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Original Contribution

GDNF modulates HO-1 expression in substantia nigra postnatal cell cultures

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Abstract

Heme oxygenase-1 (HO-1) has been strongly highlighted because of its induction in many cell types by toxic stimuli, including oxidative stress. The intense HO-1 immunostaining in the substantia nigra of Parkinson disease (PD) patients suggests its involvement in the pathogenesis of this neurodegenerative disease. In this work we investigated HO-1 expression in rat substantia nigra postnatal cell cultures under conditions mimicking dopamine toxicity and its modulation by glial cell line-derived neurotrophic factor (GDNF), a potent neuroprotective factor for dopaminergic neurons. In neuron–glia cultures, we found that H_2O_2, a product of dopamine metabolism, or L-3,4-dihydroxyphenylalanine (L-DOPA), the dopamine precursor used in the therapy of PD, induced a fast up-regulation of HO-1 mRNA and protein levels, followed by a secondary down-regulation. H_2O_2 and L-DOPA also increased HO-1 expression in astrocyte cultures, but with a delayed time course in H_2O_2-treated cultures. HO-1 expression was decreased in neuron–glia cultures under conditions under which GDNF up-regulation was observed. Because exogenously applied GDNF prevented HO-1 up-regulation in cultures treated with H_2O_2 or L-DOPA, and antibody neutralization of GDNF prevented the secondary HO-1 down-regulation observed in neuron–glia cultures, we propose that GDNF negatively modulates HO-1 expression induced by oxidative stress. To our knowledge, this is the first report showing the modulation of HO-1 expression by GDNF.

Keywords: Astrocytes; Dopaminergic neurons; Glial cell line-derived neurotrophic factor; Heme oxygenase-1; Oxidative stress; Parkinson disease; Free radicals

Up-regulation of stress proteins is a universal response to adverse conditions, and its protective role against a number of insults has been established [1–3]. Heme-oxygenase-1 (HO-1), also known as heat-shock protein 32 (HSP32) because of the heat shock element in the promoter of its gene [4], is a small stress protein with enzymatic activity involved in heme catabolism, leading to the generation of biliverdin, free iron, and CO. Three isoforms of HO, products of individual genes, have been identified: the inducible HO-1 and the constitutive HO-2 and HO-3 [5]. In the rat brain HO-1 is poorly expressed, but it is quick and highly induced by various stress-associated agents [6–8]. In the central nervous system, the HO pathway has been shown to act as a fundamental defensive mechanism for neurons exposed to an oxidant challenge [9–11].

The etiology of Parkinson disease (PD), a movement disorder characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta, is currently believed to involve the formation of dopamine-derived reactive oxygen species (ROS), by both chemical and enzymatic mechanisms, which can act as endogenous toxins if not handled properly [12]. The involvement of HO-1 in the pathogenesis of PD was suggested by the intense HO-1 immunostaining in the periphery of the Lewy bodies and a significant increase in the fraction of astrocytes expressing HO-1 in the substantia nigra of PD patients [13]. Furthermore, a relatively rapid and persistent increase in HO-1 mRNA in striatal astrocytes has been reported in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD [14]. Moreover, studies by Yoo et al. [15] showed a dramatic up-regulation of HO-1 by oxidative stress in a dopaminergic cell line.

Neurotrophic factors are also up-regulated in response to stressful conditions [16]. We have been investigating the effects of selective injury to dopaminergic neurons on the expression of glial cell line-derived neurotrophic factor (GDNF) in substantia

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; HO-1, heme oxygenase-1; HSP, heat shock protein; L-DOPA, L-3,4-dihydroxyphenylalanine; PD, Parkinson disease; ROS, reactive oxygen species.

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nigra postnatal cell cultures. GDNF was identified based on its ability to promote the survival and branching of dopaminergic neurons in culture [17]. In animal models of PD, GDNF was shown to protect nigral dopaminergic neurons against injury, to rescue them after toxin-induced injury, and to promote recovery from motor deficits [18]. Moreover, the delivery of GDNF to posterior putamen was shown to ameliorate PD symptoms in human patients [19]. It was demonstrated, both in cell culture models and in vivo, that protection of dopaminergic neurons by GDNF involves up-regulation of antioxidant enzymes and induction of antiapoptotic proteins [20,21].

Several reports uncovered a relationship between neurotrophic factors and the expression of stress proteins. Nerve growth factor was shown to protect PC12 cells against ROS by a mechanism involving the induction of HO-1 [22]. HO-1 is also induced by fibroblast growth factor-1 in spinal cord astrocytes [23]. In a model of ischemia/hypoxia-induced brain injury, GDNF was shown to significantly reduce HSP70 induction [24,25]. After spinal cord injury, up-regulation of HSP72 was considerably reduced in brain-derived neurotrophic factor- (BDNF) or insulin like growth factor-1-pretreated rats [26]. BDNF was also shown to suppress the expression of HSP27 in rat retinal ganglion cells after axotomy [27].

In the present work we examined the effects of conditions mimicking dopamine toxicity on the expression of HO-1 in substantia nigra postnatal cell cultures and its relationship to GDNF expression. Our results show that after an initial up-regulation induced by the oxidant stimuli, HO-1 is down-regulated in a process likely involving GDNF. To our knowledge, this report is the first showing modulation of HO-1 expression by GDNF. These data have been previously reported in an abstract form [28].

Material and methods

Cell culture

Animals were handled in accordance with the national ethical requirements for animal research and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Postnatal substantia nigra neuron–glia cocultures were prepared as previously described by Burke et al. [29] with some modifications. A coronal slice at the level of midbrain flexure was dissected from postnatal day 1–3 Wistar rat pups, followed by the dissection of the ventral segment of the midbrain (astrocyte preparation) or the substantia nigra (neuronal preparation). The tissue was then enzymatically dissociated under continuous oxygenation using 20 U/ml papain (Roche) in 1 mM cysteine, 116 mM NaCl, 5.4 mM KCl, 26 mM $NaHCO_3$, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, 500 μM EDTA, 25 mM glucose, and 0.001% phenol red, at pH 7.3, for about 30 min at 33°C. To stop digestion, tissue chunks were washed with culture medium. The tissue was then mechanically dissociated and the cell suspension pelleted, resuspended, and plated onto poly-D-lysine- and laminin-coated coverslips under 0.8-cm^2 holes in the bottom of 50-mm snap-top polystyrene petri dishes (BD Falcon). For midbrain astrocyte cultures, 75,000 cells were plated and fed with astrocyte culture medium M10C-G (composition described in [29]). Once the cells were confluent, 25 μM 5-fluorodeoxyuridine with 70 μM uridine (FDU) was added to the culture medium to suppress cell growth. The neuronal cultures were established by plating 80,000 cells onto confluent monolayers of midbrain astrocytes. Three days before the neuronal cell preparation, the astrocyte culture medium was changed to neuronal culture medium SF1C (composition described in [29]) to allow the conditioning by astrocytes. Proliferation of nonneuronal cells was suppressed by addition of FDU 1 day after plating. The cultures were kept at 37°C in a 5% CO_2, 95% air atmosphere. Substantia nigra neuron–glia cocultures were used after 1 week in culture, and astrocyte cultures were used after confluence was reached. The day before cell treatments, the culture medium of either neuron–glia cultures or astrocyte cultures was replaced by serum-free SF1C.

Western blot

Both GDNF and HO-1 protein levels were determined by Western blot analysis of samples obtained by cell homogenization in a lysis buffer containing 25 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and 25 μg/ml leupeptin. Protein concentration was determined using the Bradford method with bovine serum albumin as standard. Usually 15–25 μg protein was separated by SDS–PAGE using a 12% resolving gel under reducing conditions and electrotransferred onto polyvinylidene difluoride membranes (Amersham Life Sciences). After being blocked with 5% milk powder in TBS-T (0.5% Tween 20 in a 20 mM Tris and 137 mM NaCl solution), for 60 min at room temperature, the membranes were incubated overnight at 4°C with rabbit anti-HO-1 (1:1000; Stressgen) or rabbit anti-GDNF (1:1000; Santa Cruz Biotechnology) primary antibodies diluted in TBS-T containing 1% milk powder. To control for protein loading, the membranes were probed with a mouse anti-α-tubulin antibody (1:10,000; Sigma). After being rinsed, blots were incubated for 60 min, at room temperature, with an alkaline phosphatase-conjugated secondary antibody (Amersham Life Sciences), diluted 1:20,000 in TBS-T containing 1% milk powder. Protein bands were detected using the Enhanced ChemiFluorescence system (Amersham Life Sciences) and quantified by densitometric analysis using the Quantity One software (Bio-Rad).

Total RNA extraction and reverse transcription

Total RNA was extracted from substantia nigra cell cultures using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The isolated RNA was dissolved in 20 μl diethylpyrocarbonate-treated water and stored at −80°C. DNase I (Gibco BRL) was used to treat RNA samples to remove any residual genomic DNA. Samples were incubated for 15 min at room temperature with DNase I, which was then inactivated by incubation at 65°C for 10 min. To assess RNA

integrity, randomly chosen samples were analyzed in an Agilent 2100 Bioanalyzer using a RNA NanoLabChip (RNA 6000 Nano Assay; Agilent Technologies). RNA integrity numbers were greater than 7 in all analyzed samples.

Single-stranded cDNA was synthesized using TaqMan reverse transcription reagents (Roche Molecular Systems) by incubating total RNA (0.5 μg of DNase-treated RNA) for 10 min at 25°C and 30 min at 48°C in a final volume of 25 μl. Reaction was terminated by incubating for 5 min at 95°C. All samples were stored at −20°C until analysis. From the resultant cDNA sample, 5 μl was used in the real-time PCR. For control purposes, nontemplate samples were subjected to PCR amplification.

Real-time PCR

Real-time PCR was performed to monitor the expression of HO-1 and of a housekeeping gene, the 18S ribosomal RNA (TaqMan ribosomal RNA control reagents). The TaqMan technology was used and the results were analyzed in a 7900 HT sequence detector system (Applied Biosystems). Primers and the TaqMan probe were selected from GenBank (Accession No. NM_012580) and designed using the primer design software Primer Express (Applied Biosystems): forward primer—5′-TCGCATGAACACTCTGGAGATG-3′, reverse primer—5′-GGCGGTCTTAGCCTCTTCTGT-3′, TaqMan probe—5′-6-FAM-CCCCCGAGGTCAAGCACAGGGT-3′-TAMRA. Specificity of the primers was confirmed by a BLAST search. The amplification reaction mixture (25 μl) contained 5 μl of the cDNA sample, TaqMan Universal PCR Master Mix, 50 nM each primer, and 50 nM TaqMan probe. The thermal cycling conditions included 10 min at 95°C, proceeding with 40 cycles of 95°C for 15 s and 60°C for 1 min.

The size of the PCR product was determined in an Agilent 2100 bioanalyzer using a DNA 1000 LabChip Kit (Agilent Technologies). The HO-1 mRNA levels were normalized to that of 18S ribosomal RNA and expressed relative to control using the $\Delta\Delta C_t$ method.

GDNF neutralization using an antibody

Substantia nigra cell cultures were incubated for 24 h with or without 100 μM H_2O_2 or 200 μM L-DOPA, in the presence or absence of the anti-GDNF antibody (0.4 μg/ml; Santa Cruz Biotechnology). About 10 h later, the application of the antibody was repeated to ensure that only residual GDNF would be available to bind to its receptor. The cultures were then processed for Western blot analysis of HO-1 levels as described above.

Data analysis and statistics

Data are expressed as percentages of values obtained under control conditions and are presented as means ± SEM of at least three experiments, performed in triplicate, in independent cell cultures. Statistical analysis was performed using one-way ANOVA followed by Dunnett's or Bonferroni's test. Values of $p < 0.05$ were considered significant.

Results

Effects of H_2O_2 and L-DOPA on HO-1 expression in substantia nigra neuron–glia cultures

Dopaminergic neurons are highly vulnerable to oxidative stress injury because dopamine itself can undergo spontaneous or enzymatic oxidation, producing H_2O_2, superoxide, and dopamine quinone [12]. H_2O_2 can react with transition metals, like iron, generating hydroxyl radicals. In order to investigate the effects of oxidative injury on HO-1 expression in

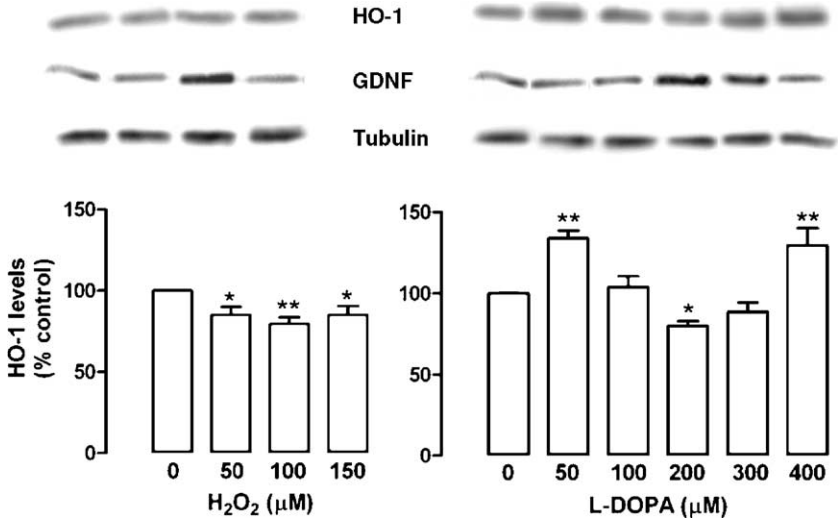

Fig. 1. Effects of H_2O_2 and L-DOPA on HO-1 protein levels in substantia nigra neuron–glia cultures. Cell cultures were incubated for 24 h with the indicated concentrations of H_2O_2 or L-DOPA. Protein extracts were prepared and separated by SDS–PAGE followed by immunoblot analysis using anti-HO-1 (1:1000; Stressgen), anti-GDNF (1:1000; Santa Cruz Biotechnology), and anti-α-tubulin (1:10,000; Sigma) antibodies. Representative Western blots for HO-1, GDNF, and tubulin are shown. Quantification was performed by densitometric analysis. The results are expressed as the percentages of control incubated in the absence of stimuli. *$p < 0.05$, **$p < 0.01$ compared to control.

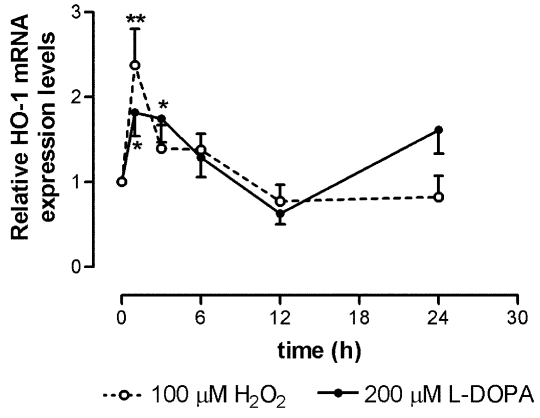

Fig. 2. Effects of H_2O_2 and L-DOPA on HO-1 mRNA levels in substantia nigra neuron–glia cocultures. Cells were incubated with 100 μM H_2O_2 or 200 μM L-DOPA for up to 24 h and total RNA was extracted. After DNase treatment, 0.5 μg of RNA from each sample was reverse transcribed and analyzed by real-time PCR for HO-1 and for a housekeeping gene, the 18S ribosomal RNA. HO-1 mRNA levels were normalized to those of 18S ribosomal RNA and expressed relative to control using the $\Delta\Delta C_t$ method. *$p < 0.05$, **$p < 0.01$ compared to control.

substantia nigra, neuron–glia cultures were exposed for 24 h to different concentrations of either H_2O_2 or L-DOPA, the dopamine precursor used as a replacement therapy for PD and also believed to increase oxidative stress. Surprisingly, HO-1 protein levels decreased with increasing H_2O_2 concentrations: HO-1 levels decreased to 84.92 ± 5.06 of control in cultures exposed to 50 μM H_2O_2 and to 84.97 ± 5.66% in cultures exposed to 150 μM H_2O_2 (Fig. 1). In contrast, exposure to L-DOPA increased HO-1 levels but revealed a very unusual concentration-dependent relationship. Incubation with 50 or 400 μM L-DOPA up-regulated HO-1 levels, whereas 200 μM significantly reduced HO-1 levels to 79.79 ± 3.04% of control (Fig. 1).

H_2O_2 has been shown to induce HO-1 expression in many systems [2,11,30], and the promoter of the HO-1 gene has binding sequences for oxidative stress-responsive transcription factors [31]. To clarify the unexpected effects of H_2O_2 and L-DOPA on HO-1 expression, we examined the time course of HO-1 mRNA and protein expression in substantia nigra cultures exposed up to 24 h to H_2O_2 or L-DOPA. HO-1 mRNA levels were determined by real-time PCR. Cell cultures treated with 100 μM H_2O_2 showed a fast increase in HO-1 mRNA levels to 2.37 ± 0.43-fold the control values at 1 h, decreasing thereafter (Fig. 2). On the other hand, in cultures incubated with 200 μM L-DOPA, the increase in HO-1 mRNA levels was smaller (1.82 ± 0.28-fold at 1 h), but more sustained over time (1.74 ± 0.28-fold the control levels at 3 h) (Fig. 2).

Regarding the effects on HO-1 protein levels in neuron–glia cultures (Fig. 3), we found an initial sustained increase followed by a decrease to levels significantly below control values after 24 h. HO-1 up-regulation caused by H_2O_2 exposure was less pronounced and more transient than that elicited by L-DOPA. At 12 h, HO-1 levels in H_2O_2-treated cultures were already decreasing, whereas in L-DOPA-treated cultures they were maximal at this time point (182.04 ± 11.64%). Despite the different temporal patterns of HO-1 expression in substantia nigra neuron–glia cocultures in response to H_2O_2 and L-DOPA, HO-1 levels were down-regulated at 24 h to about 80% of control in both cases. Fig. 3 also shows the changes in GDNF protein levels in the same experiments. We observed a significant increase in GDNF protein levels after 24 h in cultures treated with 100 μM H_2O_2 or 200 μM L-DOPA, coincidentally with a HO-1 down-regulation. Similarly, in the concentration-response studies (Fig. 1), HO-1 protein levels were decreased under conditions under which GDNF up-regulation was observed.

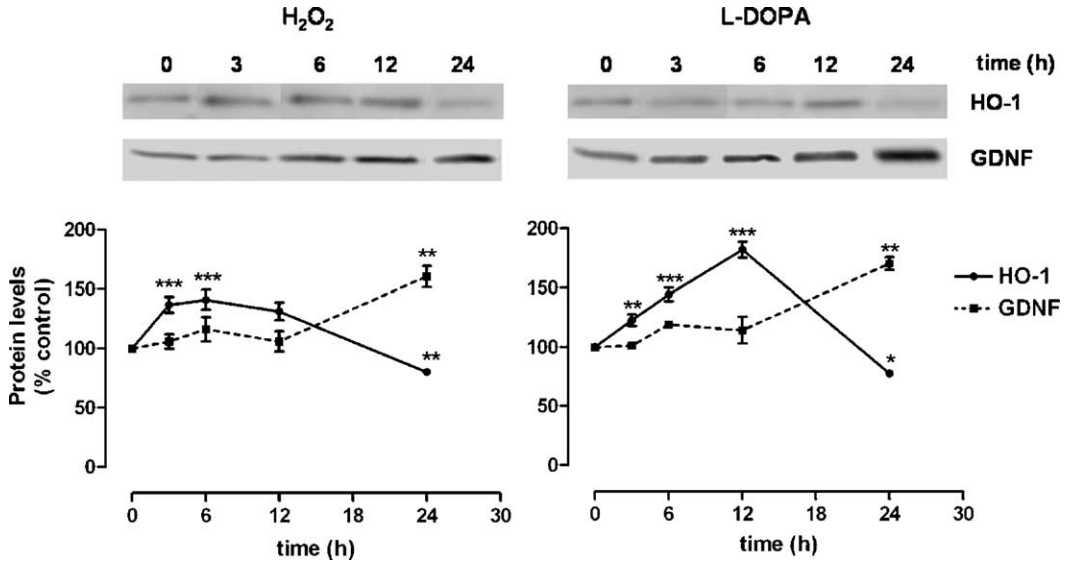

Fig. 3. Time course of H_2O_2 and L-DOPA effects on HO-1 and GDNF protein levels in neuron–glia cultures. The cultures were incubated with 100 μM H_2O_2 or 200 μM L-DOPA for the indicated periods of time. Protein extracts were prepared and analyzed by immunoblot using anti-HO-1 (1:1000; Stressgen) and anti-GDNF (1:1000; Santa Cruz Biotechnology) antibodies. Representative Western blots for HO-1 and GDNF cell content are shown. Quantification of the Western blot data was done by densitometric analysis. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to control.

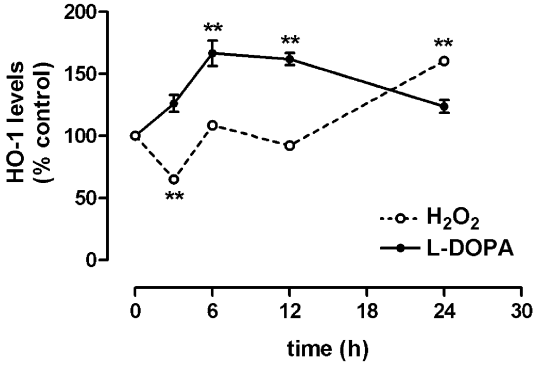

Fig. 4. Time course of H_2O_2 and L-DOPA effects on HO-1 protein levels in midbrain astrocyte cultures. The cultures were incubated with 100 μM H_2O_2 or 200 μM L-DOPA for the indicated periods of time. Protein extracts were prepared and separated by SDS–PAGE followed by immunoblot analysis using an anti-HO-1 antibody (1:1000; Stressgen). Quantification was performed by densitometric analysis. The results are expressed as the percentages of controls incubated in the absence of stimuli. $^{**}p < 0.01$ compared to control.

Effects of H_2O_2 and L-DOPA on HO-1 expression in astrocyte cultures

We also investigated the effects of H_2O_2 and L-DOPA on HO-1 levels in midbrain astrocyte cultures because stronger HO-1 induction by oxidative stress has been reported in astrocytes compared to neurons [30]. Moreover, we have found that GDNF was not up-regulated in astrocyte cultures incubated for 24 h with 100 μM H_2O_2 or 200 μM L-DOPA (unpublished data), enabling us to examine HO-1 expression without the modulation by endogenous GDNF. Fig. 4 shows the time course of the effects of 100 μM H_2O_2 or 200 μM L-DOPA on HO-1 levels in astrocyte cultures. In L-DOPA-treated cultures, HO-1 levels were elevated at all time points tested but the increases were statistically significant only at 6 and 12 h. On the other hand, incubation with H_2O_2 led to HO-1 up-regulation only at 24 h. Surprisingly, 3 h after addition of 100 μM H_2O_2, astrocyte cultures consistently exhibited reduced HO-1 levels to 64.8 ± 2.2% of control (Fig. 4). Interestingly, the temporal pattern of HO-1 changes in H_2O_2-treated astrocytes was distinct from that observed in neuron–glia cultures, in contrast to what was observed in L-DOPA-treated cultures (Figs. 3 and 4), suggesting that the mechanisms involved in HO-1 expression in response to H_2O_2 and L-DOPA might be different.

The addition of GDNF prevented HO-1 up-regulation in challenged cultures

The observation that HO-1 levels were decreased in neuron–glia cultures under conditions under which GDNF was up-regulated (Figs. 1 and 3) raised the hypothesis that GDNF could be responsible for the down-regulation of HO-1. To address this question we determined the effects of adding GDNF on HO-1 levels in astrocyte cultures treated with H_2O_2 for 24 h or L-DOPA for 12 h. These incubation periods were selected based on the maximal effect of H_2O_2 and L-DOPA on HO-1 levels in astrocyte cultures (Fig. 4). In both H_2O_2- and L-DOPA-treated cultures, the presence of 1 ng/ml GDNF prevented HO-1 up-regulation, keeping HO-1 expression at

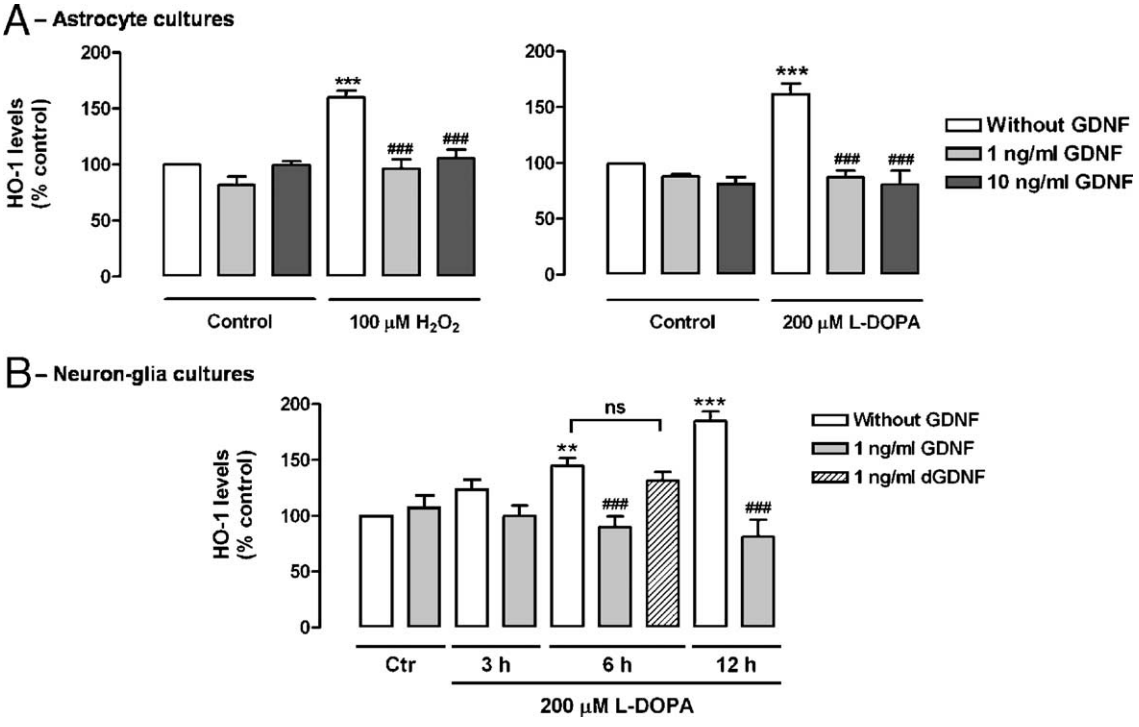

Fig. 5. Effects of adding GDNF on HO-1 levels in (A) astrocyte and (B) neuron–glia cultures challenged with H_2O_2 or L-DOPA. Astrocyte cultures were incubated with H_2O_2 for 24 h or with L-DOPA for 12 h, in the presence or in the absence of 1 or 10 ng/ml recombinant human GDNF (R&D Systems). Neuron–glia cultures were incubated with 200 μM L-DOPA for 3, 6, or 12 h, in the presence or in the absence of 1 ng/ml recombinant human GDNF. To control for specificity, heat-denatured GDNF (95°C for 10 min; dGDNF) was used. Protein extracts were then prepared and processed for HO-1 immunoblotting. ns, not significant; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control; and $^{###}p < 0.001$ compared to 100 μM H_2O_2 or 200 μM L-DOPA alone.

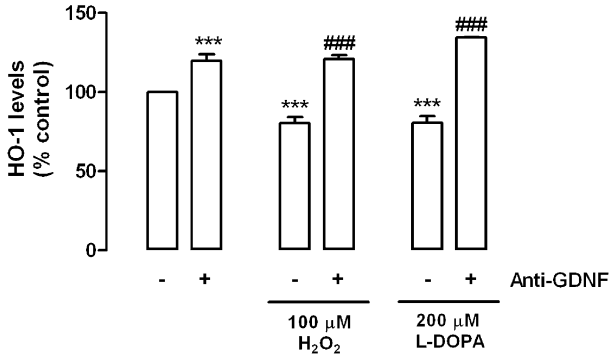

Fig. 6. Effects of GDNF neutralization during H_2O_2 or L-DOPA exposure on HO-1 levels in neuron–glia cultures. Cell cultures were incubated for 24 h with 100 μM H_2O_2 or 200 μM L-DOPA in the presence (+) or the absence (−) of the anti-GDNF antibody (0.4 μg/ml; with reinforcement after approximately 10 h) and processed for HO-1 immunoblotting. ***$p < 0.001$ compared to control and ###$p < 0.001$ compared to the same condition in the absence of the anti-GDNF antibody.

control levels (Fig. 5A). HO-1 induction decreased from 160.3 ± 5.9 to 96.3 ± 8.6% in H_2O_2-treated cultures, and from 161.8 ± 9.6 to 87.8 ± 5.6% in L-DOPA-treated cultures. We found no significant differences in the effects of 1 and 10 ng/ml GDNF on HO-1 levels in H_2O_2 or L-DOPA-challenged cultures, and neither 1 nor 10 ng/ml GDNF had significant effects on HO-1 levels in control astrocyte cultures (Fig. 5A).

We also tested the effects of adding GDNF on HO-1 expression in neuron–glia cultures at time points before significant GDNF up-regulation was observed (see Fig. 3). As shown in Fig. 5B, the presence of native GDNF, but not of heat-denatured GDNF (95°C for 10 min), prevented the increase in HO-1 protein levels observed at 3, 6, and 12 h upon treatment with 200 μM L-DOPA. Because heat-denatured GDNF did not significantly affect HO-1 induction by L-DOPA we can conclude that this is a specific effect.

Antibody neutralization of GDNF in substantia nigra neuron–glia cultures prevented HO-1 down-regulation

To further investigate whether GDNF was controlling HO-1 expression, we determined the effects of GDNF neutralization on HO-1 levels in neuron–glia cultures. Cells were incubated for 24 h with 100 μM H_2O_2 or 200 μM L-DOPA in the presence or in the absence of an anti-GDNF antibody (Fig. 6). GDNF neutralization prevented HO-1 down-regulation at 24 h both in H_2O_2- and in L-DOPA-treated cultures, increasing HO-1 levels from 80.31 ± 3.86 to 120.70 ± 2.54% and from 80.44 ± 4.33 to 134.40 ± 0.32%, respectively. Moreover, in control cells incubated with the anti-GDNF antibody, HO-1 was up-regulated to 123.31 ± 2.82% of control levels, suggesting that basal levels of GDNF were modulating HO-1 expression.

Discussion

In the present work we found that conditions mimicking dopamine toxicity triggered a transient up-regulation of HO-1 expression in substantia nigra cell cultures, and we gathered evidence showing that GDNF is involved in the secondary down-regulation of HO-1. The catabolism of heme by HO leads to the production of two potent antioxidants, biliverdin and bilirubin ([32], reviewed in [5]), that are neuroprotective at nanomolar concentrations [10]. Their antioxidant actions can be explained by consecutive cycles of oxidation–reduction between bilirubin and biliverdin [33]. In this context, the fast up-regulation of HO-1 mRNA and protein levels observed in substantia nigra cell cultures (Figs. 2 and 3) would provide a way to protect cells against H_2O_2- and L-DOPA-induced toxicity. Many studies support the importance of HO-1 in protecting different cell types, including neurons, against oxidative stress injury. Induction of HO-1 or low levels of overexpression are associated with protection against oxidative stress [9,11,22,30,34–36]. Moreover, HO-1 preinduction in different injury models confers increased resistance to oxidative stress [2,37–39]. On the other hand, suppressing HO activity using antisense transfection [37,40] or inhibitors [40,41] worsened oxidative stress. However, high levels of HO-1 expression may be toxic due to excessive cellular levels of heme-derived free iron [42]. Because the levels of HO-1 up-regulation upon H_2O_2 or L-DOPA exposure were in the low overexpression range (Figs. 1, 3, and 4) we can assume that HO-1 conferred protection against oxidative stress-induced damage. Expression of HO-1 in glia has been described in different models of brain injury [6,43–45]. H_2O_2 has been shown to induce HO-1 expression [11,46–48], and studies from Fauconneau et al. [2] and Chen et al. [9] support a role for glial HO-1 in the protection against H_2O_2-mediated oxidative stress.

The induction of HO-1 was also shown to be coupled to the induction of ferritin, which safely sequesters the iron released during heme degradation, thus avoiding its participation in the Fenton reaction and the generation of several free radical species [37,42,49–51]. The expression of HO-1 was also linked to the cellular efflux of iron [52], and HO-1 expression up-regulates an iron ATPase present in the endoplasmic reticulum, decreasing iron intracellular content [53].

Although both H_2O_2 and L-DOPA are expected to increase oxidative stress, we observed significant differences in the expression patterns of HO-1 induced by these two stimuli. L-DOPA triggered fast and sustained increases in HO-1 protein levels in both neuron–glia and astrocyte cultures, whereas H_2O_2 induction of HO-1 was higher in mixed cultures, compared to the greatly delayed induction in astrocyte cultures (Figs. 3 and 4). These differences suggest partially distinct mechanisms of HO-1 induction by H_2O_2 or L-DOPA, and dopamine derived from L-DOPA may be involved. Exogenous dopamine was shown to increase HO-1 mRNA levels in cultured astrocytes and in C6 glioma cells [45,54]. It was also suggested that dopaminergic terminals could produce substances that directly or indirectly induce HO-1 in adjacent astrocytes and that dopamine itself, or a dopamine metabolite, could be the endogenous agent triggering HO-1 expression in astrocytes [14]. It should be noted that astrocytes are capable of transporting L-DOPA and decarboxylating it to dopamine [55].

In contrast to L-DOPA, H_2O_2 induced larger and faster increases in HO-1 levels in mixed cultures than in astrocyte cultures (Figs. 2 and 4). Astrocytes were shown to have more glutathione peroxidase and catalase activities and a correspondingly greater capacity to metabolize H_2O_2 than neurons [56–58]. This ability can explain the small induction of HO-1 in astrocyte cultures (Fig. 4). On the other hand, in neuron–glia mixed cultures, incubation with H_2O_2 could decrease dopamine uptake, because oxidative stress has been shown to inhibit dopamine transport in striatal synaptosomes, thus leading to extracellular accumulation of dopamine [59]. This would in turn induce HO-1 in astrocytes [45], accounting for the higher HO-1 levels in neuron–glia mixed cultures compared to astrocyte cultures.

The down-regulation of HO-1 protein observed at longer time points (Fig. 3) could be the consequence of its own up-regulation in response to H_2O_2 or L-DOPA because Chen et al. [9] have demonstrated that increased levels of HO-1 are associated with a decrease in the accumulation and/or formation of oxygen radicals. Furthermore, Suttner and Dennery [42] have shown that, at low levels of HO-1 expression, protein carbonyl content and lipid peroxide by-products were visibly decreased compared to controls. Moreover, previous HO-1 up-regulation was shown to induce the MnSOD gene in rat astroglia cultures exposed to dopamine [60].

However, our data point to another player in the negative modulation of HO-1 expression. Several lines of evidence in the present work suggest that GDNF is involved in the down-regulation of HO-1: (i) the dependence of HO-1 expression on L-DOPA concentration was opposite to that of GDNF expression upon L-DOPA treatment (Fig. 1), (ii) there was a temporal correlation between HO-1 down-regulation and GDNF up-regulation (Fig. 3), (iii) the addition of exogenous GDNF prevented HO-1 up-regulation in astrocyte and in neuron–glia cultures treated with H_2O_2 or L-DOPA (Fig. 5), and (iv) antibody neutralization of GDNF in neuron–glia mixed cultures prevented HO-1 down-regulation at 24 h (Fig. 6).

The down-regulation of HO-1 by GDNF can be mediated by a decrease in oxidative stress levels, because GDNF was shown to suppress the accumulation of oxygen radicals induced by bleomycin sulfate in mesencephalic cultures [20] or by kainate in hippocampus [61] and to significantly elevate the activities of superoxide dismutase, catalase, and glutathione peroxidase [21,61]. Another hypothesis is that GDNF may be modulating HO-1 expression at the transcriptional level, as was suggested for other growth factors [22,23,62,63]. The promoter of the HO-1 gene contains a complex set of regulatory elements [64], some of them targets for transcription factors potentially regulated by the PI3K/Akt pathway, a GDNF signaling pathway [65]. GDNF also induces the transcription factor murine GDNF-inducible factor, which negatively regulates transcription [66].

In conclusion, we propose that cells exposed to conditions mimicking dopamine toxicity up-regulate HO-1 in response to increased oxidative stress, as a protective strategy. In a second phase, GDNF, also up-regulated in response to injury, would down-regulate HO-1, keeping its expression at a protective level. To our knowledge, this is the first report showing modulation of HO-1 by GDNF, with potential relevance to PD. The lower levels of GDNF reported in the substantia nigra of PD patients [67] may contribute to the increased HO-1 levels observed in this brain region, where a significant increase in the fraction of astrocytes expressing HO-1 has been reported and neurons exhibit intense HO-1 immunostaining in the periphery of the Lewy bodies ([13], reviewed in [5]). The up-regulation of HO-1 in response to conditions of oxidative stress would then become overwhelming, and the release of iron from heme degradation by HO-1 would exacerbate oxidative injury to dopaminergic neurons.

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