solution in a shaking water bath during 1 hour at 37° C in the presence of L-ARG, L-NARG, QUIN or some combinations of them. Lipid peroxidation, expressed as percentage vs control values (100 %), was measured in synaptosomes by detection of lipid fluorescent products. Percentages of n=6-11 experiments ± S.E.M. are shown. *a* $p<0.05$, QUIN

treatment different to control value; *b* p<0.05, different from QUIN treatment; one-way ANOVA followed by Tukey's test.


Lipid Fluorescent Products (F.U./mg prot)
0
1
2
3
4
5
6
7
a
b
b
QUIN + APV
APV (250 μM)
QUIN (100 μM)
Control



Lipid Fluorescent Products (F.U./mg prot)
0
1
2
3
4
5
6
7
a
a
b
b
b
b
QUIN + L-NARG (400 μM)
QUIN + L-NARG (200 μM)
QUIN + L-NARG (100 μM)
QUIN + L-NARG (40 μM)
QUIN + L-NARG (10 μM)
QUIN (100 μM)
Control



Lipid Fluorescent Products (% of Lipid Peroxidation)
350
300
250
200
150
100
50
0
a
a
a
b
b
a
QUIN + L-ARG + L-NARG
QUIN + L-NARG
L-NARG (200 μM)
QUIN + L-ARG
L-ARG (100 μM)
QUIN (100 μM)
Control

DISCUSION

Los principales hallazgos de este trabajo están relacionados a los efectos específicos de diferentes moléculas con actividad fisiológica sobre el daño oxidativo inducido por el ácido quinolínico en el SNC de la rata. De esta manera podemos diferenciar, mediante el empleo de técnicas bioquímicas sencillas que evidencian la peroxidación de lípidos, aquellos factores promotores del estrés oxidativo de aquellos que lo inhiben. Entre los primeros, nuestros resultados sugieren que los cambios en la homeostasis del fierro cerebral o su adición como factor exógeno, así como un incremento en la producción del radical óxido nítrico y de sus especies tóxicas, están ambos contribuyendo de manera significativa en el estrés oxidativo después de la activación selectiva de receptores para NMDA por el ácido quinolínico; mientras que entre aquellos factores que resultaron potencialmente inhibidores de la lipoperoxidación podemos mencionar el bloqueo de dichos receptores por el empleo de antagonistas específicos tales como la dizocilpina y el ácido 2-amino-5-fosfonovalérico, así como la inactivación de especies reactivas del oxígeno por el empleo de glutatión y cobre.

Un aspecto importante en este trabajo fue la selección de las condiciones experimentales bajo las cuales serían probados los efectos de cada uno de los factores potencialmente moduladores de estrés oxidativo inducido por el quinolinato. Tanto *in vitro* como *in vivo*, el quinolinato resultó promover de manera significativa la lipoperoxidación, sugiriendo que en ambas condiciones se expresan los mecanismos necesarios para producir el estrés oxidativo generado por el incremento en la actividad de los receptores para aminoácidos excitadores.

La importancia de los radicales libres como mediadores de daño celular y como promotores de procesos patológicos ha sido ampliamente descrita en la literatura (Kehrer, 1993; Olanow & Arendash, 1994; Dawson *et al.*, 1995; Simonian & Coyle, 1996). Más aún, la participación de procesos de excitación de receptores para aminoácidos excitadores, tales como el glutamato y

el aspartato, en la generación de eventos de estrés oxidativo ha recibido también gran atención como una alternativa para explicar la naturaleza de ciertas enfermedades neurodegenerativas (Olney, 1989; Pellegrini-Giampietro *et al.*, 1990; Coyle *et al.*, 1991; Coyle & Puttfarcken, 1993; Schulz *et al.*, 1995).

En particular, el estudio de los efectos de los metabolitos de la vía de la kinurenina en el SNC (misma que se encuentra a nivel cerebral ubicada en células gliales y en células endoteliales de capilares) tiene gran importancia pues tan solo esta vía emplea aproximadamente el 80 % del triptofano en el cerebro (Stone, 1993). En esta vía metabólica que transforma al L-triptofano en NAD+, después de formar múltiples metabolitos intermediarios, destacan por su acción sobre los receptores glutamatérgicos para NMDA el ácido kinurénico y el ácido quinolínico. El primero constituye un antagonista selectivo endógeno de dichos receptores, mientras que el segundo representa el correspondiente agonista endógeno (Stone, 1993). Esta relación se vuelve aún más interesante cuando se considera por un lado que ningún otro metabolito de la vía parece desempeñar un papel tan importante como el que tienen los dos mencionados en cuanto a sus efectos en el Sistema Nervioso, y por otro lado, a que en condiciones fisiológicas, la acción de estas dos moléculas sobre los receptores NMDA se dá conservando una proporción de 3:1 (3 moléculas de kinurenato son necesarias para mantener inhibido al receptor por cada molécula de quinolinato). Si este balance se ve alterado bajo circunstancias que favorezcan la síntesis de quinolinato más que la de kinurenato, entonces se manifestarán condiciones de sobre-excitación neuronal que conducirán eventualmente a la generación de procesos patológicos como los que se observan en enfermedades tales como la encefalopatía hepática y el complejo SIDA-demencia (Heyes *et al.*, 1992). De esta manera, podría decirse que la vía de la kinurenina no solo es responsable de la modulación de la actividad de estos receptores, sino

que además parece estar genéticamente "programada" para conservar este balance en términos fisiológicos.

La participación del ácido quinolínico como posible factor etiológico en neuropatías degenerativas (Heyes *et al.*, 1992) condujo a un especial interés por estudiar los efectos neurotóxicos de esta molécula. Entre estos posibles eventos involucrados en su patrón de daño neuronal, la participación del estrés oxidativo comenzó a cobrar interés a partir del hallazgo de que su metabolito precursor inmediato en la misma vía metabólica de la kinurenina, el 3-hidroxiantranilato, es capaz de alterar el status oxidativo en eritrocitos humanos por efectos que bien podría compartir con otros intermediaros de la vía, incluyendo al quinolinato: la inducción de oxidación de productos de la hemoglobina, por su reactividad auto-oxidativa catalizada y por incremento en el flujo de la red glucolítica oxidativa (Dykens *et al.*, 1989). Estos hallazgos sugiriendo que los metabolitos de la vía de la kinurenina podrían estar involucrados en el incremento tóxico de la actividad redox, se vieron reforzados por reportes posteriores, como el de Moroni y colaboradores (1992), quienes describieron poco tiempo después que los derivados del kinurenato y del tiokinurenato, conocidos genéricamente como tiokinureninas (ácidos 7-Cl-kinurénico y 7-Cl-tiokinurénico), son capaces de proteger contra el daño excitotóxico y contra la peroxidación de lípidos producidos *in vitro* e *in vivo* por quinolinato y glutamato, tanto en cultivos primarios de células granulares cerebelosas como en cuerpo estriado de ratas lesionadas por microinyección, actuando específicamente como antagonistas sobre los receptores para NMDA y posiblemente como atrapadores de radicales libres. En este contexto, la posible participación de mecanismos de producción de radicales libres en el patrón tóxico del quinolinato fue nuevamente apoyada por Nakao y colaboradores (1996), quienes demostraron que el empleo de dos agentes antioxidantes específicos con acción de atrapadores de radicales libres, el α-fenil-tert-butil nitronio y el fármaco experimental

U-83836E, previnieron las lesiones estriatales producidas *in vivo* por la microinyección de quinolinato a ratas.

Sin embargo, uno de los reportes más reveladores e integrativos que hacen referencia a la acción oxidativa del quinolinato y a aquellos factores involucrados en dicho efecto fue publicado por Lipton y colaboradores (1994). En este artículo, los autores postulan que los eventos de daño neuronal derivados de procesos inflamatorios e infecciosos de origen viral involucran la microgliosis y producción de quinolinato inducida por activación de macrófagos (Heyes *et al.*, 1992) con la consecuente producción de óxido nítrico y ácido araquidónico después de la excitación de receptores para NMDA, todo lo cual conduce a un estrés oxidante, el cuál es requisito para la muerte neuronal.

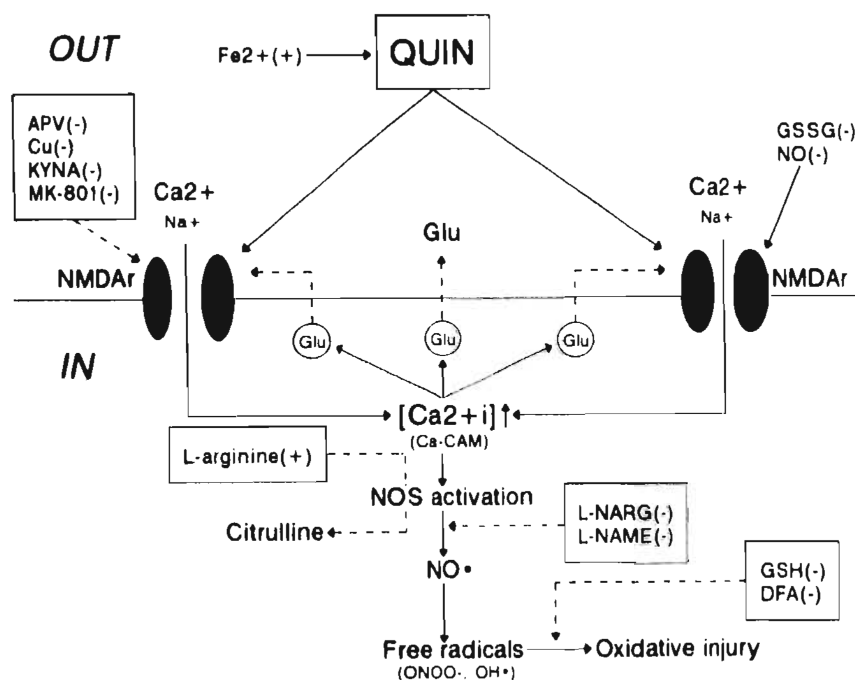
Recientemente, Stípek y colaboradores (1997), a partir de hallazgos previos de Goda y colaboradores (1996), han demostrado que el efecto peroxidativo del quinolinato en el cerebro es completamente dependiente de fierro endógeno, argumentando la capacidad de esta toxina de formar complejos con ciertos metales de transición, entre los cuales, el fierro destaca por su abundancia en el tejido cerebral. Adicionalmente, este complejo quinolinato-fierro favorecería la permanencia del fierro en su estado Fe^{2+}, constituyéndose en un sustrato idóneo para la generación de radicales libres por la reacción de Fenton, en presencia de peróxido de hidrógeno.

Esta serie de evidencias a favor de una posible implicación de procesos de daño oxidativo como mediadores de la neurotoxicidad del quinolinato, aunada a nuestros propios reportes demostrando la potencia pro-oxidante de esta molécula, prueban que el incremento en la actividad redox cerebral por acción de la excitación prolongada de receptores glutamatérgicos es un aspecto clave para la expresión de daño neuronal en capítulos de isquemia-hipoxia, alteraciones auto-inmunes y procesos excitotóxicos con componentes hereditarios (Goda *et*

al., 1996). Entre los efectos más notables producidos por el complejo quinolinato-fierro (II) destacan el incremento en la transferencia de electrones desde el complejo hacia moléculas del oxógeno, la generación de radicales superóxido e hidroxilo con la consecuente ruptura de cadenas de DNA y la alteración de otras biomoléculas.

Sea cual sea el mecanismo de acción tóxica del QUIN en el cerebro, actuando como agonista tipo NMDA o en su forma de complejo con el fierro endógeno, el papel que desempeñan los factores evaluados en este trabajo constituyen una explicación alternativa al problema de los radicales libres y el estrés oxidativo en las diferentes patologías del SNC.

A continuación se presenta un diagrama integrativo que contempla todos aquellos aspectos de la neurotoxicidad del QUIN que fueron caracterizados en este estudio:



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Brevemente, el daño oxidativo producido por el QUIN en el SNC puede asociarse a la formación de complejos pro-oxidantes con el fierro libre endógeno. Dicho complejo puede ser responsable directo de la generación de radicales libres, o bien unirse a receptores para NMDA y activar el canal asociado permeable a calcio y sodio. La prevención de la formación del complejo, y consecuentemente, del daño oxidativo mediado por el QUIN, está dada por moléculas antioxidantes, tales como el glutatión y la desferrioxamina, o por metales de transición, como el cobre, mismos que fueron probados en nuestro estudio. A este nivel, la protección de la neurotoxicidad del QUIN también puede observarse por la acción directa de distintas moléculas sobre el receptor para NMDA, las cuales inhibirán su respuesta. Entre dichas moléculas, en este estudio de probaron los efectos de los conocidos antagonistas del NMDA, el APV, el KYNA y el MK-801, todos los cuales resultaron ser antioxidantes. También el cobre podría estar actuando en el bloqueo de dichos receptores. Una vez activado el receptor, el incremento en los niveles intracelulares de calcio será responsable tanto de la liberación de glutamato desde la terminal sináptica (contribuyendo así en mayor grado a la activación de receptores para EAA), como a la activación de múltiples enzimas, entre las que destaca la calcio-calmodulina, misma que incrementará los niveles de NO por activación directa de la NOS. En este punto, la adición de L-arginina resultará en la activación selectiva de la NOS, incrementando los niveles de NO·. El empleo de inhibidores de la actividad de la NOS, tales como la L-NARG, resultaron en la prevención de la lipoperoxidación inducida por QUIN, evidenciando la participación del NO en dichos eventos por acción directa, o bien por generación de otras especies reactivas del oxígeno, tales como el radical hidroxilo o el peroxinitrito. Como se observa, este esquema sugiere una serie de eventos secuenciales responsables, a distintos niveles, de la generación de eventos oxidativos y neurotóxicos. Su secuenciación será objeto de estudio profundo durante los próximos años.

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