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Heme Oxygenase: A Font of Multiple Messengers

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Neurotransmitters and related messenger molecules abound in the brain with numbers ranging between 50 and 100 depending on who is doing the counting. Their chemical structures vary from peptides and large proteins to metal ions such as zinc and the diatomic gases nitric oxide (NO) and carbon monoxide (CO). Most are formed by enzymatic processing although there are notable exceptions. The neurotransmitter pool of zinc comes from a zinc transporter that concentrates the metal into synaptic vesicles together with glutamate. Neurotransmitter pools of amino acids are probably sequestered largely by transporters although this is not altogether clear. For the most part, neurotransmitter peptides are not generated by selective enzymes but by generalized peptide-processing enzyme systems. Highly selective neurotransmitter forming enzymes exist for the biogenic amines; for D-serine, which is formed by serine racemase that converts L-serine to D-serine (Wolosker et al. 1999, 2000); for NO, generated from arginine by NO synthase (NOS); and for CO, formed from heme by heme oxygenase (HO). In most cases the biosynthetic enzyme or series of enzymes yield a single end product. For instance, tryptophan hydroxylase coupled with aromatic amino acid decarboxylase is solely concerned with forming serotonin, while tyrosine hydroxylase, aromatic amino acid decarboxylase, and dopamine beta-hydroxylase together generate norepinephrine. The focus of this essay is a notable exception. Heme oxygenase gives rise to three discrete products, CO, ferrous iron and biliverdin-bilirubin. Only recently has there been an appreciation that all three of these products have important physiologic roles which may be complementary.

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CARBON MONOXIDE

HO was first identified as an enzyme that degrades heme in aging red blood cells. The enzyme was highly concentrated in the spleen, the graveyard of erythrocytes. It is induced by heme, enabling it to respond to hemolysis or tissue destruction, which releases heme from mitochondrial enzymes. Maines and associates (Maines et al. 1986; Trakshel et al. 1986) identified a second enzyme, which is not inducible and is most highly concentrated in the brain and testes. The non-inducible enzyme is designated HO2, while the inducible form is HO1.

Following the identification of NO as a neurotransmitter, we wondered whether there might exist other gaseous transmitter molecules and explored CO as a possibility. If CO were a transmitter, a form of HO, presumably HO2, should be localized to neurons. *In situ* hybridization reveals HO2 in discrete neuronal populations in the brain with localizations closely resembling those of soluble guanylyl cyclase (Verma et al. 1993). CO as well as NO can bind to and activate guanylyl cyclase, which is presumably their second messenger. HO2 localizations in the brain resemble soluble guanylyl cyclase better than the localizations of NO synthase. In olfactory neuronal cultures inhibition of HO2 depletes cyclic GMP levels while inhibition of NO synthase is without effect (Verma et al. 1993; Ingi and Ronnett 1995; Ingi et al. 1996).

Direct evidence for CO as a neurotransmitter came from studies of intestinal non-adrenergic, non-cholinergic transmission (NANC), which accounts for the relaxing phase of peristalsis (Zakhary et al. 1997). Various molecules, including ATP and vasoactive intestinal polypeptide, had been suggested as NANC neurotrans-

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mitters, but there was no definitive evidence. Immunohistochemical staining reveals HO2 and nNOS often localized to the same myenteric plexus neurons. Mice with targeted deletion of HO2 ($HO2^{-/-}$) display about a 50% reduction in NANC transmission with a similar reduction in $nNOS^{-/-}$ intestine (Zakhary et al. 1997). NANC transmission is virtually abolished in mice with combined deletion of nNOS and HO2 (Zakhary et al. 1997).

CO may also have a transmitter role in the vas deferens where it is localized to neuronal populations that regulate ejaculation (Burnett et al. 1998). Ejaculatory reflexes mediated by the bulbospongiosus muscle are abolished in $HO2^{-/-}$ mice, and ejaculation is markedly diminished in intact $HO2^{-/-}$ mice. By contrast with the colocalization of the nNOS in the gut, these enzymes have different localizations and functions in the male reproductive track. nNOS is not evident in vas deferens neurons but instead is localized to the penile innervation which does not possess HO2. NO appears to be the neurotransmitter subserving penile erection. Erection, elicited in rats by stimulation of the cavernous nerves, is markedly reduced by treatment with NOS inhibitors (Burnett et al. 1992).

HO2 is also important in blood vessel function. Immunohistochemical studies reveal HO2 localized to endothelial layers of blood vessels, analogous to endothelial NOS (Zakhary et al. 1996). In some blood vessels NO is the sole endothelial-derived relaxing factor. However, in many blood vessels relaxation is only partially antagonized by inhibitors of NOS. HO inhibitors reverse the NO-independent relaxation (Zakhary et al. 1996). Thus, in blood vessels, as in the intestinal myenteric plexus, CO and NO appear to work synergistically.

How is HO2 regulated? CO, like NO, cannot be stored in synaptic vesicles so that it must presumably be formed upon demand. In the case of NO, this involves exquisitely fine tuning of nNOS. Glutamate, acting through NMDA receptors, very rapidly activates the formation of NO in the following way. Calcium, entering cells through NMDA receptor channels, binds to calmodulin which stimulates nNOS (Bredt and Snyder 1990). Might there be a comparably rapid activation of HO2? We found that protein kinase C phosphorylates HO2 and augments its catalytic activity (Doré et al. 1999a). Phorbol esters, which activate protein kinase C, stimulate HO2 activity and bilirubin staining in neuronal cultures. This appears to take place on a very rapid time scale. Hence, calcium entry into cells following neuronal activation might be expected to activate protein kinase C and thereby HO2.

How does CO act as a transmitter? Thus far, its only known target is soluble guanylyl cyclase. Like NO, CO binds to heme in the active site of the enzyme altering its conformation and leading to enzymatic activation. NO is up to 100 times more potent than CO in stimulating soluble guanylyl cyclase, so much so that some workers have questioned whether the enzyme is a physiologic target of CO. However, Friebe et al. (1996) have established that treatment of preparations with the drug YC1, which binds to guanylyl cyclase, increases the potency of CO up to 100-fold. Perhaps in intact tissues comparable conformational alterations of guanylyl cyclase render it sensitive to CO. The finding that intestinal cyclic GMP levels are markedly reduced in $HO2^{-/-}$ mice establishes persuasively that CO regulates cyclic GMP levels in intact organisms.

BILIRUBIN

Biliverdin is the direct product of HO activity. The enzyme biliverdin reductase occurs in almost all tissues of the body in great abundance so that biliverdin levels themselves rarely accumulate. Instead, biliverdin is almost immediately reduced to bilirubin (Figure 1). Bilirubin has long been regarded as a toxic end product of heme metabolism, which is rapidly conjugated to glucuronic acid for urinary excretion. The immaturity of the conjugating system in newborns leads to "physiologic" jaundice, while a substantial minority of human infants develop high enough levels of bilirubin that brain damage from kernicterus is a notable risk.

The brain lacks conjugating enzymes so that bilirubin presumably accumulates from HO activity in neurons. We wondered whether it might have a physiologic role. Ames and collaborators (Stocker et al. 1987a, 1987b) noted that bilirubin is an antioxidant. We explored possible antioxidant-neuroprotectant effects of bilirubin in cerebral cortical cultures of embryonic rats (Doré et al. 1999a). We elicited oxidative damage with hydrogen peroxide. Protoporphorin derivatives that are inhibitors of HO worsen neurotoxicity. Neurotoxicity is also greatly accentuated in cultures made from $HO_2{}^{-/-}$ mice. Bilirubin added to the cultures is markedly neuroprotective. Surprisingly, as little as 10 nM bilirubin concentration protects against 10,000 times higher concentrations of hydrogen peroxide. This apparent paradox can be explained by an oxidative-reductive cycling between biliverdin and bilirubin (Figure 1). When bilirubin acts as an antioxidant, it is itself oxidized to biliverdin, which is in turn immediately reduced back to bilirubin by the tissue excess of biliverdin reductase. We have been able to directly demonstrate this process utilizing mixtures of the key chemicals and enzymes (S.H.S. and D.E.B., unpublished observation.)

Compelling evidence that bilirubin is a physiologic neuroprotectant emerges from studies of protein kinase C regulation and neuroprotection by phorbol ester. It is well known that low concentrations of phorbol esters, such as phorbol myristyl acetate (PMA), augment protein kinase C, while higher concentrations down-regulate enzyme expression. In hippocampal and cortical

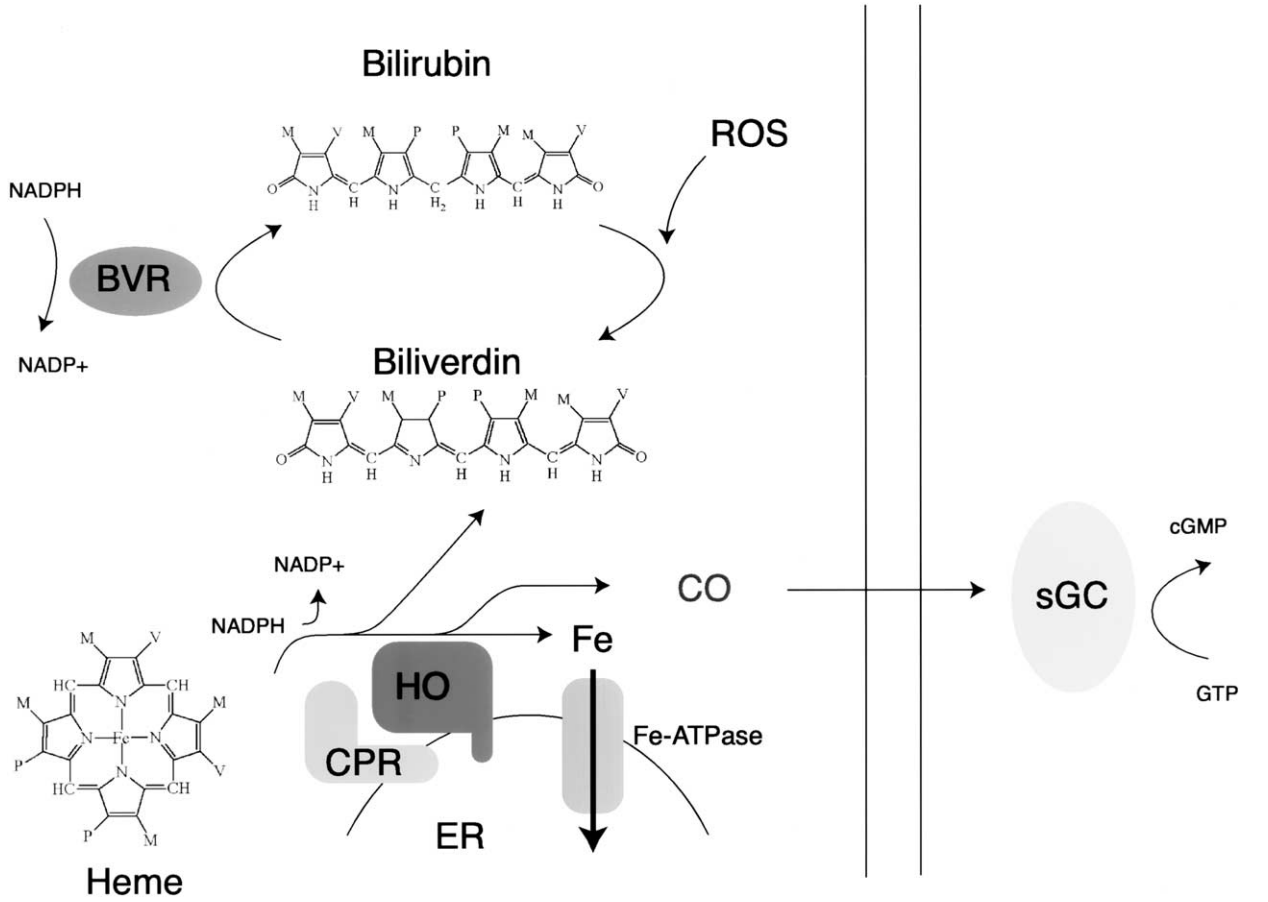

Figure 1. Heme oxgenase (HO) degrades heme to produce three products: iron, carbon monoxide, and biliverdin. Heme is a highly conjugated porphyrin ring (M = methyl, V = vinyl, P = propionate) that chelates an iron molecule. HO, acting with NADPH cytochrome P450 reductase (CPR), cleaves the α-meso bond of the heme by a mixed oxidative-reductive reaction, releasing a one-carbon fragment as carbon monoxide (CO). CO acts to stimulate soluble guanylyl cyclase (sGC). The free iron molecule (Fe) is removed from the cytoplasm by an Fe-ATPase. Biliverdin is rapidly reduced to bilirubin by the soluble enzyme biliverdin reductase. Reactive oxygen species (ROS) can be scavenged by bilirubin, protecting the cell from oxidative stress. ROSs oxidize bilirubin to form biliverdin, which can be reduced to bilirubin by BVR, completing a catalytic cycle which can account for the non-stoichiometric protection of bilirubin against hydrogen peroxide toxicity.

cultures, 0.1 μM PMA augments HO2 activity and bilirubin staining and prevents hydrogen peroxide elicited neurotoxicity (Doré et al. 1999a). By contrast, 1.0 μM PMA down-regulates protein kinase C, fails to activate HO2 and is not neuroprotective (Doré et al. 1999a).

The physiologic neuroprotectant role of bilirubin in intact animals is evident from studies showing accentuation of vascular stroke damage in $HO2^{-/-}$ mice (Doré et al. 1999b, 2000) This is not a nonspecific effect of "debility" in the $HO2^{-/-}$ animals who appear outwardly healthy. $HO1^{-/-}$ mice, who are far more debilitated than $HO2^{-/-}$ mice and die at a young age, do not manifest increased stroke damage. This specificity is further emphasized by the worsening of stroke damage elicited by the HO inhibitor tin protoporphyrin IX and the absence of additivity in neural damage in $HO2^{-/-}$ mice treated with tin protoporphyrin IX (Doré et al. 1999b). The accentuated neural damage in $HO2^{-/-}$ brain following stroke displays a marker profile reflecting apoptotic rather than necrotic cell death, and resembles the apoptotic profile of augmented cell death in cerebellar granule cell cultures of $HO2^{-/-}$ mice (Doré et al. 2000).

Thus, bilirubin is a physiologic neuroprotectant which may lessen neural damage following various types of hypoxic insults associated not only with stroke but also with neurodegenerative disease. We have recently obtained direct evidence for a neuroprotective role of HO2 linked to Alzheimer's disease (Takahashi et al. 2000). Yeast 2-hybrid and other protein-binding studies reveal HO2 and HO1 binding to the amyloid precursor protein (APP). This interaction has no effect on APP processing but does result in inhibition of HO activity. The APP derivatives that occur in familial forms of Alzheimer's disease are much more potent than normal APP in inhibiting HO activity. To examine in vivo consequences of this finding, we utilized mice with the

"Swedish" mutant APP. HO2 activity in cerebral cortical cultures from these mice is markedly reduced as is evident by profoundly diminished staining for bilirubin. Moreover, neurotoxicity elicited by hemin or hydrogen peroxide is markedly increased in cultures from Swedish mutant brain. While the HO inhibitor tin protoporphyrin IX worsens neurotoxicity in control cultures, it is ineffective in Swedish cultures in which NO activity is already diminished. We presume that the loss of physiologic protection by bilirubin in brains of patients with familial Alzheimer's disease increases sensitivity to the neurotoxic effects of the high levels of amyloid beta peptide generated in these patients.

IRON

Very little attention had been devoted to the third product of HO, ferrous iron. However, a little reflection reveals that cells must have some mechanism to deal with the iron released by HO. Ferrous iron is notoriously toxic even in low concentrations. It participates in the Fenton reaction giving rise to hydroxyl free radical, one of the most toxic biological species, which accounts for a major portion of cell death in stroke and septic shock. We noted a striking gap in the literature on cellular iron dynamics. Much was known about how iron enters cells. It is bound in the plasma to transferrin, enters cells when the transferrin receptor is endocytosed and is stored in cells bound to ferritin. As with other cations such as calcium, one would expect there to exist a pump that extrudes iron from cells, but none had been described in the literature.

Our first hint of a link between HO and iron extrusion came from studies characterizing $HO1^{-/-}$ mice (Poss and Tonegawa 1997). These animals die at an early age from massive iron overload in the liver and kidney. Yet, their plasma iron levels are subnormal. The iron overload in tissues was surprising, considering that HO is responsible for extracting iron out of the mitochondrial heme-containing proteins, so that in the absence of HO1 we might have expected less iron in cells. Presumably, in the absence of HO1 mitochondrial proteins continue to turn over but iron fails to leave cells, because HO1 somehow is associated with iron extrusion. A role for HO1 in removing iron from cells would also explain the low serum iron in $HO1^{-/-}$ mice.

We speculated that HO1 is closely linked to cellular iron extrusion mechanisms. This would prevent iron generated by HO from damaging cells. To test this hypothesis, we labeled endogenous iron pools of fibroblasts with $^{55}Fe^{2+}$ and monitored physiologic influx and efflux of iron (Ferris et al. 1999). Transfection of HEK 293 cells with HO1 markedly accelerates efflux. By contrast, primary fibroblasts cultures from $HO1^{-/-}$ mice manifest a markedly decreased iron efflux.

We wondered whether regulation of iron efflux by HO1 determines cellular viability in the presence of oxidative stresses. HO1 knockout fibroblasts are much more sensitive to apoptotic death elicited by serum deprivation (Ferris et al. 1999). Cell death is prevented by iron chelators but not by 8-bromo-cyclic GMP or bilirubin, indicating that iron, but not the other two products of NO, regulates viability of these cells.

Our studies of iron efflux imply the existence of a pump mediating iron extrusion, analogous to calcium ATPases. No such iron pump had been previously described, conceivably because of difficulties with ferric iron salts that readily precipitate. Utilizing $^{55}Fe^{2+}$ salts in potassium gluconate buffer, we successfully identified ATP-dependent iron accumulation into microsomal vesicles (Barañano et al. 2000). Binding is absolutely dependent upon ATP with the relative activity of various nucleotides paralleling known affinities for P-type ATPases. Like HO1, the iron pump is most highly concentrated in the spleen, the repository of senescent erythrocytes. Both HO1 and the iron pump appear localized to the endoplasmic reticulum. We were struck with the impressive inducibility of the iron pump. In macrophage cultures iron itself causes a 10-fold enhancement of pump activity. There is a doubling in the first two hours followed by a 16-hour plateau and then a quintupling within another two hours. The first phase of induction appears independent of new protein synthesis while the second phase requires transcriptional and translational activity, being blocked by actinomycin D and cycloheximide. Inducibility is evident in intact animals treated with glycerol that causes muscle degradation with a massive release of iron into the circulation.

What is the disposition of the iron pump in the brain? Under basal conditions HO1 levels in the brain are quite low, although high levels can be elicited following various stresses that induce HO1. Because HO2 is intimately linked to neural protection, we presume that in neurons in the brain HO2 would also be involved in regulating iron efflux, although there is not yet any direct evidence for this.

CONCLUSIONS

Is there any biological significance to the fact that HO produces three biologically active substances? Might they act in some coordinated fashion? In terms of the direct actions of CO, those that we know relate to its stimulation of cyclic GMP formation leading to relaxation of intestinal smooth muscle and vasodilatation. We do not know the consequences of comparable actions in the brain. One might reason by analogy with NO, which also stimulates cyclic GMP. Synaptic NO may mediate behavioral inhibition, at least in the area of aggression and sexual activity, because nNOS knockout mice are extraordinarily aggressive and display ex-

cess, inappropriate sexual activity (Nelson et al. 1995). Relaxation of smooth muscle certainly conserves energy which would be cytoprotective in relationship to oxidative and other stressors that kill cells by energy depletion. Cytoprotective muscle relaxation might be synergistic with the cytoprotective effects of bilirubin. The link of HO1 to iron efflux may provide another means of cytoprotection. Thus, a model emerges whereby the three products of HO could work in concert to preserve cellular integrity.

Are there other precedents whereby multiple products of an enzyme work in coordination, especially in the neurotransmitter arena? NOS gives rise to NO and citrulline. Thus far, there has not been any major evidence for neural actions of citrulline. Among neuropeptides, protein precursors sometimes contain multiple peptides. One of the two enkephalin precursors encodes both methionine-enkephalin and leucine-enkephalin. Another precursor encodes only leucine-enkephalin. The two enkephalins tend to be stored in different neurons, although in some cases they occur in the same neurons. Whether or not they function synergistically is not altogether clear. Pro-opiomelanocortim (POMC) encodes ACTH and beta-endorphin. While there is minimal evidence of ACTH and beta endorphin working together, there have not been extensive studies to evaluate this possibility.

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