S100β Stimulates Inducible Nitric Oxide Synthase Activity and mRNA Levels in Rat Cortical Astrocytes*

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The glia-derived, neurotrophic protein S100β has been implicated in development and maintenance of the nervous system. However, S100β has also been postulated to play a role in mechanisms of neuropathology, because of its specific localization and selective overexpression in Alzheimer's disease. To begin to address the question of whether S100β can induce potentially toxic signaling pathways, we examined the effects of the protein on nitric oxide synthase (NOS) activity in cultures of rat cortical astrocytes. S100β treatment of astrocytes induced a time- and dose-dependent increase in accumulation of the NO metabolite, nitrite, in the conditioned medium. The S100β-stimulated nitrite production was blocked by cycloheximide and by the NOS inhibitor N-nitro-L-arginine methyl ester, but not by the inactive D-isomer of the inhibitor. Direct measurement of NOS enzymatic activity in cell extracts and analysis of NOS mRNA levels showed that the NOS activated by S100β addition is the calcium-independent, inducible isoform. Furthermore, the specificity of the effects of S100β on activation of NOS was demonstrated by the inability of S100α and calmodulin to induce an increase in nitrite levels. Our data indicate that S100β can induce a potent activation of inducible NOS in astrocytes, an observation that might have relevance to the role of S100β in neuropathology.

The normal development and maintenance of the brain involves the temporal and spatial coordination and proper functioning of a number of intracellular and cell-cell signaling events, and the contribution of glial cells to these signaling processes is becoming more widely appreciated. The classical concept of the role of glia in brain function is rapidly changing with newer evidence of the crucial nature of these cells in controlling neurotransmitter levels, maintaining calcium homeostasis, and synthesizing and releasing neurotrophic and growth factors (for review, see Ref. 1). One such glia-derived factor is S100β, a protein that promotes neuritic outgrowth of specific neuronal populations (e.g. cortical (2, 3), dorsal root ganglia (4), serotonergic (5, 6), and motoneurons (7)) and enhances survival of neurons during development (7, 8) and after insult (9). S100β is also a glial mitogen, inducing phosphoinositide hydrolysis, increases in intracellular calcium, and protooncogene expression (10, 11). These trophic functions require nanomolar concentrations of a disulfide-linked S100β dimer (see Ref. 12). Thus, S100β may be beneficial during development of the nervous system, and increased S100β expression and secretion following acute glial activation in response to central nervous system injury may be one mechanism the brain uses in attempts to repair injured neurons.

However, S100β may also reach concentrations that are deleterious, e.g. in neurodegenerative diseases like Alzheimer's disease and Down syndrome where chronic glial activation occurs (13). It has been found that S100β levels in severely affected brain regions of Alzheimer's disease patients are severalfold higher than in age-matched control samples (13, 14), that S100β is increased selectively in regions that exhibit the most neuropathological involvement (15), and that S100β overexpression is correlated with the prevalence of neuritic plaques (16). These data raise the possibility that high concentrations of S100β may be detrimental, an idea supported by the recent demonstration that an S100 isoform can induce apoptosis in PC12 cells through sustained increases in intracellular calcium (17, 18).

As a first approach to addressing the question of whether S100β can induce potentially toxic signaling pathways, we examined the effect of S100β on nitric oxide (NO) generation. NO is synthesized by the enzyme nitric oxide synthase (NOS)1 through the conversion of L-arginine to L-citrulline. In the nervous system, NO has been implicated in the regulation of cerebral blood flow, synaptic plasticity, and cell growth (see Ref. 19). A large amount of data also supports the concept that NO production in the central nervous system may be involved in the neuropathology associated with ischemia, traumatic insults, and neurodegeneration (see Refs. 19–21). We report here that treatment of rat cortical astrocytes with S100β results in a stimulation of NOS activity and generation of NO.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Calmodulin and S100α were purified as described previously (22, 23). Bovine S100β was expressed from a synthetic gene in Escherichia coli (24). Purification was achieved by DEAE anion exchange chromatography followed by calcium-dependent phenyl-Sepharose chromatography as described previously (11). Elution of S100α from phenyl-Sepharose was done with buffer containing 20 mM Tris-Cl, 500 mM NaCl, 1 mM EGTA, pH 7.4 (elution buffer). For experiments with tissue-isolated protein, S100β was isolated from bovine brain (Pel-Freez, Rogers, AR) by a similar procedure. In addition, E. coli lysates (lacking the S100 expression vector) were taken through the same purification protocol. The purity of the S100β was analyzed by electrophoresis on 15% acrylamide-SDS minigels (Idea Scientific, Minneapolis MN) in the absence of reducing agents. S100β was stored at 20°C in storage buffer (elution buffer plus 4 mM CaCl2). Protein concentrations were determined by the method of Lowry et al. (25) using bovine serum albumin as standard or by amino acid analysis as the abbreviations used are: NOS, nitric oxide synthase; LPS, lipopolysaccharide; L- and D-NAME, N-nitro-L- and (D)-arginine methyl ester; PBS, phosphate-buffered saline.

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L-NAME. Reactions were terminated by adding 2 ml of 20 mM HEPES, 2544 dithiothreitol, 0.32M sucrose). Cells were homogenized by brief sonication and trypsinized, and cells were passed through a 136-
Carthy(27). Briefly, the cerebralcortex fromoneratpupwasdissected homogenization buffer containing 200
arginine. Aliquots of the unbound material (300-
DowexAG50W-X8(Na
microcentrifuge. The resulting supernatant was passed through a
agarose-formaldehyde gel, transferred to Hybond-N membranes (Am-
scintillation counting and was expressed as the radioactivity (cpm/

Fig. 1. Time course of S100β-stimulated nitrite accumulation in rat astrocyte cultures. Cells were incubated with 40 μg/ml S100β (squares) or control storage buffer alone (circles) for the indicated times, and nitrite concentrations in the conditioned media were determined. Data shown are from one of three independent experiments, each done in triplicate. Values shown are the mean ± S.E. from the triplicate determinations. Error bars are shown only when larger than the symbol. S100β-stimulated nitrite accumulation was significantly different from control values (p < 0.05) at 24-, 48-, and 72-h treatment times.

RESULTS
The ability of S100β to stimulate NO production was determined initially by measuring the accumulation of nitrite (a stable NO metabolite) in the conditioned medium of astrocytes following exposure to S100β. Tertiary cultures of neonatal rat cortical astrocytes were prepared as described under “Experimental Procedures.” These cultures contained approximately 98% astrocytes, as assessed by positive staining for the astrocyte intermediate filament protein, GFAP (data not shown). This result was consistent with the observation that only 2–3% of the cells showed positive staining for OX-42 (data not shown), a marker for microglia (33). Fig. 1 shows the time course of nitrite accumulation following exposure of these cells to S100β (40 μg/ml S100β for 6–72 h). S100β-treated astrocytes showed increased nitrite accumulation in their conditioned medium compared to control cultures treated with storage buffer alone. The increased nitrite levels were evident after a 12 h exposure of cells to S100β and reached a plateau at about 48 h. The maximal level of nitrite accumulation in response to S100β was 3–4-fold higher than that generated by exposure of
cells to buffer alone.

The response to S100β was dose-dependent in the range of 1–40 μg/ml S100β, assayed after 48 h treatment of cells (Fig. 2). The concentration of S100β required to elicit 50% of the maximal nitrite increase was 20–25 μg/ml, and the maximal response was achieved by 35–40 μg/ml S100β.

To test whether the S100β-induced nitrite accumulation was dependent on NOS activity, we examined the effect of S100β in the presence of the NOS inhibitor, L-NAME. Incubation of cells for 48 h with S100β in the presence of L-NAME almost completely abolished the nitrite accumulation, whereas the inactive inhibitor isofrom, D-NAME, was ineffective (Fig. 3). The basal level of nitrite accumulated during 48 h was also affected by L-NAME but not D-NAME.

Incubation of cells with S100β in the presence of the protein synthesis inhibitor cycloheximide prevented the S100β stimulation of nitrite accumulation (Table I). Moreover, the specificity of the response to S100β was examined by comparing the ability of two other related calcium binding proteins, S100α and calmodulin, to enhance nitrite accumulation. Table I shows that neither S100α nor calmodulin stimulated nitrite accumulation, under conditions where the LPS positive control induced nitrite accumulation to a similar extent as S100β. To exclude the possibility that the S100β stimulation of nitrite accumulation was a result of contaminating LPS in the recombinant S100β preparation, we isolated S100β from bovine brain tissue and we took an E. coli lysate lacking the S100 expression vector through the same purification protocol as S100. We found that bovine brain S100β induced nitrite accumulation to a similar extent as recombinant S100β, and that the E. coli lysate showed no induction above the buffer control (data not shown).

The ability of S100β to enhance nitrite accumulation that was inhibited by NOS inhibitors suggested an activation of NOS enzyme activity. To test this directly, we examined NOS enzyme activity in cytosolic extracts of astrocytes treated with S100β for 24 h, by measuring the conversion of L-arginine to L-citrulline. As shown in Fig. 4, S100β treatment of astrocytes resulted in a 4-fold stimulation of NOS activity. The S100β-evoked NOS activity was independent of the presence of calcium in the enzyme assay, suggesting that the NOS stimulated by S100β is the inducible enzyme (iNOS). This was further confirmed by the demonstration that S100β-treated astrocytes showed increased levels of iNOS mRNA, as measured by Northern blot analysis (Fig. 5).

**DISCUSSION**

We have demonstrated that treatment of astrocytes with S100β enhances NOS activity and release of NO, as demonstrated by the increase in accumulation of the stable NO metabolite nitrite in the conditioned medium. Measurement of
Fig. 5. S100β stimulation of iNOS mRNA. Cells were incubated for 24 h with S100β (40 μg/ml) or control storage buffer, and iNOS mRNA levels were determined by Northern blot analysis of 10 μg total RNA, as described under “Experimental Procedures.” The blot was re-probed with cyclophilin cDNA, and the iNOS mRNA levels were expressed as the ratio of density of iNOS mRNA versus cyclophilin mRNA. Inset shows the iNOS mRNA bands from control (C) versus S100β-treated (S) astrocytes.

NOS enzyme activity and mRNA levels confirm that the NOS stimulated by S100β addition is the calcium-independent inducible iNOS isoform. The specific NOS inhibitor L-NAME, but not the inactive d-NAME, suppressed the S100β-induced NOS activity. Moreover, the specificity of the effects of S100β on iNOS activation was demonstrated by the inability of S100α or calmodulin to induce an increase in NO. These data demonstrate that S100β induces a stimulation of astrocytic iNOS activity and generation of NO.

The NOS enzyme responsible for synthesizing NO from arginine exists in three major forms: two constitutive cNOS isoforms (eNOS and nNOS) that are calcium/calmodulin dependent and present in a variety of cells, including endothelial cells, neurons, platelets, and astrocytes; and an inducible form (iNOS) that is calcium-independent and expressed after gene induction in a variety of cells, including macrophages, endothelial cells, and astrocytes (see Ref. 34). Induction of iNOS in astrocytes has been well documented previously by exposure of cells in vitro to bacterial endotoxin (LPS) or combinations of cytokines, such as interleukin-1β, interferon-γ or tumor necrosis factor-α (1, 35). Our data represent the first report that S100β is another potent inducer of astrocytic iNOS activity. The S100β-stimulated NO production exhibits similar characteristics to those reported for induction by LPS or other cytokines in terms of long (hour) time frame and narrow dose-response curve (31, 35). The response occurs over several hours of continuous application of S100β, and nitrite concentrations continue to increase up to 48 h after S100β exposure. Similar to IL-1β (35), the effect of S100β on iNOS activity was dose-dependent through a small concentration range. The magnitude of the nitrite accumulation in response to S100β also varied somewhat among cultures and among S100β preparations, suggesting that endogenous coordinators or specific S100β conformational states might be required for maximal S100β induction of iNOS.

We considered the possibility that our results might reflect an activation of NOS activity in microglia, rather than astrocytes, because microglia express high levels of iNOS activity (36). However, this possibility seems unlikely because our terry cultures are ~98% astrocytes, as determined by GFAP and OX-42 immunoreactivity, and because the intensity of the iNOS mRNA on Northern blots and the iNOS enzyme activity from cytosolic extracts are too high to reflect a signal from only 2% of the cells in the culture. Thus, our data support an S100β-induced activation of iNOS activity in astrocytes.

The mechanisms involved in activation of iNOS by S100β addition are unknown at present. Our observation that S100β stimulates iNOS mRNA levels suggests that regulation may be at the level of transcription of the iNOS gene, as has been shown previously for iNOS stimulation by LPS and interferon-γ (34, 37, 38). However, iNOS has also been found to be regulated at the level of mRNA and protein stability (39, 40). Definition of the mechanisms by which S100β induces iNOS activity, and whether S100β interacts with other cytokines to modulate iNOS activity awaits further investigation.

The consequences of S100β-induced NO release from astrocytes are not known. There is a wealth of sometimes conflicting evidence in the literature that NO can be beneficial or detrimental to nervous system function (see Refs. 19 and 21). For example, it has been found that NO can act as a diffusible messenger to mediate cell-cell signaling pathways involved in synaptic plasticity and regulation of cerebral blood flow. However, there is also a large amount of biochemical and pharmacological data to suggest that NO is involved in the neuropathology associated with traumatic or ischemic insults, autoimmune diseases, and neurodegenerative disorders. Relevant to our studies, it has been reported (41) that stimulation of astrocytic iNOS activity and release of NO leads to enhanced NMDA receptor-mediated neurotoxicity. We are currently pursuing studies with astrocytic/neuronal co-cultures to evaluate the effects of S100β-stimulated NO release on the neuronal cell. In this regard, it is interesting to note that in some experiments, the S100β-treated astrocytes exhibited morphological changes consistent with toxicity, such as release of lactate dehydrogenase and cell rounding and detachment from the substrate (data not shown). The reason for this observation has not been defined yet, but appeared to correlate with the levels of nitrate accumulation in response to S100β. It is probable that the toxic potential of NO in vivo depends, at least in part, on the concentration released from cells. However, the biology of NO is complex and the susceptibility of a cell to NO-mediated toxicity likely depends on a number of variables, such as acute versus chronic exposure to NO, the array of redox forms in which NO can exist, availability of reactive oxygen species and anti-oxidant defenses, changes in ion homeostasis, presence of other cytoprotective or cytotoxic agents, and the immune status of the organism.

Our data demonstrate that treatment of astrocytes with S100β results in a potent induction of iNOS activity and NO production. Stimulation of iNOS required micromolar concentrations of S100β, unlike the nanomolar concentrations required for neurotrophic and mitogenic effects. The requirement of higher concentrations of S100β for activation of iNOS suggests that S100β might possess dual roles in regulation of cell function, being beneficial to cells at low doses and detrimental at higher doses. If S100β stimulates astrocytic iNOS activity in vivo, this might be relevant to the role of S100β in neurodegenerative disorders like Alzheimer’s disease and Down syndrome, where S100β levels are increased severalfold in temporal lobe samples compared to age-matched control samples (13). It has been found that S100β levels are elevated in specific brain regions from Alzheimer patients (15), that the overexpression of S100β correlates with the pattern of regional neuropathology and neuritic plaque involvement (15, 16), and that S100β is localized primarily in activated astrocytes surrounding neuritic plaques (15). It should also be noted that the local concentration of S100β in astrocytes surrounding neuritic plaques in Alzheimer’s disease has not been determined, although the tissue levels are in the micromolar range (15). The relationship between increased S100β levels and neuropathology is not known. It may be that the up-regulation of S100β after acute central nervous system injury or perhaps in neurodegenerative disease is part of a compensatory response the brain uses in attempts to repair injured neurons, through an action of S100β in its neurotrophic and neuroprotective roles.
However, the chronic overexpression of S100\textsuperscript{b} or expression of S100\textsuperscript{b} above some threshold level as in Alzheimer’s disease may have deleterious consequences leading to neuronal dysfunction and eventual death, perhaps through altered calcium homeostasis or inappropriate neuritogenesis. Our data that S100\textsuperscript{b} can stimulate astrocytes to produce NO provide another possible mechanistic scenario by which the high levels of S100\textsuperscript{b} seen in Alzheimer’s disease and other neurodegenerative disorders might contribute to neuropathology.

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