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Hydrogen peroxide-mediated neuronal cell death induced by an endogenous neurotoxin, 3-hydroxykynurenine

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ABSTRACT **3-Hydroxykynurenine (3-HK) is a tryptophan metabolite whose level in the brain is markedly elevated under several pathological conditions, including Huntington disease and human immunodeficiency virus infection. Here we demonstrate that micromolar concentrations (1–100 μM) of 3-HK cause cell death in primary neuronal cultures prepared from rat striatum. The neurotoxicity of 3-HK was blocked by catalase and desferrioxamine but not by superoxide dismutase, indicating that the generation of hydrogen peroxide and hydroxyl radical is involved in the toxicity. Measurement of peroxide levels revealed that 3-HK caused intracellular accumulation of peroxide, which was largely attenuated by application of catalase. The peroxide accumulation and cell death caused by 1–10 μM 3-HK were also blocked by pretreatment with allopurinol or oxypurinol, suggesting that endogenous xanthine oxidase activity is involved in exacerbation of 3-HK neurotoxicity. Furthermore, NADPH diaphorase-containing neurons were spared from toxicity of these concentrations of 3-HK, a finding reminiscent of the pathological characteristics of several neurodegenerative disorders such as Huntington disease. These results suggest that 3-HK at pathologically relevant concentrations renders neuronal cells subject to oxidative stress leading to cell death, and therefore that this endogenous compound should be regarded as an important factor in pathogenesis of neurodegenerative disorders.**

As a possible cause of the neuronal degeneration found in the brains of patients with various neurological disorders, aberrant metabolism of endogenous substances that leads to the production of excess amounts of neurotoxic compounds has provoked much recent interest. The kynurenine pathway, the major metabolic pathway of the amino acid tryptophan, has been a focus of attention in this context, since this pathway produces quinolinic acid as a metabolic intermediate. Quinolinic acid has an ability to activate the *N*-methyl-D-aspartate subtype of glutamate receptors; thus an excess of this compound is presumed to cause excitotoxic damage in neuronal cells (1). Indeed, the hypothesis that quinolinic acid may act as an endogenous excitotoxin was supported by the finding that injections of quinolinic acid into the striatum appear to replicate the histopathological and neurochemical features of the brains of Huntington disease (HD) patients, such as destruction of medium spiny neurons with relative preservation of large somatostatin/neuropeptide Y neurons (2). The argument for the endogenous quinolinic acid neurotoxicity hypothesis, however, is not yet convincing, since measurement of the levels of quinolinic acid in the brain and cerebrospinal fluid has failed to demonstrate any changes that are consistent with the hypothesis (3).

In the course of these investigations, 3-hydroxykynurenine (3-HK), another metabolic intermediate of the kynurenine pathway, has been found to be increased in HD brain (4, 5). Subsequent studies revealed that the levels of 3-HK in the brain are also significantly elevated in other pathological conditions such as dementia associated with human immunodeficiency virus (HIV) infection (6), hepatic encephalopathy (7), and Parkinson disease (8). All the above syndromes are characterized by severe neurological dysfunctions. Moreover, earlier studies using a neuronal hybrid cell line have shown that 3-HK exerts cytotoxicity and causes cell lysis (9, 10). Nevertheless, the argument that 3-HK may be involved in brain pathogenesis has long been dismissed (11). This dismissal is largely due to the fact that the concentrations of 3-HK required for exerting cytotoxicity on the cell line were over 100 μM (9), approximately 100 times higher than the brain concentrations of this compound even under pathological conditions. Since toxicity of 3-HK has not yet been determined using central nervous system neurons, it is worth investigating whether 3-HK can exert toxic actions at concentrations comparable to those found in diseased brain.

In the present study, we evaluated cytotoxic potency of 3-HK by using primary neuronal cultures prepared from the striatum, a brain region where marked neuronal loss is observed in HD brain. Our results reveal that brain neurons are highly vulnerable to 3-HK toxicity.

MATERIALS AND METHODS

Neuronal Culture, Drug Treatments, and Determination of Neuronal Survival. Dissociated neuronal cultures of the striatum were prepared from embryonic day 19 Wistar rats (Charles River Japan) according to the methods described previously with some modifications (12). Cells were suspended by enzymatic digestion of striatal tissue with 0.25% trypsin/0.01% deoxyribonuclease I and subsequent mechanical dissociation. Cells were plated in polylysine-coated plastic 48- and 24-well plates at a density of 1×10^5 cells per cm^2 (for determination of neuronal survival and NADPH diaphorase staining, respectively) or on polyethylenimine-coated glass coverslips in 35-mm dishes at a density of 4×10^4 cells per cm^2 (for the measurement of intracellular peroxide levels). Drugs dissolved in N2 hormone-supplemented serum-free medium were applied at 2 days *in vitro* (DIV), except for NADPH diaphorase staining experiments. After a certain period (usually 48 hr after the change of the medium), the cultures were fixed and stained with cresyl violet, and the surviving neurons were counted as described (12).

Measurement of Intracellular Peroxide Levels. The levels of intracellular peroxides were quantified by confocal laser scanning microscope imaging of cultured striatal neurons loaded with 2′,7′-dichlorofluorescin diacetate (2′,7′-dichlorodihydrofluorescein diacetate, DCFH-DA) as described (13). DCFH-DA is a nonpolar compound that is converted into a

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Abbreviations: 3-HK, 3-hydroxykynurenine; HD, Huntington disease; DIV, days *in vitro*; DCFH-DA, 2′,7′-dichlorofluorescin diacetate (2′,7′-dichlorodihydrofluorescein diacetate); DCF, 2′,7′-dichlorofluorescein; SOD, superoxide dismutase; NDGA, nordihydroguaiaretic acid.

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non-fluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. DCFH is membrane-impermeant and rapidly oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides. DCFH loading was carried out by incubating the cells with 100 μM DCFH-DA for 1 hr. After being washed three times with Hanks' balanced salt solution containing 5.6 mM glucose and 20 mM Hepes (pH 7.4), cells were located under visible light on a Nikon Diaphot inverted microscope coupled to a confocal laser scanning microscopic system (Bio-Rad MRC-600) and scanned once with the laser (excitation 488 nm, emission 514 nm). Average fluorescence intensity for every cells was quantified by using the Bio-Rad software comos.

NADPH Diaphorase Staining. Striatal neurons were used at 7 DIV for NADPH diaphorase staining. To prevent proliferation of non-neuronal cells, cultures were treated with 1 μM cytosine arabinonucleoside from 3 days after plating. The cells were treated with drugs for 48 hr, then fixed by paraformaldehyde and incubated for 1 hr at 37°C with NADPH diaphorase staining solution (14) containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100, 1.2 mM sodium azide, and 0.1 M Tris·HCl (pH 7.2). The staining reaction was terminated by washing the cultures with phosphate-buffered saline. The positively stained neurons were counted by using low-power (×100) bright-field optics. The total number of neurons was measured under a phase-contrast microscope. Finally, The number of NADPH diaphorase-positive and total neurons was normalized by setting the number of surviving neurons in nondrug control cultures as 100%.

Materials. Superoxide dismutase (SOD) was purchased from Wako Chemicals, Osaka, Japan. Indomethacin, nordihydroguaiaretic acid (NDGA), and deprenyl were from Funakoshi, Tokyo, Japan. DCFH-DA was from Molecular Probes. Other chemicals were purchased from Sigma.

Statistics. Data are expressed as mean ± SEM. Statistical significance of difference was determined by one-way analysis of variance followed by Tukey's method.

RESULTS

Micromolar Concentrations of 3-HK Are Toxic to Cultured Striatal Neurons. Exposure of striatal cultures to 3-HK resulted in a concentration- and time-dependent reduction in the number of surviving neurons (Fig. 1). Marked cell death was apparent in cultures treated with 3-HK at a concentration as low as 1 μM. 3-HK did not show significant toxic effect at 0.1 μM (data not shown). Cell death appeared with a delay of about 6 hr after the addition of 3-HK, and then the percentage of lysed cells in 3-HK-treated cultures increased steadily, reaching a maximum after ≈24 hr of exposure. In the presence of 1 and 10 μM 3-HK, about 50% and 65% of total striatal neurons had degenerated after 48 hr, respectively. The data in Fig. 1 also show that 100 μM 3-HK was toxic to virtually all cultured cells over the course of 48 hr.

Catalase and Desferrioxamine, but Not SOD, Block 3-HK Toxicity. Previous reports suggest that 3-HK produces reactive oxygen species, probably H_2O_2, through its autoxidation (15, 16). Because generation of H_2O_2 plays a critical role in neurotoxic actions of several endogenous substances (17, 18), we examined possible involvement of H_2O_2 in 3-HK neurotoxicity by extracellular application of catalase, an enzyme that decomposes H_2O_2 into H_2O and O_2. H_2O_2 is freely permeant in the cell membrane (19), and therefore it readily diffuses out of the cells if generated intracellularly. Since the volume of extracellular medium in the culture is much bigger than that of cells, the reduction of extracellular H_2O_2 by catalase is expected to decrease intracellular H_2O_2, thereby blocking the toxicity. As shown in Fig. 2*A*, simultaneous application of catalase (200 units/ml) almost completely blocked the toxicity of 3-HK at all tested concentrations (1–100 μM). On the other hand, SOD (200 units/ml) did not show any significant protective effect against the neuronal death induced by 3-HK (Fig. 2*B*).

H_2O_2 itself is a weak oxidant among reactive oxygen species (19). However, in the presence of Fe^{2+} it can decompose to hydroxyl radical, an extremely reactive species responsible for most of the covalent modification and damage to macromolecules, including DNA, proteins, and lipid membranes (19, 20). To investigate the involvement of hydroxyl radical, we tested the effect of an iron chelator, desferrioxamine. Desferrioxamine protected striatal neurons from 3-HK-induced degeneration in a concentration-dependent manner (Table 1). Taken together, these results suggest that neurotoxicity of 3-HK is mediated by production of H_2O_2 and its subsequent decomposition to hydroxyl radical.

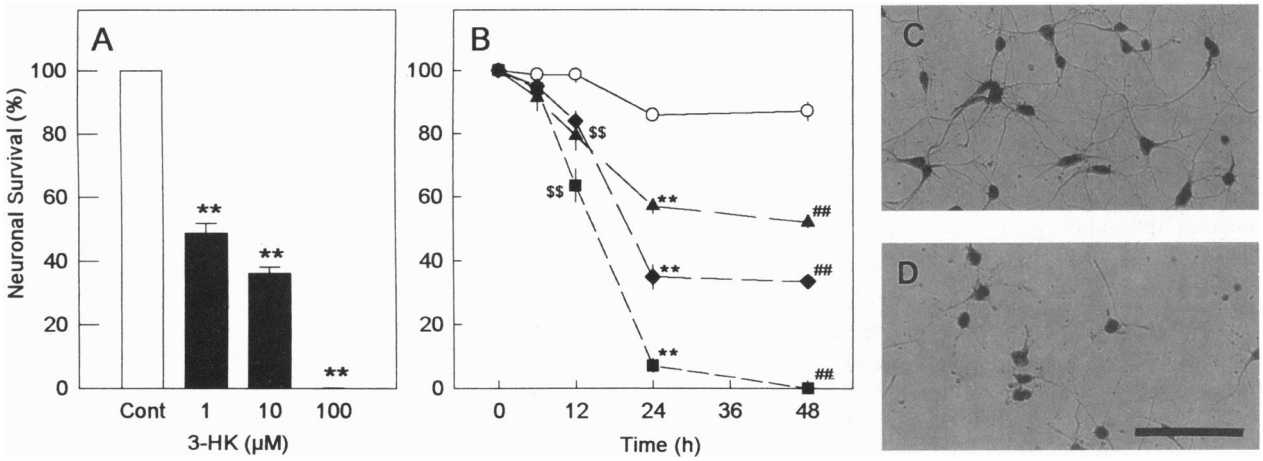

FIG. 1. (*A*) Concentration dependence of 3-HK toxicity for cultured striatal neurons. The surviving neurons were counted 48 hr after the addition of 3-HK at indicated concentrations. (*B*) Time course of 3-HK neurotoxicity. Cell cultures were fixed before and 6, 12, 24, and 48 hr after the addition of 3-HK, and the numbers of surviving neurons were counted. ○, control; ▲, 1 μM 3-HK; ◆, 10 μM 3-HK; and ■, 100 μM 3-HK. The values were normalized by setting the number of surviving neurons in nondrug control cultures (*A*) or before the addition of the drug (*B*) as 100%. Data are expressed as means ± SEM of 20 independent experiments (*A*) and 4 observations of one representative experiment (*B*). Similar results were obtained from two other sets of experiments like those in *B*. (*A*) **, $P < 0.01$ versus control (Cont). (*B*) $$, **, and ##, $P < 0.01$ versus control (12, 24, and 48 hr after the addition of the drug, respectively). (*C* and *D*) Representative photomicrographs of cultured striatal neurons stained with cresyl violet. (*C*) Control culture. (*D*) Culture exposed to 10 μM 3-HK for 48 hr. (Bar = 100 μm.)

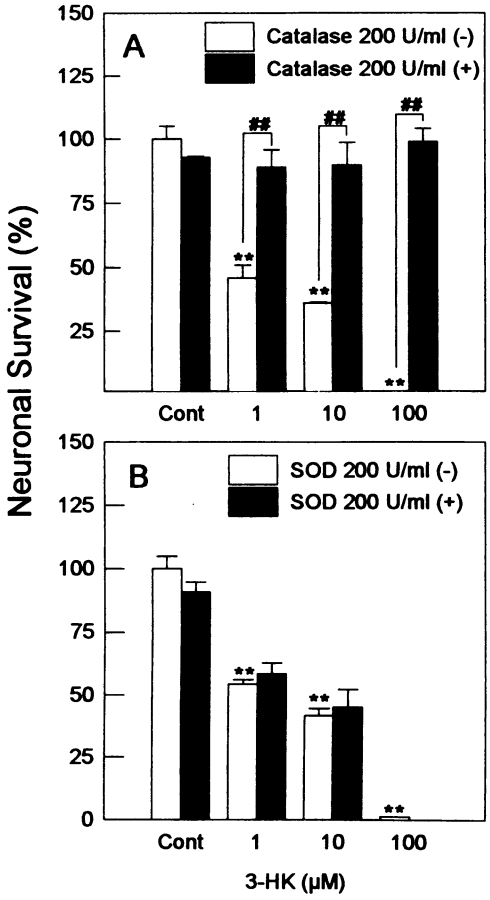

FIG. 2. Catalase (*A*) but not SOD (*B*) inhibits 3-HK toxicity. Enzymes were applied simultaneously with 3-HK and were present during the entire period of 3-HK exposure. The number of surviving neurons was counted and normalized as described in the legend of Fig. 1. Data are expressed as means ± SEM (n = 3–4). **, $P < 0.01$ versus control; ##, $P < 0.01$ versus 3-HK alone.

Xanthine Oxidase Activity Is Involved in 3-HK Toxicity. The above data suggest that the generation of H_2O_2 is primarily responsible for the neurotoxicity of 3-HK. Indeed, H_2O_2 is expected to be generated through the autoxidation of 3-HK (15, 16). However, the slope of the concentration-dependence curve for 3-HK neurotoxicity (Fig. 1*A*) was not so steep as that reported in a cell line study (9) and those described with other compounds that show cytotoxicity through autoxidation (18). Therefore, we wondered if some additional components are involved in 3-HK neurotoxicity. In this context, we inferred possible roles of endogenous enzymes related to cellular oxidative metabolism.

Table 1. Effect of desferrioxamine on 3-HK-induced neurotoxicity

Treatment	No. of surviving neurons, % of control
Control	100.0 ± 4.4
3-HK (10 μM)	19.6 ± 3.5**
3-HK (10 μM) + DFX (1 μM)	26.6 ± 2.3##
3-HK (10 μM) + DFX (10 μM)	51.1 ± 3.4##
3-HK (10 μM) + DFX (100 μM)	85.1 ± 0.2##

Desferrioxamine (DFX) was applied simultaneously with 3-HK and was present during the entire period of 3-HK exposure. The number of surviving neurons was counted and normalized as described in the legend of Fig. 1. Data are expressed as means ± SEM (n = 3–4). **, $P < 0.01$ versus control; ##, $P < 0.01$ versus 3-HK alone.

There are a number of enzymatic mechanisms that can generate reactive oxygen species. These include enzymes involved in arachidonic acid metabolism, mitochondrial energy metabolism, and microsomal cytochrome systems, and also various other oxidases. Several inhibitors of these enzymes were applied simultaneously with 1–100 μM 3-HK to test for their ability to prevent the cell death. Inhibitors of cyclooxygenase (indomethacin) and lipoxygenase (NDGA) did not protect striatal neurons from 3-HK neurotoxicity. Rather, these inhibitors significantly decreased the number of surviving neurons when applied with 3-HK (Table 2). Inhibitors of monoamine oxidase A (deprenyl), monoamine oxidase B (clorgyline), and NADPH oxidase (neopterin) had no effect on 3-HK neurotoxicity. In contrast, a xanthine oxidase inhibitor, allopurinol, showed partial but significant protection against 3-HK toxicity. Since allopurinol is reported to have poor permeance into cell membranes (21), we also performed pretreatment experiments with allopurinol. Pretreatment of striatal neurons with allopurinol for 24 hr markedly suppressed the neuronal cell death induced by subsequent exposure to 1 and 10 μM 3-HK (Table 2). Similar results were obtained with oxypurinol (100 μM), another potent xanthine oxidase inhibitor (data not shown). The toxicity of higher concentration (100 μM) of 3-HK was not prevented by pretreatment with these xanthine oxidase inhibitors. Thus, the concentration-response curve for 3-HK neurotoxicity in the presence of xanthine oxidase inhibitors had a steep slope, which resembled the slopes reported in the cell line study (9). These results suggest that endogenous xanthine oxidase activity is involved in exacerbation of 3-HK toxicity for striatal neurons.

3-HK Causes Intracellular Accumulation of Peroxides. To further examine the involvement of H_2O_2 in 3-HK-induced cell death, a fluorometric assay with DCFH-DA was performed to quantify intracellular levels of H_2O_2. DCF is a relatively selective indicator of the levels of H_2O_2 (22), and generated

Table 2. Influences of inhibitors generating superoxide and H_2O_2 enzymatically on 3-HK-induced neurotoxicity

Treatment	No. of surviving neurons, % of control						
	Allopurinol (300 μM)	Indomethacin (100 μM)	NDGA (1 μM)	Neopterin (100 μM)	Deprenyl (100 μM)	Clorgyline (1 μM)	Allopurinol (300 μM) pretreatment
Control	100 ± 2.8	100 ± 2.3	100 ± 0.9	100 ± 4.9	100 ± 3.6	100 ± 3.6	100 ± 7.2
Inhibitor alone	105 ± 4.6	104 ± 5.3	93 ± 3.3	96 ± 5.1	104 ± 0.9	107 ± 5.4	103 ± 6.5
3-HK (1 μM)	25 ± 3.1**	57 ± 7.3**	42 ± 4.4**	30 ± 5.7**	26 ± 1.3**	26 ± 1.3**	34 ± 3.0**
3-HK (1 μM) + inhibitor	58 ± 5.8##	29 ± 1.1##	33 ± 0.9	43 ± 3.0	29 ± 3.3	25 ± 0.1	88 ± 3.9##
3-HK (10 μM)	18 ± 1.9**	34 ± 2.1**	37 ± 1.7**	34 ± 0.5**	26 ± 2.1**	26 ± 2.1**	34 ± 8.5**
3-HK (10 μM) + inhibitor	34 ± 4.2	18 ± 3.7	8 ± 1.1##	36 ± 2.4	28 ± 0.9	27 ± 1.3	89 ± 8.9##
3-HK (100 μM)	0**	0**	0**	0**	0**	0**	0**
3-HK (100 μM) + inhibitor	2 ± 0.9	0	0	0	0	0	0

Inhibitors were applied simultaneously with 3-HK and were present during exposure of the culture to 3-HK. The number of surviving neurons was counted and normalized as described in the legend of Fig. 1. In the case of allopurinol pretreatment, the drug was added 24 hr before 3-HK treatment. Data were expressed as means ± SEM (n = 3–4). **, $P < 0.01$ versus control; ##, $P < 0.01$ versus each concentration of 3-HK without inhibitors.

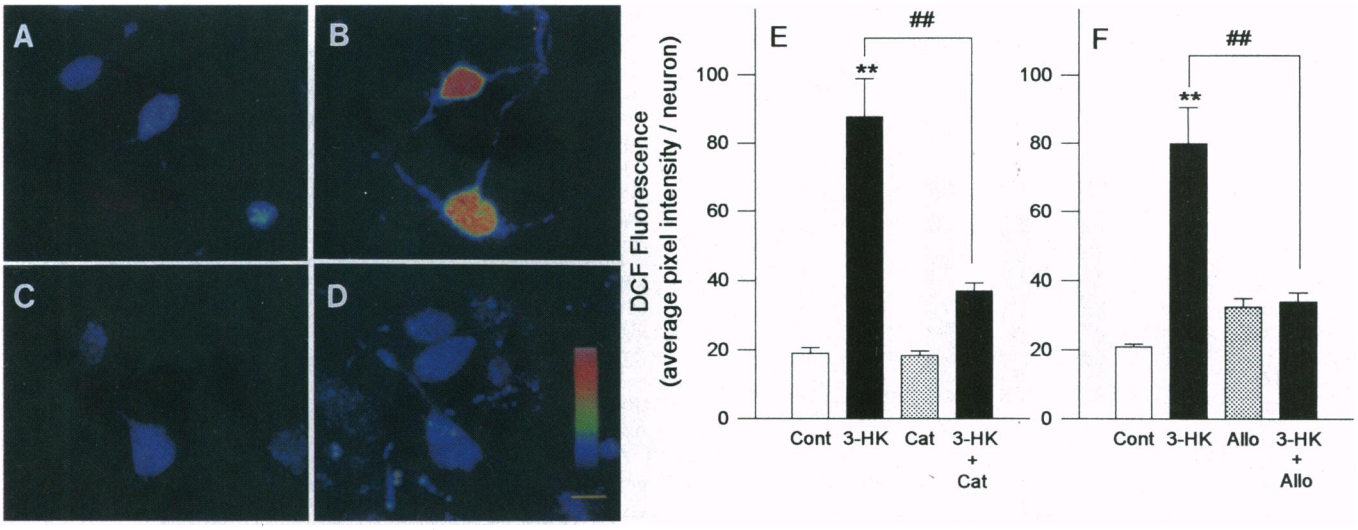

FIG. 3. Intracellular accumulation of H_2O_2 induced by 3-HK. Cells were treated with drugs for 20 hr, and the intensity of DCF fluorescence at cell bodies was quantified by using confocal laser scanning microscopy. (*A-D*) Images of DCF fluorescence in striatal neurons: control (*A*), in the presence of 10 μM 3-HK (*B*), in the presence of catalase at 200 units/ml (*C*), and in the presence of 10 μM 3-HK + 200 units/ml catalase (*D*). Fluorescence intensity is represented by a pseudocolor scale (indicated in *D*) with blue being low, green medium, and red high levels of fluorescence. (Bar = 10 μm.) (*E*) 3-HK-induced increase in DCF fluorescence and its blockade by catalase (Cat). Mean values of DCF fluorescence intensity are shown (n = 31–65). (*F*) Allopurinol pretreatment blocked 3-HK-induced peroxide generation. Cells were treated with 300 μM allopurinol (Allo) for 24 hr, and then 10 μM 3-HK was applied. Cultures were incubated for 20 hr, and DCF fluorescence was quantified (n = 39–44). **, $P < 0.01$ versus control; ##, $P < 0.01$ versus 3-HK alone.

peroxides can be readily visualized and analyzed by using a confocal laser microscope. As shown in Fig. 3 *A*, *B*, and *E*, when cells were treated with 10 μM 3-HK for 20 hr, the intensities of DCF fluorescence showed significant increases, about 4 times larger values than those in untreated cells. The increase in DCF fluorescence was largely attenuated by application of catalase at 200 units/ml (Fig. 3 *C*, *D*, and *E*), indicating that the increased fluorescence reflects increased levels of intracellular H_2O_2. DCF assay was also performed on the cells pretreated with 300 μM allopurinol for 24 hr. Allopurinol pretreatment virtually abolished the increase in DCF fluorescence induced by 10 μM 3-HK (Fig. 3*F*).

NADPH Diaphorase-Containing Neurons Are Spared from 3-HK Toxicity. Among various types of neuronal subsets specified by morphological and neurochemical characteristics, a small subset of neurons labeled with NADPH diaphorase staining is selectively spared in several neurodegenerative disorders, including Alzheimer disease (23, 24), Parkinson disease (24), and HD (25, 26). Therefore, we performed NADPH diaphorase staining in 3-HK-treated striatal neuronal cultures to determine whether these neurons were also resistant to 3-HK toxicity. In this experiment we used 7-DIV cultures, because NADPH diaphorase-positive neurons did not appear in younger (2-DIV) cultures, in agreement with the observation of Dawson *et al.* (14). In 7-DIV cultures NADPH diaphorase-positive neurons represented about 1% of the total number of cultured neurons (Fig. 4 *A*, *C*). Exposure to 1 or 10 μM 3-HK markedly reduced the total number of surviving neurons (Fig. 4*B*), consistent with the findings using 2-DIV cultures. However, the number of NADPH diaphorase-positive neurons remained unchanged. As shown in Fig. 4*C*, selective preservation of NADPH diaphorase-positive neurons

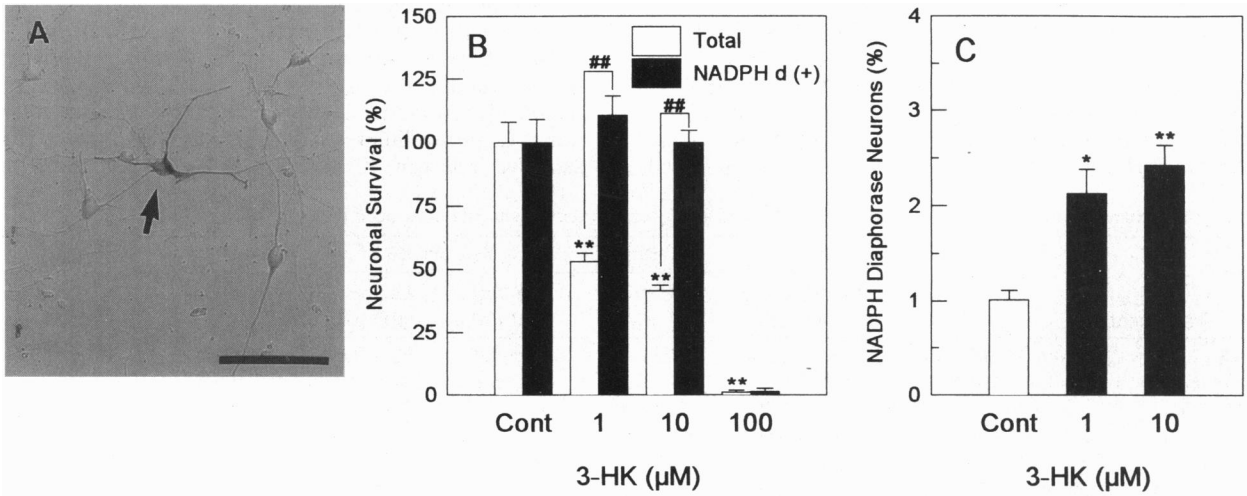

FIG. 4. NADPH diaphorase-containing neurons are spared from 3-HK toxicity. (*A*) Representative photomicrograph showing NADPH diaphorase staining. A stained cell is indicated by an arrow. (Bar = 100 μm.) (*B*) Effect of 3-HK on the survival of total and NADPH diaphorase-containing neurons [NADPH d (+)]. The number of surviving neurons was determined 48 hr after the addition of 3-HK. The values were normalized by setting the number of surviving total neurons and NADPH d (+) neurons in nondrug control cultures as 100%, respectively. (*C*) Percentage of NADPH diaphorase-positive neurons in total surviving neurons after 3-HK exposure. n = 4 determinations. *, $P < 0.05$; **, $P < 0.01$ versus control; ##, $P < 0.01$ versus 3-HK alone.

after 3-HK treatment resulted in a significant increase in their relative proportion over the total surviving neurons. On the other hand, virtually all neurons, including the NADPH diaphorase-positive population, died when challenged with 100 μM 3-HK.

DISCUSSION

Increased levels of the endogenous tryptophan metabolite 3-HK have been reported in brains under various pathological conditions, including HD (4, 5), human immunodeficiency virus (HIV) infection (6), and hepatic encephalopathy (7). Notably, these syndromes are associated with severe neurological dysfunctions. In the case of HIV-1-positive patients, the increase in 3-HK levels is greater in individuals with dementia than in those without dementia (6). The concentrations of 3-HK under these pathological conditions are estimated to be 0.3–1.2 μM, whereas those in nondiseased brains are 0.08–0.3 μM (5–7). In the present study, we provide evidence that 3-HK at a concentration as low as 1 μM, a concentration comparable to that in the brain under pathological conditions, is toxic to brain neurons. Brain 3-HK may be derived from peripheral tissues through blood–brain barrier (27) as well as from *de novo* synthesis in brain, possibly by astrocytes and microglia (28). Although no information is available concerning cellular localization of 3-HK in brain, 3-HK derived from these sources is expected to accumulate at significant levels in neuronal cells by active uptake mechanisms (29).

Two lines of evidence indicate that the toxic actions of 3-HK are mediated by generation of H_2O_2. First, application of catalase almost completely blocked 3-HK neurotoxicity. Second, fluorometric assay revealed that DCF fluorescence markedly increased within the cultured neurons upon exposure to 3-HK. This increase in DCF fluorescence probably reflects intracellular accumulation of H_2O_2, because treatment with catalase markedly reduced the fluorescence increase. This view is also consistent with previous reports suggesting the involvement of H_2O_2 in 3-HK-induced cell lysis in a neuronal hybrid cell line (9) and in 3-HK-induced DNA double-strand breaks (16). H_2O_2 may be formed as a result of the autoxidation of 3-HK. Such oxidative reactions of 3-HK and structurally related *o*-aminophenol compounds have been demonstrated (15, 30).

Experiments using several enzyme inhibitors revealed that in addition to the nonenzymatic autoxidation of 3-HK, endogenous xanthine oxidase activity is involved in peroxide generation, in particular, at lower concentrations (1–10 μM) of 3-HK. Indeed, xanthine oxidase inhibitors almost completely blocked H_2O_2 accumulation by 10 μM 3-HK, and also prevented neuronal cell death induced by 1–10 μM 3-HK. It is unlikely that the effect of xanthine oxidase inhibitor allopurinol was exerted through its action as a hydroxyl radical scavenger (31), since cell death induced by direct application of H_2O_2 was not inhibited by pretreatment with 300 μM allopurinol (data not shown). Therefore, our results suggest that "neuronal" xanthine oxidase exacerbates cell damage by 3-HK. In this context, kainate toxicity in cerebellar granule cell culture has been reported to be dependent on endogenous xanthine oxidase (21), which, along with our present results, suggests that xanthine oxidase plays a key role in neuronal cell death. On the other hand, a previous study has demonstrated that neuronal xanthine oxidase activity is very low, less than 1/100 of that in glial cells (32). Further investigations are required to determine whether neuronal cells possess appreciable levels of xanthine oxidase.

The mechanisms by which xanthine oxidase accelerates 3-HK-induced generation of peroxides are not clear. One possibility is that this enzyme can utilize 3-HK as a substrate and directly catalyze the reaction of 3-HK autoxidation. Another possibility is that low concentrations of 3-HK are capable of stimulating the activities of endogenous xanthine oxidase, thereby enhancing free radical generation during the oxidative reaction catalyzed by this enzyme. These alternatives should be addressed in future studies. On the other hand, neurotoxicity of 100 μM 3-HK was not prevented by xanthine oxidase inhibitors, suggesting that at this high concentration, nonenzymatic autoxidation of 3-HK can produce a sufficient amount of peroxides to cause cell death. This concentration of 3-HK has also been shown to be toxic to a neuronal hybrid cell line (9).

Pretreatment experiments with xanthine oxidase inhibitors also verified intracellular localization of a toxic pool of H_2O_2. In these experiments 3-HK challenge was carried out in the absence of an extracellular xanthine oxidase inhibitor. Thus, the ability of this treatment to attenuate toxicity of 1–10 μM 3-HK most likely reflects the inhibition of H_2O_2 generation in the intracellular compartment. Indeed, 3-HK has been reported to be taken up, via the neutral amino acid carrier, in a neuronally derived hybrid cell line and tissue slices prepared from rat brain (29).

The reasons why NADPH diaphorase-positive neurons are selectively spared from 3-HK neurotoxicity are not yet clear. A likely explanation is that these neurons have a large capacity to detoxify oxygen free radicals, and therefore they are generally resistant to oxidative stress. This view is consistent with the previous findings that these neurons are also resistant to nitric oxide-mediated injury (14). Whatever the precise cellular mechanisms, this observation serves an interesting coincidence with pathological characteristics of brains of patients with neurodegenerative disorders such as HD (25, 26). The specific aspiny neuronal subpopulation containing NADPH diaphorase, which is colocalized with somatostatin and neuropeptide Y (33), is selectively spared in the degenerated striatum of HD patients (25, 26).

Brain neuronal cells are at particular risk from damage caused by free radicals, since the brain has an extremely high rate of oxygen consumption and neuronal membranes have high contents of polyunsaturated fatty acids that are susceptible to lipid peroxidation. Under physiological conditions, there is a well-balanced equilibrium between free radical generation and various enzymatic and nonenzymatic antioxidant defense systems. An imbalance in this status leading to free radical accumulation is defined as oxidative stress, which is implicated in various neurodegenerative disorders (20). Our results indicate that 3-HK should be regarded as a potent endogenous neurotoxin which renders neuronal cells to oxidative stress, and that the elevated levels of this compound in the brain may be at least in part relevant to the pathological consequences of various neurodegenerative disorders.

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