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Overexpression of Heme Oxygenase in Neuronal Cells, the Possible Interaction with Tau*

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Increased expression of heme oxygenase-1 (HO-1) is a common feature in a number of neurodegenerative diseases. Interestingly, the spatial distribution of HO-1 expression in diseased brain is essentially identical to that of pathological expression of tau. In this study, we explored the relationship between HO-1 and tau, using neuroblastoma cells stably transfected with sense and antisense HO-1 constructs as well as with the vector alone. In transfected cells overexpressing HO-1, the activity of heme oxygenase was increased, and conversely, the level of tau protein was dramatically decreased when compared with antisense HO-1 or CEP transfected cells. The suppression of tau protein expression was almost completely reversed by zinc-deuteroporphyrin, a specific inhibitor of heme oxygenase activity. The activated forms of ERKs (extracellular signal-regulated kinases) were also decreased in cells overexpressing HO-1 although no changes in the expression of total ERK-1/2 proteins were observed. These data are in agreement with the finding that the expression of tau is regulated through signal cascades including the ERKs, whose activities are modulated by oxidative stresses. The expression of tau and HO-1 may be regulated by oxidative stresses in a coordinated manner and play a pivotal role in the cytoprotection of neuronal cells.

Heme oxygenase (HO)[1] is a microsomal enzyme that cleaves heme, a pro-oxidant, to produce biliverdin and carbon monoxide (1). Biliverdin is converted to a potent antioxidant (2), bilirubin, and carbon monoxide has been implicated to be a physiological regulator of cGMP and vascular tone (3, 4). The HO system is the rate-limiting step in heme degradation. To date, three HO isoforms (HO-1, HO-2, and HO-3) have been identified that catalyze this reaction. HO-1 is a 32-kDa heat shock protein (1, 5), which is inducible by numerous noxious stimuli (6, 7). HO-2 is a constitutively synthesized 36-kDa protein which is abundant in brain and testis (8). HO-3 is related to HO-2 but is the product of a different gene, and its ability to catalyze heme degradation is much less than that of HO-1 (9). Within the brain, the majority of HO activity is attributed to the HO-2 isozyme (8). The expression of HO-1 is normally very low in the brain but increases markedly after heat shock, ischemia, or glutathione depletion (8, 10–12). In the normal rat brain, HO-1 is restricted to select neuronal and non-neuronal cell populations in the forebrain, diencephalon, cerebellum, and brainstem (10). However, after heat shock or ischemia, increased HO-1 is seen in neuronal and glial cells throughout the brain (10, 12–14).

While the long-lived lesions of Alzheimer disease (AD) provide ample opportunity for oxidative modification because of their low rate of turnover (15), the more important issue is when, during the course of AD, oxidative abnormalities actually develop. Recent studies show that the oxidative damage associated with AD is represented by lipid peroxidation (16), nitration (17), reactive carbonyls (18), and nucleic acid oxidation (19) that are all increased in vulnerable neurons whether or not they contain neurofibrillary tangles (NFT). NFT are composed of a hyper-phosphorylated form of the tau protein. In fact, an inverse relationship between oxidative damage and NFT was noted in one case (19). Thus, these findings raise the question as to the relationship between tau abnormalities and oxidative stress.

We have previously demonstrated that HO-1 is associated with degenerating neurons (20–22) and that specific induction of HO-1, but not HO-2, parallels the regional susceptibility of neuronal degeneration (23). Moreover, the spatial distribution of HO-1 expression in AD brain is essentially identical to that of the pathogenic conformational changes of tau protein, the major component of the intraneuronal lesion of AD, neurofibrillary tangles (24). In this study, we demonstrated that when HO-1 is overexpressed, physiological expression of tau was suppressed and, at the same time, the mitogen-activated protein kinase (MAPK)-cascade was inactivated. This suggests that a change in oxidation state not only induces HO-1 expression but may also play a role in the regulation of the tau gene.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Transfection into Neuroblastoma Cells—The *Hin*dIII fragment from pRc/CMV-human HO-1 (25) containing the human heme oxygenase-1 coding sequence was cloned into the episomal vector pCEP-4β (see Fig. 1*A*). This places the coding sequence in either a sense or antisense orientation under the control of the cytomegalovirus immediate-early gene enhancer-promoter. The orientation of positive clones was determined by digestion with *Sac*II. Sense and antisense clones were transfected into the human neuroblastoma cell line M-17, using the cationic lipid DOTAP (Roche Molecular Biochemicals)

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[1] The abbreviations used are: HO, heme oxygenase; AD, Alzheimer disease; ERK, extracellular signal-regulated kinase; G6PDH, glucose 6-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFT, neurofibrillary tangles; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase-polymerase chain reaction.

in serum-free Opti-MEM (Life Technologies, Inc.) as described previously (26). Transfected cells were maintained at 37 °C in Opti-MEM supplemented with 5% fetal calf serum, penicillin/streptomycin, and 500 μg/ml hygromycin (Calbiochem). For the HO inhibition study, cells were cultured in the same medium with the addition of 5 μM of zinc-deuteroporphyrin (Porphyrin Products) following the transfection.

Cell Viability and HO Activity—Cells were transferred to serum-free Opti-MEM with or without hydrogen peroxide (0.3 mM). After 24 h of incubation at 37 °C, cell viability was evaluated by trypan blue exclusion. Data were obtained from four separate experiments and expressed as the percentage of dead (stained) cells compared with total (stained and unstained) cells.

HO activity was measured using a microassay that measures the conversion of [^{14}C]heme to [^{14}C]bilirubin as described previously (27). Briefly, cells were homogenized in 100 μl of ice-cold KPB (0.1 M potassium phosphate buffer, pH 7.5) containing 50 μM PMSF and centrifuged at 14,000 × *g* for 1 min. The supernatants (10–15 μg of protein) were incubated for 30 min at 37 °C with 11 μM [^{14}C]heme (specific activity 52 Ci/mol) and 2 mM NADPH. The resultant [^{14}C]bilirubin was extracted into toluene (1 ml), and the radioactivity was determined using a Wallac LKB 1214 Rackbeta with 95.5% counting efficiency. HO activity was expressed as pmol [^{14}C]bilirubin formed/hr/mg of protein. Statistical analyses were using one-way analysis of variance.

Immunoblots—Cells were lysed in a buffer (lysis buffer B) consisting of 0.5% Nonidet P-40, 0.5% deoxycholate, and 10 mM EDTA in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.4), containing 10 μg/ml each of leupeptin, antipain, and pepstatin, and 1 mM PMSF. Cell debris was removed by centrifugation at 300 × *g* for 10 min. Protein from the supernatants was precipitated by adding 5 volumes of methanol, and the resulting pellet was then dissolved in sample buffer and separated on 10% SDS-polyacrylamide gel. For immunoblotting, the separated proteins were electrophoretically transferred to Immobilon-P (Millipore) for 16 h at 25 V. The membranes were blocked with 10% non-fat dry milk for 1 h at room temperature and probed with each primary antibody for 16 h at 4 °C. The primary antibodies used were mouse monoclonal antibodies, Tau1, to a dephosphorylated epitope of tau, and G7 (Santa Cruz Biotechnology), to a phosphorylated epitope of c-Jun N-terminal kinase-1/2 (JNK-1/2), rabbit antisera to HO-1, HO-2, extracellular signal-regulated kinase 1 (ERK-1), ERK-2, ERK-1/2 (StressGen), or ferritin (Dako), and affinity purified antisera specific to phosphorylated (activated) ERKs (Promega). Prior to the incubation with Tau1, the membrane was treated with 10 units/ml alkaline phosphatase (P5931; Sigma) in 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 0.2 mM dithiothreitol, and 100 mM Tris-HCl (pH 8.0) at 37 °C for 2 h. After 1 h incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase, the membranes were extensively washed in TBST (TBS with 0.1% Tween-20) and visualized on x-ray film after reaction with chemiluminescent reagent (Renaissance; NEN Life Science Products). Quantification of the results was performed using a computer-assisted scanning system (PDI, Huntington Station, NY). The data obtained were expressed as optical densities and analyzed statistically using one-way analysis of variance.

Northern Blotting and RT-PCR—Total RNA was extracted from cells using Ultraspec RNA (Biotecx), size separated by electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to nylon membranes (GeneScreen Plus Membranes; NEN Life Science Products) and hybridized with G6PDH (glucose 6-phosphate dehydrogenase) or HO-1 cRNA labeled with [^{32}P]cytidine triphosphate. For RT-PCR experiments, cDNAs were prepared with random hexamers (First-strand cDNA synthesis kit; Amersham Pharmacia Biotech) and used as templates for subsequent PCR. For HO-1 and HO-2 cDNA amplification, primers and PCR procedures were as described previously (28). For tau cDNA amplification, samples were heated at 94 °C for 2 min and then subjected to thermocycling (18 cycles of 30 s at 94 °C, 30 s at 63 °C, and 30 s at 72 °C), using the specific primers 5′-CGCCAGGAGTTCGAAGTGATG-3′ and 5′-TGTCATCGCTTCCAGTCCCGTC-3′. The PCR products were separated on a 5% polyacrylamide gel in 1× Tris borate-EDTA buffer and visualized with UV light after ethidium bromide staining.

RESULTS

HO-1 Expression—Human neuroblastoma cells, M-17, were transfected with a full-length human HO-1 cDNA subcloned into a mammalian expression vector driven by the cytomegalovirus promoter (Fig. 1*A*) (26). Transfected cells expressed high levels of HO-1 mRNA (Fig. 1*B*) and protein (Fig. 3). The HO activity of cells transfected with sense HO-1 cDNA was

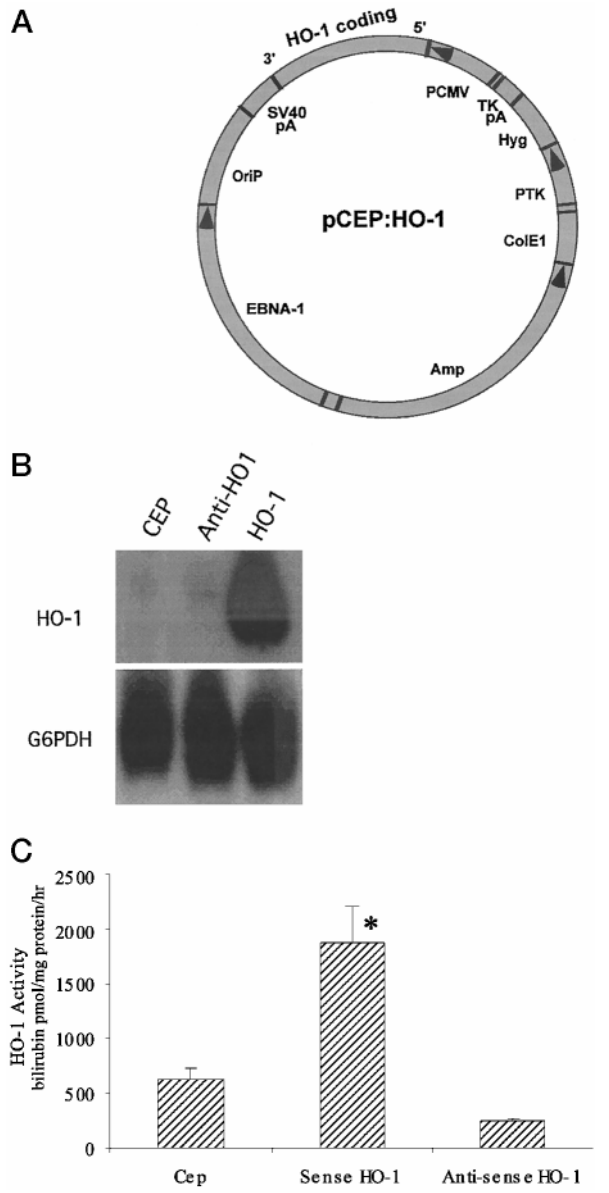

FIG. 1. **Overexpression of human HO-1 cDNA in M-17 neuroblastoma cells.** *A*, schematic representation of the cDNA construct using pCEP vector. The sense clone is shown. *B*, Northern blot analysis demonstrated a significant increase in HO-1 transcripts in HO-1 transfected cells. G6PDH was used as a standard for normalization. *C*, HO activity was significantly increased in HO-1 transfected cells. *, $p < 0.05$.

significantly higher ($p < 0.05$) than mock (CEP) transfected cells (Fig. 1*C*), while cells transfected with the antisense clone were not significantly different from CEP-transfected cells with respect to HO activity (Fig. 1*C*). There were no obvious differences in the growth rate or morphology in any of the transfected cells. To test whether overexpression of HO-1 could protect the cells from oxidative insult, the transfected cells were exposed to 0.3 mM H_2O_2 in serum-free Opti-MEM for 24 h. The HO-1 overexpressing cells showed a significantly higher survival rate compared with the CEP transfected control cells (Fig. 2). Interestingly, there was a significant difference in cell survival between HO-1 overexpression and controls even under the basal experimental conditions without H_2O_2 exposure (Fig. 2).

Suppression of Tau Expression—To explore a possible relationship between HO-1 expression and cytoskeletal abnormalities, we compared tau protein expression in normal cells or

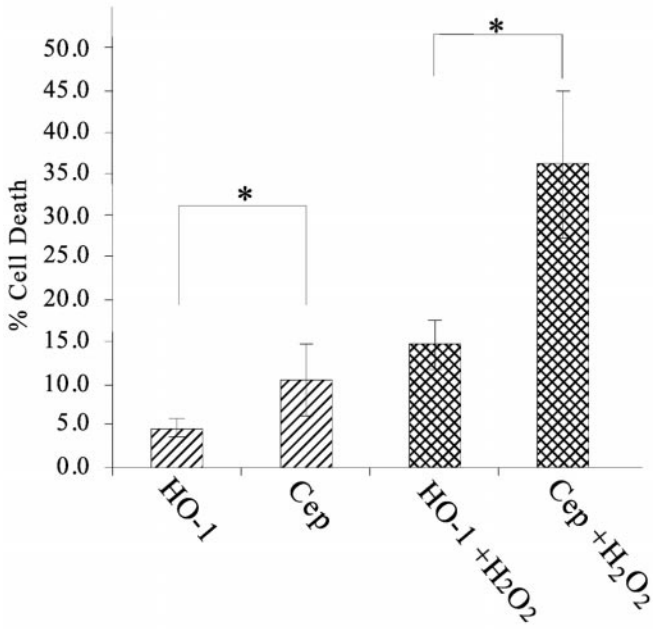

FIG. 2. **Changes in cell viabilities induced by oxidative stress.** HO-1 overexpression significantly improved cell viability in serum-free media with or without the addition of hydrogen peroxide (0.3 mM). *, $p < 0.05$.

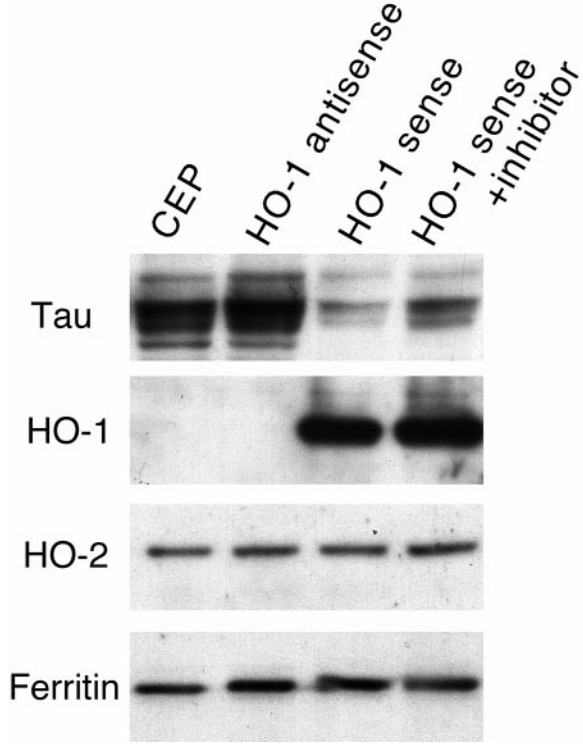

FIG. 3. **Representative immunoblots of cell lysates from the transfected cells.** HO-1 overexpression specifically suppresses tau expression, which was partially alleviated by the addition of an HO inhibitor to the media. Data are representative of two independent experiments.

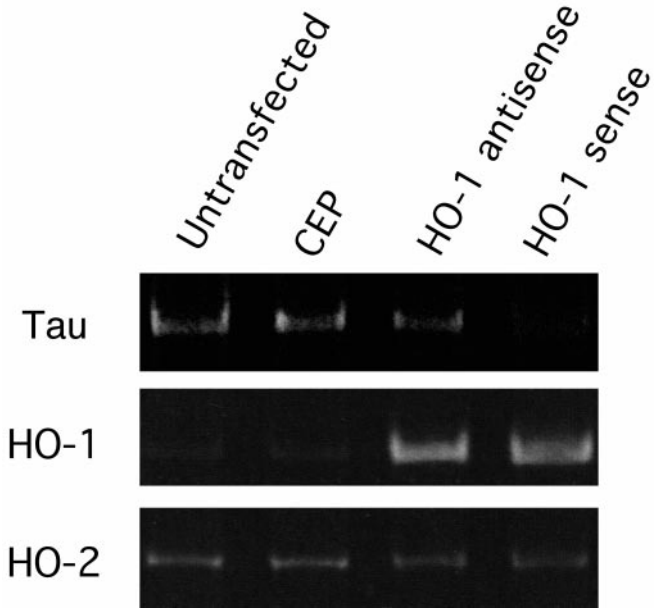

FIG. 4. **RT-PCR experiments using total RNA from each line of cells.** Not only sense HO-1 transcripts but also comparable levels of antisense HO-1 transcripts were detected using the same primer pair. The amount of tau mRNA showed a decrease in HO-1 transfected cells. There were no changes in the level of HO-2 transcripts. Data are representative of two separate experiments.

cells overexpressing HO-1. In untransfected M-17 cells (data not shown) and CEP transfected cells (Fig. 3), the monoclonal antibody Tau1 recognized presumptive tau protein around 50 kDa. In the cells overexpressing HO-1, the expression of tau protein was dramatically suppressed (Fig. 3). The suppression of tau protein was partially alleviated by the addition of zinc-deuteroporphyrin, an HO inhibitor, to the medium (Fig. 3). The antisense-transfected cells showed no obvious change in tau expression (Fig. 3). The addition of HO inhibitor did not affect the HO-1 protein level nor were there any apparent changes in HO-2 and ferritin expression (Fig. 3). RT-PCR experiments demonstrated decreased expression of tau transcripts in HO-1 transfected cells (Fig. 4). In contrast, in the antisense HO-1 cells, a comparable amount of tau transcript to that of untransfected or CEP transfected cells was detected (Fig. 4).

Changes in MAPK Activation—In immunoblots, rabbit antisera specific for activated (dually phosphorylated) ERK-1/2 recognized two bands, the size of which were consistent with those of ERK-1 (p44 in Fig. 5, *A* and *B*) and ERK-2 (p42 in Fig. 5, *A* and *B*). Reprobing of the same membrane with anti-ERK-1 or anti-ERK-2 serum revealed that each band could be specifically recognized, respectively (data not shown). The expression of the activated form of ERK-1 was decreased in HO-1 transfected cells; however, with the addition of an HO-inhibitor, activated ERK-1 was increased over basal levels when compared with CEP and antisense transfected cells (p44 in Fig. 5*A*). The changes in expression of activated ERK-2 showed a similar pattern (p42 in Fig. 5*A*). The amount of total (phosphorylated and unphosphorylated) ERK-1/2 protein was unchanged among these cells (Fig. 5*B*). In contrast, no changes were observed in JNK-1/2 expression or activation (p46 and p54 in Fig. 5*C*). The quantification of each band revealed that expression changes in the activated form of ERK-1 were statistically significant ($p < 0.05$) both in sense HO-1 cells and in HO-1 cells with an HO-1 inhibitor (Fig. 5*D*).

DISCUSSION

In the present study, we successfully transfected sense- and antisense-human HO-1 cDNA into human neuroblastoma cells using an episomal vector. The transfection of the DNA constructs did not affect the morphology or growth rate of the cells. Cells transfected with the sense HO-1 cDNA exhibited a severalfold induction of HO-1 transcripts (Fig. 1*B*) and protein (Fig. 3), which was mirrored in a more than 3-fold increase in HO activity (Fig. 1*C*). Overexpression of the HO-1 gene consid-

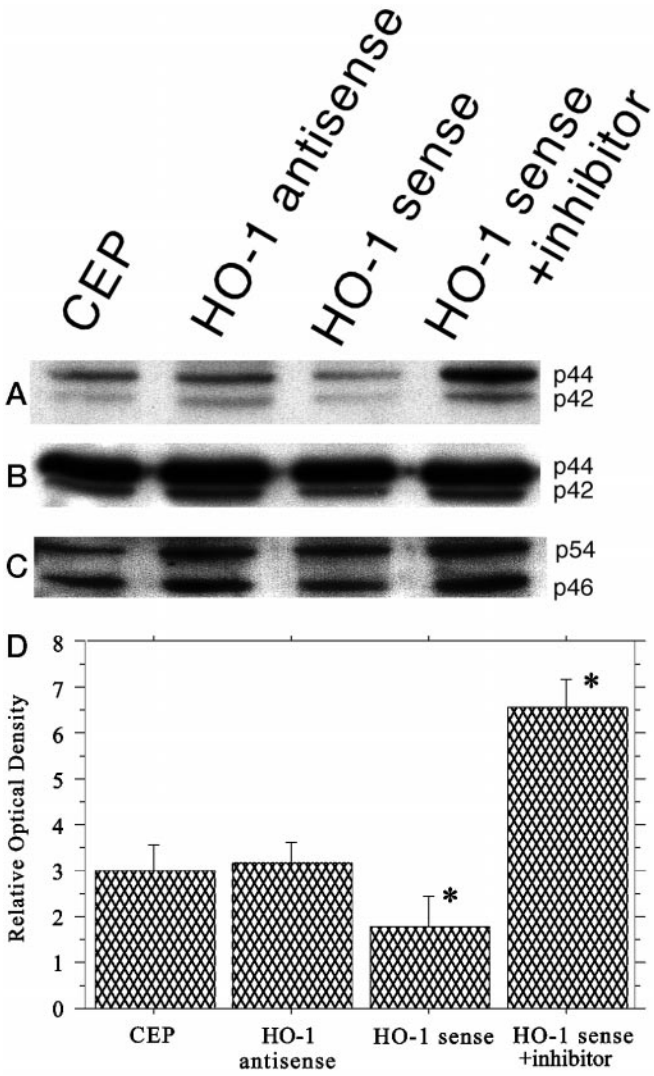

FIG. 5. **Changes in ERK and JNK activation in transfected cells.** The expression changes of dually phosphorylated-active form of ERK-1 (p44) and ERK-2 (p42) (*A*) and of total (phosphorylated and unphosphorylated) ERK-1/2 proteins (*B*) were shown. *Lower panel* (*C*) demonstrates the expression of dually phosphorylated-active form of JNK-1/2 (p46 & p54). A representative result of two independent experiments is shown. The *bar graph* (*D*) showed quantified values of activated ERK-1 expressions. Data were obtained from three independent experiments and are expressed as optical density. *, $p < 0.05$

erably enhanced resistance of the cells to oxidative injury produced by H_2O_2, which is consistent with previous reports showing that HO-1 overexpression is cytoprotective in non-neuronal cells (25, 29). Although heme catabolism mediated by HO-1 produces a potential pro-oxidant, iron, the present data suggest that HO-1 protects neuronal cells as well from oxidative stresses. The increased iron released from heme catabolism has been thought to induce expression of ferritin, which sequesters excess iron and may protect cells against oxidative stresses (30); however, our data clearly show that overexpression of HO-1 does not affect ferritin expression. On the other hand, in the antisense transfected cells, there was residual HO activity below the basal level that is probably because of the expression of intrinsic HO-2. In fact, HO-2 expression was not affected by transfection of the present series of cDNA constructs (Fig. 3).

One notable effect of HO-1 overexpression was that expression of tau protein was specifically suppressed. This suppression was partially alleviated by treatment with an HO inhibitor, suggesting that the regulation of tau expression might depend on HO activity. Tau is a microtubule-associated protein and is expressed predominantly in neurons and neuron-derived cells (31). The detailed regulatory mechanism for tau gene expression is still unknown, but recently, ERKs, which are prominent members of the MAPK family, were shown to induce tau promoter activity (32). In our study, HO-1 overexpression decreased the phosphorylated (activated) forms of ERK-1 and ERK-2, which is consistent with the observed suppression of tau expression. When the HO inhibitor was added, the phosphorylated form of ERK-1 increased over the control levels, suggesting that ERK-1 activation was down-regulated by the increased HO activity and that the HO inhibitor suppressed not only the overexpressed HO-1 activity but also the intrinsic HO-2 activity. Although the level of the activated form of ERK-1 was increased and that of ERK-2 was restored to control levels by the addition of HO inhibitor, only partial recovery of tau protein expression was observed. This suggests that tau gene expression is not directly controlled by ERK cascades. In fact, tau has been classified as a "late gene" and the effect of MAPK cascades on its expression is thought to be indirect and mediated by additional downstream transcription factors (32). Interestingly, changes in cell growth and morphology were not observed in HO-1 overexpressing cells even though tau protein expression was suppressed. This implies that other molecules can substitute for the tau protein, which has also been suggested in previous reports using a cell culture model (33) and in knockout mice (34).

ERK-1 (p44) and ERK-2 (p42) are major components of the ERK family and are believed to be functionally redundant. They play a pivotal role in stress signaling and are activated in cells exposed to environmental stresses, such as UV irradiation and H_2O_2 (35). HO-1 is also a stress-inducible protein, and its product, bilirubin, is a potential antioxidant. The data, demonstrating the increase in survival of HO-1 overexpressing cells under normal conditions (Fig. 2), imply that there is a basal level of oxidative stress to cells in culture. This finding is also in good agreement with the results showing a basal level of activation of ERKs in control cells. The HO-1 overexpressing cells showed increased resistance to H_2O_2 exposure, suggesting that HO-1 can potentiate the ability of the cell to withstand such oxidative stresses. By its ability to reduce stress, activation of the ERKs may be suppressed by HO-1 overexpression. Alternatively, carbon monoxide, which is another product of HO-1-mediated heme catabolism, may play a role in ERK inactivation. Carbon monoxide is a potential signaling molecule that functions by activating guanyl cyclase (3). Elevation of cGMP was shown to block the activation of MAPK cascades (36), and therefore, carbon monoxide may also be a suppressor of ERK activation.

In AD brain, the expression of HO-1 is closely associated with pathological changes in the tau protein (24), suggesting that there is a common underlying mechanism mediating tau pathology and HO-1 expression. Recently, widespread distribution of the activated form of ERKs was demonstrated in vulnerable neurons in AD brain that preceded the accumulation of phosphorylated tau (37). These data suggest that, in the AD brain, ERKs may play pivotal roles in tau gene regulation and phosphorylation. ERKs also up-regulate HO-1 gene expression (38), and nearly all vulnerable neurons that exhibit induction of the HO-1 gene show ERK activation.[2] In each cell, tau protein is located not only in the cytosol but also in the nucleus and nucleolus (31), and its expression is inducible by neurotoxins such as glutamate (39) or MPTP (40), whose toxicities are mediated by oxidative stress. Thus, it is plausible that tau has

[2] A. Takeda and M. A. Smith, unpublished observation.

additional functions beyond its well known role in the dynamics of microtubules. We hypothesize that the co-localization of HO-1 and the pathological form of the tau protein may be protective responses against oxidative stress in neuronal cells (41), which may be mediated through ERK cascades. In fact, neurons that express phosphorylated tau are more resistant to cell death (41) supporting this hypothesis. Further studies on the tau/HO-1 relationship will be required before a comprehensive understanding of neurodegenerative processes involving cytoskeletal abnormalities is achieved.

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