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The Role of Hydrogen Peroxide in the In Vitro Cytotoxicity of 3-Hydroxykynurenine

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Previous studies have indicated that the generation of H_2O_2 may be a key step in the mechanism mediating the in vitro cytotoxicity of 3-hydroxykynurenine (3HK). An exposure protocol resulting in a delayed toxicity was utilized in order to further examine the role of H_2O_2 in the in vitro toxicity of 3HK in a neural hybrid cell line. 3HK-induced cell lysis was significantly attenuated by administration of catalase after termination of 3HK exposure and was abolished when intracellular peroxidase activity was elevated by pretreatment of cultures with horseradish peroxidase. In addition, a dose-dependent attenuation of 3HK toxicity was observed when cultures were exposed to 3HK in the presence of the iron chelator, desferrioxamine (DFO). Pretreatment with DFO also resulted in a significant attenuation of 3HK toxicity. These data suggest a direct role for H_2O_2 and metal ions in the cytotoxic action of 3HK and indicate that cell lysis results from the intracellular accumulation of toxic levels of H_2O_2.

KEY WORDS: 3-Hydroxykynurenine; toxicity; vitamin B6 deficiency; oxidative stress; N18-RE-105 cells.

INTRODUCTION

Adequate vitamin B6 nutrition is critical for the normal function and development of the immature central nervous system (CNS). In human infants (1,2) and neonates of several species (3–5), vitamin B6 undernutrition results in marked neurological impairment including ataxia, tremor, irritability and seizures, and in neuropathological changes consistent with altered development or neuronal damage (6,7). Pyridoxal phosphate, the predominant coenzyme form of vitamin B6, plays a central role in the metabolism of amino acids and biogenic amines including several neurotransmitters. In addition to altering parameters of GABAergic (8,9,10) and monoaminergic (4,11,12) neurotransmission, neonatal vitamin B6 deficiency results in a dramatic increase in CNS levels of 3-hydroxykynurenine (3HK) – an endogenous metabolite of tryptophan which has been reported to possess convulsant (13,14) and cytotoxic (15) properties. Increased 3HK levels have also been measured in CNS tissue from Huntington's disease patients (16) and in rat CNS following bacterial endotoxin exposure (17).

Dose-response data indicate that 3-hydroxykynurenine, at concentrations within the range of those measured in CNS tissue from vitamin B6 deficient rat pups (18), is toxic to a neuronally derived hybrid cell line (15). Since the in vitro toxicity of 3HK is attenuated by antioxidant treatments and is abolished when catalase (CAT) is present in the incubation medium, it was suggested that H_2O_2 plays a critical role in 3HK toxicity (15). In 3HK-exposed cultures, toxic levels of H_2O_2 may be produced intracellularly by the action of cellular oxidases or on either side of the membrane by the metal-catalyzed "auto-oxidation" of 3HK (19). Alternatively, H_2O_2 may be required as a cosubstrate for the peroxidative metabolism of 3HK to a toxic quinoneimine.

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In the present study, we have exploited the observation that the toxicity of 3HK is delayed following a time-limited exposure (15) in order to examine the effects of treatments administered before and after exposure to 3HK such that the observed effects on toxicity must reflect actions confined to the intracellular compartment. The data support a direct role for H_2O_2 in 3HK toxicity and suggest that 3HK-induced cell lysis results from toxic levels of H_2O_2 in the intracellular compartment.

EXPERIMENTAL PROCEDURE

Materials. Dulbecco's modified Eagle's medium (430–2100) and fetal bovine serum were obtained from Grand Island Biological Co. (Grand Island, NY). Catalase was obtained from Boehringer Mannheim (Indianapolis, IN) Iron salts ($FeCl_3$ and $FeSO_4$) were of reagent grade. Desferrioxamine mesylate was generously provided by CIBA-Geigy (Summit, NJ). All other reagents were purchased from Sigma (St. Louis, MO). Mouse neuroblastoma x rat embryonic retina (N18-RE-105) cells were kindly supplied by Dr. R. Schnaar of the Department of Pharmacology and Experimental Therapeutics of the Johns Hopkins University School of Medicine.

Cell Culture Methods. N18-RE-105 cells were maintained as described by Malouf et. al. (20). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 3.7 g/l $NaHCO_3$, 5% fetal bovine serum, 100 μM hypoxanthine, 1 uM aminopterin, and 16 μM thymidine. Cultures were incubated at 37°C in a humidified 9%–10% CO_2 atmosphere. The cells were split weekly.

All experiments employed cells which were subcultured at confluence from 75 cm^2 flasks and plated on 35 mm culture dishes at a density of 20,000 cells per dish in 1.0 ml culture medium 20–24 hours prior to experimental treatment. Cultures were exposed to potential toxic and/or protective agents by aspirating the incubation medium and replacing it with identical medium containing the agent(s) of interest. Exposure solutions were prepared immediately before use by dissolving experimental compounds in culture medium adjusted to pH 7.2–7.3 with HCl. Cultures were washed with 1.0 ml fresh culture medium between successive treatments. Except where noted otherwise, exposure to 3HK (500 uM) was for a period of 2 hours and 20 minutes. This exposure duration was chosen as the minimum exposure time required to reliably achieve greater than 80% cell death in the absence of protective agents. The durations of pre- and post- treatments were 2 hours and 22 hrs, respectively. Except where noted, cultures were harvested for quantitative determination of cell death 24 hours after the start of 3HK exposure. All culture plates were examined microscopically prior to harvest.

Cytotoxicity Assay. Cytotoxicity was quantitated by measuring the release of the constitutive cytosolic enzyme, lactate dehydrogenase (LDH) into the culture medium (21). LDH was assayed in the culture medium and in lysates of cells remaining attached to the culture dishes. Previous experiments have indicated that 3HK exposure does not result in detachment of viable cells, as reflected by the consistently insignificant proportions of LDH activity associated with pellet fractions from centrifugation of culture medium from 3HK exposed cell cultures. Percent cell death was estimated as LDH activity in the culture medium divided by the total LDH activity in the medium and cell lysate.

For measurement of LDH, the incubation medium was withdrawn from each culture dish and the cells remaining adhered to the surface of each dish were lysed in 1 ml of lysis buffer (0.5% Triton X-100 in 0.1 M potassium phosphate buffer,pH 7.0). LDH activity was measured spectrophotometrically in the culture medium and in the lysate by monitoring the decrease in absorbance at 340 nm in the presence of excess pyruvate and reduced nicotine adenine dinucleotide (NADH).

Horseradish Peroxidase (HRP) Histochemistry. The uptake of HRP into N18-RE-105 cells was demonstrated by exposing cultures to diaminobenzidine (DAB) and H_2O_2 to produce a colored precipitate in the presence of peroxidase. Fresh stock solutions of H_2O_2 (25 mM) and DAB (1 mg/ml) in Hanks Balanced Salts Solution (HBSS) were prepared daily. The DAB solution was adjusted to pH 5.5 with 1M Na_2HPO_4. H_2O_2 and DAB stocks were combined 1:9 immediately before use yielding a staining solution of 2.5 mM each.

Prior to staining, cultures were washed three times with HBSS. After the final wash, 1 ml staining solution was added and cultures were incubated for 20 min at 37°C. Stained cells were washed with HBSS afer incubation in staining solution and viewed with HBSS as the bathing medium.

RESULTS

Time course of delayed cytotoxicity of 3HK. We have previously reported (15) that exposure to 500 uM 3HK for a period of two hours results in delayed cell lysis as reflected by the release of LDH into the culture medium. While cell lysis measured immediately upon termination of exposure did not differ between exposed and unexposed cultures, near complete cell lysis was evident 22 hours later in 3HK exposed cultures. The time course of cell lysis was determined following a discrete 2 hour or a continuous exposure to 500 uM 3HK. Continuously exposed cultures were assayed for cell lysis at 2, 4, 8, 12, and 24 hours after the start of 3HK exposure, and cultures exposed for two hours were assayed for cell lysis at 2, 4, 8, and 24 hours after the start of the exposure period. 3HK exposure was terminated with a wash (1 ml fresh culture medium) and replacement of exposure medium with fresh culture medium.

In Figure 1, percent cell lysis is plotted as a function of time for cultures exposed to 3HK continuously or for two hours, only. Except at the 4 hour time point, the time course of cell lysis appears to be nearly identical for both exposure conditions. The fact that cultures exposed to 3HK for only 2 hours, but not those that were continuously exposed, were washed following exposure may contribute to the apparent difference in the level of cell lysis observed at 4 hours. It appears that the time course of cell lysis is little affected by continued exposure beyond the two hour period that we have previously found to produce maximum levels of cell death. This time-limited exposure protocol was chosen for further study both for its convenience and its adaptability to

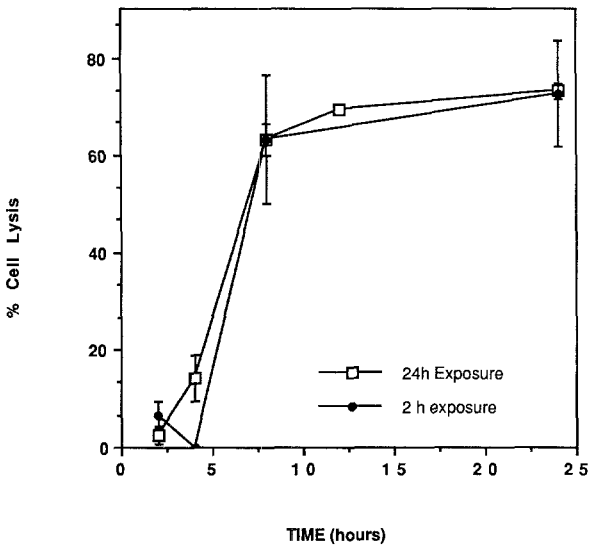

Fig. 1. Time course of cell lysis in cultures exposed to 3HK for 2 or 24 hours. Symbols represent mean ± SEM of 3 experiments performed in triplicate.

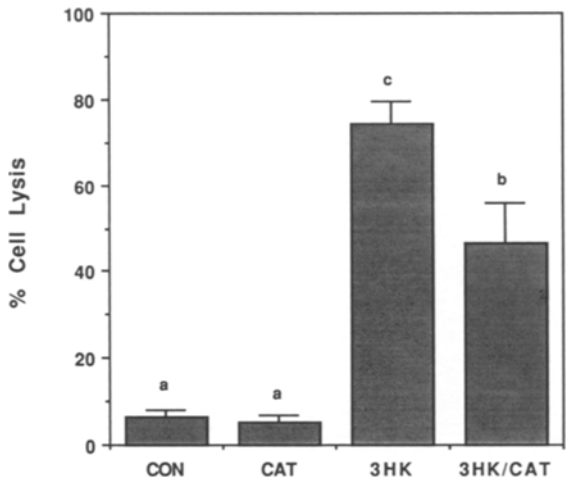

Fig. 2. Effect of catalase post-treatment on 3HK induced cell lysis. Cultures were incubated in medium containing 0 or 5 U/ml catalase following exposure to 0 or 500 uM 3HK for 2 h 20 min. Displayed data represents mean + SEM of 4 experiments performed in triplicate. Means were compared with Duncan's multiple range test following one-way ANOVA. Treatment group means marked with differing superscripts are significantly different ($p < 0.01$) from one another.

examining the effects of treatments applied before, during, and after exposure.

Catalase (CAT) Post-treatment. The observation that the cytotoxic effects of 3HK are abolished when CAT is present in the exposure medium (15) suggests a central role for H_2O_2 in 3HK toxicity. It is not clear from our previous data whether exogenously added CAT, which may be largely excluded from the cellular cytoplasm, acts to detoxify intracellular H_2O_2 or scavenges H_2O_2 produced in the culture medium. We have examined this issue by adding exogenous catalase to cultures after termination of 3HK exposure, such that any protective effect must be due to scavenging of intracellular H_2O_2.

Cultures incubated for 2 hours 20 minutes in the presence or absence of 500 μM 3HK were washed and incubation was continued in either fresh medium or medium containing 40 U/ml CAT. The results shown in Figure 2 indicate that the toxicity of 3HK is significantly attenuated by post-exposure addition of CAT. Post-treatment of control (no 3HK exposure) cultures with CAT had no significant effect on cell survival.

Elevation of Intracellular Peroxidase Activity. The neuronal uptake of horseradish peroxidase (HRP) forms the basis of several versatile neuroanatomical techniques (see 22). We have exploited the uptake of HRP by N18-RE-105 cells to further probe the role of H_2O_2 in 3HK toxicity. Catalase-sensitivity 3HK toxicity could result from the generation of toxic levels of H_2O_2 or if H_2O_2 was required as a cosubstrate for the peroxidative metabolism of 3HK to a toxic quinoneimine. In the former case, elevation of cellular peroxidase activity would be predicted to protect cells from the harmful effects of 3HK exposure by scavenging H_2O_2. In the latter case, the effect of increased peroxidase activity would depend on the availability of H_2O_2. In the presence of ample H_2O_2 increased peroxidase activity would enhance toxicity by increasing the production of the toxic metabolite. However, the production of the toxic metabolite would be little affected by increased peroxidase activity if H_2O_2 was rate-limiting.

The effect of elevated intracellular peroxidase activity on vulnerability to 3HK exposure was evaluated in cells plated either in medium alone or in medium containing 5 U/ml HRP. Control and HRP treated cultures were incubated overnight and washed three times prior to 3HK exposure. The HRP pretreatment by itself had no effect on cell survival (Figure 3). However, the level of cell lysis measured in HRP treated cultures after 3HK exposure did not differ significantly from control levels (Figure 3a). Pretreatment of cultures with HRP also attenuated the toxic effect of H_2O_2 exposure (Figure 3b).

In order to verify that the HRP treated cells had taken up HRP from the incubation medium, cultures were treated for histochemical demonstration of peroxidase activity. Peroxidase activity was weak or absent in control cultures while HRP treated cultures were heavily

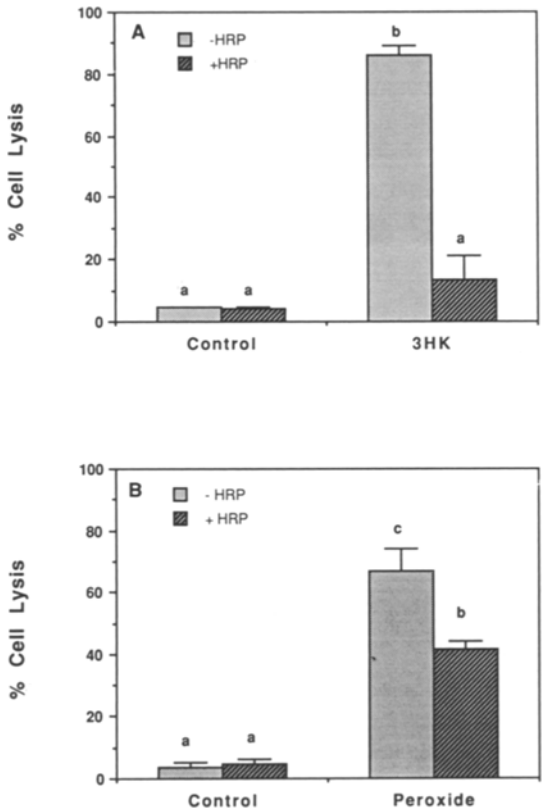

Fig. 3. Effect of elevated intracellular peroxidase activity on 3HK- or H_2O_2-induced cell lysis. Cultures were incubated overnight in the presence or absence of 5 U/ml horseradish peroxidase (HRP) prior to exposure to fresh medium (Control), 500 uM 3HK (a), or 25 uM H_2O_2 (b) for 2 h 20 min. Displayed data represent means + SEM of 3 (a) or 4 (b) experiments performed in triplicate. Means were compared with Duncan's multiple range test following one-way ANOVA. Treatment group means marked with differing superscripts are significantly different ($p < 0.01$) from one another.

stained. Staining was never observed in acellular regions on the surface of the culture dish, even when stained cultures were not rinsed prior to viewing (data not shown). Therefore, it is unlikely that HRP adsorbed to the surface of the culture dishes could have contributed to the observed protective effect of HRP-pretreatment.

Iron Chelation with Desferrioxamine (DFO). The effect of the iron chelator, DFO, on 3HK-induced cell lysis was examined in order to assess the role of iron in 3HK toxicity. We have previously shown that generation of H_2O_2 is a critical step in the process resulting in 3HK induced cell lysis, and proposed that H_2O_2 may be produced as the result of the oxidation of 3HK to the corresponding quinoneimine (15). Iron has been implicated as having a role in the toxicity of structurally similar oxidizable compounds such as catecholamines and their 6-hydroxy- and 6-amino- analogues (23).

Cultures were exposed to 0 (CON) or 500 μM 3HK for 2 hours 20 minutes in the presence of 0, 0.5, 1.0, or 1.5 mM DFO, and cell lysis was assayed 24 hours after the start of 3HK exposure. Exposure to DFO at concentrations up to 1.5 mM had no effect on cell survival in CON cultures. In contrast, cell lysis in cultures exposed to 3HK was inhibited by DFO in a dose dependent manner (Figure 4). As shown in Figure 5, 3HK toxicity could be restored in the presence of DFO by addition of iron at a concentration (500 μM) that had no effect on cell survival in the absence of 3HK.

The effects of pre- and post-treatment of 3HK exposed cultures with DFO are shown in Figure 6. Pretreated cultures were incubated in medium containing 3 mM DFO for two hours immediately prior to exposure to 3HK. Medium containing 3mM DFO was added to post-treated cultures immediately upon termination of 3HK exposure. Treatment with DFO after exposure to 3HK had no effect on the toxic endpoint (Figure 6b). However, the toxicity of 3HK was significantly attenuated in cultures pretreated with 3mM DFO (Figure 6a), suggesting that iron chelation in a (intracellular) compartment inaccesible to dilution by the wash and change of medium was responsible for the attenuated toxicity in DFO pretreated cultures. A control experiment was conducted in order to rule out the possibility that the protective effect of DFO pretreatment might be due to the presence of a large pool of DFO remaining adsorbed to the surface of the culture plate after the washes and change of medium (data not shown).

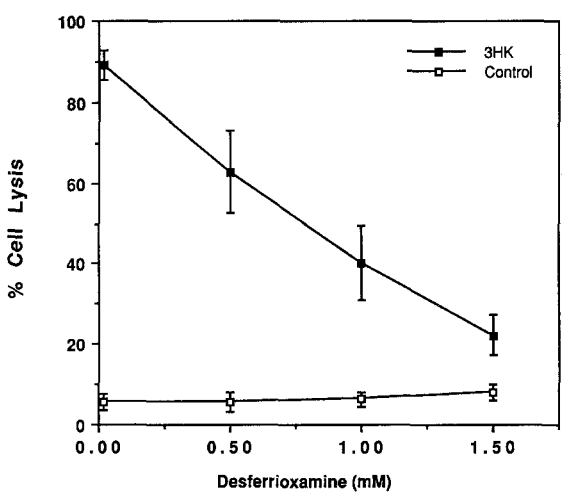

Fig. 4. Dose-response relationship for the attenuation of 3HK toxicity by desferrioxamine (DFO). Cultures were exposed to 0 or 500 μM 3HK in the presence of the indicated concentrations of DFO. Data represent mean ± SEM of three triplicate determinations.

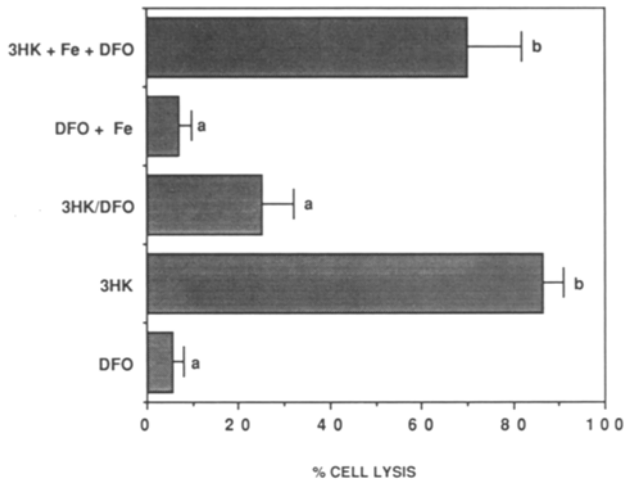

Fig. 5. Effect of exogenous iron on the protective effect of DFO. Cultures were exposed to 1.5 mM DFO or 500 uM 3HK alone (DFO, 3HK), or 1.5 mM DFO in combination with 500 uM 3HK (3HK/DFO), 500 uM $FeCl_3$ (DFO + Fe), or both $FeCl_3$ and 3HK (3HK + Fe + DFO). Data represent means ± SEM of three experiments performed in triplicate. Means were compared with Duncan's multiple range test following one-way ANOVA. Treatment group means marked with differing superscripts are significantly different ($p < 0.01$) from one another.

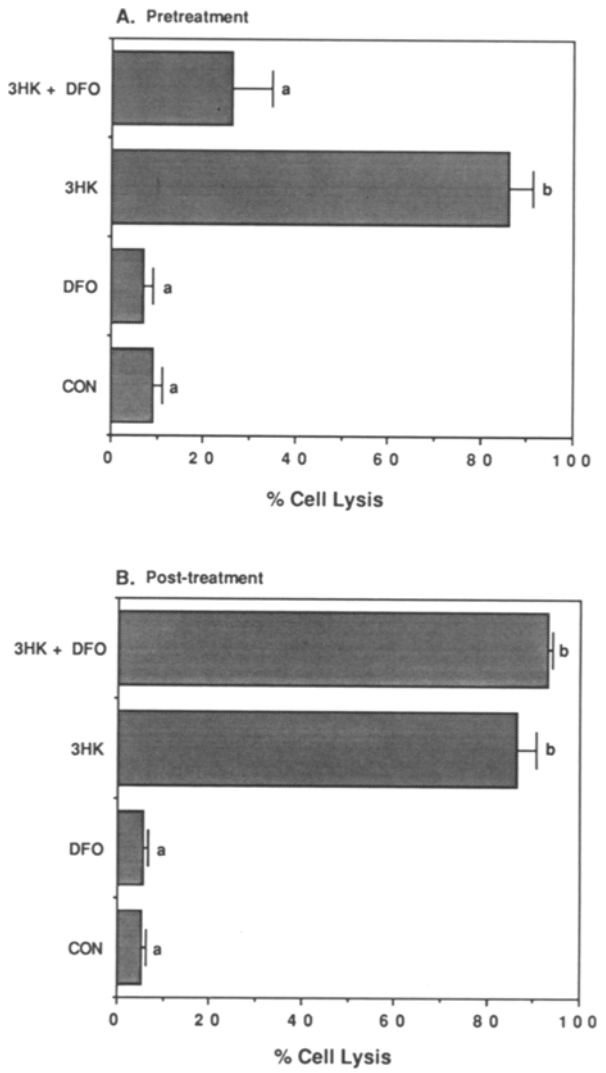

Fig. 6. Effect of DFO Pre- (a) and post-treatment (b) on 3HK-induced cell lysis. Cultures were treated with 3 mM DFO before or after exposure to 500 uM 3HK (see Results, Experiment IV). Data represents means ± SEM of three experiments performed in triplicate. Means were compared with Duncan's multiple range test following one-way ANOVA. Treatment group means marked with differing superscripts are significantly different ($p < 0.01$) from one another.

DISCUSSION

The experimental results reported here provide further support for the direct involvement of H_2O_2 in 3HK toxicity, and specifically implicate an intracellular pool of H_2O_2 as having a role in the observed toxicity. Since H_2O_2 is a permeant species, the methods employed in the present study do not permit discrimination between intracellular generation of H_2O_2 and its accumulation from the bathing medium.

We have used an experimental protocol under which the toxicity of 3HK, as assessed by LDH efflux, is delayed following termination of 3HK exposure. It is unlikely that the observed delay in cell death is attributable to a temporal lag between irreversible damage and its expression as a loss of membrane integrity because cell lysis can be attenuated by treatment with CAT after termination of 3HK exposure. Rather, the delay in 3HK-induced cell lysis appears to reflect an ongoing, catalase-sensitive toxic process.

The ability of neuronal cells, including N18-RE-105 cells, to accumulate HRP was exploited in order to further investigate the role of H_2O_2 in 3HK toxicity. Catalase-sensitive 3HK toxicity could result either directly from the generation of toxic levels of H_2O_2, or if H_2O_2 was required for the oxidation of 3HK, by endogenous peroxidase enzymes, to a toxic metabolite (eg. a quinoneimine). These two possibilities are readily distinguishable on the basis of the effect of altered peroxidase activity on 3HK-induced cell lysis. Increased peroxidase activity would be expected to attenuate 3HK toxicity if H_2O_2 was directly toxic. On the other hand, enhanced toxicity would be expected if H_2O_2 was required for the peroxidase-mediated oxidation of 3HK to a toxic metabolite. The results obtained are consistent with a direct role for H_2O_2 in 3HK toxicity since 3HK-

induced cell lysis is abolished by elevation of intracellular peroxidase activity (fig. 3a). Furthermore, HRP pretreatment significantly attenuated the toxic effects of exogenously administered H_2O_2. Since HRP catalyzes the oxidation of 3HK in the presence of H_2O_2, as reflected by an increase in absorbance at 430–460 nm (data not shown), these data also indicate that the observed toxicity cannot be attributed to the peroxidative production of a toxic metabolite.

The intracellular localization of a toxic pool of H_2O_2 is indicated by the results of experiments in which antioxidant treatments were administered before initiation or after termination of 3HK exposure. Post-treatment with CAT was initiated after removal of extracellular 3HK and H_2O_2 with the exposure medium. Thus, the ability of this treatment to attenuate 3HK toxicity most likely reflects the detoxication of H_2O_2 residing in, or generated within, the intracellular compartment after termination of 3HK exposure. Pretreatment of cultured cells with HRP resulted in increased intracellular peroxidase activity, as indicated by increased diaminobenzidine staining, in HRP-treated cultures. The abolition of 3HK toxicity by pretreatment with HRP suggests that the scavenging of H_2O_2 from intracellular pools is sufficient to protect cells from the adverse effects of 3HK exposure, which is in agreement with results obtained with catalase.

Finally, we have shown that 3HK toxicity is attenuated by pretreatment with DFO, presumably as a result of chelation of intracellular iron. Iron is thought to have an obligatory catalytic role in "auto-oxidation" reactions (23). Accordingly, we observed a dose-dependent attenuation of 3HK toxicity in cultures exposed to 3HK in the presence of DFO at concentrations ranging up to 1.5 mM. The toxicity of 3HK is restored, in the presence of DFO, by addition of exogenous iron. The requirement for millimolar concentrations of DFO is somewhat surprising in view of its very high affinity for ferric iron (24). On the other hand, such high concentrations may be required to attain intracellular DFO levels sufficient to chelate intracellular iron. In addition, iron can contribute to oxidative damage in biological systems by catalyzing the reduction of H_2O_2 to form the highly reactive hydroxyl radical (25). Thus, chelation of iron could attenuate 3HK toxicity either by inhibiting the "auto-oxidative" generation of H_2O_2, or by inhibition of hydroxyl radical formation via the Fenton reaction.

While the evidence supports a primary role for an intracellular pool of H_2O_2 in 3HK toxicity, the site of H_2O_2 production remains unknown. Preliminary results of uptake studies using [^{3}H]3HK suggest that, in this cell line, 3HK may gain access to the cellular cytoplasm via a saturable process. Similar studies conducted by Speciale et. al. (26) have shown that L-kynurenine, an immediate precursor and close structural analog of 3HK, is actively accumulated by cultured astrocytes via the neutral amino acid transporter. Thus, it is likely that a pool of 3HK may be available for intracellular oxidation.

On the basis of these studies and those previously reported (15), it can be speculated that the cytotoxic effect of 3HK results from the intracellular accumulation of H_2O_2 generated by the iron-catalyzed "auto-oxidation" of 3HK. The understanding of a mechanism of action of 3HK toxicity gains greater importance in view of recent evidence that CNS concentrations of 3HK are also elevated in Huntington's disease patients (16) and in experimental animals following bacterial endotoxin exposure (17).

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