Antioxidative Effect of Vitamin D3 on Zinc-Induced Oxidative Stress in CNS

ANYA M. Y. LIN

Department of Medical Research and Education, Veterans General Hospital–Taipei, and Department of Physiology, National Yang-Ming University, Taipei, Taiwan

ABSTRACT: Antioxidative mechanisms of vitamin D3 were evaluated both *in vitro* and *in vivo*. A 4-h incubation of brain homogenates at 37°C increased the formation of Schiff base fluorescent products of malonaldehyde, an indicator of lipid peroxidation. Incubation with vitamin D3 dose-dependently suppressed auto-oxidation. The antioxidative potency for inhibiting zinc-induced lipid peroxidation was as follows: vitamin D3 > Trolox (a water-soluble analogue of vitamin E) ≥ β-estradiol > melatonin. In the presence of high dose of desferrioxamine, a metal chelator, vitamin D3 attenuated auto-oxidation. These *in vitro* data indicate that vitamin D3 may act as a terminator of the lipid peroxidation chain reaction. The antioxidative effect of vitamin D3 on zinc-induced oxidative injury was verified using local infusion of vitamin D3 *in vivo*. Intranigral infusion of zinc elevated lipid peroxidation in the infused substantia nigra and depleted striatal dopamine content at 7 days after infusion. Furthermore, elevated cytosolic cytochrome c and DNA ladder, indicative of apoptosis, were demonstrated in the infused substantia nigra. Simultaneous infusion of vitamin D3 and zinc prevented oxidative injury and apoptosis induced by zinc alone. The involvement of glia-derived neurotrophic factor (GDNF) expression was excluded since vitamin D3 did not alter GDNF level in the infused substantia nigra at 24 h or 4 days after intranigral infusion of vitamin D3. Our results suggest that vitamin D3, independent of upregulation of GDNF expression, may acutely prevent zinc-induced oxidative injuries via antioxidative mechanisms.

KEYWORDS: vitamin D3; antioxidative action; zinc; transition metal; lipid peroxidation

INTRODUCTION

Vitamin D3, an active metabolite of vitamin D, reportedly possesses a variety of biological functions.[1,2] Recently, a neuroprotective role of vitamin D3 has been suggested. For example, vitamin D3 reportedly reduced neuronal damage induced by H_2O_2 in the ventral mesencephalic neuronal culture.[3] Furthermore, several *in vivo* studies have showed that systemic vitamin D3 attenuated cortical infarction[3] and neurotoxicity induced by 6-hydroxydopamine or iron of rat brain.[4,5] Upregulation of neurotrophic factors, including glia-derived neurotrophic factor (GDNF), has been

Address for correspondence: Anya M. Y. Lin, Department of Medical Research and Education, Veterans General Hospital–Taipei, Taipei 112, Taiwan. Voice: +886-2-28712121, ext. 2688; fax: +886-2-28751562.
myalin@vghtpe.gov.tw

Ann. N.Y. Acad. Sci. 1053: 1–11 (2005).
doi: 10.1196/annals.1344.028

proposed for the vitamin D3–induced neuroprotection.[3–6] Nevertheless, vitamin D3 may also exert its protection via antioxidative actions. In the past decade, several studies have shown that vitamin D3 possesses an antioxidative activity. Vitamin D3 has been demonstrated as a membrane antioxidant that inhibited iron-induced lipid peroxidation of brain liposomes.[7] Systemic vitamin D3 suppressed the elevated lipid peroxidation observed in vitamin D3–deficient rats.[8] A vitamin D3 analogue has been found to inhibit endotoxemia via regulation of free radical formation.[9] Furthermore, vitamin D3 attenuated inducible nitric oxide synthase activity and thus decreased free radical formation in rat primary astrocytes.[10] Moreover, vitamin D3 has been reported to reduce oxidative stress by upregulating antioxidative defense systems, including glutathione content, glutathione peroxidase, and superoxide dismutase in cultured astrocytes[10] and in liver.[11]

Zinc, one of the transition metals, has been reported to induce neurotoxicity in CNS. Indeed, clinical studies have shown accumulation of zinc in the nigrostriatal dopaminergic system in Parkinson's disease patients.[12] Incubation with exogenous zinc has been found to induce cell death in cortical neuronal culture.[13] Furthermore, releases of endogenous zinc were reportedly responsible for the hippocampal neuronal damages induced by kainate[14] or transient forebrain ischemia.[15] Moreover, our previous studies showed that local infusion of zinc induced neurotoxicity in the nigrostriatal dopaminergic system.[16,17] Oxidative stress may be one of the causes for the zinc-induced neurotoxicity. Further, apoptosis and necrosis have been suggested to be responsible for zinc-induced neurotoxicity.[17]

In the present study, the involvement of antioxidative mechanisms in vitamin D3–induced neuroprotection was investigated against zinc-induced oxidative stress. Antioxidative effect of vitamin D3 on both auto-oxidation and zinc-induced lipid peroxidation was performed by incubation of zinc in brain homogenates. The potency of vitamin D3 in suppressing zinc-induced lipid peroxidation was compared with several well-known antioxidants. Desferrioxamine was used as a positive control to study the free radical scavenging property of vitamin D3. Furthermore, the antioxidative effect of vitamin D3 was investigated using local infusion of vitamin D3 in the substantia nigra of anesthetized rats. Several oxidative injuries, including lipid peroxidation in the infused substantia nigra and striatal dopamine content, were evaluated. The effect of intranigral infusion of vitamin D3 on the cytosolic cytochrome c level, an indicator of apoptosis, was investigated using Western blot and immunohistochemical studies.

METHODS

In Vitro *Studies*

Cortical samples were homogenized in chilled Ringer's solution (50 mg/mL) and treated for either auto-oxidation or zinc-induced lipid peroxidation as follows. *Auto-oxidation*: The homogenates were incubated at 37°C for 4 h. Vitamin D3 (Abbot, MI) and desferrioxamine (Sigma, St. Louis, MO) were included in each experiment as described in the results. *Zinc-induced lipid peroxidation*: The homogenates were incubated at 37°C for 4 h following an addition of zinc (200 μM) ± vitamin D3, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich, Milwaukee,

WI), β-estradiol, and melatonin (Sigma, St. Louis, MO). A 400-μL sample was transferred to a tube containing 300 μL chloroform and 100 μL methanol. After centrifugation at 8000*g* for 5 min, an aliquot of chloroform extract was transferred to another tube containing 100 μL methanol scanned using a spectrofluorometer. Lipid peroxidation was determined by measuring the levels of malondialdehyde and its dihydropyridine polymers, which emit fluorescence at 426 nm when activated by UV at 356 nm.[18]

In Vivo *Study*

Adult, male Sprague-Dawley rats, weighing 250–350 g, were used. These animals were maintained according to the guidelines established in the "Guide for the care and use of laboratory animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (U.S.A.).

Chronic Surgery and Drug Infusion

To induce oxidative stress in the nigrostriatal system, rats were anesthetized with chloral hydrate (450 mg/kg, ip, Sigma, St. Louis, MO) and placed in a stereotaxic instrument (David Kopf Instruments, Palo Alto, CA). One μL of Krebs-Ringer solution of zinc chloride (zinc, 40 nmol) ± vitamin D3 (0.6 pmol) was infused stereotaxically into substantia nigra (coordinates: 3.2 mm anterior and 2 mm above the interaural zero; 2.1 mm lateral to the midline; 3.5 mm below the incisor bar). Drug solutions were infused at a rate of 0.2 μL/min through a 30-gauge stainless steel needle. The injection needle was held in place for an additional 3 min following drug infusion. After the surgery, rats recovered from anesthesia and were placed in home cages for 7 days.

Fluorescence Assay of Lipid Peroxidation in Substantia Nigra

At the end of each *in vivo* experiment, rats were sacrificed by decapitation. Substantia nigra dissected from both hemispheres was homogenized in chilled 400 μL chloroform and 200 μL methanol. After centrifugation, an aliquot of the chloroform and methanol layer was scanned using a spectrofluorometer. The relative fluorescent intensities of samples in a cuvette were measured as mentioned above.

HPLC-EC Analysis of Striatal Dopamine Content

Rats were decapitated. Regional dissections were performed and striata were immediately frozen in liquid nitrogen and stored at −70°C until analysis. An HPLC with EC detection procedure was used to quantify dopamine content in striatum.[19]

Western Blot Analysis of Cytochrome c

Substantia nigras were dissected and homogenized in 100 μL of ice-cold mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris-HCl, 1 mM EDTA, 20 mM fluorocitrate, pH 7.4). After homogenization, the suspension was centrifuged at 600*g* for 5 min at 4°C, and the supernatant transferred to a chilled Eppendorf tube and centrifuged at 17,000*g* for 10 min at 4°C. Purity of the cytosolic

fraction was determined by measuring cytochrome oxidase. The cytosolic protein samples (50 μg) were run on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a nitrocellular membrane (Bio-Rad, Richmond, CA) at 80 V for 75 min. Blots were probed with a mouse monoclonal antibody (7H8.2C12, BD Pharmingen, San Diego, CA) against the denatured form of cytochrome c at a dilution of 1:500 at 4°C for 45 min. After primary antibody incubation, the membrane was washed and incubated with horseradish peroxidase–conjugated goat antimouse IgG (Chemicon, Temecula, CA) for 40 min at room temperature. The immunoreaction was visualized using Amersham enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). After this detection, the bound primary and secondary antibodies were stripped by incubating the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate) at 50°C for 30 min. The membrane was reprobed with a mouse cytochrome oxidase subunit IV antibody or β-actin (1:1000) and then visualized with a procedure similar to that for cytochrome c.

Immunostaining of Cytochrome c

Rats were perfused transcardially with chilled saline and followed by 4% paraformaldehyde–PBS solution. Brains were frozen-sectioned coronally at 16 μm using a cryostat. A mouse monoclonal antibody against the native form of cytochrome c (6H2.B4, BD Pharmingen, U.S.A.) was used *in situ*. Sections were then incubated with 0.3% Triton-100 and 1% goat serum, blocked with 3% goat serum, and incubated with mouse anticytochrome c (20 μg/mL) in 1% GS-PBS-T at 4°C overnight. The sections then were incubated with FITC-conjugated secondary antibody for anticytochrome c and visualized under a fluorescence microscope.

Measurement of GDNF by Enzyme-Linked Immunosorbent Assay (ELISA)

Substantia nigra was homogenized in a lysis buffer and centrifuged at 12,000*g* for 20 min. The supernatant was acidified according to the method described.[20] Samples were neutralized to pH 7.4, adjusted to contain the same amount of protein, and assayed for GDNF using GDNF ELISA kits (R&D, Minneapolis, MN). For measurement of GDNF, mouse monoclonal anti-GDNF antibody was used as a capture antibody, and biotinylated goat anti-GDNF antibody was used for detection. A 96-well microplate reader was used to measure the optical densities.

RESULTS

Incubation of brain homogenates at 37°C for 4 h increased the formation of peroxidized lipids compared with those incubated at 0°C (as basal level). Vitamin D3 dose-dependently suppressed the elevated lipid peroxidation (FIG. 1A). Addition of zinc (200 μM) further increased lipid peroxidation. Incubation with vitamin D3 inhibited zinc-induced increase in lipid peroxidation in a concentration-dependent manner (FIG. 1B). Furthermore, the antioxidative capacity of vitamin D3 was compared with that of Trolox (a water-soluble analogue of vitamin E), β-estradiol, and melatonin. Vitamin D3 was found to be about 10^3-fold more potent than Trolox and

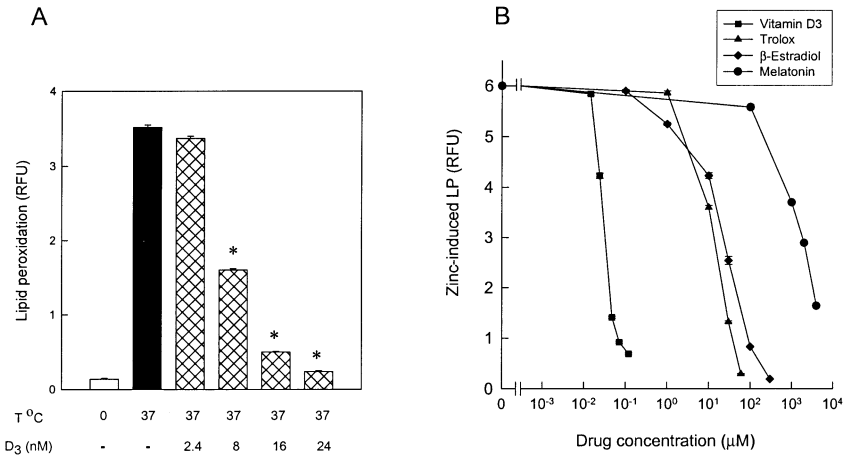

FIGURE 1. Effects of vitamin D3 on auto-oxidation and zinc-induced lipid peroxidation of cortical homogenates. **(A)** A dose-dependent inhibition by vitamin D3 of auto-oxidation of cortical homogenates. Brain homogenates were incubated at 37°C for 4 h with or without addition of vitamin D3. **(B)** Dose-response curves for vitamin D3, Trolox, β-estradiol, and melatonin on zinc-induced lipid peroxidation (LP) of cortical homogenates. Brain LP was reported as relative fluorescence units (RFU). Values are the mean ± SEM (n = 4–5) from a representative experiment that was replicated with similar results. *$P < 0.05$ in vitamin D3 group compared with the control group (37°C) by one-way ANOVA followed by post-hoc analyses.

β-estradiol and 10^5 times more potent than melatonin in preventing the zinc-induced lipid peroxidation (FIG. 1B).

Desferrioxamine was used as a metal ion chelator to study the antioxidative mechanisms of vitamin D3. Both vitamin D3 (24 nM) and desferrioxamine (10 μM) prevented auto-oxidation in a time-dependent manner (FIG. 2). Auto-oxidation was inhibited with a 4-h incubation of vitamin D3 or desferrioxamine. In contrast, no significant inhibition by vitamin D3 or desferrioxamine of auto-oxidation was observed when vitamin D3 or desferrioxamine was included in the brain homogenates for the last hour during a 4-h incubation (FIG. 2). At this time point, auto-oxidation was suppressed when high dose of vitamin D3 (240 nM), but not desferrioxamine (100 μM), was coincubated (FIG. 2A). In the presence of high dose of desferrioxamine (100 μM), which did not attenuate auto-oxidation, coincubation of 240 nM vitamin D3 actually suppressed auto-oxidation (FIG. 2B).

To evaluate the antioxidative effect of vitamin D3 on zinc-induced neurotoxicity, zinc with or without vitamin D3 was locally infused in the substantia nigra of anesthetized rats. Seven days after intranigral infusion of zinc, lipid peroxidation was elevated in the infused substantia nigra and the dopamine content was depleted in the ipsilateral striatum (FIG. 3). While intranigral infusion of vitamin D3 alone altered neither basal lipid peroxidation in substantia nigra nor dopamine content in the ipsilateral striatum, coinfusion of vitamin D3 and zinc prevented zinc-induced oxidative injuries (FIG. 3).

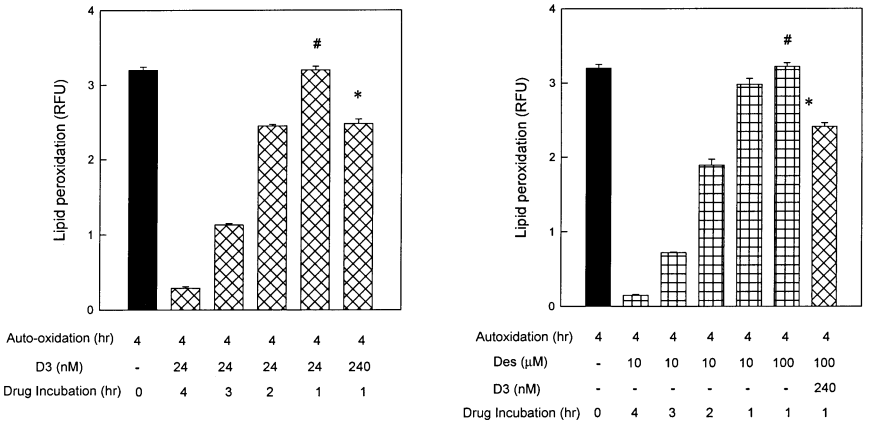

FIGURE 2. The antioxidative action of vitamin D3 on auto-oxidation in the presence of desferrioxamine. Desferrioxamine (10 nM) time-dependently inhibited auto-oxidation. No significant inhibition of auto-oxidation was observed when 10 μM or 100 μM desferrioxamine was included in the brain homogenates for the last hour during a 4-h incubation. At this time point, 240 nM vitamin D3 actually attenuated auto-oxidation in the presence of 100 μM desferrioxamine. #Not significant in high dose of desferrioxamine (100 μM) compared with low dose of desferrioxamine (10 μM). *$P < 0.05$ in desferrioxamine (100 μM) + vitamin D3 compared with desferrioxamine (100 μM) alone by one-way ANOVA followed by post-hoc analyses. Brain LP was reported as relative fluorescence units (RFU). Values are the mean ± SEM ($n = 3$) from a representative experiment that was replicated with similar results.

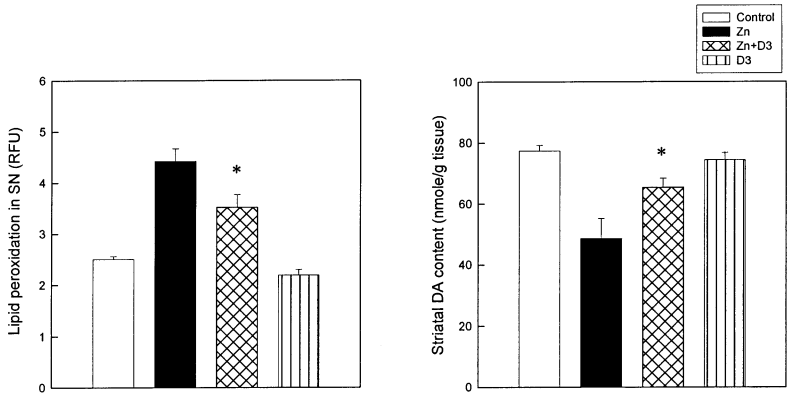

FIGURE 3. Effects of vitamin D3 on zinc-induced oxidative injuries in the nigrostriatal dopaminergic system at 7 days after an intranigral infusion of zinc. LP in the microdissected substantia nigra was measured and reported as relative fluorescence units (RFU). Striatal dopamine content was determined using HPLC-EC detection. Values are the mean ± SEM ($n = 4$–6). *$P < 0.05$ in vitamin D3 + zinc group compared with zinc group by one-way ANOVA followed by post-hoc analyses.

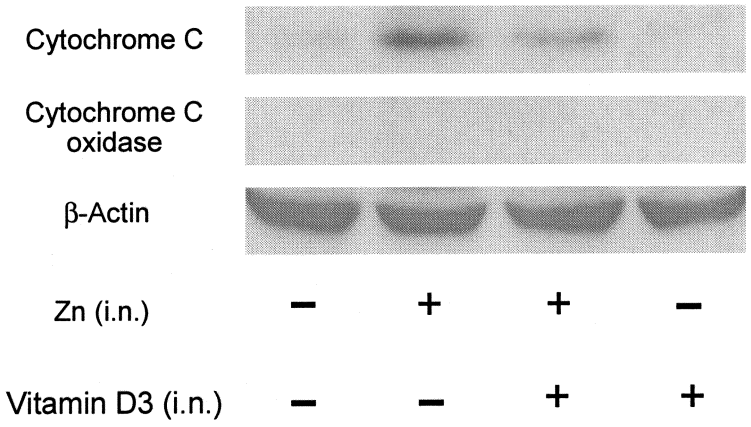

FIGURE 4. Effects of vitamin D3 on zinc-induced elevation in cytosolic cytochrome c level of substantia nigra at 4 h after an intranigral infusion of zinc. A representative result of cytochrome c in the cytosolic fraction of substantia nigra was detected by Western blotting at 4 h after an intranigral infusion of zinc ± vitamin D3. Cytochrome oxidase was not detected, indicating the purity of the cytosolic fraction. Fifty μg of protein was loaded in each lane in all experiments. Similar results were obtained from three independent experiments.

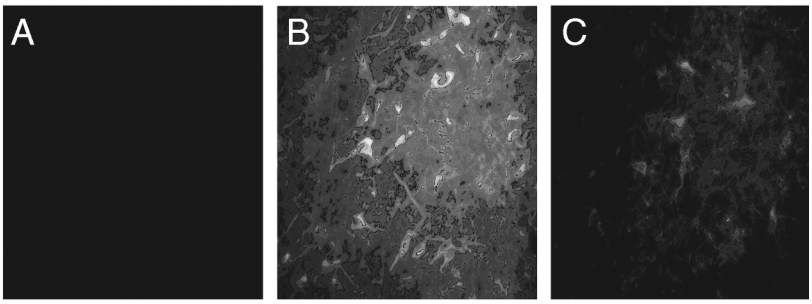

FIGURE 5. Fluorescent microscopic images show reduction in cytosolic cytochrome c immunofluorescence in the substantia nigra infused with zinc and vitamin D3 mixture. Four hours after intranigral infusion, a strong immunofluorescence was obtained in the zinc-infused substantia nigra **(B)** compared with the intact control **(A)**. The cytoplasmic cytochrome c fluorescence was attenuated in the substantia nigra receiving zinc and vitamin D3 mixture **(C)**. Scale bar: 20 μm.

To evaluate the effect of local vitamin D3 on zinc-induced apoptosis, the cytosolic cytochrome c of substantia nigra was examined using Western blot analysis and immunofluorescent staining. Four hours after intranigral infusion of zinc, cytosolic cytochrome c levels were increased in the infused substantia nigra, whereas cytochrome c oxidase (subunit IV) was nearly absent (FIG. 4). The absence of cytochrome c oxidase in the cytosolic extract indicated that the cytosolic extracts were free of mitochondrial contamination. While vitamin D3 alone did not elevate the cytochrome c in the cytosolic extracts of the zinc-infused substantia nigra, infusion

TABLE 1. GDNF levels in intact SN or SN receiving zinc, vitamin D3, or zinc plus vitamin D3 mixture

GDNF level (pg/mg tissue)	Intact SN	Zn	Zn + D3	D3
24 h after intranigral infusion	29.9 ± 3.3 (4)	37.0 ± 3.5 (4)	32.2 ± 4.1 (4)	34.9 ± 5.3 (4)
4 days after intranigral infusion	24.6 ± 1.9 (4)	28.6 ± 4.6* (4)	25.1 ± 3.7 (4)	30.7 ± 3.1 (4)

NOTE: Local vitamin D3 had no effect on basal GDNF levels measured by ELISA assay. Values are the mean ± SEM. Numbers in parentheses represent sample size. Similar results were obtained in duplicates. *$P < 0.05$ in zinc-infused substantia nigra at 4 days after infusion compared with substantia nigra at 24 h after the infusion by one-way ANOVA followed by post-hoc analysis.

of vitamin D3 and zinc mixture inhibited zinc-induced increase in cytosolic cytochrome c in the infused substantia nigra (FIG. 4). The distribution of cytosolic cytochrome c was confirmed using immunofluorescent staining. A less intensive cytoplasmic distribution of cytochrome c fluorescence in the substantia nigra infused with zinc and vitamin D3 mixture was observed compared with that with zinc (FIG. 5).

Using the technique of ELISA, the basal GDNF level in the intact substantia nigra averaged 29.9 ± 3.3 pg/mg tissue ($n = 4$). Twenty-four hours after intranigral infusion, the GDNF level was not significantly changed in the zinc-infused substantia nigra. Intranigral infusion of vitamin D3 alone did not elevate GDNF level in the infused substantia nigra. Furthermore, the GDNF level was not increased in the substantia nigra receiving local infusion of vitamin D3 and zinc mixture. Four days after intranigral infusion, the GDNF level in the zinc-infused substantia nigra was insignificantly decreased compared with that after 24 h. At the same time, the GDNF level was not increased in the substantia nigra receiving the zinc and vitamin D3 mixture (TABLE 1).

DISCUSSION

Due to the pathophysiology of oxidative stress in the CNS neurodegenerative diseases,[21–23] several neuroprotective strategies have been proposed,[24–28] including supplementation with antioxidants,[26,27] upregulation of antioxidative defensive enzymes,[27] and intermittent hypoxia.[28] In the past decade, vitamin D3 has been found to be antioxidative[6–10] and may be neuroprotective. Our present study further supports this notion in that D3 dose-dependently suppressed auto-oxidation and zinc-induced lipid peroxidation in cortical homogenates. Moreover, our data showed a time-dependent inhibition of auto-oxidation by vitamin D3. In the presence of 100 μM desferrioxamine, which chelated metal ion and had no effect on the elevated lipid peroxidation, 240 nM vitamin D3 significantly attenuated auto-oxidation. These data indicate that vitamin D3 may be a terminator of the lipid peroxidation chain reaction.

Compared with several well-known antioxidants, vitamin D3 may be one of the most powerful antioxidants in biological organisms as shown in the present study.

The antioxidative potencies of vitamin D3 and vitamin E have been compared and the results varied. Vitamin D3 has been found equally potent as that of vitamin E in suppressing lipid peroxidation in liver.[11] However, our *in vitro* study demonstrated that vitamin D3 was 10^3-fold more potent than Trolox (a water-soluble analogue of vitamin E) in inhibiting zinc-induced lipid peroxidation. Similar results were observed in suppressing iron-induced lipid peroxidation in the cortical homogenates (unpublished observation). The reasons for the discrepancy may be due to different tissues and/or the different routes of drug administration used in our studies and Sardar's study.[11] In Sardar's study, vitamin E was supplied in diet, while vitamin D3 was orally applied, and bioavailabilities[11] of vitamins D3 and E may result in different dose responses. In contrast, our *in vitro* study used the exact doses of vitamin D3 and vitamin E and these drugs were incubated in identical conditions. Melatonin, which has been used as a lipid-soluble antioxidant,[26,27] was found to be 10^5 times less potent than vitamin D3 in our study. β-Estradiol, a well-known steroid, possessing antioxidative actions via metal-chelating activity and radical scavenging activity, was used as a positive control.[29,30] Our data showed that vitamin D3 is antioxidative, with similar mechanisms as, but 10^3 times more potent than, β-estradiol in inhibiting zinc-induced lipid peroxidation.

Two well-known pathways participate in activation of vitamin D3 receptors. One is a nuclear receptor–mediated genomic pathway that regulates gene transcription and the other is a membrane receptor–mediated nongenomic pathway that rapidly opens calcium channels.[1] Upregulation of GDNF levels has been reported in the neuroprotection by chronic administration of vitamin D3 (ip) in our previous study[5] and others.[3,4] These data suggest that receptor activation through genomic and nongenomic pathways[1] may be involved in this vitamin D3–induced neuroprotection.[3–5] In contrast, cortical homogenates in which cellular integrity was disrupted were used in the present study to rule out the possibility of receptor activation, and our *in vitro* data showed an inhibition by vitamin D3 of auto-oxidation and zinc-induced lipid peroxidation. Furthermore, our *in vivo* data demonstrated no upregulation of GDNF expression when local infusion of vitamin D3 diminished the elevation in cytosolic cytochrome c and the subsequent oxidative injuries induced by zinc alone in the nigrostriatal dopaminergic system. These data directly exclude the involvement of GDNF in the vitamin D3–induced neuroprotection observed in the present study. While vitamin D3 reportedly activated the receptor-mediated calcium translocation[1,31,32] that may further induce apoptosis,[33,34] local infusion of vitamin D3 (0.6 pmol) alone did not increase the cytosolic cytochrome c in the infused substantia nigra. Moreover, vitamin D3 altered neither lipid peroxidation in the infused substantia nigra nor striatal dopaminergic content at 7 days after the infusion. These data indicate that vitamin D3 may attenuate zinc-induced oxidative injury via antioxidative mechanisms instead of through genomic and/or nongenomic activation.[1]

So far, our study has shown neuroprotection by vitamin D3; however, vitamin D3 has been reported to reduce cell proliferation and increase apoptosis in response to oxidative stress.[35] One of the possibilities may be due to vitamin D3–induced production of thioredoxin-binding protein-2/vitamin D3 upregulated protein-1 (TBP-2/VDUP-1),[36] which suppresses thioredoxin function,[35,37] an antioxidative defense system in biological organisms. The dose of vitamin D3 required to upregulate the TBP-2/VDUP-1 was 1 μM and a significant upregulation of TBP-2/VDUP-1 was observed 16 h after the incubation.[35] In contrast, the doses of vitamin D3 were 24 nM

in our *in vitro* study or 0.6 pmoles in our animal study, and both treatments significantly reduced zinc-induced lipid peroxidation at 4 h after incubation or local infusion of zinc. Thus, TBP-2/VDUP-1 may not be involved in the neuroprotection of vitamin D3 in the present study.

In conclusion, our *in vitro* data showed that vitamin D3 possesses a very powerful antioxidative property and is capable of suppressing auto-oxidation and zinc-induced lipid peroxidation. Furthermore, the *in vivo* study showed that local vitamin D3 alone did not cause deleterious effects, but efficiently prevented zinc-induced oxidative injuries. These data suggest that, in addition to upregulation of neurotrophic factors, antioxidative mechanisms of vitamin D3 may be considered in counteracting oxidative injuries in the CNS.[1]

ACKNOWLEDGMENTS

This study was supported by Grant Nos. NSC90-2420-B-075-017 and VGH90-373 (Taipei, Taiwan).

REFERENCES

1. NORMAN, A.W. *et al*. 1992. 1,25$(OH)_2$-Vitamin D3, a steroid hormone that produces biological effects via both genomic and nongenomic pathway. J. Steroid Biochem. Mol. Biol. **41:** 231–240.
2. REICHEL, H. *et al*. 1989. The role of the vitamin D endocrine system in health and disease. N. Engl. J. Med. **320:** 980–991.
3. WANG, J.Y. *et al*. 2001. Vitamin D3 attenuated 6-OHDA-induced neurotoxicity in rats. Brain Res. **904:** 67–75.
4. WANG, Y. *et al*. 2000. Vitamin D3 attenuates cortical infarction induced by middle cerebral arterial ligation in rats. Neuropharmacology **39:** 873–880.
5. CHEN, K.B. *et al*. 2003. Systemic vitamin D3 attenuated oxidative injuries in the locus coeruleus of rat brain. Ann. N.Y. Acad. Sci. **993:** 313–324.
6. NAVEILHAN, P. *et al*. 1996. 1,25$(OH)_2$-Vitamin D3, an inducer of glial cell-line–derived neurotrophic factor. Neuroreport **7:** 2171–2175.
7. WISEMAN, H. 1993. Vitamin D is a membrane antioxidant: ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol, and tamoxifen and relevance to anticancer action. FEBS J. **326:** 285–288.
8. KUZMENKO, A.I. *et al*. 1997. Effects of vitamin D3 and ecdysterone on free-radical lipid peroxidation. Biochemistry (Mosc.) **62:** 609–612.
9. HORIUCHI, H. *et al*. 1999. Protective effect of vitamin D3 analogues on endotoxin shock in mice. Agents Actions **33:** 343–348.
10. GARCION, E. *et al*. 1999. 1,25-Dihydroxyvitamin D3 regulates the synthesis of γ-glutamyl transpeptidase and glutathione levels in rat primary astrocytes. J. Neurochem. **73:** 859–866.
11. SARDAR, S. *et al*. 1996. Comparative effectiveness of vitamin D3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidants system in Sprague-Dawley rats. Int. J. Vitamin Nutrition Res. **66:** 39–45.
12. DEXTER, D.T. *et al*. 1989. Increased nigral iron content and alteration in other metal ions occurring in brain in Parkinson's disease. J. Neurochem. **52:** 1830–1836.
13. MANEV, H. *et al*. 1997. Characterization of zinc-induced neuronal death in primary cultures of rat cerebellar granule cells. Exp. Neurol. **146:** 171–178.
14. ASSAF, S.Y. & S.H. CHUNG. 1984. Release of endogenous Zn from brain tissue during activity. Nature **308:** 734–736.
15. KOH, J.Y. *et al*. 1996. The role of zinc in selective neuronal death after transient global cerebral ischemia. Science **272:** 1013–1016.

16. LIN, A.M.Y. 2001. Coexistence of zinc and iron augmented oxidative injuries in the nigrostriatal dopaminergic system of SD rats. Free Radical Biol. Med. **30:** 225–231.
17. LIN, A.M.Y. *et al*. 2003. Zinc-induced apoptosis in substantia nigra of rat brain: neuroprotection by vitamin D3. Free Radical Biol. Med. **34:** 1416–1425.
18. KIKUGAWA, K. *et al*. 1989. Fluorescent and crosslinked protein formed by free radical and aldehyde species generated during lipid peroxidation. Adv. Exp. Med. Biol. **266:** 345–356.
19. CHIUEH, C.C. *et al*. 1983. 6-Fluorocatecholamines as false adrenergic neurotransmitters. J. Pharmacol. Exp. Ther. **225:** 529–533.
20. OKRAGKY, A.J. & M. HAAK-FRENDSCHO. 1997. An acid treatment method for enhanced detection of GDNF in biological samples. Exp. Neurol. **145:** 592–596.
21. HALLIWELL, B. & J.M. GUTTERIDGE. 1984. Oxygen toxicity, oxygen radicals, transition metals, and neurodegenerative diseases. Biochem. J. **219:** 1–14.
22. KOUTSILIERI, E. *et al*. 2002. Free radicals in Parkinson's dieease. J. Neurol. **249**(S2)**:** II1–II5.
23. COHEN, G. & P. WERNER. 1994. Free radicals, oxidative stress, and neurodegeneration. *In* Neurodegenerative Diseases, pp. 139–162. Saunders. Philadelphia.
24. BEAL, M.F. 2003. Bioenergetic approaches for neuroprotection in Parkinson's disease. Ann. Neurol. **53**(suppl. 3)**:** S39–S47.
25. ALLEGRA, M. *et al*. 2003. The chemistry of melatonin's interaction with reactive species. J. Pineal Res. **34:** 1–10.
26. REITER, R.J. 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog. Neurobiol. **56:** 359–384.
27. LIN, A.M.Y. & L.T. HO. 2000. Melatonin suppresses iron-induced neurodegeneration in rat brain. Free Radical Biol. Med. **28:** 904–911.
28. LIN, A.M.Y. *et al*. 2002. Neuroprotective effect of intermittent hypoxia on iron-induced oxidative injury in rat brain. Exp. Neurol. **176:** 328–335.
29. MERCE, A.L.R. *et al*. 1996. Potentiometric study of vitamin D3 complexes with manganese (II), iron (II), iron (III), and zinc (II) in water-ethanol medium. J. Inorganic Biochem. **73:** 167–172.
30. ROMER, W. *et al*. 1997. Novel "scavestrogens" and their radical scavenging effects, iron-chelating, and antioxidative activities and total antioxidative activities. Steroids **62:** 304–310.
31. BOLAND, R.L. *et al*. 1991. Single cell analysis of changes in cytosolic calcium induced by vitamin D3 metabolites in cultured rat mesangial cells. Biochem. Biophys. Res. Commun. **180:** 833–840.
32. TASAKA, T. *et al*. 1991. Mechanism of transient increase in intracellular concentration of free calcium ions in HL-60 cell differentiation induced by vitamin D3 and phorbol ester. Biochem. Int. **23:** 137–143.
33. BUDD, S.L. 1998. Mechanism of neuronal damage in brain hypoxia/ischemia focus on the role of mitochondrial calcium accumulation. Pharmacol. Ther. **80:** 203–229.
34. VIRAG, L. *et al*. 1999. Requirement of intracellular calcium mobilization for peroxynitrite-induced poly(ADP-ribose) synthetase activation and cytotoxicity. Mol. Pharmacol. **56:** 824–833.
35. JUNN, E. *et al*. 2000. Vitamin D3 upregulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. J. Immunol. **164:** 6287–6295.
36. CHEN, K.S. *et al*. 1993. Isolation and expression of human 1,25-dihydroxyvitamin D3 24-hydroxylase cDNA. Proc. Natl. Acad. Sci. USA **90:** 4543–4547.
37. NISHIYAMA, A. *et al*. 1999. Identification of thioredoxin-binding protein-2/vitamin D3 upregulated protein 1 as a negative regulator of thioredoxin function and expression. J. Biol. Chem. **274:** 21645–21650.