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Up-Regulation of cDK5/p35 by Oxidative Stress in Human Neuroblastoma IMR-32 Cells

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Abstract Cdk5, a member of the cyclin-dependent kinase (cdk) family, is predominately active in neurons, where its activity is tightly regulated by the binding of its neuronal activators p35 and p39. Cdk5 is implicated in regulating the proper neuronal function; a deregulation of cdk5 has been found associated with Alzheimer's disease and amyotrophic lateral sclerosis. As oxidative stress products have been seen co-localized with pathological hallmarks of neurodegenerative diseases, we studied the effect of oxidative stress on the cdk5 enzyme in human neuroblastoma IMR-32 cells. We evaluated the effects of 4-hydroxynonenal and Ascorbate plus $FeSO_4$ on cdk5 activity and on the expression of cdk5 and p35 proteins. We report here that oxidative stress stimulates cdk5 activity and induces an upregulation of its regulatory and catalytic subunit expression in IMR-32 vital cells, showing that the cdk5 enzyme is involved in the signaling pathway activated by oxidative stress. J. Cell. Biochem. 88: 758–765, 2003. © 2003 Wiley-Liss, Inc.

Key words: cdk5; p35; kinase activity; oxidative stress; neuroblastoma cells

Cdk5 is a serine/threonine kinase in the cyclin-dependent kinase (cdk) family, which is structurally related to other cdks known for their role in regulating the cell cycle [Lew and Wang, 1995, review]. Unlike others cdks, cdk5 is not dependent on its association with cyclins for activation, rather its activity requires association with one of two brain-specific regulatory subunits, called p35 and p39 [Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995; Humbert et al., 2000].

Cdk5/p35 activity has been implicated in a wide range of cellular functions. Cdk5/p35 is involved in neuronal development and migration, neurite outgrowth, synaptic transmission, dopamine signaling in striatum, exocytosis, differentiation of muscle cells, and in the organization of acetylcholine receptors at the neuromuscular junctions [Dhavan and Tsai, 2001; Maccioni et al., 2001; Paglini and Caceres, 2001; Smith et al., 2001, reviews]. Results from a number of studies including dominant negative mutant forms of cdk5 and knock-out mice demonstrate that the cdk5/p35 complex plays an essential role in neurite outgrowth and neuronal differentiation [Nikolic et al., 1996; Ohshima et al., 1996]. At the cellular level, cdk5/p35 regulates the cytoarchitecture of the cells through the modulation of actin dynamics, microtubules, and neurofilaments functions [Grant et al., 2001; Smith et al., 2001]. Improper cdk5 activity has been detected in the brain of Alzheimer's disease [Patrick et al., 1999] and amyotrophic lateral sclerosis [Bajaj, 2000; Patzke and Tsai, 2002, reviews]. Pathological hallmarks of different neurodegenerative diseases partially overlap with staining for cdk5 and with oxidative stress products [Yamaguchi et al., 1996; Pei et al., 1998; Bajaj, 2000; Takahashi et al., 2000; Adams et al., 2001; Sayre et al., 2001; Sherer et al., 2001; Borghi et al., 2002].

To explore the possibility that oxidative stress may function as a regulator of the cdk5 enzyme we examine cdk5/p35 activity during the cellular response to oxidative stress in human neuroblastoma IMR-32 cells.

Using 4-hydroxynonenal (HNE) or Ascorbate plus $FeSO_4$ (Asc/$FeSO_4$) as oxidative

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insults, we demonstrate that sub-millimolar concentrations of these agents lead to upregulation of cdk5 activity, as well as catalytic and p35 regulatory subunit expression in vital human neuroblastoma cells, showing that oxidative stress is involved in the regulation of the cdk5/p35 enzyme.

MATERIALS AND METHODS

Cell Cultures

Monolayer human neuroblastoma IMR-32 cell line [Tumilowicz et al., 1970] was maintained under subconfluent conditions in RPMI 1640 medium (Gibco), supplemented with 0.5% non-essential aminoacids, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 10% (v/v) fetal bovine serum in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere with 5% CO_2. The growth medium was changed twice a week.

Experimental Treatments

HNE (Calbiochem) and Asc/$FeSO_4$ (Sigma) were used to induce oxidative stress. HNE is a potent lipid peroxidation-derived aldehyde; it can be produced in response to oxidative insults and, in turn, is a potential inducer of intracellular oxidative stress through peroxide production [Uchida et al., 1999]. Asc/$FeSO_4$ is an oxidant agent which induces lipid peroxidation and HNE production [Zhang et al., 1993; Tamagno et al., 2000].

The cells were plated 48 h prior to chemical exposure and were at 70–80% confluence at the time of treatment. The medium was changed and HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM) was added. The effect was analyzed 6 and 24 h after the addition of the oxidant agents. Controls without oxidant exposure were run simultaneously.

The viability of the IMR-32 cells was measured 6 and 24 h after chemical treatment. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay was used for the quantification of metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye and MTT reduction is a measure of the mitochondrial function that is generally correlated with cell viability [Mosmann, 1983; Green et al., 1984]. MTT-assay was performed following the manufacturer's instructions (Sigma).

Antibodies

The following antibodies were used: anti-cdk5 (C-8; Santa Cruz Biotechnology, Inc, CA), an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of cdk5 of human origin, which can immunoprecipitate cdk5 as active kinase and does not react with cdk2 or other cdks; anti-p35 (C-19; Santa Cruz Biotechnology, Inc, CA), an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of p35 of human origin, p35(C-19)P; anti-p35 (N-20; Santa Cruz Biotechnology, Inc, CA), an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of p35 of human origin; anti-rabbit IgG conjugated with peroxidase as a secondary antibody (Santa Cruz Biotechnology, Inc., CA).

Immunoprecipitation and cdk5 Kinase Activity Assay

The medium was removed 6 and 24 h after treatment; cells were washed with cold phosphate buffer saline (PBS), scraped and collected by centrifugation for analysis.

Cell extracts were obtained by lysing cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, pH 8.0, 0.1% NP-40, 5 mM DTT) supplemented with cocktails of protease and phosphatase inhibitors (Sigma) for 30 min at 4°C [Nikolic and Tsai, 2000]. Cell lysates were recovered and centrifuged at 10,000*g* for 5 min at 4°C. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce). One hundred fifty micrograms of total proteins were incubated with rabbit IgG anti-cdk5 (C-8) at 4°C with gentle shaking for 1 h, followed by precipitation with Protein A/G PLUS-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4°C. The immunoprecipitates were washed three times in lysis buffer, then twice in kinase buffer (50 mM Hepes at pH 7.9, 5 mM $MgCl_2$, 1 mM DTT). For each sample, a total volume of 20 μl of the kinase assay mixture, containing 50 mM HEPES, pH 7.9, 1 mM DTT, 5 mM $MgCl_2$, 20 μM ATP, 2 μg of histone H1(Type III, from calf thymus, Sigma) was added to the cdk5 immunoprecipitates. The phosphorylation reaction was initiated with the addition of [γ^{32}P]ATP (2 μCi) (New England Nuclear, Dupont NEN, Boston,

MA) and incubated at 30°C for 30 min. The reaction was terminated by adding two times Laemmli sample buffer and boiled for 5 min prior to loading on the gel. Phosphorylated histone H1 was separated by 10% SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, visualized and quantified by PhosphoImager (BioRad). The lack of labeled protein in the gel autoradiography after blotting demonstrated the efficiency of the transfer.

Western Blot Analysis

Cdk5 and p35 steady-state levels were detected in total cell lysates by Western blot analysis using specific antibodies. Briefly, cells were lysed in hot SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 1% 2β-mercaptoetanol, 10% glycerol) and boiled for 5 min. Total proteins (50 μg of protein/lane) were resolved on a 10% SDS-polyacrylamide gel [Laemmli, 1970] and blotted on PVDF membranes (Hybond-P, Amersham Pharmacia). The membranes were blocked for 1 h at room temperature (RT) with blocking solution (25 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1%Tween-20, 5% dry milk and 3% BSA) and incubated with anti-cdk5 polyclonal antibody (C-8, 1:1,000) or anti-p35 polyclonal antibody (C-19, 1:1,000) for 2 h at RT. The membranes were washed four times with blocking solution and exposed to peroxidase-conjugated secondary antibody (1:10,000) in blocking solution for 1 h at RT. The reactions were developed with enhanced chemiluminescence following the manufacturer's instructions (ECL plus kit, Amersham Pharmacia, Chicago, IL). The protein concentration was determined by the BCA method. Quantification of the band signals was carried out by densitometry (FluorS-Max Imager, Bio Rad).

Identification of cdk5 Regulatory Subunit in Human Neuroblastoma IMR-32 Cells

To identify the cdk5 regulatory subunit, we analyze the anti-cdk5 immunoprecipitates (C-8) used for the kinase assay. Western blot analysis was determined using specific antibodies against the carboxy terminus of p35 (C-19, 1:20,000) and the amino terminus of p35 (N-20, 1:5,000). Competition of the p35 antibody (C-19) with the immunogen peptide p35(C-19)P was carried out by incubating the antibody with the peptide prior to immunodetection.

Statistical Analysis

Data are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Significance was established at a P-value < 0.05 (*) or < 0.01(**) or < 0.001(***).

RESULTS

Cell Morphology and Viability Are not Modified by Oxidative Stress

Morphology of human neuroblastoma IMR-32 cells was observed at the phase contrast microscope 6 and 24 h after exposure to HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM). IMR-32 cells exposed to the vehicle alone were flat, showed intact cell bodies and exhibited a fibroblast-like morphology with short cytoplasmatic processes. We did not observe morphological changes either at 6- and 24-h post-treatments (Fig. 1A).

To validate the morphological observations in assessing IMR-32 cell viability, the cells were also examined by MTT-assay. Exposure of IMR-32 cells to HNE or Asc/$FeSO_4$ did not affect cell viability, as indicated by an unmodified level of MTT reduction (Fig. 1B).

Cdk5 Activity Is Increased by Oxidative Stress

Cdk5 activity appeared increased in the treated cells, as measured by in vitro phosphorylation of histone H1. Figure 2A shows an autoradiogram of ^{32}P-labeled histone H1; quantification of radioactivity incorporated in histone H1 was performed by phosphoimager (Fig. 2B). Our analysis revealed a threefold increase in cdk5 kinase activity in cells exposed to HNE or Asc/$FeSO_4$, 6 h after treatment, as compared with untreated cells. Cdk5 kinase activity returns to control levels 24 h post-treatment with Asc/$FeSO_4$, while it remains higher for up to 24 h with HNE treatment.

p35 Is the Regulatory Subunit of cdk5 in Human Neuroblastoma IMR-32 Cells

Cdk5 kinase activity depends on the association of the cdk5 catalytic subunit and the regulatory subunit. To identify the cdk5 regulatory subunit expressed in IMR-32 cells, we analyzed the cdk5 immunoprecipitates of untreated and treated cells by Western blotting using two different p35 specific antibodies, one recognizing the C-terminus (C-19) and the other recognizing the N-terminus (N-20) of the

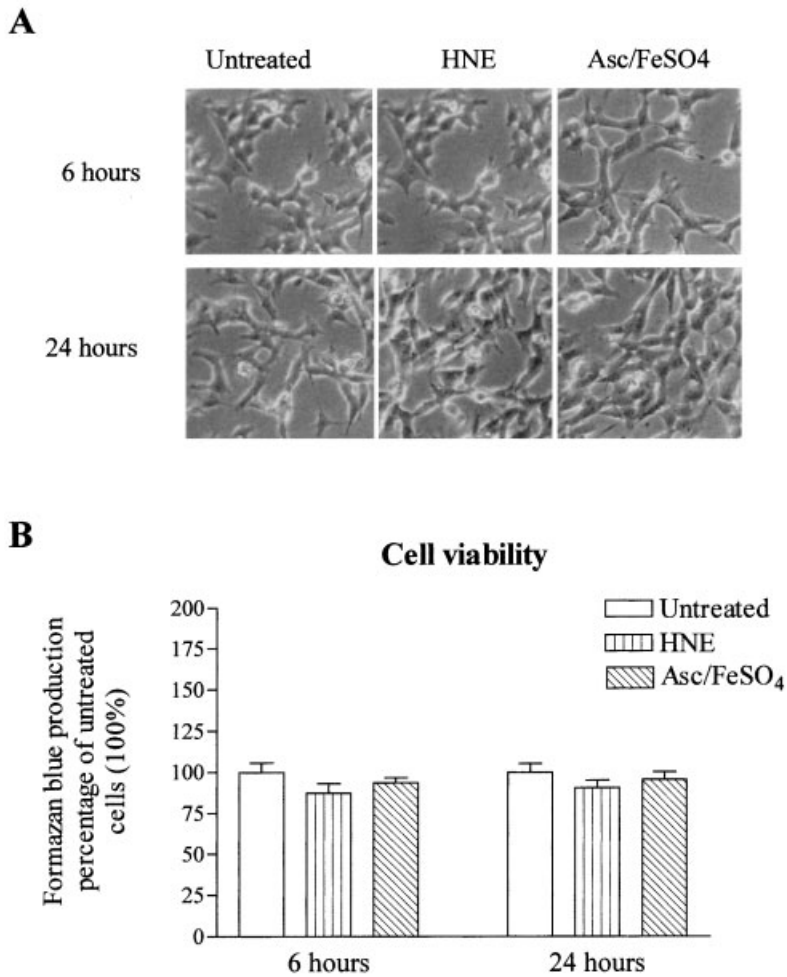


Fig. 1. Cell morphology and viability. **A**: Morphology of human neuroblastoma IMR-32 cells under phase contrast microscope 6 and 24 h after exposure to HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM). **B**: Quantitative assessment of cell viability by MTT-assay. Results are expressed as the percentage of formazan production relative to control, which was defined as 100%. Each data point represented the mean ± SEM; n = 3; $P > 0.05$ (Bonferroni's Multiple Comparison Test).

human protein p35 (Fig. 3). Our results indicate that the two antibodies recognize the identical protein p35 on the basis of the same apparent molecular weight. Moreover, competition with the immunogenic peptide p35C-19P abolishes the detection of the protein present in the membranes used for Western blot analysis (Fig. 3).

Cdk5 and p35 Steady-State Levels Are Increased by Oxidative Stress

To investigate the expression of the cdk5 and p35 proteins, equal amounts of total protein extracts from IMR-32 cells were analyzed by Western blotting using specific antibodies that are able to recognize the C-terminus of both proteins, C-8 and C-19, respectively. The cdk5 and p35 proteins were constitutively expressed in this cell line; the cdk5 and p35 steady-state levels in total IMR-32 cell extracts detected 6 h post-treatment were higher than those from untreated cells (Fig. 4A,B). Quantitative analysis of the band signals from the total cell extracts revealed a twofold increase in cdk5 protein levels in response to HNE or Asc/$FeSO_4$, a threefold increase and a twofold increase in p35 protein levels in response to HNE and Asc/$FeSO_4$ respectively, 6 h after treatment (Fig. 4C,D). These responses persisted for at least 24 h in the HNE treated cells, while they returned to basal levels in the Asc/$FeSO_4$ treated cells (Fig. 4A,B,C,D). Using the antibody (C-19), which recognizes both p35 and its C-terminal fragment p25, we detected an

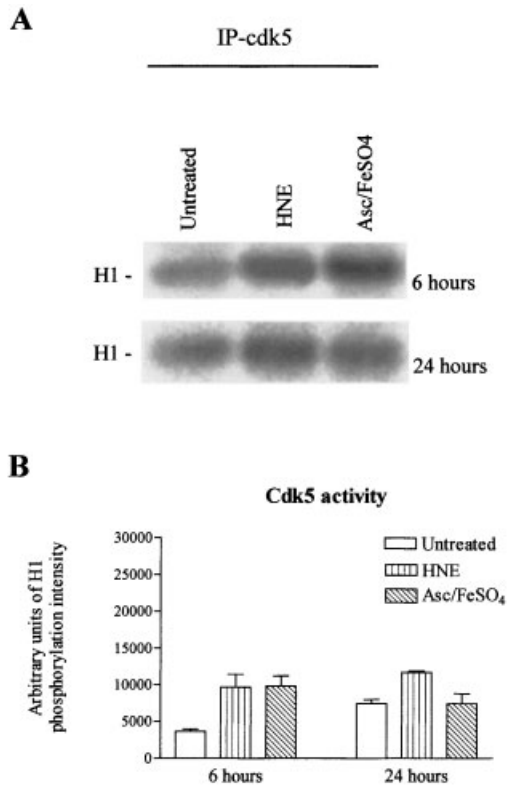


Fig. 2. Cdk5 kinase activity. IMR-32 cells were treated with HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM) for the indicated times. **A**: Cdk5 activity was determined after immunoprecipitation of IMR-32 cell lysates with cdk5 antibody (C-8), followed by incubation with ^{32}P-ATP in the presence of histone H1, as exogenous substrate. Kinase assay showed a threefold increase in cdk5 kinase activity 6 h after HNE or Asc/$FeSO_4$ treatment. The activity is increased for at least 24 h in the HNE treated cells, while it returns to control levels in Asc/$FeSO_4$ treated cells after 24 h. **B**: Quantification of cdk5 activity: arbitrary units were used for the y-axis to indicate the relative intensity. Each data point represented the mean ± SEM; n = 3; $^{**}P < 0.01$ (Bonferroni's Multiple Comparison Test).

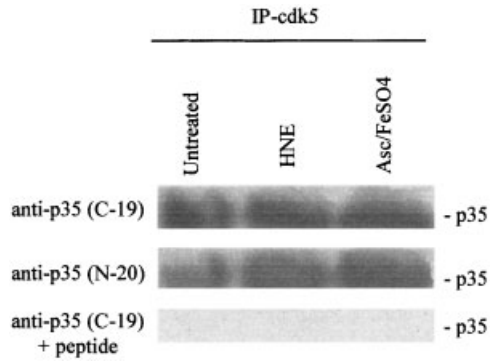


Fig. 3. Identification of the cdk5 regulatory subunit. Cdk5 regulatory subunit was identified in IMR-32 cells 6 h after exposure to vehicle (untreated) and after treatment with HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM). Cdk5 was immunoprecipitated from 150 μg of cell lysates and the immunoprecipitates were probed with anti-p35 C-terminus, anti-p35 N-terminus, or anti-p35 C-terminus in the presence of the corresponding antigenic peptide C-19P.

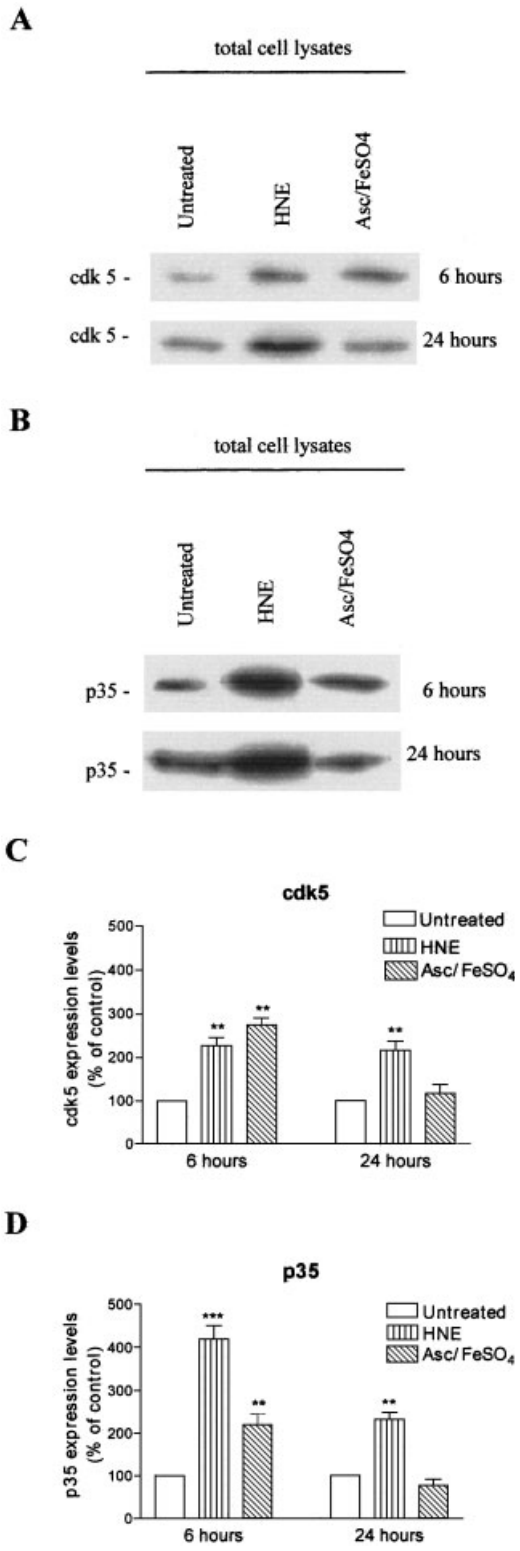
increased level of p35 protein and p25 absence in our experimental conditions.

DISCUSSION

In the present study, human neuroblastoma IMR-32 cells were analyzed for cdk5 enzyme regulation in response to oxidant stimuli, such as HNE and Asc/$FeSO_4$.

We found that cdk5 is active in IMR-32 cells because the regulatory subunit p35 is constitutively expressed in these cells, as has been seen in PC12 cells [Yan and Ziff, 1995]. Here we report that oxidative stress induces an overactivation of cdk5 kinase, as well as an upregulation of catalytic and regulatory subunits. Cdk5 kinase activity, as well as catalytic and p35 regulatory subunit expression, are increased at 6 h after both treatments; HNE formation is induced by iron-ascorbate as the main aldehyde lipid peroxidation product [Tamagno et al., 2000], therefore exogenous and endogenous HNE induced by Fe^{2+} appear to be key mediators of cdk5 and p35 regulation. The persistence of the effects observed 24 h after HNE treatment may be due to the preservation of this stable aldehyde in the culture medium, which may represent a continuous stimulus [Kruman et al., 1997]. The single treatments with two agents did not modify cell morphology and cell viability. Moreover, we observed an increased level of p35 protein without cleavage of p35 to p25, suggesting that the preservation of mitochondrial functions does not lead to the activation of the calpain required for proteolytic cleavage of p35 to p25 [Patrick et al., 1999; Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000].

An interesting result from the present study is the demonstration that the cdk5 enzyme activity parallels the level of the p35 regulatory subunit more closely than the level of the cdk5 catalytic subunit. We observed a threefold increase in cdk5/p35 activity, as well as in the p35 protein level, while the cdk5 protein level showed a twofold increase. These results suggest that cdk5/p35 kinase activity primarily depends on the regulation of the p35 protein level, which, in turn, may be regulated at the transcription level.



In vivo studies demonstrated that the levels of both p35 mRNA, protein and cdk5 kinase activity present the same temporal profile during development, as well as in adult and aged rat brains [Tomizawa et al., 1996; Delalle et al., 1997; Wu et al., 2000]. Recently, it has been demonstrated that the neuronal specific activity of the cdk5/p35 kinase is achieved through the regulation of p35 expression due to modulation of Sp transcription factor levels and activity during the neuronal differentiation of P19 cells [Ross et al., 2002]. Moreover, it has been reported that oxidative stress enhances the DNA binding of the transcription factors Sp1 and Sp3 to their cognate GC-box in embryonic cortical neurons [Chatterjee et al., 2001]. The p35 promoter contains not only the functionally important GC-box, but also the consensus Egr (early growth response) binding site. Although, the Egr binding sites are not required for the expression of p35 in P19 or primary cortical neurons [Ross et al., 2002], the Egr transcription factors may regulate the expression of p35 in response to neuronal stimulation [Beckmann and Wilce, 1997; O'Donovan et al., 1999]. A recent study suggests that the nerve growth factor contributes to the increased expression of p35 through the ERK-dependent induction of Egr1 in PC12 cells [Harada et al., 2001]. Egr-1 transcription, DNA-binding activity, and the protein level have been found to be upregulated by oxidative stress induced by H_2O_2 in mouse osteoblasts [Nose et al., 1991; Ohba et al., 1994; Nose and Ohba, 1996]. Therefore, the p35 protein level may be modulated through the activation of the transcription factor Egr1 in our experimental model represented by IMR-32 cells exposed to oxidative stress. In addition, oxidative stress modulates transcription factors, such as AP1, which, in turn, can act on the cdk5 promoter [Ohshima et al., 1995].

Fig. 4. Cdk5 and p35 steady-state levels. IMR-32 cells were treated with HNE (10 μM) or Asc/$FeSO_4$ (500 μM/5 μM) for the indicated times. Western blots of total cell extracts (50 μg of total proteins per well) with antibodies recognizing cdk5 (C-8) (**A**) or p35 (C-19) (**B**). We observe an increase in cdk5 and p35 steady-state levels at 6 h after both treatments. This response persists for at least 24 h in the HNE treated cells, while it returns to basal levels in Asc/$FeSO_4$ treated cells after 24 h. Quantitative analysis of cdk5 (**C**) and p35 (**D**) proteins: untreated cells were considered as 100%. Each data point represented the mean ± SEM; n = 3; $^{**}P < 0.01$ (Bonferroni's Multiple Comparison Test).

At present, the exact sequence of events leading to the upregulation of cdk5 and p35 proteins after HNE or Asc/$FeSO_4$ remains unclear. Although, many pathways are known to be redox-sensitive, the mechanism for the redox regulation of transcription factors is not fully understood [Allen and Tresini, 2000, review]. Future works will elucidate the role of oxidative stress on the transcription factors, which regulate the cdk5 and p35 protein levels.

Our study reveals that oxidant insult upregulates cdk5 activity, as well as cdk5 and p35 expression, in human neuroblastoma IMR-32 cells, suggesting that cdk5/p35 may be a kinase member of a signaling pathway activated by oxidative stress.

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