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Corticosterone Regulates Heme Oxygenase-2 and NO Synthase Transcription and Protein Expression In Rat Brain

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Abstract: Heme oxygenase (HO)-1 and -2 produce carbon monoxide, which is suspected, as is nitric oxide (NO), to function as a neuronal messenger. We report on glucocorticoid-mediated modulation of HO-2 and NO synthase expression in brain and the differential response of the two proteins to corticosterone in different brain regions. Corticosterone treatment (40 mg/kg, 20 days) had opposing effects on HO-2 and NO synthase transcript levels: increasing the 1.3- and 1.9-kb HO-2 mRNAs and decreasing that of the brain-specific 10.5-kb NO synthase. Corticosterone did not uniformly affect HO-2 protein expression in all regions, but appeared to cause a universal reduction in NO synthase, e.g., HO-2 was decreased in hippocampus (CA1 and dentate gyrus), but not in cerebellum. In contrast, NADPH diaphorase staining was reduced in hippocampus and in molecular and granule layers of cerebellum (not detected in Purkinje cells). Striking deficits in neuronal morphology and number of diaphorase-staining neurons were observed in the lateral tegmental area, paraventricular nucleus, and frontal cortex; HO-2 expression was only selectively affected. In cerebellum, activity of NO synthase, but not that of HO, was reduced. Consistent with the possibility that carbon monoxide can generate cyclic GMP, the change in cyclic GMP level did not mirror the decrease in NO synthase. We suggest that glucocorticoid-mediated deficits in hippocampal functions may reflect their negative effect on messenger-generating systems. **Key Words:** Heme oxygenase-2—Nitric oxide synthase—Corticosterone—Carbon monoxide—Protein expression—Rat brain.
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Synthesis of carbon monoxide (CO), the putative neurotransmitter (Ewing and Maines, 1992; Maines et al., 1993; Stevens and Wang, 1993; Verma et al., 1993; Zhuo et al., 1993), is catalyzed by the heme oxygenase (HO) enzyme system (for review, see Maines, 1992). The α-meso carbon of heme tetrapyrrole (Fe-protoporphyrin IX) is the source of CO. This system, which constitutes the only means for the formation of CO in mammalian species, consists of two isozymes, HO-1 and HO-2 (Maines et al., 1986; Cruse and Maines, 1988). The isozymes are different gene products and differ in many ways, including their regulation and tissue distribution. To elaborate, HO-1, which is also known as HSP32, is a stress protein and under normal conditions is present only at minute levels in the brain (Sun et al., 1990; Ewing and Maines, 1991; Ewing et al., 1992). In contrast, HO-2, which for the most part is associated with neurons, is expressed in most brain regions (Ewing and Maines, 1992) and is by far the major component of the impressive CO-generating capability of this organ (Trakshel and Maines, 1989). In fact, the CO-generating activity of the brain equals that of the spleen (Maines, 1988); the spleen is the primary site of hemoglobin heme degradation in mammals. To date, except for development and maturation of the brain (Sun et al., 1990), no stimulus has been identified as being effective in regulating HO-2 (Maines et al., 1986; Trakshel et al., 1986). The refractoriness of HO-2 gene regulation most likely reflects the sparsity of identifiable regulatory elements in the promoter region of the HO-2 gene, wherein only a single copy of the consensus sequence of a glucocorticoid (GC) response element (GRE) was identified recently (McCoubrey and Maines, 1994).

Previously, we have shown (Ewing and Maines, 1992; Ewing et al., 1993) that HO-2 is present in brain areas that are susceptible to excess GC levels, including the CA1, CA2, and CA3 regions of the hippocampal complex and dentate gyrus (Pulsinelli et al., 1982; Munck et al., 1984; Sapolsky, 1987; Stein and Sapolsky, 1988; Woolley et al., 1990). The hippocampal complex is, in particular, sensitive to excess GCs wherever GC receptors are concentrated (McGimsey et al., 1991). The complex is involved in learning and memory. Dysfunctions of the complex are associated with certain neurodegenerative disorders involved in long-

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Abbreviations used: CO, carbon monoxide; GC, glucocorticoid; GRE, glucocorticoid response element; HO, heme oxygenase; NO, nitric oxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

term potentiation and certain forms of learning (for review, see Hawkins et al., 1993). Furthermore, in most brain regions, HO-2 colocalizes with guanylate cyclase (de Vente et al., 1989; Southam et al., 1992), including the hippocampal complex, the Purkinje cells, and the molecular and granule layers of the cerebellum, where a robust expression of the protein and its transcripts are detected (Ewing and Maines, 1992; Ewing et al., 1993; Maines, 1993; Verma et al., 1993). In fact, this colocalization, in addition to the undetectable levels of nitric oxide (NO) synthase in Purkinje cells and in CA1 pyramidal cells (Schmidt et al., 1992; Vincent and Kimura, 1992), has been a strong argument for the suspected role of CO as a regulator of cyclic GMP. NO synthase generates NO, the known activator of the heme-dependent isozyme of guanylate cyclase (Ignarro et al., 1984; Garthwaite and Garthwaite, 1987; Palmer et al., 1987; Marletta, 1989; Dawson et al., 1992).

The effect of excess GCs on HO and NO synthase activity and expression is not known. Presently, we have examined HO-2 and NO synthase response in vivo to excess corticosterone. We have taken the approach of examining expression of the enzymes at transcript, protein, and activity levels in response to chronic exposure to excess corticosterone. We have observed an opposite regulatory effect of corticosterone on HO-2 and NO synthase gene expression and documented by immunohistochemical analysis the consequences of excess corticosterone exposure to expression of HO-2 and NO synthase, as determined by NADPH diaphorase staining (Dawson et al., 1991) in different areas of the brain. We show a differential effect of corticosterone on HO-2 and NO synthase in different brain regions. A negative effect of elevated steroid levels is observed on both enzyme proteins in the hippocampus. In cerebellum, on the other hand, the absence of a negative effect of the steroid on HO-2 expression coincides with decreases in NO synthase. We propose that diminished hippocampal complex ability to generate the gaseous heme ligands, CO and NO, may be a contributing factor to deficits in hippocampal functions caused by exposure to excess GCs, and suggest that spared CO-generating capability of the cerebellum is important to maintenance of cyclic GMP in this region.

MATERIALS AND METHODS

Materials

Oligonucleotide probes were obtained from Research Genetics (Huntsville, AL, U.S.A.). [α-^{32}P]dCTP (>3,000 Ci/mmol) and cyclic GMP radioimmunoassay kit were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.), and goat anti-rabbit γ-globulin and rabbit peroxidase-antiperoxidase were purchased from Organon Teknika-Cappel (Durham, NC, U.S.A.). Adult male Sprague–Dawley rats (125–150 g), obtained from Harlan Industries (Madison, WI, U.S.A.), were used as the tissue source for the studies. Random Primers DNA labeling system was purchased from United States Biochemical (Cleveland, OH, U.S.A.). DNA-ligase and T4 polynucleotide kinase were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Oligo-(dT)-cellulose, DNase I, salmon testis DNA, dextran sulfate, Triton X-100, diaminobenzimide, nitro blue tetrazolium, NADPH, and corticosterone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nytran membranes for northern blot hybridization and nitrocellulose for western blot analysis were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). All other chemicals were of the highest purity commercially available.

Animals

All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals,* as approved by the local animal care committee. Adult male rats were used for all experiments. Rats were given daily subcutaneous injections of corticosterone (40 mg/kg) dissolved in commercial corn oil for a period of 20 days, essentially as described by Woolley et al. (1990). On the day following the last injection, animals were killed, and the brains removed and processed for mRNA extraction, enzyme assay, analysis of cyclic GMP level, or immunocytochemistry, as detailed below.

HO-2 antisera preparation and HO-2 immunohistochemistry

Rat testis HO-2 protein was purified as previously described (Trakshel et al., 1986). Protein preparations were used to raise polyclonal antisera in New Zealand white rabbits, as detailed before (Cruse and Maines, 1988). For immunohistochemistry, rats were anesthetized using a mixture of chloral hydrate and pentobarbital before being perfused through the heart with 0.9% (wt/vol) saline, followed by 4% (wt/vol) paraformaldehyde for 1 h at 25°C. Following fixation, brains were equilibrated sequentially with 10, 20, and 30% (wt/vol) sucrose solutions in 0.1 *M* phosphate buffer (pH 7.2) at 4°C. Thirty-micrometer sections were obtained using a sliding microtome and preserved in 0.1 *M* phosphate buffer containing 30% (wt/vol) sucrose and 30% (vol/vol) ethylene glycol at −20°C until immunohistochemical staining. HO-2 immunohistochemical staining of brain sections was carried out using peroxidase-antiperoxidase as described previously (Ewing et al., 1992). A 1:1,000 dilution of primary serum in 0.1 *M* phosphate buffer (pH 7.2) containing 0.3% (vol/vol) Triton X-100 and 10% (vol/vol) normal goat serum was used for 4 days at 4°C. Authenticity of antibody-antigen complexes was confirmed by preadsorption of serum with excess rat testis HO-2 protein. Staining observed under normal immunohistochemical conditions was nearly abolished when additional brain sections were incubated in this serum.

NADPH diaphorase immunohistochemical staining

Identical neurons stain for NO synthase and NADPH diaphorase in fixed brain, as well as peripheral tissues (Dawson et al., 1991; Hope et al., 1991; Matsumoto et al., 1993). Therefore, NO synthase localization was achieved using NADPH diaphorase histochemistry essentially as described by Hope et al. (1991); brain tissue from control and treated animals was prepared as described for HO-2 immunohistochemistry. The incubation mixture contained 50 m*M* Tris-Cl, (pH 7.6), 0.2% (vol/vol) Triton X-100, 0.5 m*M* nitro blue tetrazolium, and 1 m*M* NADPH at 37°C for 15–30 min. Authenticity of NADPH diaphorase staining was confirmed

by the lack of color formation in tissue incubated in the above buffer in the absence of NADPH. All immunohistochemical analyses were carried out using identical conditions for the control and treated brain sections, and observations were confirmed using three to five animals per group. Cresyl Violet Nissl staining for neurons was carried out by standard methodology (Paxinos and Watson, 1986).

Probes

The HO-2 hybridization probe was the full-length (1,300 bp) rat testis HO-2 cDNA, and the HO-1 hybridization probe was an HO-1 fragment corresponding to nucleotides +71 to +833, as reported by Shibahara et al. (1985) generated via an adaptation of the polymerase chain reaction as previously described (Rotenberg and Maines, 1990). The NO synthase hybridization probe was a polymerase chain reaction product consisting of NO synthase cDNA nucleotides +2 to +685 (Bredt et al., 1991*a*). All cDNA probes used in northern blot analysis, including mouse α-actin cDNA probe (Minty et al., 1981), were labeled according to the manufacturer's instructions with [α-^{32}P]dCTP by the random priming method and further purified by spin chromatography, as previously described (Sambrook et al., 1982).

RNA preparation and northern blot analysis for HO-1, HO-2, and NO synthase

Total RNA was prepared from rat brain by the method of Chirgwin et al. (1979), and polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography (Kingston, 1987). Formaldehyde-denatured poly(A)$^+$ RNA was fractionated on a 1.2% agarose gel, transferred to Nytran (Kingston, 1987), and subsequently baked in vacuo (80°C for 1.5 h). Prehybridization, hybridization of the appropriate ^{32}P-labeled DNA, and posthybridization treatment of filters were performed essentially as described before (Sun et al., 1990).

Measurement of HO and NO synthase activities and level of cyclic GMP

Microsomal fractions were prepared to determine HO activity in the presence of purified biliverdin reductase (Kutty and Maines, 1981), as described previously (Trakshel et al., 1988); enzyme activity is expressed as nanomoles of bilirubin produced per hour per milligram of protein. NO synthase activity of frozen brain (−80°C) tissue was measured as the conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline as described by Salter et al. (1991). Levels of cyclic GMP were measured by immunoassay in accordance with the manufacturer's instructions. For this analysis, brain tissue was homogenized in 50 m*M* Tris buffer (pH 7.5) containing 5 m*M* EDTA, 0.5 m*M* 3-isobutyl-1-methylxanthine and subsequently incubated in a boiling water bath for 3 min. After centrifugation, the clarified supernatant was kept at −20°C and used for assay of cyclic GMP content. Protein estimations were performed by the method of Lowry et al. (1951).

Western blot analysis

Rat brain microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli, 1970) and transferred to nitrocellulose for western blot analysis (Towbin et al., 1979). HO-2 protein was visualized using antibody against rat testis HO-2 protein as detailed before (Cruse and Maines, 1988).

Statistical analysis and quantitation of northern and western blot signals

Differences between control and treated animals were assessed for significance by using Student's *t* test. Differences between means of $p \leq 0.05$ were considered significant. The intensity of signals on the northern and western blots were quantitated by laser densitometry using an LKB-XL densitometer. The intensity of bands on the northern blots were normalized using α-actin as the standard.

RESULTS

To examine whether corticosterone treatment affects HO-2 gene regulation and to gain insight to the possibility that the GRE in the upstream promoter region of the HO-2 gene is functional, we examined the effect of chronic corticosterone administration on HO-2 gene expression in the rat brain. Transcriptional analysis of HO-2 1.3- and 1.9-kb homologous mRNAs was carried out by northern blot analysis. As shown in Fig. 1a, both HO-2 transcript levels were elevated (twofold) following steroid treatment relative to control. To our knowledge, this constitutes the first reporting of an agent increasing HO-2 transcription in any tissue. In the HO-1 promoter region, the GRE consensus sequence is not detected, consistent with the present observation that the level of its 1.8-kb transcript was not affected by corticosterone treatment (Fig. 1b). Cyclic GMP has been implicated in a number of physiological functions in the brain, and NO has been postulated as the activator of guanylate cyclase. Hence, we examined the effects of corticosterone treatment on brain NO synthase transcription. The results of analysis of transcription level are depicted; as shown in Fig. 1c, the level of brain-specific 10.5-kb NO synthase transcript was notably decreased in treated animals (50%). The already low levels of the transcript in normal animals is noteworthy.

We examined the consequence of corticosterone treatment on the pattern of HO-2 and NO synthase protein expression in various brain regions. Expression of immunoreactive HO-2 protein in cerebellum of normal and treated animals is shown in Fig. 2; corticosterone treatment did not decrease either the relative intensity or number of cells displaying immunoreactivity for HO-2. As noted, intense staining of Purkinje cells, glial cells in the granule layer, and basket cells in the molecular layer of this region occurred in both control (Fig. 2a) and steroid-treated (Fig. 2b) animals. No distinguishable differences in cell morphology or number of HO-2–expressing Purkinje cells between control and treated animals were detected.

We undertook immunohistochemical analysis using NADPH diaphorase staining to evaluate NO synthase protein response to corticosterone treatment. As shown in Fig. 3a and b, there was a marked reduction of NADPH diaphorase staining in cerebellum of treated animals. Consistent with the reported information on the absence of NO synthase in Purkinje cells (Bredt et al., 1991*b*; Southam et al., 1992; Vincent and Kimura,

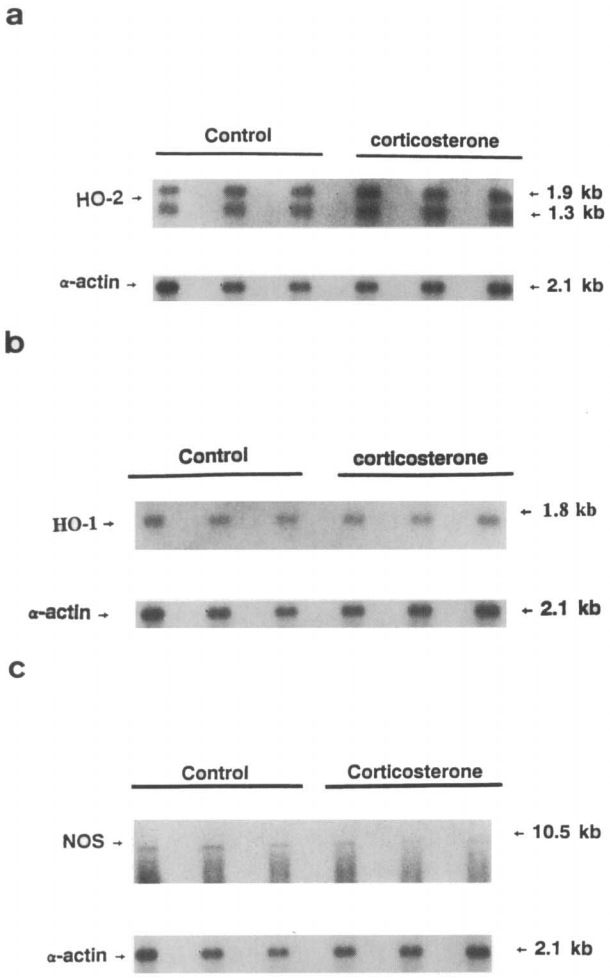

FIG. 1. Effects of GC treatment on brain HO-1, HO-2, and NO synthase mRNAs as assessed by northern blot analysis. Poly(A)$^+$ RNA was isolated from brains of control rats or rats treated subcutaneously with 40 mg/kg corticosterone for 20 days, and subjected to northern blot analysis, as described in Materials and Methods. Each lane contained 4 μg of poly(A)$^+$ RNA isolated from individual rats. α-Actin cDNA was used as a control for sample loading. **a:** Blot was hybridized with a full-length ^{32}P-labeled HO-2 cDNA (Rotenberg and Maines, 1990). **b:** Blot was probed with ^{32}P-labeled HO-1 cDNA (Shibahara et al., 1985). **c:** Blot was hybridized with ^{32}P-labeled NO synthase (NOS) probe. Quantification of NO synthase mRNA levels by laser densitometry, using α-actin signal as the standard, revealed a 50% reduction in transcript level; quantification of the two HO-2 mRNA levels yielded a twofold increase in corticosterone-treated animals for both 1.3- and 1.9-kb transcripts.

1992), no staining in control or treated animals was detected. Staining for NADPH diaphorase in control cerebellar tissue was rather intense in the granule and molecular layers, whereas intensity of the staining was pronouncedly lowered in treated animals.

We investigated whether the effects of corticosterone extended to the cortical regions of the brain. Some examples of the striking and, as far as we know, never reported before effects of the treatment on neuronal morphology in the cortex are provided in Fig. 3. When compared with controls (Fig. 3c, e, and g), after exposure to excess levels of corticosterone (Fig. 3d, f, and h), neurons in the lateral tegmental area, paraventricular nucleus, and frontal cortex showed vivid deficits in both the number displaying NADPH diaphorase and the dendritic morphology. In the paraventricular nucleus, HO-2 was also markedly affected (Fig. 3i and j, control and treated, respectively). The morphology of neurons that stain for HO-2 and NO synthase appear to differ. HO-2 neurons in the lateral tegmental area, however, were not noticeably affected (Fig. 3k and l, control and treated, respectively) by corticosterone treatment, suggesting the regional specificity of HO-2 response to the steroid.

As noted above, the hippocampal complex in particular is sensitive to excess GCs. Therefore, the effect of corticosterone treatment on NO synthase, assessed by NADPH diaphorase staining, and HO-2 protein immunoreactivity in the complex was examined. As shown in Fig. 4, staining for diaphorase, which was detected in neurons of the hilus of the dentate gyrus in the control hippocampal formation (Fig. 4a), was essentially absent in tissue of treated rats (Fig. 4b). However, although diminished when compared with control (Fig. 4c), HO-2 immunoreactivity in corticosterone-treated animals (Fig. 4d) was readily evident in the dentate gyrus of the hippocampal complex, where a number of cells displayed HO-2 immunoreactivity. Similarly, in the CA1 region of the hippocampal complex, where a relatively large number of cells normally express HO-2 (Fig. 4e), subsequent to exposure to excess levels of corticosterone, the number of positive-staining cells and the intensity of staining observed under identical immunohistochemical conditions were both noticeably diminished (Fig. 4f). Staining for NADPH diaphorase was not detected in CA1. The decrease in neuronal staining for HO-2 and NO synthase appears to be rather specific, as there was no obvious reduction in the population of cells in the hippocampal formation that can be detected by general neuronal Cresyl Violet Nissl staining (Fig. 4g and h, control and treated, respectively).

We examined consequences of the differential effect of corticosterone on HO-2 and NO synthase in cerebellum on cyclic GMP production. To this end, we measured the level of cyclic GMP and evaluated cerebellar capacity to produce gaseous heme ligands by measuring NO synthase and HO activities in control and corticosterone-treated rats. As shown in Fig. 5, the response of the enzyme systems to steroid treatment distinctly differed. Specifically, whereas total HO activity was essentially refractory to steroid treatment, there was a notable and statistically significant decrease of ~35% in cerebellar NO synthase activity. Despite this rather large magnitude of reduction, the level of cyclic GMP was modestly decreased and the decrease was not statistically significant.

Assessment of total HO-2 protein by western blot analysis demonstrated that, despite the localized dimin-

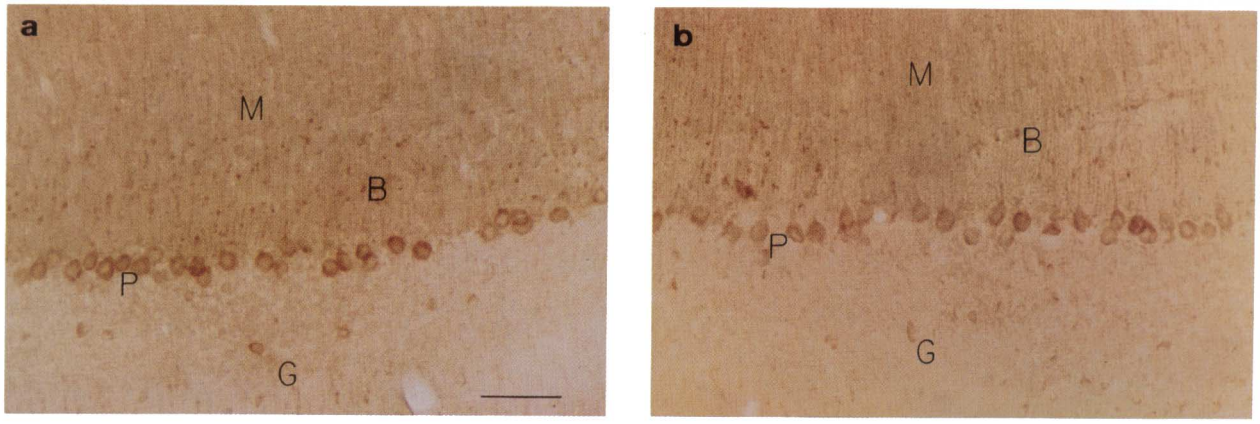

FIG. 2. Immunohistochemical localization of HO-2 protein in normal and GC-treated cerebellum. Immunohistochemical analysis was performed on brains of control or corticosterone-treated (40 mg/kg for 20 days) rats as detailed in the text. HO-2–immunoreactive protein was localized in Purkinje cells, as well as basket cells scattered throughout the molecular layer and glia in the granule cell layer of cerebellum in the control (**a**) and GC-treated (**b**) rat brain. Note that expression of HO-2 protein after GC treatment is nearly identical to HO-2 localization in control rat brain. P, Purkinje cells; M, molecular layer; G, granule cell layer; B, basket cells. Bar = 100 μm.

ishment in HO-2–immunoreactive protein in hippocampus and in paraventricular nucleus, there was no decrease in the overall level of HO-2 protein detectable by this technique in the cortex (Fig. 6). This finding is consistent with the above suggested regional specificity of HO-2 response to corticosterone. Also shown in the figure is the western blot analysis of HO-2 in cerebellum. Consistent with the immunohistochemical analysis shown in Fig. 2, there was an absence of a change in HO-2 protein in this region.

DISCUSSION

The present findings with corticosterone demonstrate the effect of the agent positively modulating HO-2 gene expression and negatively affecting that of NO synthase in the brain. The HO system and NO synthase are the sources of CO and NO production, respectively; gaseous heme ligands are suspected of being neurotransmitter molecules in the brain. The observation that corticosterone modulates expression of HO-2 and NO synthase in the brain is not surprising, and inhibition and enhancement by GCs have been documented in various systems (Cavanaugh and Thompson, 1983). Adrenal GCs interact with cellular receptors, which, in turn, influence gene expression. Because the HO-2 gene has a GRE in its promoter region (McCoubrey and Maines, 1994), the increase in HO-2 mRNA in the brain most likely reflects a receptor-mediated event. Although the promoter region of NO synthase has not been mapped, there is evidence that GCs, also by a receptor-mediated process, inhibit mRNA synthesis for this enzyme (Radomski et al., 1990). In addition, in brain, GCs control neuronal activity, as well as the cellular structure and morphology. The regimen of GC treatment used in the present study has been shown previously to alter dendritic morphology of neurons in the adult rat hippocampus (Woolley et al., 1990), whereas a more prolonged exposure to the steroids (3 months) is known to reduce neuronal number (Sapolsky et al., 1985). Consistent with those reports are the present findings: enhanced levels of GCs dramatically alter dendritic morphology of NADPH diaphorase-containing cells in various regions of the brain; and a general reduction in the number of neurons was not detected (Fig. 4g and h). Therefore, it appears that decreases in HO-2 and NO synthase reflect changes in cellular structure and morphology, rather than cell loss that would occur with prolonged exposure to adrenal GCs.

In the hippocampus, consistent with previous observations (Ignarro et al., 1984; Schmidt et al., 1992), we detected few NO synthase-expressing neurons; these cells are primarily concentrated in the hilus of the dentate gyrus. As noted in Fig. 4b, staining for NADPH diaphorase is essentially lost subsequent to exposure to excess corticosterone. This attenuation could be a reflection of a decrease in transcription rate, as well as loss of protein. The former possibility is consistent with the data shown in Fig. 1c; although a diminished translation and/or stability of NO synthase protein could account for the protein loss, the same line of reasoning could be used to explain loss of NO synthase in other brain regions. GC stimulation of protein degradation and proteolysis is an established phenomenon (MacDonald et al., 1980). Moreover, it is well known that GCs cause cellular apoptosis (Schwartzman and Cidlowski, 1993), and as presently noted, those regions of the brain that are known to be enriched with GC receptors, such as the hippocampus and paraventricular nucleus (McGimsey et al., 1991), display a pronounced decrease in both NADPH diaphorase and HO-2 level. The corticosterone-mediated neuronal apoptosis is vividly shown in Fig. 3d, f, and h, denoting loss of den-

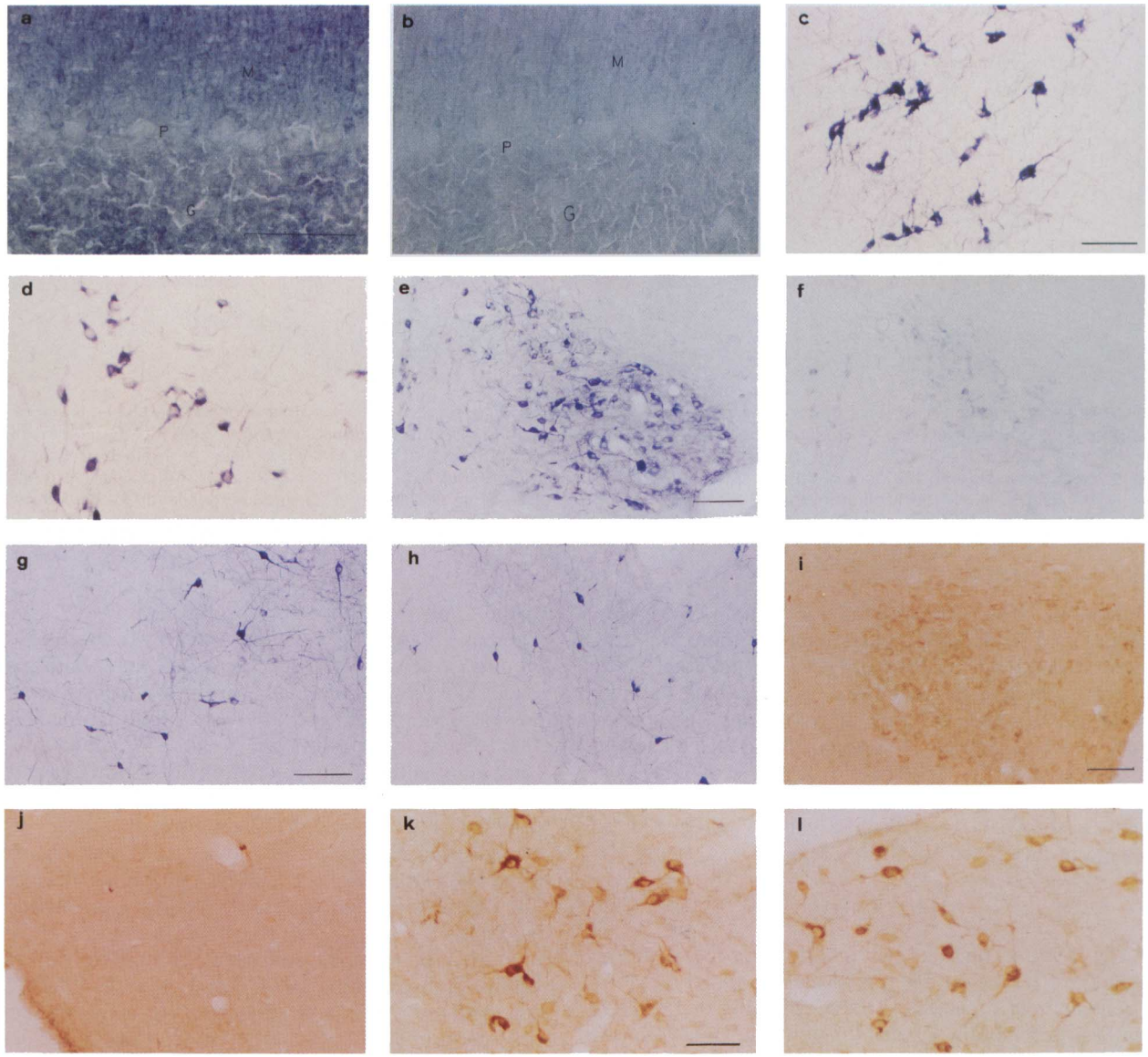

FIG. 3. Photomicrographs detailing NADPH diaphorase staining in different regions of brain in control and GC-treated animals. Rats were treated with corticosterone as described in the legend to Fig. 1. Immunohistochemical staining for NADPH diaphorase was carried out as described in the text. **a** and **b** show NO synthase expressed in the cerebellum of control and GC-treated animals, respectively. Intense staining observed in the molecular (M) and granule cell (G) layers of this region in control animals was notably diminished after corticosterone administration under identical immunohistochemical conditions. **c, e,** and **g** show Intense neuronal diaphorase staining in the lateral tegmental area, paraventricular nucleus, and frontal cortex, respectively, in control brain. **d, f,** and **h** are Identical regions in corticosterone-treated animals. Although intensity of staining is rather comparable, neurons in the lateral tegmental area (d) and in frontal cortex (h) show notable distortion and loss of their dendritic arborization after exposure to GC treatment. In paraventricular nucleus (f), intensity of staining was also considerably attenuated. **i** (control) and **j** (treated) show attenuated HO-2 immunoreactivity in cells of paraventricular nucleus of treated animals, whereas neurons in lateral tegmental area (**k** and **l;** control and treated, respectively) did not display notable changes. P, Purkinje cells; M, molecular layer; G, granule cell layer. Bar = 100 μm.

dritic arborization of neurons stained for diaphorase in the lateral tegmental region, paraventricular nucleus, and frontal cortex. Because GC receptors have not been completely mapped in the brain, a correlation between receptor occurrence and loss of staining for diaphorase and HO-2 in other brain regions cannot be made.

Clearly, not all HO-2-containing neurons in the brain are affected adversely by excess corticosterone. For example, the number of Purkinje neurons that express HO-2 appear unaffected by the treatment in the cerebellum. Also, the intensity of immunohistochemical staining for the protein does not appear lessened in these neurons. This stands in contrast to the apparent widespread negative effect of corticosterone on NO

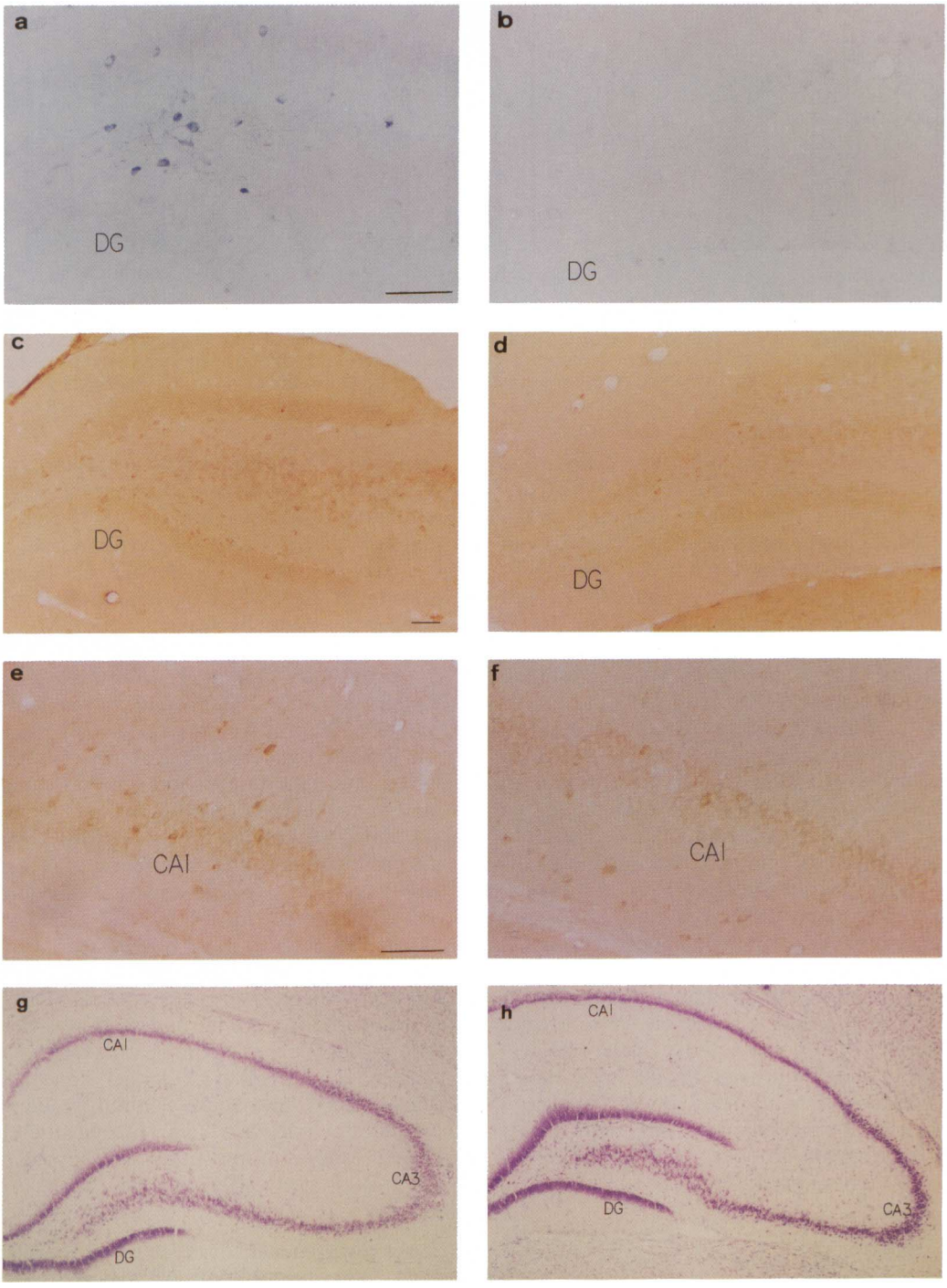

FIG. 4. Immunohistochemical staining of hippocampal complex of normal and GC-treated rats for HO-2 and NADPH diaphorase. Immunohistochemical analysis for HO-2 and NADPH diaphorase was performed as outlined in Materials and Methods. **a** and **b** show diaphorase staining in the hilus of the dentate gyrus in control and corticosterone-treated animals, respectively. A pronounced reduction in staining of neurons in treated tissue is observed. The same region was examined for HO-2 immunoreactivity. As shown, when compared with control (**c**), immunoreactivity in treated rats (**d**), although diminished, is readily evident. **e** and **f** show HO-2 immunohistochemical analysis in the CA1 pyramidal layer of the hippocampal complex in control and GC-treated animals, respectively. A noticeable decrease in positive-staining cells is noted. General neuronal Cresyl Violet Nissl staining of the hippocampal formation is shown in **g** and **h** (control and treated, respectively). As shown, a discernible decrease in neuronal elements is not apparent in treated animals. DG, dentate gyrus. Bar = 100 μm.

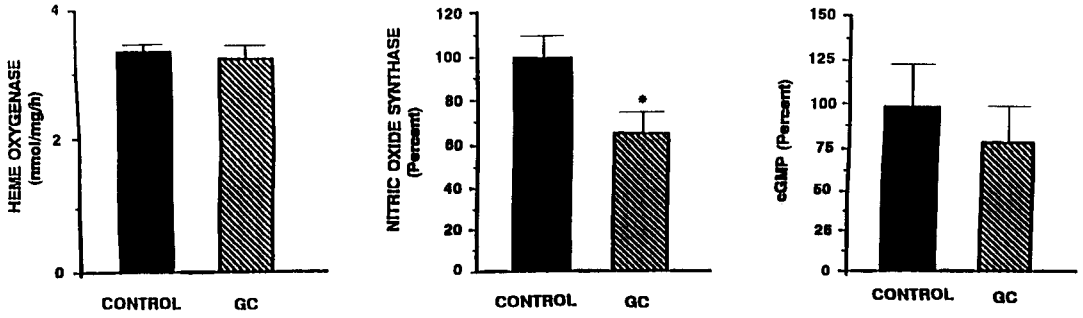

FIG. 5. Effect of GC administration on cerebellar level of cyclic GMP and activities of HO and NO synthase. Animals were treated with daily injections of corticosterone as detailed in the text. Cerebellum was isolated and used for assessment of cyclic GMP level and activities of HO and NO synthase, as detailed in Materials and Methods. Cerebellum from one animal was used for one determination. Data were analyzed by *t* test and are presented as the means ± SE of four determinations. $^*p \leq 0.05$, statistical significance.

synthase in cerebellum. For example, the cells in the granule and molecular layers of this region, which under normal conditions display intense immunostaining for NADPH diaphorase, show a dramatic reduction in staining, resembling the response noted in the hippocampal complex. A possible explanation for the overall lowered negative impact of corticosterone on HO-2 relative to that on NO synthase proteins could reflect the inducibility of HO-2 transcription by corticosterone and its inhibition of that of NO synthase.

The effects of GCs are paradoxical and range from degeneration of neuronal components, as described above, to protection (Landfield et al., 1978; Sapolsky et al., 1985; Sloviter et al., 1989). For example, in the adult rat brain, GCs act by an unknown mechanism to inhibit damage related to lipid peroxidation and facilitate recovery after head trauma (McEwen and Gould, 1990). In this case, based on the present findings and the previous observations, it would appear reasonable to suspect a role for the HO system, and in particular its HO-2 component, in this unknown mechanism of protection. This suggestion is based on the following considerations: (a) the known activity of the HO system to degrade hemoglobin heme, as the heme molecule is the most potent catalyst for lipid peroxidation (Tappel, 1961); (b) the recently discovered antioxidant activity of bile pigments (Stocker et al., 1987), with the sole source of bile pigment formation in mammals being the HO system; (c) the overall relative resilience of the HO-2 protein to corticosterone; (d) the positive regulating effect of corticosterone on HO-2 gene expression (Fig. 1); and (e) the immense capability of the enzyme system to generate the potential neurotransmitter, CO, which, as noted in the present study, appears to essentially sustain cyclic GMP levels in face of a marked reduction in NO synthase activity (Fig. 5). Given the differential affinity of CO and NO for the heme moiety of guanylate cyclase (for review, see Maines, 1993), it would be reasonable to suggest that when the NO-generating capability of brain is compromised, the opportunity would be provided for CO to bind to the heme moiety and activate the enzyme, thus sustaining the cellular levels of cyclic GMP. Based on the observations with the response of NO synthase and HO-2 systems, it would appear reasonable to suspect that a decrement in ability to generate the heme ligands, NO and CO, may well be a contributing factor to the degeneration of hippocampal complex functions known to be caused by excess levels of GCs, including deficiencies in learning and memory. Recent studies with the hippocampal complex have provided evidence for the hypothesis that CO, either alone or in combination with NO, may serve as a retrograde message that produces activity-dependent presynaptic enhancement during long-term potentiation (Stevens and Wang, 1993; Zhuo et al., 1993).

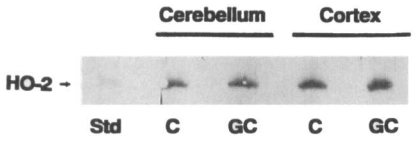

FIG. 6. Effect of GC treatment on brain HO-2 protein as assessed by western blot analysis. Microsomes were isolated from rat brain cerebellum and the cortex and subjected to SDS-PAGE. Each lane contained 100 μg of protein. Protein was transferred subsequently to a nitrocellulose filter as described in Materials and Methods, and the filter was probed with rabbit anti-rat testis HO-2 antisera, with 5 ng of purified rat testis HO-2 used as the standard.

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