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Studies of aminochrome toxicity in a mouse derived neuronal cell line: is this toxicity mediated via glutamate transmission?

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Summary. Aminochrome was found to be toxic in a mouse-derived neuronal cell line (CNh). The effect was concentration dependent (10–150μM). The issue whether aminochrome toxicity involves glutamate transmission was studied with several glutamate receptors antagonists. Incubation of the cells with aminochrome (150μM) in the presence of 100μM of the AMPA antagonist, NBQX resulted in an increase of cell survival, from 52 to 73%. However, this protective effect did not seem to be related to activation of ionotropic glutamate receptors since incubation of CNh cells with 200μM of glutamate resulted in only 10% decrease of cell survival. However, NBQX was found to inhibit *in vitro* the autoxidation process. One hundred μM AP-5 did not have any effect on aminochrome toxicity. The toxic effect of aminochrome on CNh cells seems to be dependent of extracellular activation since addition of dicoumarol, a specific inhibitor of DT-diaphorase, did not affect that toxicity, which can be explained perhaps by a lack of a transport system for aminochrome into the CNh cells.

Keywords: Amino acids – Dopamine – Toxicity – Aminochrome – Semiquinone – NMDA – AMPA – Kainate – Glutamate – Quinone – Excitotoxicity

Introduction

A relationship among free radicals and excitatory amino acids transmission has been suggested (Zeman et al., 1994). Excitatory amino acid receptor-activation results in the generation of harmful free radicals (Pazdernik et al., 1992) and these in turn can enhance excitatory amino acid release (Pellegrini-Giampietro et al., 1990). The overexpression of Cu-Zn-superoxide dismutase in transgenic mice significantly reduces glutamate neurotoxicity (Chan et al., 1990), and the inhibition of the free radical-generating enzyme xanthine

oxidase protects the olfactory cortex from excitotoxic injuries induced by kainic acid (Facchinetti et al., 1992; Dykens et al., 1987). Mannitol, a scavenger of hydroxyl radicals, has been reported to protect cerebellar neurons from kainate-induced death (Dykens et al., 1987). Carvedilol, a antihypertensive agent, has been found to inhibit lipid peroxidation and also to provide protection against glutamate-mediated excitotoxicity (Lysko et al., 1992). Furthermore, the antioxidants, vitamin E and reduced glutathione (GSH) have been reported to provide protection against glutamate-induced excitotoxicity on PC12 cells (Pereira and Oliveira, 1997), and melatonin has showed protective effects against the elevation of lipid peroxidation induced by either kainate or NMDA (Kim and Kwon, 1999).

The possible role of reactive oxygen species generated during one-electron reduction of aminochrome in the activation of ionotropic glutamate receptors is an open question. One-electron reduction of aminochrome to leukoaminochrome o-semiquinone radical has been suggested to be the reaction responsible for the formation of reactive oxygen species in the neurodegenerative process of the dopaminergic system in Parkinson's disease (Baez et al., 1995; Segura-Aguilar et al., 1998; Segura-Aguilar et al., 1999). Leukoaminochrome o-semiquinone is very reactive with oxygen, resulting in the reduction of dioxygen to superoxide radicals. DT-diaphorase has been proposed to play a neuroprotective role in the presence of superoxide dismutase and catalase by preventing the autoxidation and the redox cycling process during aminochrome metabolism (Segura-Aguilar and Lind, 1989; Baez et al., 1995). One-electron quinone reductases and DT-diaphorase compete to reduce aminochrome to leukoaminochrome o-semiquinone and leukoaminochrome (the products of one- and two-electron reduction), respectively.

We have recently developed a cell line (CNh) from mouse cerebral cortex expressing several neuronal properties observed in vivo, including N-methyl-D-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA)/kainate receptors. CNh cells exhibit increments of intracellular Ca^{2+} in response to externally administered glutamate, NMDA, AMPA and kainate (Cardenas et al., 1999). Thus, we have used this cell line to study if the mechanism of aminochrome neurotoxicity includes that of activation of ionotropic glutamate receptors. The role of one and two-electron reduction of aminochrome in the activation of these receptors and cellular localization of aminochrome activation was also studied.

Material and methods

Chemicals

Dopamine, Dulbecco's modified eagle medium (DMEM), nutrient mixture HAM F-12, 6-nitro 7-sulfamobenzo(f)quinoxaline-2,3-dione (NBQX) and 2-amino-5-phosphonopentanoic acid (AP-5) were purchased from Sigma Chemical Co. (St Louis, MO, USA). ^{3}H-dopamine was from Amersham (England) and ^{3}H-choline was from NEN (Boston, USA). Aminochrome was prepared by oxidizing dopamine with Mn^{3+}-pyrophosphate complex according to Segura-Aguilar and Lind, 1989. ^{3}H-Aminochrome

was prepared by oxidizing ^{3}H-dopamine in the presence of excess of Mn^{3+}-pyrophosphate complex. The Mn^{3+}-pyrophosphate complex was prepared according to Archibald and Fridovich (1982).

Cell culture

The CNH cell line was originally prepared from cerebral cortex dissected from mouse fetuses. The cells are positive to several neuronal markers such as MAP-2, cynaptophysin, neuron specific enolase (NSE), choline acetyl transferase by immunohistochemistry and lack glial traits, such as glial hibrillary acidic protein (GSAP), S-100 and galacto cerebroside, after almost 2 years in continuous culture (Cardenas et al., 1999). CNh cells were seeded in 35 mm plastic petri dishes to get density of 5,000 cells/cm^2. The culture medium consisted of DMEM/Ham F12 nutrient mixture (1:1) containing 6 g/l glucose, 10% bovine serum, 1 g/L bicarbonate, 2.5% fetal bovine serum, and 40 μg/ml gentamicine (Cardenas et al., 1999). The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO_2. The toxicity experiments were performed in the absence of bovine serum and the dishes were incubated during 120 min. Aminochrome was formed by mixing 150 μM dopamine with 4 mM manganese^{3+}-pyrophosphate before been applied to the cells medium. In the experiments with different concentrations of aminochrome the ratio of dopamine to manganese was correspondly modified. Toxicity was measured by marking five squares of 1 $\times$ 1 mm under the petri dish before the cells were seeded. The cells were counted under contrast microscopy in the marked areas before and after the treatments. After the treatments Trypan Blue was added to the plate before the second counting. The incorporation of trypan blue to the cells and the cell detached from the plate was considered to indicate toxicity. The amount of positive cells was then compared to the total counts prior to the incubation periods with the corresponding toxic agents.

RT-PCR

The total RNA was isolated by using a RNeasy kit (QIAGEN). One μg of the total RNA was used for the synthesis of the single strand DNA with the reverse transcriptase reaction during 50 min at 42°C. The primers were designed from the cDNA sequence of DT-diaphorase (EC 1.6.99.2) reported by Bayney et al. (1989). The amplification of ssDNA of DT-diaphorase was performed by PCR reaction using the following primers 5′CAGAAACGACATCACAGGGGAG-3′ (upstream) and 5′ CAAGCACTCTCT CAAACCAGCC-3′ (downstream). The PCR reaction was performed in three steps: (i) 95°C for 5 min; (ii) one cycle at 94°C for 40 sec, 65°C for 45 sec, 72°C for 1 min, and eleven cycles with the annealing temperature decreased one degree/cycle from 65°C until 55°C; (iii) 30 cycles at 94°C for 40 sec, 62°C for 45 sec, 72°C for 1 min. The PCR incubation contained 8 μl of RT-incubation, 0.2 mM dNTP each, 1.5 μM $MgCl_2$, 0.5 μM primers, 5 μl 10X PCR-buffer (GibcoBRL), 29 μl H_2O and 1 U Taq polimerasa (GibcoBRL). The region amplified by PCR was between the bases 230 and 438, which resulted in a fragment of 209 bp (Bayney et al., 1989).

Tracer uptake into CNh cells

The uptake of ^{3}H-dopamine and ^{3}H-aminochrome into CNh cells was measured according to Allen et al. (1997) with slight modifications. ^{3}H-dopamine and ^{3}H-aminochrome (1 μCi) were added to each dish and the uptake was assessed after an incubation period of 10 min at 37°C in KREBS medium. The final concentration of dopamine and aminochrome was 100 μM. At the end of the uptake period, extracellular medium was removed and the cells

were rapidly washed 5 times with 2 ml ice-cold KREBS medium to remove residual ^{3}H-tracers. Cell membranes were disrupted with 1 ml of 1% Triton X-100 and after 3 minutes incubation 900 ul of the cell/Triton X-100 extract was removed and analyzed for ^{3}H-tracer content by liquid scintillation counting. The protein concentration was measured according to Smith et al. (1985) (Pierce, Rockford, IL, USA). Control experiments were performed with 1 μCi ^{3}H-choline (NEN Dupont, Boston, USA) (100 μM final concentration). The results are expressed in nmole/mg protein/10 min).

DT-diaphorase activity

DT-diaphorase was determined according to Ernster et al. (1962) by measuring the reduction of NADH at 340 nm. The cells were sonicated in 50 mM Tris-HCl, pH 7.5, during 10 sec and centrifugated at 10,000 xg for 20 min. The enzyme activity was measured in the pellet and supernatant fraction. The incubation contained 50 mM Tris-HCl, pH 7.5, 0.08% Triton X100, 500 μM NADH, 77 μM cytochrome C, 10 μM menadione. After obtaining the initial rate, 10 μM dicoumarol was added to inhibit DT-diaphorase completly (Beyer et al., 1987).

Results

Incubation of CNh cells with 150 μM dopamine during 2 h resulted in a low but statistically significant 7% decrease of survival (Fig. 1). The addition of 150 μM aminochrome to the incubation mixture resulted in 48% decrease of cell survival (Fig. 1A). The toxic effect of aminochrome was found to be concentration dependent as shown in Fig. 1B.

A possible involvement of activation of glutamate ionotropic receptors in the observed cytotoxic effects of aminochrome was investigated by incubating CNh cells with AP-5 or NBQX antagonists to NMDA and AMPA/kainate receptors, respectively, and 150 μM aminochrome. It was found that 100 μM NBQX produced a significant increase of survival, from 52 to 73% (Table 1). 100 μM AP-5 did not have any effect on aminochrome toxicity. Furthermore, a concentration of 200 μM glutamate, which induced a rapid and transient increase in $[Ca^{2+}]$ in CNh cells (Cardenas et al., 1999) produced only a 10% decrease of survival, which was not affected by NBQX (Table 1A).

The possibility that inhibitory effect of NBQX on aminochrome toxicity was due to a scavenger property of this antagonist was also investigated. This idea was tested by using the reductive activation of aminochrome with DT-diaphorase to leukoaminochrome *in vitro*. We added 100 μM NBQX to an incubation mixture containing 30 μM aminochrome, 500 μM NADH and 1 μg DT-diaphorase in 50 mM phosphate buffer (pH 6.5). The autoxidation was recorded by following the continous NADH oxidation at 340 nm. It was found that 100 μM of NBQX completely inhibited the autoxidation process of aminochrome by DT-diaphorase (not shown).

To study whether aminochrome toxicity was dependent on one- or two-electron reductive activation, dicoumarol was added to the culture medium finding that 50 μM dicoumarol had no effect on aminochrome toxicity (Fig. 2A). The lack of effect of dicoumarol on aminochrome toxicity opened questions regarding (i) the expression of DT-diaphorase in CNh cells, and (ii) the location of the metabolic activation of aminochrome.

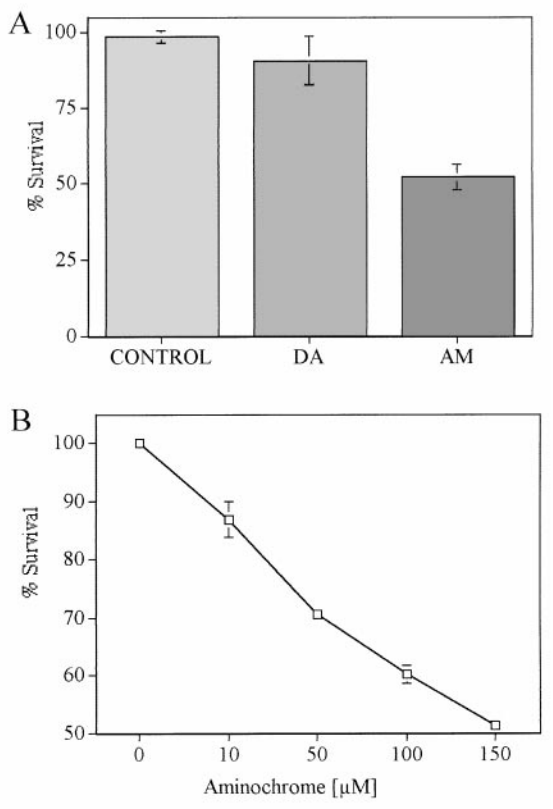

Fig. 1. Effect of aminochrome on cerebral cortex neuronal cells. **A** The incubation conditions and toxicity determination are described under Materials and methods. The concentration of dopamine (*DA*), and aminochrome (*AM*) were 150μM. Values are means ± SD (n = 6). **B** Percent survival as function of aminochrome concentration

Table 1. Effect of glutamate (A) and glutamate antagonists on CNh cells. Survival was measured as described under Materials and methods. Statistic significance was estimated by Student t-test (P** < 0.01)

A Addition	% Survival
Glutamate (200μM)	90 ± 7
Glutamate (200μM) + AP5 (100μM)	88 ± 4
Glutamate (200μM) + NBQX (100μM)	95 ± 3
B Addition	% Survival
Aminochrome	52 ± 4
Aminochrome + NBQX (100μM)	73 ± 5**
Aminochrome + AP-5 (100μM)	50 ± 2

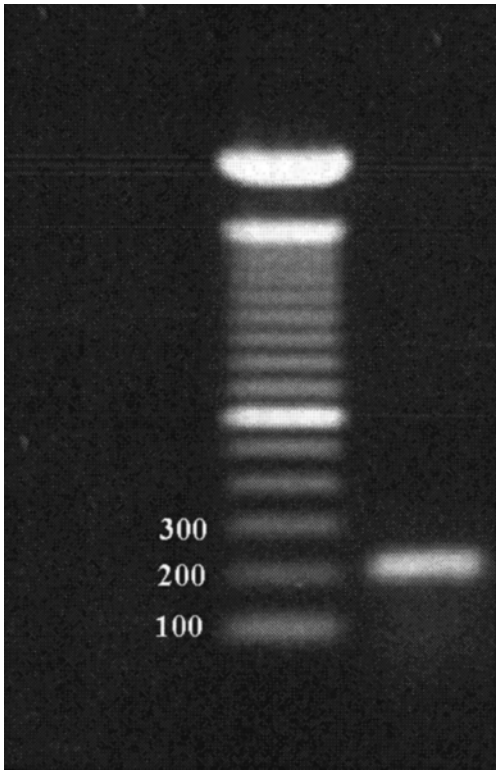

Fig. 2. Expression of DT-diaphorase mRNA in CNh cells. The expression of DT-diaphorase mRNA was determined by using RT-PCR as described under Materials and methods

The mRNA expression of DT-diaphorase in CNh cells was determined by using a RT-PCR technique. Figure 3 shows that the DT-diaphorase mRNA was expressed in CNh cells. In addition, the enzymatic activity of DT-diaphorase was also determined. The enzymatic activity in the 10,000xg pellet was 43nmole/min/mg protein and in the 10,000xg supernatant was 546nmole/min/mg.

The lack of effect of dicoumarol could be explained by an extracelular reduction of aminochrome and further uptake into the cells. To answer this possibility we performed uptake experiments of ^{3}H-dopamine and ^{3}H-aminochrome into CNh cells. Table 1 shows that there was a low transport for both ^{3}H-dopamine and ^{3}H-aminochrome, as compared to ^{3}H-choline in CNh cells. The observed uptake of ^{3}H-dopamine was 35-fold lower than that of ^{3}H-choline after 10min. In addition, about 97% of the total ^{3}H-dopamine or ^{3}H-aminochrome was found in the extracellular fraction (not shown).

Another experiment to demonstrate that aminochrome was extracellularly reduced to reactive oxygen species was that of inhibiting this metabolic activation by adding pure DT-diaphorase to the medium in the presence or absence of superoxide dismutase and catalase. The presence of DT-diaphorase, superoxide dismutase and catalase inhibited the amino-

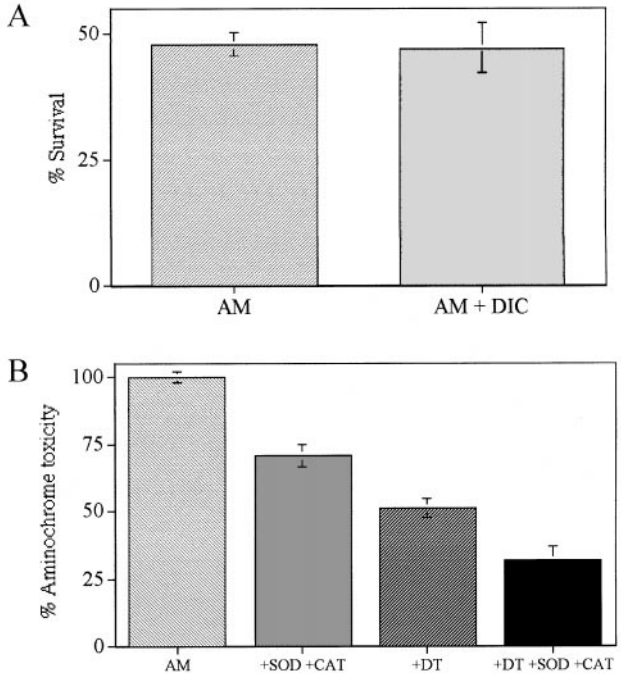

Fig. 3. Role of one- and two-electron reduction on aminochrome toxicity. **A** The effect of dicoumarol on aminochrome-dependent on CNh cells. The values are the mean ± SD (n = 6). **B** Extracellular inhibition of aminochrome toxicity. One microgram of pure DT-diaphorase, superoxide dismutase and catalase were added to the incubation medium. The values are the mean ± SD (n = 5)

chrome toxicity by approximatelly 70%. The presence of DT-diaphorase alone or superoxide dismutase and catalase together decreased the toxic effects of aminochrome by approximately 50% and 30%, respectively (Fig. 3B).

Discussion

Activation of ionotropic glutamate receptors (NMDA and AMPA/Kainate) induces excitoxicity by increasing the Ca^{2+} influx, thus impairing Ca^{2+} homeostasis (Meldrum and Garthwaite, 1990). A link between excitotoxicity and free radical has been proposed (Pazdernik et al., 1992). Antioxidants and/or antioxidant enzymes, such as superoxide dismutase and catalase, have been reported to inhibit glutamate-dependent excitotoxicity (Dykens et al., 1987; Pereira and Oliveira, 1997; Kim and Kwon, 1999; Chen et al., 1990).

Previous reports have linked the cytotoxic and apoptotic effects of dopamine in cell cultures to activation of glutamate receptors, since the toxic effects of dopamine can be inhibited by antagonist to NMDA receptors (AP-5) (Zhang et al., 1998; Lieb et al., 1995; Cheng et al., 1996). However, no effect of NMDA (MK-801) or non-NMDA (CNQX) antagonists has been found on dopamine-dependent toxicity of striatal neurons (Cheng et al., 1996).

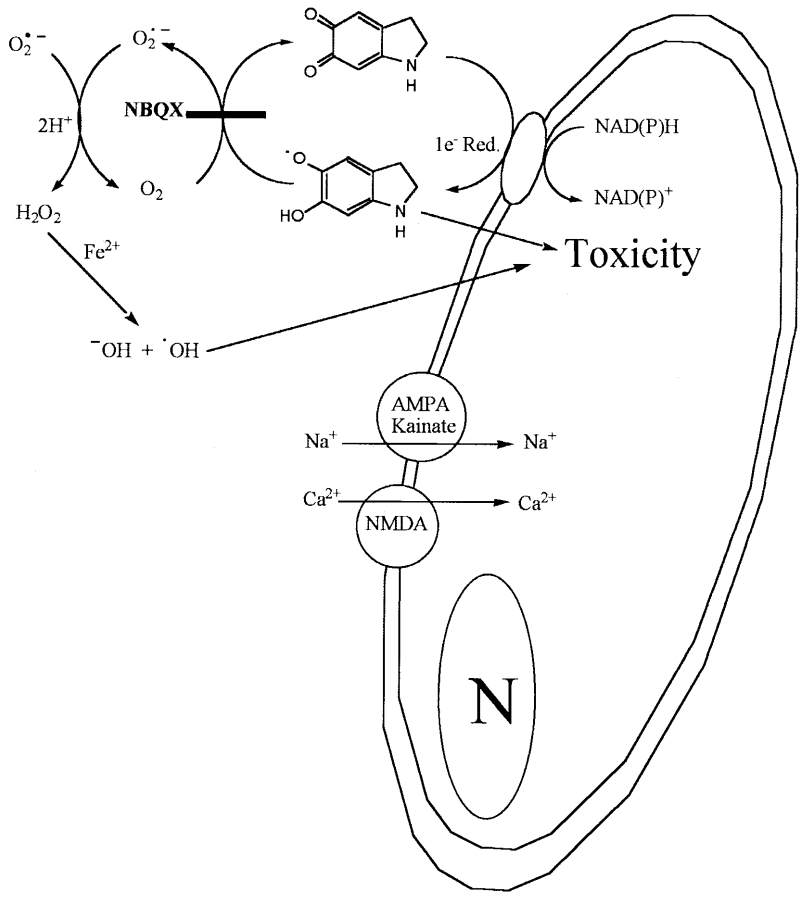

Fig. 4. Possible mechanism of toxicity of aminochrome in cerebral cortex neurons. Aminochrome is activated extracellularly to reactive oxygen species by flavoenzymes present in the cell membrane using electrons from intracellullar NADH or NADPH. Aminochrome is exclusively reduced by one-electron transfer flavoenzymes to leukoaminochrome *o*-semiquinone radicals since DT-diaphorase is mainly located in the cytosol and associated to mitochondria and endoplasmatic reticulum. Formation of leukoaminoachrome *o*-semiquinone radicals gives rise a redox cycling proccess resulting in the formation of large amounts of superoxide which dismutate and form hydrogen peroxide. Hydrogen peroxide is a precursor of hydroxyl radicals, which react with the majority of biomolecules. This redox cycle will deplete NADH required for ATP formation and/or deplete NADPH required for reduction of GSSG to GSH, which is an important antioxidant. The inhibitory effect of NBQX on aminochrome seems to be due to its ability to inhibit the redox cycling process

Although, AP-5 has been reported to prevent dopamine-dependent toxicity in female, but not in male dopaminergic neurons (Lieb et al., 1995). The mechanism of cytotoxic and apoptotic effects of dopamine has been proposed to be linked to dopamine oxidation and its reduction to leukoaminochrome o-semiquinone radical and formation of reactive oxygen species (Baez et al., 1995; Segura-Aguilar et al., 1998).

The possibility that aminochrome-dependent toxicity is mediated via a glutamate mechanism was studied in experiments with CNh cells. This cell

line has been proposed to be a useful tool for investigating dopamine-related toxicity (Cardenas et al., 1999). The phenotype of this cell line is neuronal, expressing receptors for severals putative neurotransmitters including glutamate and, in agreement, it has been found that 60–300μM glutamate induces a rapid and transient increase in Ca^{2+} concentration (Cardenas et al., 1999). In the present study, it was observed that the presence of NBQX produced an increase in the survival of cells treated with aminochrome (Table 1A). It seems plausible that the metabolic activation of aminochrome to leukoaminochrome o-semiquinones initiate a toxic process and that the reactive oxygen species formed during the redox cycling of aminochrome targeted the cell membrane allowing a release of glutamate, activating NMDA and AMPA/kainate receptors. However, incubation of CNh cells with 200μM glutamate showed only a slight decrease in cell survival (10%). In addition, NBQX did not appear to modify the effect of glutamate. A possible explanation to the lack of toxic effects of glutamate is that higher concentrations and longer time of incubation are required to induce toxicity, as reported previously (Pereira and Oliveira, 1997). Furthermore, it was found that NBQX inhibited the autoxidation and redox cycling process *in vitro* when aminochrome was reduced by DT-diaphorase.

No specific transport of aminochrome was observed in CNh cells (Table 2), suggesting that aminochrome may act extracellulary, and therefore not available for DT-diaphorase, which is considered to be an intracellular enzyme (Ernster et al., 1962). The addition of pure DT-diaphorase together with superoxide dismutase and catalase showed to inhibit aminochrome toxicity, which is in agreement with previous reports (Segura-Aguilar and Lind, 1989; Baez et al., 1995). These results support the idea that one-electron reduction of aminochrome is involved in the decrease of CNh cell survival observed in the presence of aminochrome. It seems plausible that flavoenzymes located in the outer membrane transfer electrons received from intracellular NADH or NADPH to extracellular aminochrome. Redox cycling of extracellular aminochrome catalyzed by flavoenzyme(s) may result in the formation of reactive oxygen species and leukoaminochrome o-semiquinone. These reactive oxygen species may induce lipid peroxidation, deactivation of enzymes, oxidation of thiol groups in proteins and reaction with nucleophiles.

Table 2. ^{3}H-dopamine and ^{3}H-aminochrome uptake into CNh cells. The uptake of ^{3}H-dopamine into CNh cells was measured as described under Materials and methods. The statistic significance was measured by using Student t-test ($P^{***} < 0.001$)

	Uptake (nmole/mg/10 min)
^{3}H-Choline	158 ± 52
^{3}H-Dopamine	16 ± 4***
^{3}H-Aminochrome	28 ± 6***

Although, the mechanism of aminochrome toxicity in CNh cells seems to be independent of glutamate receptors, the possible involvement of glutamate receptors cannot be discarded. Evidently, further experiments have to be performed for exploring this hypothesis.

Acknowledgements

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