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13. Lymphoblasts (*14*) were maintained in suspension culture at 37°C in a 5% CO_2 incubator in RPMI 1640 medium supplemented with 15% (v/v) heat-inactivated fetal calf serum, 1 mM sodium pyruvate, penicillin G at 100 unit/ml, and streptomycin at 100 μg/ml. On day 0, 1×10^7 cells were suspended in a 75-cm^2 flask containing 20 ml of the above medium with 10% fetal calf serum. On day 2, each flask received 20 ml of additional fresh medium of identical composition. On day 3, the cells in each flask were collected by centrifugation (2000 rpm, 10 min, 4°C), washed with 8 ml of ice-cold Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), and centrifuged as above. The cell pellet was frozen at −70°C and used within 2 weeks. To prepare a cell extract, each thawed pellet was resuspended in 0.5 to 1.5 ml of ice-cold buffer containing 50 mM sodium Hepes (pH 7.2), 0.3 mM Nonidet-P40, 1 mM dithiothreitol, and 10 mM NaCl. The suspension was passed ten times through a 21-gauge needle and then centrifuged at $10^5 g$ for 1 hour at 4°C. The supernatant was used for all enzyme assays.
14. Permanent lymphoblast cell lines were established by Epstein-Barr virus transformation of blood lymphocytes. Cell lines from normal subjects were established in Dallas and designated as follows: Control-1 (male, age 26; culture designation L430); control-2 (female, age 24; L429); control-3 (male, age 38; L389). Cell lines from CHM subjects were established by R. Nussbaum in Philadelphia or by F. Cremers in Nijmegen, the Netherlands, and are designated as follows: CHM-1 [male, age 26; patient 45-06 in (*3*); large deletion of CHM gene with no detectable mRNA; culture L659]; CHM-2 [male, age 45; patient 95-02 in (*3*); no rearranged CHM gene by Southern gel analysis, but reduced mRNA; culture L660]; CHM-3 (male, age 54; patient 7.6 in (*2*); large deletion with no detectable mRNA; culture L665); CHM-4 [male, age 35; patient 25.6 in (*2*); large deletion with no detectable mRNA; culture L669]. Cell lines from Usher syndrome type II were established by S. D. van der Velde-Visser, A. van Aarem, and C. Cremers in Nijmegen and designated as follows: Usher II-1 (male, age 59; culture L663); and Usher II-2 (male, age 52; culture L664).
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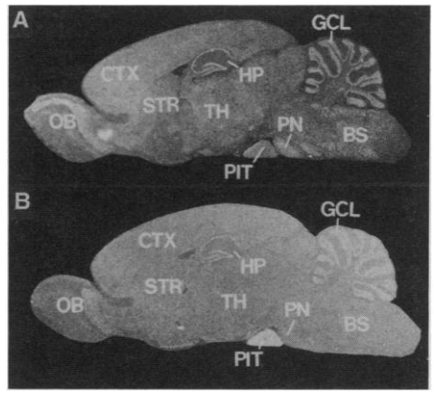

Fig. 1. In situ hybridization of (**A**) heme oxygenase-2 and (**B**) cytochrome P-450 reductase mRNA in brain sagittal sections. Ribonuclease (RNase) pretreatment of sections (10 μg ml^{-1} for 1 hour at 37°C) and 100-fold excess of unlabeled probe each abolished labeling, which indicates specificity. A high stringency wash (70°C) did not reduce labeling. Abbreviations: OB, olfactory bulb; CTX, cortex; STR, striatum; TH, thalamus; HP, hippocampus; PN, pontine nucleus; PIT, pituitary; GCL, granule cell layer; and BS, brain stem.

Carbon Monoxide: A Putative Neural Messenger

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Carbon monoxide, an activator of guanylyl cyclase, is formed by the action of the enzyme heme oxygenase. By in situ hybridization in brain slices, discrete neuronal localization of messenger RNA for the constitutive form of heme oxygenase throughout the brain has been demonstrated. This localization is essentially the same as that for soluble guanylyl cyclase messenger RNA. In primary cultures of olfactory neurons, zinc protoporphyrin-9, a potent selective inhibitor of heme oxygenase, depletes endogenous guanosine 3′,5′-monophosphate (cGMP). Thus, carbon monoxide, like nitric oxide, may be a physiologic regulator of cGMP. These findings, together with the neuronal localizations of heme oxygenase, suggest that carbon monoxide may function as a neurotransmitter.

Abundant evidence indicates that nitric oxide (NO) is a physiologic messenger molecule that mediates the regulation of blood vessel tone (it is also known as endothelial-derived relaxing factor). NO also regulates the tumoricidal and bactericidal actions of macrophages and serves as a putative neurotransmitter in the central and peripheral nervous systems (*1*). Molecular cloning reveals that distinct proteins account for neuronal (*2*), macrophage (*3*), and endothelial (*4*) NO synthase (NOS). NO is a short-lived free radical gas that can activate soluble guanylyl cyclase by binding tightly to the heme moiety of the enzyme (*1*). If a normally noxious gas such as NO is a physiologic messenger, perhaps other gases serve similar functions. Our clone of brain NOS revealed close homology with only one other mammalian protein, cytochrome P-450 reductase (*2*). Cytochrome P-450 reductase is best known as the source of electrons for the P-450 class of enzymes, although it also serves as the electron donor for heme oxygenase, which degrades heme to biliverdin and releases carbon monoxide (CO) in the process (*5–7*). Like NO, CO is a noxious gas that activates guanylyl cyclase (*8*). In this study, we used in situ hybridization to examine the localization of mRNA for heme oxygenase-2, the constitutive form of heme oxygenase in the brain, and the ability of heme oxygenase in neuronal cultures to regulate cGMP.

Two forms of heme oxygenase have been characterized (*9*). Heme oxygenase-1 is induced by heme and is expressed at high concentrations in the spleen and liver, where it is responsible for destruction of heme from red blood cells. Heme oxygenase-2 is not inducible and is widely distributed throughout the body with high concentrations in the brain (*9*). Our initial in situ hybridization studies failed to detect heme oxygenase-1 in rat brain, which agrees with previous findings that this protein is not normally evident in the brain [although after heat shock some heme oxygenase-1 is expressed in glia and certain neurons (*10*)]. By contrast, we observed heme oxygenase-2 mRNA in discrete sites

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throughout the brain (Figs. 1 to 3) (*11*). The highest concentrations of heme oxygenase-2 mRNA occur in neurons of the olfactory epithelium and in the neuronal and granule cell layer of the olfactory bulb (Figs. 1 and 2). In the hippocampus, heme oxygenase-2 mRNA is prominently expressed in the pyramidal cell layer and dentate gyrus. In the cerebellum, high concentrations are evident in the granule and Purkinje cell layers, and the pontine nucleus is also heavily labeled (Fig. 1). In coronal sections, intense staining of the habenula is apparent, with large concentrations of heme oxygenase-2 also seen in the piriform cortex, tenia tecta, olfactory tubercle, and islands of Callejae (Fig. 2). Higher magnification reveals that the mRNA is contained in neurons such as the granule cells of the cerebellum, hippocampus, and olfactory bulb as well as the pyramidal cells of the hippocampus and Purkinje cells of the cerebellar cortex. Unilateral olfactory bulbectomy, which causes retrograde degeneration of olfactory neurons, depletes heme oxygenase-2 mRNA in the olfactory epithelium, which indicates a localization to olfactory neurons (*12*).

The rate-limiting enzyme in porphyrin biosynthesis is δ-aminolevulinate synthase (ALAS). In brain (*12*) and olfactory epithelia (Fig. 2), we observed virtually identical localizations for ALAS and heme oxygenase-2 mRNA, which reflects a mechanism for the biosynthesis of porphyrin precursors of heme in the same structures that cleave to form CO. Heme oxygenase-2 localizations are also closely similar to cytochrome P-450 reductase (Fig. 1). This observation suggests that a major role of cytochrome P-450 reductase in the brain is to provide electrons for heme oxygenase activity.

The activation of soluble guanylyl cyclase by NO accounts for the ability of excitatory amino acids to stimulate cGMP activity in the brain and for vasodilators to enhance cGMP concentrations in blood vessels (*13*). Major discrepancies in localizations of NOS and guanylyl cyclase in the brain indicate that a substantial portion of guanylyl cyclase may not serve as a target for NO (*13*). Guanylyl cyclase can also be activated by CO, which may explain the ability of CO to relax smooth muscle and to block platelet aggregation (*8*). To determine whether CO is a major physiologic regulator of guanylyl cyclase in the brain, we localized mRNA for the guanylyl cyclase-2 subunit of soluble guanylyl cyclase (Fig. 3). In numerous regions, guanylyl cyclase localization appeared virtually identical to that of heme oxygenase-2. For instance, in discrete areas such as the tenia tecta, habenula, islands of Callejae, and olfactory tubercle, which lack NOS, both guanylyl cyclase and heme oxygenase-2 mRNA were highly enriched.

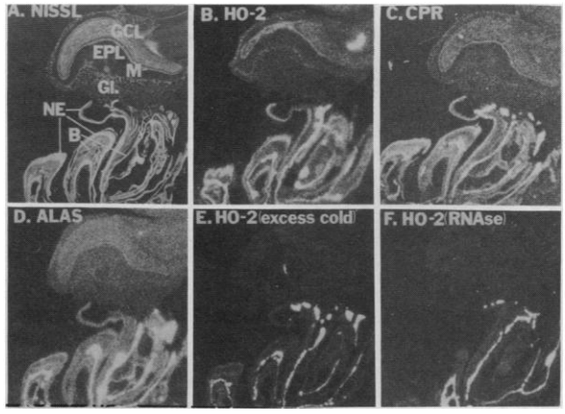

Fig. 2. In situ hybridization of nasal epithelium and olfactory bulb. (**A**) Nissl stain, (**B**) heme oxygenase-2 (HO-2), (**C**) cytochrome P-450 reductase (CPR), (**D**) ALAS, (**E**) heme oxygenase-2 with 100-fold excess unlabeled probe, and (**F**) heme oxygenase-2 pretreated for 1 hour at 37°C with RNase (10 μg ml^{-1}). Labeling was not reduced by a high stringency wash (70°C). The RNase and unlabeled probe controls were also performed on cytochrome P-450 reductase and ALAS with results similar to those seen in (E) and (F). Abbreviations: B, bone; NE, nasal epithelium; Gl, glomeruli; EPL, external plexiform layer; M, mitral cell layer; GCL, granule cell layer.

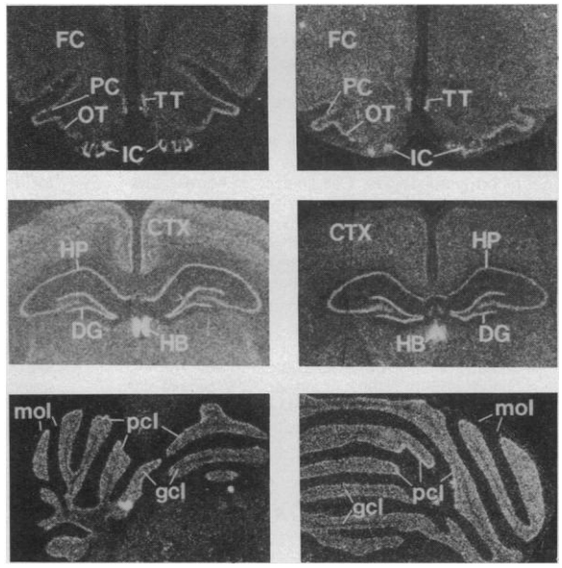

Fig. 3. In situ hybridization in coronal sections for (left column) guanylyl cyclase and (right column) heme oxygenase-2. The excess unlabeled probe, RNase, and high-stringency wash controls were performed on the sections stained for guanylyl cyclase as described in the caption to Fig. 2. Abbreviations: FC, frontal cortex; PC, piriform cortex; OT, olfactory tubercle; TT, tenia tecta; IC, Islands of Callejae; CTX, cortex; HP, hippocampus; DG, dentate gyrus; HB, habenula; gcl, granule cell layer of the cerebellar cortex; mol, molecular layer; and pcl, Purkinje cell layer.

The absence of NOS in these areas (*13*) suggested that there may be another endogenous regulator of guanylyl cyclase. The colocalization of heme oxygenase-2 and guanylyl cyclase in these regions suggests that CO normally mediates cGMP concentrations. In the cerebellum, NOS inhibition blocks glutamate stimulation of cGMP values, which indicates a role for NO in cGMP regulation (*13*). Cerebellar NOS is localized to granule cells and basket cells (*13*), which synapse on Purkinje cells. The Purkinje cells have large concentrations of guanylyl cyclase (Fig. 3), which provides a cellular basis for NO influences on cGMP. Because guanylyl cyclase and heme oxygenase-2 mRNA are localized to granule cells and Purkinje cells (Fig. 3), CO may also participate in cerebellar cGMP regulation, possibly by way of nonglutamatergic synapses. Throughout the rat brain, the localization of guanylyl cyclase resembles that of heme oxygenase-2 much more closely than it does any known neurotransmitter or second messenger (*14*). The colocalization of heme oxygenase-2 and guanylyl cyclase is not absolute; the NOS concentrations in the corpus striatum parallel those of guanylyl cyclase better than heme oxygenase-2 concentrations do (*2*) (Fig. 1). The results of a recent report of in situ hybridization localizations of soluble guanylyl cyclase agree with our findings (*15*).

Because there are high densities of heme oxygenase-2, cytochrome P-450 reductase, and ALAS in olfactory neurons, we used primary olfactory neuronal cultures to examine the relation of CO activity to cGMP concentration (Table 1) (*16*). We monitored cGMP values in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) to ensure that any observed effects did not simply involve changes in phosphodiesterase activity. Basal cGMP concentrations in olfactory neurons are high (7.2 pmol per milligram of protein), similar to those of rod outer segments (*17*). In several tissues, NO that was gen-

Table 1. Regulation of endogenous cGMP in cultures of primary olfactory neurons by agents that affect CO formation. Methods were as described in (*16*). All experiments included the phosphodiesterase inhibitor IBMX. In experiments with CO, cell culture medium was bubbled with 100% CO gas for 1 min. Data are means of 7 to 12 independent determinations ± SEM. Control concentrations of cGMP were 56 pmol per milligram of protein.

Treatment	cGMP (% control)
Zn PP-9	
10 μM	10.5 ± 0.5
1 μM	16.5 ± 4.8
750 nM	14 ± 2.4
500 nM	16.2 ± 5
100 nM	17.1 ± 4.6
50 nM	42 ± 6.4
10 nM	76 ± 5.5
Hemoglobin	
50 μM	3 ± 2.5
10 μM	3 ± 3.0
5 μM	3 ± 1.9
1 μM	22 ± 4.0
0.5 mM NAME	
	88 ± 2.4
+1 μM Zn PP-9	16 ± 3.5
+1 μM Zn PP-9 + 1 μM hemoglobin	7.6 ± 1.9
SNP and CO	
1 μM Zn PP-9	15 ± 3.5
10 μM SNP	120 ± 3.1
CO	105 ± 2.1
10 μM SNP + 1 μM Zn PP-9	120 ± 2.5
CO + 1 μM Zn PP-9	102 ± 2.8

erated by NOS regulates cGMP concentrations (*1*). However, the NOS inhibitor nitroarginine methyl ester (NAME) (0.5 mM) failed to alter cGMP concentrations in the olfactory cultures. Furthermore, the cultured cells lacked NOS, as determined by RNA blot, protein immunoblot, and catalytic assay (*12*). Hemoglobin, which binds NO, blocks NO-mediated responses such as cGMP production (*1*). A hemoglobin concentration as low as 1 μM depleted cGMP levels (Table 1).

Because hemoglobin binds CO tightly and there are high concentrations of heme oxygenase-2 mRNA in olfactory neurons, we tested whether CO that was generated by heme oxygenase-2 regulates cGMP in these cultures. We used zinc protoporphyrin-9 (Zn PP-9), which inhibits heme oxygenase activity in brain microsomal membranes with an inhibitory constant of approximately 3 nM (*16*), a value that resembles its potency as an inhibitor of purified heme oxygenase (*18*). In olfactory neuronal cultures, Zn PP-9 lowered cGMP concentrations with a 60% decrease apparent at 50 nM (Table 1). To evaluate whether Zn PP-9 directly inhibits guanylyl cyclase, we added sodium nitroprusside (SNP), which generates NO that in turn can stimulate guanylyl cyclase. In the presence of 1 μM Zn PP-9, which completely depletes cGMP, SNP restored cGMP concentrations, which indicates the absence of direct guanylyl cyclase inhibition by Zn PP-9 in our system (Table 1). Furthermore, CO itself restored Zn PP-9–inhibited cGMP to control concentrations (Table 1B).

Table 2. Conditioned media from donor cells restore cGMP concentration in recipient cells treated with Zn PP-9. Methods and data analysis were as in Table 1.

Treatment of recipient cells	Addition of donor media	cGMP in recipient cells (% Control)
	No	100
	Yes	98 ± 4
Zn PP-9	No	45 ± 6
Zn PP-9	Yes	93 ± 5.3
Zn PP-9*	Yes	51 ± 10

*Donor cells were treated with Zn PP-9 in this last trial.

Table 3. Odorant stimulation of cGMP concentration blocked by Zn PP-9 and hemoglobin. Methods and data analysis were as in Table 1, except that IBMX was not included in order to decrease the cGMP concentrations.

	cGMP (pmol mg^{-1} of protein)
Control	7.2 ± 0.11
0.1 μM IBMP	13.9 ± 0.15
1 μM Zn PP-9	4.47 ± 0.12
1 μM Zn PP-9 + 0.1 μM IBMP	4.48 ± 0.07
1 μM hemoglobin	4.55 ± 0.11
1 μM hemoglobin + 0.1 μM IBMP	4.95 ± 0.28

To establish further that cGMP is regulated by CO release, we conducted a conditioned media transfer experiment (Table 2). In the recipient cells, cGMP was depleted by Zn PP-9. The addition of medium from donor cells restored cGMP concentrations to control values. However, if the donor cells had also been pretreated with Zn PP-9, their medium failed to restore cGMP concentrations. Thus, the factor in the medium of donor cells that stimulates cGMP may be formed as a product of enzymatic activity that can be inhibited by low concentrations of Zn PP-9, presumably the generation of CO.

Odorants markedly enhance cGMP concentrations in cultures of primary olfactory neurons with peak increases at about 20 s (*19*). The potent odorant 1-isobutyl-3-methoxypyrazine (IBMP) (Table 3) and several other odorants (*12*) also raised cGMP values with a slower time course that peaked at about 1 to 2 min. The enhancement of cGMP concentrations after IBMP treatment was blocked by hemoglobin and Zn PP-9 but not by nitroarginine, which indicates that mediation was by CO and not NO.

In preliminary experiments, the incubation of rat cerebral cortex brain slices with Zn PP-9 only minimally lowered cGMP values. One explanation is that tissues of brain slices are much less accessible to Zn PP-9 than are cell cultures. Also, the enrichment of ALAS in olfactory epithelium provides much more heme precursor for CO in this tissue under basal conditions than does the same treatment in cortex. ALAS is a highly inducible enzyme, which suggests that physiological activation by neurotransmission leads to CO-stimulated cGMP formation in the brain. Whether a specific neurotransmitter regulates the CO pathway as glutamate acts on the NO pathway remains to be established.

Several lines of evidence indicate that CO is a neural messenger associated with physiologic maintenance of endogenous cGMP concentrations. In the brain, heme oxygenase-2 mRNA occurs in discrete neuronal populations whose localizations closely resemble those of guanylyl cyclase. Moreover, a selective, potent inhibitor of heme oxygenase depletes endogenous cGMP in neuronal preparations. Direct stimulation of guanylyl cyclase activity by CO has been demonstrated (*8, 20, 21*).

The colocalization of ALAS with heme oxygenase-2 in the brain indicates that the machinery for porphyrin turnover and CO biosynthesis occurs together. The colocalizations of cytochrome P-450 reductase mRNA with heme oxygenase-2 imply a major role for brain cytochrome P-450 reductase in electron donation for heme oxygenase activity.

Both CO and NO can be formed by distinct constitutive and inducible enzymes, with the first type predominating in brain tissue. Biosynthesis of both requires electron donation that results from activity between the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome P-450 reductase. For NO, the activity of cytochrome P-450 reductase is accomplished by the NOS protein. Although both NOS and heme oxygenase-2 display neuronal localizations in the brain, regional distributions differ markedly for the two enzymes. Outside the brain NO relaxes blood vessels, and peripheral heme oxygenase-2 mRNA is localized in smooth muscle of blood vessels (*12*). This activity and localization agrees with the ability of CO to relax smooth muscle through the activation of guanylyl cyclase (*8*) or other mechanisms (*22*).

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