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INDUCTION OF INDUCIBLE NITRIC OXIDE SYNTHASE AND HEME OXYGENASE-1 IN RAT GLIAL CELLS

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Summary

Recent observations suggest a possible interaction between the nitric oxide (NO)/NO synthases and carbon monoxide (CO)/heme oxygenases systems. We examined the effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ), and NO donor such as *S*-nitroso-*N*-acetylpenicillamine (SNAP) on induction of inducible NO synthase (iNOS) and heme oxygenase-1 (HO-1) in mixed glial cells and in rat hippocampus. In *in vitro* glial cells, treatment with LPS induced the expression of 130-kDa iNOS after 6 h, and NO_2^- accumulation and enhancement of the protein level of 33-kDa HO-1 after 12h. In addition, treatment with SNAP induced HO-1 expression after 6 h. Although a NOS inhibitor, such as N^G-nitro-L-arginine (NNA), did not change LPS-induced iNOS expression, the inhibitor suppressed both NO_2^- accumulation and the enhancement of HO-1. Immunocytochemistry showed that LPS-treatment induced iNOS-immunoreactivity predominantly in microglia, while this treatment induced HO-1-immunoreactivity in both microglia and astrocytes. These results suggest that endogenous NO production by iNOS in microglia causes autocrine- and paracrine-induction of HO-1 protein in microglia and astrocytes in rat brain.

Key Words: inducible nitric oxide synthase, heme oxygenase-1, microglia, astrocyte, glial cells

Similarity between the functional machinery of NO synthases and heme oxygenases

One function of heme oxygenase (HO) is the oxidation of the heme molecule in concert with NADPH-cytochrome P450 reductase (CPR) and then specific cleavage of the heme molecule into biliverdin, carbon monoxide (CO), and iron (1). In addition, biliverdin is rapidly metabolized by biliverdin reductase to bilirubin, an antioxidant (2). Recent studies have indicated that there are at least two isozymes of HO, *i.e.*, an inducible type (HO-1) and a constitutive type (HO-2) (1). Similarly, nitric oxide synthases (NOSs) consist of an inducible type (iNOS) and constitutive types (nNOS and eNOS) (3). It is well known that NOS coupled with calmodulin (CaM) catalyzes the production of nitric oxide (NO) and L-citrulline from L-arginine in an NADPH-dependent manner (3). In this process, NADPH-derived electrons are shuttled from flavins to NOS-bound heme (4). In fact, consensus sequences of recognition sites for FMN, FAD, and NADPH are localized within the carboxyl-terminal half of NOS, which is structurally homologous to other dual-flavin enzymes such as CPR which transfer electrons to hemoprotein (5). On the other hand, CPR transfers NADPH-derived electrons to heme in HO, and then HO forms CO and biliverdin from heme.

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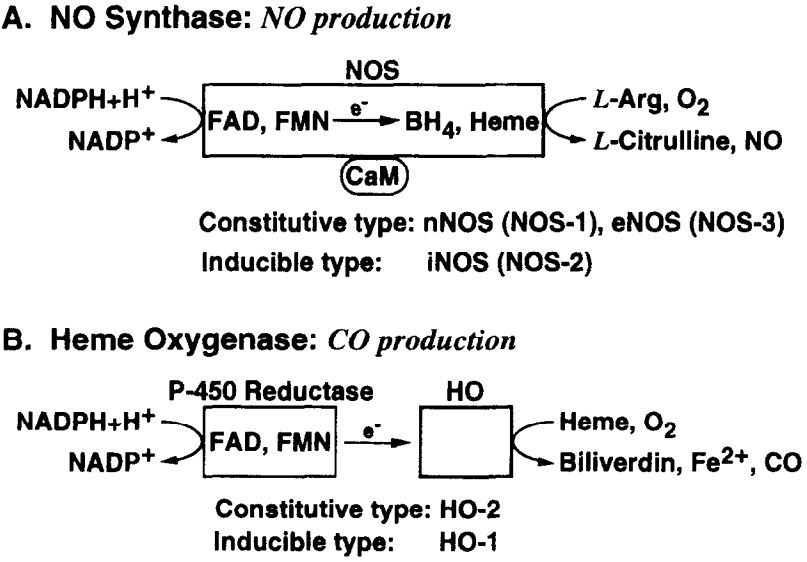

FIG. 1

Functional machinery of NO synthases (A) and heme oxygenases (B). See text for details. NOS, NO synthase; CaM, calmodulin; HO, heme oxygenase; e⁻, electron.

Thus, the functional machinery of HO/CPR is similar to NOS/CaM, although the substrates (heme or L-arginine) and resulting molecules (CO/biliverdin or NO/L-citrulline) are different (Fig. 1). In addition, both the stable gas CO and the unstable gas NO activate guanylyl cyclase (6) and may play a role in the brain as biological messengers and regulators of cGMP levels (7, 8).

Recent reports have indicated that endogenous NO and NO donors induce an increase in HO activity and HO-1 mRNA expression in cultured rat hepatocytes (9) and human glioblastoma cells (10), and that HO-2 activity is reduced by NO donors and increased by NOS inhibitors (11). These observations suggest a possible interaction between the NO/NOS and CO/HO systems. Therefore, we examined the effects of endogenous NO produced by iNOS induction and NO donors on HO-1 induction in glial cells.

Induction of iNOS in microglia

In *in vitro* culture, several laboratories reported that endotoxins and cytokines induced iNOS in glial cells (12, 13). The activation was caused by the *de novo* synthesis of iNOS proteins with a molecular size of 130-kDa in cultured glial cells. We also reported that after *in vitro* glial cells were treated with lipopolysaccharide (LPS), expression of 130-kDa iNOS protein began after 5 h and peaked at 24 h after stimulation and that NO_2^- in culture media accumulated linearly from 6 h to 48 h in the presence of L-arginine (14). When we used mixed glial cells (approximately 7% of microglia and over 90% of astrocytes), iNOS was expressed by LPS and/or interferon-γ (IFN-γ) almost entirely in microglia (15). This may be assumed because Jak2-tyrosine kinase, which is activated by IFN-γ receptors, exists in microglia rather than in

astrocytes (15). In addition, the amount of iNOS expression in microglia was more intense than in astrocytes (16), and a combination of LPS and cytokines is necessary to induce intensive iNOS expression in secondary cultures of rat astrocytes (17) and rat astrocyte-derived C6 glioma cells (18). Therefore, even if contamination by microglia were less than 1%, iNOS expression might be detectable. From these observations, we concluded that iNOS expression occurred mostly in ameboidal microglia rather than in astrocytes (15, 19).

Recently, the 5'-flanking region of iNOS gene was cloned from the mouse genomic cosmid library (20). They described that the promoter region of mouse iNOS gene involves the IFN-γ-activation site (GAS), the IFN-γ response element (γ-IRE), the nuclear factor κB (NF-κB)-binding site, etc. It is known that GAS is activated by IFN-γ-activated factor (GAF), which is the homodimer of tyrosine phosphorylated signal transducer and activator of transcription-1α (STAT1α). iNOS induction by LPS requires the translocation of NF-κB into the nucleus (21). In contrast, its induction by IFN-γ required tyrosine phosphorylation of STAT1α in cultured microglia (15).

On the other hand, the class II of major histocompatibility complex (MHC) was also induced in numerous ameboidal microglia by intrahippocampal microinjection of IFN-γ plus LPS. At that time, the iNOS-immunopositive cells were observed in a part of MHC class II-immunoreactive ameboidal microglia. The induction of MHC class II in the ameboidal microglia may be required to induce iNOS in brain *in vivo*. (19). Also, reactive microglia that express MHC class II have been observed phagocytosing degenerated neuronal elements in Alzheimer's disease, Parkinson's disease, acquired immunodeficiency syndrome (AIDS), and some other neuronal degenerative disorders (22, 23). Thus, MHC class II-immunopositive reactive microglia may play a key role in neurodegeneration.

NO-induced expression of HO-1 in microglia and astrocytes

In *in vitro* culture, LPS-treatment induced enhancement of HO-1 protein in both microglia and astrocytes following iNOS induction in microglia, although HO-1 protein was present in some microglia under normal culture conditions. LPS-induced enhancement of HO-1 levels was inhibited by a NOS inhibitor such as N^G-nitro-L-arginine (NNA). In addition, NO donor such as *S*-nitroso-*N*-acetylpenicillamine (SNAP) enhanced the HO-1 level in both microglia and astrocytes (24). These results suggest that NO produced by iNOS in microglia causes autocrine- and paracrine-induction of HO-1 protein in microglia and astrocytes (Fig. 2). On the other hand, since microglia were activated to the ameboid type by cultivation, HO-1 protein might be induced by another activation pathway in normal culture.

The 5'-flanking region of the rat HO-1 gene was cloned from a rat liver genomic DNA library, which suggests that the promoter region of the rat HO-1 gene involves the metal-response element (MRE), the heat-shock element (HSE), the activator protein-1 (AP-1), etc. (25). In addition, recent reports have noted that NO amplified Ca^{2+}-induced activation of cAMP responsive element binding protein (CREB) and resulted in c-Fos induction and AP-1 activation in rat PC12 cells (26), and that oxidative stress activated metal-responsive transcription factor-1 (MTF-1) in an *in vitro* system (27). These observations suggest that NO-induced HO-1 expression may be mediated by activation of AP-1 and/or MTF-1. Further studies of the detailed mechanisms of HO-1 induction are necessary before definite conclusions can be made.

Possible functions of iNOS and HO-1 in brain damage

Since iNOS is activated in a Ca^{2+}/CaM-independent manner, massive NO was produced (3, 12, 14). In addition, NO causes neurotoxicity (8), and it also induces *S*-nitrosylation and ADP-ribosylation of cysteine residues in several enzymes, which results in a reduction in enzyme activity (28, 29, 30).

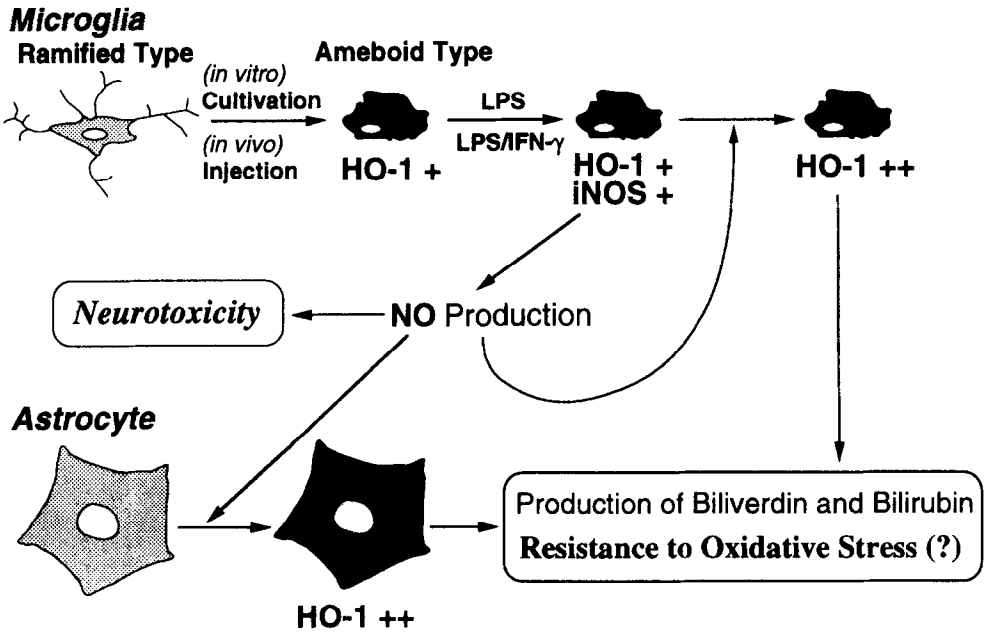

FIG. 2

Possible mechanisms of induction of iNOS and HO-1 in rat glial cells. Expression intensity: +, low level; ++, high level.

In contrast to rat HO-2 protein which contains three cysteine residues, rat HO-1 protein lacks a cysteine residue (25), suggesting that HO-1 proteins may not be influenced by NO. Recently, it was suggested that HO also functions as a defense system against oxidative stress, since biliverdin and bilirubin produced locally may act as physiological antioxidants and potent scavengers of oxygen radicals (2). In addition, CO is now suggested to play a role in neurotransmission (7) and in vascular tone regulation (31). In fact, overexpression of HO-1 was associated with marked decreases in cell growth and DNA synthesis, and increased survival in response to hyperoxic oxidant injury (32). These findings suggest that numerous glial cells in which HO-1 protein is induced may be resistant to NO-induced cell toxicity and that neurons near these glial cells are also protected against oxidative stress (Fig. 2). In addition, it is tempting to speculate on the possibility of an interaction between the CO/HO-1 and NO/iNOS systems in the brain under some pathological conditions.

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