EXPRESSION, BUT NOT ACTIVITY, OF NEURONAL NITRIC OXIDE SYNTHASE IS REGIONALLY INCREASED IN THE ALCOHOLIC BRAIN†

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Abstract — The regional distribution of nitric oxide synthase (NOS) was investigated in alcoholic post-mortem brains compared with brains of non-alcoholic control individuals. Total enzyme activity in 28 brain regions was determined using the [3H]-citrulline formation assay, whereas Western blot analyses were used for semi-quantitative measurement of the neuronal isoform of NOS (nNOS). In the alcoholic brain, nNOS protein expression was increased in the following regions: frontal cortex (85%), the cingulate gyrus (294%), the nucleus accumbens (54%), the entorhinal cortex (85%) and the thalamus (51%). These increases were, however, not associated with higher total NOS activity. Interestingly, nNOS protein content was increased in the frontal cortex and the nucleus accumbens, brain regions which are suggested to be involved in the dopaminergic mesolimbic reward system. It is concluded that upregulation of signal transduction pathways, such as the adenosine 3’,5’-monophosphate and the protein kinase C-dependent pathway, due to stimulation of G-protein-coupled neurotransmitter receptor regulation, as a form of functional tolerance, could be responsible for increased nNOS protein expression, and downregulation of NOS enzyme activity in these brain regions.

INTRODUCTION

Nitric oxide (NO) is a small, gaseous messenger molecule with a very short half-life (3–6 s) synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Palmer et al., 1988). NO is freely diffusible across membranes, and thus cannot be stored in vesicles, unlike classical neurotransmitters, and is released by exocytosis upon membrane depolarization. NO seems to be terminated primarily by reaction with its targets. The major target for NO in the central nervous system (CNS) is soluble guanylyl cyclase (sGC), a heterodimeric haemoprotein to which NO binds, thereby triggering the synthesis of the second messenger molecule cyclic guanosine 5’-monophosphate (cGMP) (Murad, 1986). Thus, control of NO synthesis, by NOS, is the primary regulatory mechanism.

Based on molecular, biological, and biochemical criteria, three isoforms of NOS have been identified (Förstermann et al., 1991): neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS), which differ in their subcellular distribution and regulation mechanisms. The constitutive cytosolic nNOS and the membrane-associated eNOS are both calcium (Ca2+)/calmodulin-regulated enzymes, while cytosolic iNOS is independent of Ca2+/calmodulin and is induced by cytokines or endotoxins. In the human brain, a regional pattern of NOS distribution with the highest levels of NOS activity in the substantia innominata, cerebellar cortex, nucleus accumbens, and subthalamic nucleus was demonstrated, whereas the lowest levels were estimated in the corpus callosum, thalamus, occipital cortex, and dentate nucleus (Blum-Degen et al., 1999b). nNOS protein is distributed throughout the brain, in contrast to iNOS and eNOS, which are not detectable (Blum-Degen et al., 1999b). It is likely, therefore, that nNOS is primarily responsible for NOS activity in the human brain.

The biological effects of NO are diverse; in the brain, it serves as a second messenger in neuronal signal transduction following activation of excitatory amino acid receptors, such as the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor (East and Garthwaite, 1991). Some of the emerging roles of NO in the CNS include regulation or control of neuronal morphogenesis, short- or long-term synaptic plasticity, regulation of neurotransmitter release, regulation of gene expression and modification of sexual and aggressive behaviour (Yun et al., 1997). Deregulated formation of NO from all three NOS isoforms is implicated in neural injury in a range of neurological diseases including ischaemic conditions, Parkinson’s disease, and Alzheimer’s disease (Yun et al., 1997; Gerlach et al., 1999). In addition, experimental evidence suggests that NO may play a role in voluntary ethanol consumption (Lancaster, 1995; Rezvani et al., 1995; Calapai et al., 1996) and in neurobiological alterations induced by chronic ethanol consumption during the development of ethanol tolerance, dependence and withdrawal (Khanna et al., 1993; Adams et al., 1995). Finally, NO appears to mediate glutamate neurotoxicity, which may be involved in alcohol-induced brain damage (Lancaster, 1992).

The objective of the present study was to investigate the regional distribution of NOS in alcoholic brains, compared with brains of non-alcoholic control individuals. We estimated total enzyme activity in 28 brain regions using the [3H]-citrulline formation assay, Western blot analyses were used for the semi-quantitative measurement of nNOS protein. Some of the results of this study have been presented in preliminary form elsewhere (Blum-Degen et al., 1999a,c).
MATERIALS AND METHODS

The methods used in this investigation have been reported in detail elsewhere (Gsell et al., 1993; Gerlach et al., 1995; Blum-Degen et al., 1999b).

Collection and dissection of human brain tissue

Brains were provided from the Austro-German Brain Bank in Würzburg. At autopsy, the right hemisphere was routinely removed for neuropathological examination and the left hemisphere was frozen at minus 80°C for biochemical analysis.

Clinical diagnosis and autopsy diagnosis (including cause of death, agony duration, death-to-autopsy time interval, and records of all medications administered within 8 days of death) were obtained from the hospital charts (which included pre-admission medications) and entered into computer data files. Criteria for inclusion in this study were the absence of coma preceding death and the absence of localized clinical brain disease, including brain tumour and stroke. Alcohol consumption histories were obtained in a standardized, systematic way from resident and intern physician’s admitting histories. Alcohol consumption was tentatively classified as: none, social (on special occasions only), moderate (usually daily consumption of less than 3 drinks), or heavy consumption (the cut-off point was 5 drinks or 80 g of absolute alcohol or more daily for at least 10 years). All alcoholics included in this study had a history of documented multiple episodes of intoxication, various types and degrees of alcohol withdrawal illnesses, or in-patient detoxification. Exclusion criteria included use of other drugs and heavy cigarette smoking (more than 10 cigarettes per day).

Control tissue was sourced from diseased subjects without any history of neurological or psychiatric disorders, lacking neuropathological abnormalities, and who never drank or drank on special occasions only. Moderate drinkers, users of other drugs and heavy cigarette smokers (more than 10 cigarettes per day) were excluded.

Tissue preparation

Prior to biochemical measurements of NOS, the left hemispheres were dissected on a cooled Teflon surface (–20°C) to obtain the following brain regions according to the protocol of Gsell et al. (1993): prefrontal cortex, frontal cortex, temporal cortex, parietal cortex, occipital cortex, cingulate gyrus, corpus callosum, hypothalamus, habenula, mammillary bodies, raphe nucleus, locus coeruleus, dentate nucleus, caudate nucleus, putamen, nucleus accumbens, globus pallidus, substantia innominata, nuclei septi, amygdala, entorhinal cortex, hippocampus, substantia nigra, red nucleus, subthalamic nucleus, thalamus, cerebellar cortex, and ventral tegmental area. Tissue from all 28 regions was available for the majority of cases.

Meninges and blood vessels were removed from the frozen brain tissue. Prior to the [3H]-l-citrulline formation assay and Western blot analysis, samples were weighed and homogenized in 5 vol of homogenization buffer (Tris-HCl 50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM) β-mercaptoethanol (12 mM), leupeptin (1 μM), pepstatin A (1 μM), and trasylool (0.2%).

Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. Sample aliquots were stored at –80°C pending biochemical analysis. All tissue samples from the same brain were analysed simultaneously and under identical assay conditions.

Measurement of NOS activity

NOS activity was determined in triplicate in crude homogenates of the 28 brain regions using the formation of [3H]l-citrulline from [3H]l-arginine as previously described (Gerlach et al., 1995).

Western blot analysis

Western blot analysis was performed by a method slightly modified from Gerlach et al. (1995). Briefly 25 μg of crude homogenate were separated on a 6% SDS Tris-glycine gel. The standard for the quantification of nNOS was recombinant rat brain nNOS (22.5 ng lane) purchased from Biomol (Hamburg Germany).

Membranes were blocked with 5% BSA in phosphate-buffered saline and subsequently incubated with the specific monoclonal antibody against nNOS (purchased from Transduction Laboratories, Lexington, KY, USA). The cross-reactivity of the antibody was tested by incubation with two alternative isoforms of NOS. No cross-reactivity was seen using our conditions. After incubation with the secondary antibody (rabbit anti-mouse IgG–horseradish peroxidase conjugate, Amersham, Buchler, Germany) the immune complexes were detected on X-ray films using the ECL (Enhanced Chemiluminescence) Protein Detection Kit (Amersham, Buchler, Germany). Specific bands were semi-quantified using a laser scanner and protein amounts were calculated compared with densitometric values of the corresponding standard.

Statistics

Data are displayed as means ± SEM. Two groups, controls and alcoholics, as defined in collection and dissection of human brain tissue were selected for this study. A comparison between two groups of continuous variables, such as NOS activity and nNOS protein content (alcoholics compared with non-alcoholic controls), was performed using the non-parametric Mann Whitney U test.

RESULTS

Reported data were obtained from brains of 40 subjects: 23 alcoholics (18 men and 5 women) and 17 controls (10 men and 7 women). The mean age, post-mortem intervals and storage time (± SEM) for the alcoholics were 58.7 ± 2.9 years (range: 37–90 years), 18.7 ± 2.7 h (range 3–40.5), and 89.8 ± 8.7 months, while for the controls the mean age, post-mortem intervals and storage time were 73.6 ± 2.5 years (range 55–90 years), 21.0 ± 2.6 h (range 7–48 h), and 38.8 ± 7.5 months. It is apparent that the age was significantly higher in the control group (P = 0.0012 and 0.4944, respectively), whereas the mean storage time was higher in the alcoholic group (P = 0.0019).

Western blot analyses were used for semi-quantitative measurement of nNOS protein content in the 28 brain regions, whereas total enzyme activity was estimated using the [3H]-l-citrulline formation assay. nNOS protein content (Fig. 1) appears to be increased selectively in some brain regions of alcoholics such as the frontal cortex (85%), the cingulate
gyrus (294%), the nucleus accumbens (54%), the entorhinal cortex (85%), and the thalamus (51%). This increase was, however, not associated with a higher total NOS activity (Fig. 2).

**DISCUSSION**

Chronic alcohol consumption can induce alterations in the function and morphology of most, if not all, brain systems and structures. Alcohol affects several brain neurotransmitters including dopamine, γ-aminobutyric acid, glutamate, serotonin, adenosine, noradrenaline, and opioid peptides and their receptors (Swift, 1999). In addition, there is some experimental evidence suggesting that NO plays a role in voluntary ethanol consumption (Lancaster, 1995; Rezvani et al., 1995; Calapai et al., 1996) and in neurobiological alterations induced by chronic ethanol consumption during the development of ethanol tolerance, dependence, and withdrawal (Khanna et al., 1993; Adams et al., 1995). These effects and the association with NO are suggested to be potential targets for drug therapy of alcohol dependence (Lancaster, 1995; Calapai et al., 1996; Adams and Cicero, 1998). Accordingly, NOS inhibitors may prove to be effective in the treatment or prevention of alcohol-induced brain damage. Furthermore, intervention in NO pathways might be beneficial in reducing alcohol consumption and craving behaviour of alcoholics.

Indeed, studies in the rat have demonstrated that NOS inhibition prevents the development of alcohol tolerance (Khanna et al., 1993). Furthermore, the inhibition of NOS is associated with suppression of alcohol consumption in favour of water by otherwise alcohol-preferring rats (Beaugé et al., 1994; Rezvani et al., 1995). It would therefore be of considerable interest to ascertain whether these molecular mechanisms may contribute to the biological actions in man, in particular in terms of whether the NO-generating enzyme NOS is altered in the alcoholic brain.

Our current results demonstrate that the nNOS protein content is increased selectively in some brain regions of...
alcoholics, compared with non-alcoholic controls. We previously reported that NOS activity and nNOS protein content remain unaltered during ageing and that these parameters are independent of post-mortem delay, gender or storage time (Blum-Degen et al., 1999b); therefore, we believe that our results are reliable, although the two groups studied were not matched by sex, age, post-mortem delay or storage time.

Interestingly, nNOS protein content was increased in the frontal cortex and the nucleus accumbens, brain regions which are thought to be involved in the dopaminergic mesolimbic reward system (Koob, 1992). Repeated excessive alcohol ingestion sensitizes this pathway and might lead to the development of addiction (Robinson and Berridge, 1993). In rats, an increase of nNOS protein content has been demonstrated in the nucleus accumbens after chronic alcohol ingestion, while in the frontal-parietal cortex, hippocampus, and striatum, a decrease was reported (Fitzgerald et al., 1995). The molecular mechanisms underlying an increased nNOS protein synthesis in specific brain regions following chronic alcohol consumption are unknown. nNOS is a Ca^{2+}/calmodulin-dependent, and, under most conditions, constitutively expressed, enzyme ( Förstermann et al., 1991 ). However, recent studies indicate that it can be induced (i.e. new protein synthesis) under conditions of stress, injury and cellular differentiation (Meyer, 1995; Zweier et al., 1995). In PC12 cells, it has been shown that ethanol alone had no effect on nNOS expression; however, in the presence of nerve growth factor (NGF), nNOS expression was amplified (Phung and Black, 1999). This increase was eliminated by pre-treatment with staurosporine, suggesting that the effect of alcohol on nNOS expression is mediated by a protein kinase C (PKC)-dependent pathway. Upregulation of signal transduction pathways, such as the adenosine 3′,5′-monophosphate (cAMP) and the PKC pathway, due to stimulation of G-protein-coupled neurotransmitter receptors, has been considered as a form of functional tolerance (Nestler, 1992), and could be responsible for the increased nNOS gene expression.

The increase in nNOS protein concentration is, however, not associated with a higher total NOS enzyme activity. As iNOS and eNOS were not detectable in human brain tissue using our conditions, we suggest that nNOS is primarily responsible for NOS activity in the human brain. Because nNOS protein content is only a minor part of the total protein content, we did not find any differences using total NOS enzyme activity. When activity was calculated as pmol/mg nNOS protein min, however, we were able to demonstrate a reduction (data not shown).

NOS activity also appears unchanged in vivo. In the cerebrospinal fluid of alcohol-dependent subjects the amount of the NO metabolites, nitrite, and nitrate, were not different from those in control subjects (Neiman and Benthin, 1997). Further, NOS activity in various brain regions of mice remained similar to controls following acute (3 g/kg, i.p.) and chronic (33 g/mg/day orally for 3.5 days) administration of ethanol ( Ikeda et al., 1999 ). Naassila et al. (1997) reported, however, that NOS catalytic activity is increased in the striatum, but not in the cerebellum, cortex or hippocampus of rats following chronic alcohol intake (around 20 g/kg/day for 30 days). These findings suggest that the regulation of NO generation might be disturbed in regions where increased nNOS protein is demonstrated. NO synthesis is regulated in a complex manner (Snyder et al., 1998). NOS has recognition sites for calmodulin, NADPH, FAD, and FMN and also utilizes haem and tetrahydrobiopterine as oxidative-reductive cofactors. nNOS is also regulated by phosphorylation. nNOS can be phosphorylated by PKC, Ca^{2+}/calmodulin dependent protein kinase, cAMP-dependent protein kinase, and cGMP-dependent protein kinase. Although PKC phosphorylation clearly inhibits enzyme activity, the effects of phosphorylation by the other enzymes are, as yet, unknown, although they may elicit some inhibition (Snyder et al., 1998). The upregulation of these enzymes following chronic alcoholism might explain why we found no increased NOS activity despite an increased nNOS content.

In summary, the nNOS protein content appears to be selectively increased in some brain regions involved with the dopaminergic mesolimbic reward system. This increase is, however, not associated with a higher total NOS enzyme activity. We suggest that upregulation of signal transduction pathways, possibly as a form of functional tolerance, could be responsible for the increased nNOS protein content, and the down-regulation of total NOS enzyme activity in these brain regions. Further studies are necessary to investigate the molecular mechanisms underlying the different NOS regulations during chronic alcoholism for understanding the rewarding and sensitizing effects of alcohol.

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