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Inhibition of Astroglial Nitric Oxide Synthase Type 2 Expression by Idazoxan

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ABSTRACT

Binding of idazoxan (IDA) to imidazoline receptors of the I_2 subtype in astrocytes influences astroglial gene expression as evidenced by increased expression of glial fibrillary acidic protein and mRNA. To determine whether IDA affected glial inflammatory gene expression, we tested the effects of IDA on astroglial nitric oxide synthase type-2 (NOS-2) expression. NOS-2 was induced in primary rat astrocytes and C6 glioma cells by incubation with 1 μg/ml lipopolysaccharide (LPS) plus three cytokines (tumor necrosis factor-α, interleukin-1β, and interferon-γ) or three cytokines alone. Cells were incubated with 1–100 μM IDA, and at 24 h NOS-2 expression assessed. In astrocytes and C6 cells, preincubation with IDA dose-dependently inhibited nitrite accumulation (IC_{50} ~25 μM), accompanied by a reduction in NOS-2 protein levels and L-citrulline synthesis activity in cell lysates. IDA also inhibited nitrite production in LPS stimulated RAW 264.7 macrophages. In astrocytes, but not C6 cells, longer preincubation times with IDA yielded significantly greater suppression, and maximal suppression (>90%) was achieved after a 8 h preincubation in 100 μM IDA. The degree of inhibition was diminished whether IDA was added after LPS plus cytokine mixture. In contrast to NE, continuous incubation with IDA was required to achieve suppression. IDA reduced induction of NOS-2 protein levels, steady state NOS-2 mRNA levels, and activity of a NOS-2 promoter construct stably transfected in C6 cells. These results show that IDA inhibits NOS-2 activity and protein expression in glial cells and macrophages, and suggest that this occurs by decreasing transcription from the NOS-2 promoter.

Incubation of primary astrocytes or of C6 glioma cells with bacterial endotoxin lipopolysaccharide (LPS) and/or a combination of cytokines nominally including interleukin-1 (IL-1) induces de novo expression of nitric oxide synthase type 2 (NOS-2) (Galea et al., 1992; Hewett et al., 1993; Lee et al., 1993; Simmons and Murphy, 1993; Feinstein et al., 1994). Regulation of NOS-2 expression is primarily achieved at the level of gene expression, because the activity of this enzyme is independent of intracellular calcium concentration. A role for NO produced by the NOS-2 enzyme has been demonstrated in the pathology and symptoms associated with several neurological conditions, including multiple sclerosis (Bo et al., 1994) and cerebral ischemia (Iadecola, 1997; Galea et al., 1998). We have previously shown that incubation of astrocytes with neurotransmitters such as norepinephrine (NE) or dopamine significantly attenuate NOS-2 induction (Feinstein et al., 1993; Feinstein, 1998). These neurotransmitter also activate adenylate cyclase, elevate intracellular cAMP levels, and increase expression of glial fibrillary acidic protein (GFAP) (Goldman et al., 1984), a marker of astroglial cell maturation.

Idazoxan (IDA) is an imidazoline derivative originally shown to be an antagonist at *alpha*-2 adrenergic receptors (ARs). However, IDA also binds to a class of binding sites termed imidazoline receptors (I-receptors or IRs) that can be found both in the cytoplasmic membrane (I_1-receptors) as well as within the mitochondrial membrane (I_2-receptors). These two subclasses are described based on their affinities for several imidazolines and are differentially expressed in mammalian tissues (Regunathan and Reis, 1996a). Although the function of the IRs is not fully established, it has been shown that 1) binding of imidazolines to IRs, but not to ARs, mediates the hypotensive effects of this class of drugs (Regunathan and Reis, 1996a); 2) treatment of rats with IDA protects the brain from focal cerebral ischemia (Gustafson et al., 1990; Maiese et al., 1992); and 3) IDA inhibits proliferation of vascular smooth muscle cells (Regunathan et al., 1996b). Both in vitro binding (Regunathan et al., 1993) and in vivo immunohistochemical studies have localized IRs in astrocytes (Ruggiero et al., 1998), suggesting that some of IDA effects may be mediated by this cell type. We have previously reported that primary astrocytes express functional I_2-

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ABBREVIATIONS: NOS, nitric oxide synthase; IL, interleukin; IFN, interferon; CM, cytokine mixture; GFAP, glial fibrillary acidic protein; IDA, idazoxan; NE, norepinephrine; CAT, chloramphenicol acetyl transferase; LPS, lipopolysaccharide; ARs, adrenergic receptors; IRs; imidazoline receptors (I-receptors); LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

receptors since incubation with IDA increases GFAP expression (Regunathan et al., 1993). Systemic administration of similar imidazoline agents binding to I_2 sites also increases astroglial GFAP expression in rat brain (Olmos et al., 1994; Alemany et al., 1995). These results demonstrated that IDA, as the case for some neurotransmitters, could modify astrocyte gene expression. We therefore tested the possibility that IDA, as found for NE, could also modify induction of inflammatory gene expression (NOS-2) in these cells.

Materials and Methods

Reagents. Cell culture reagents [Dulbecco's modified Eagle's medium (DMEM)], antibiotics, and LPS (*Salmonella typhimurim*) were from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Atlanta Biologicals, Inc. (Norcross, GA). Recombinant human tumor necrosis factor-α (10^7 U/mg) was from Genzyme Corp. (Cambridge, MA). Recombinant human IL-1β (10^7 U/mg) was from the NIH human reagents program. Recombinant rat interferon-γ (IFN-γ) (4×10^6 U/mg) and geneticin were from GIBCO (Gaithersburg, MD). Peroxidase-conjugated goat secondary antibodies were from Vector Labs (Burlingame, CA). Rabbit polyclonal antibody against NOS-2 was from Chemicon International, Inc. (Temecula, CA). IDA was obtained from Research Biological International (Natick, MA), dissolved in sterile water, and added directly to cells from a 10-mM stock solution. A lactate dehydrogenase (LDH) assay kit was from Promega (Madison, WI).

Cell Culture. Primary astrocytes were prepared from cerebral cortices of postnatal day 1 Sprague–Dawley rats (Galea et al., 1992). These cultures consist of greater than 95% astrocytes as determined with antibodies to the astrocyte specific marker GFAP, and between 1–3% microglial cells. C6 cells (Dr. C. Naus, University of Ontario, London, Canada) were passaged once per week, and maintained in DMEM with 10% FCS. The mouse macrophage RAW 264.7 cell line was a gift of Dr. Carl Nathan (Department of Medicine, Cornell University Medical College, Ithaca, NY) and was maintained in DMEM with 10% FCS. The stable cell line C6-p1, which has a 1588-bp mouse NOS-2 promoter driving expression of the bacterial chloramphenicol acetyl transferase (CAT) reporter gene (Xie et al., 1993), was maintained by passaging in DMEM plus 10% FCS containing 1.2 mg/ml Geneticin. For experiments, transfected lines were passaged into 6 or 12 well plates in the absence of Geneticin, and used within 3 to 4 days.

NOS-2 Induction Protocol. The growth media was removed, the cells washed once in serum free media, and then NOS-2 inducers added in fresh serum free media. Astrocytes were incubated with 1 μg/ml LPS plus a three-cytokine mixture (CM; 20 U/ml IFN-γ, 2 ng/ml IL-1β, and 10 ng/ml tumor necrosis factor-α). NOS-2 was induced in C6 cells and the C6-p1 transfected cells with 1 μg/ml LPS plus 20 U/ml IFN-γ that gives comparable induction in C6 cells as does LPS plus CM. RAW 264.7 cells were incubated with 1 μg/ml LPS. After overnight incubation with LPS, cytokines, and IDA, an aliquot of the cell culture media was assayed for LDH activity. In all cases the extracellular LDH levels amounted to <5% of total (intracellular plus extracellular) LDH activity, and this amount was not affected by overnight incubation with up to 100 μM IDA.

NOS Activity Measurements. NOS-2 activity was indirectly assessed by accumulation of NO_2 in the cell culture media, from 18 to 48 h after addition of inducers. An aliquot of the media (100 μl) was mixed with 50 μl of Griess reagent (Green et al., 1982), incubated 5 min at room temperature, and the absorbance at 546 nm determined. Solutions of $NaNO_2$ diluted in DMEM served as standards. The absorbance readings due to incubation of cells in DMEM alone were subtracted from sample values. Activity is calculated as nmol of NO_2 accumulated per 24 h per mg of total cellular protein. NOS-2 activity was measured directly by the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline (Galea et al., 1992). After 24 h incubation, cell lysates were prepared from astrocytes by homogenization in 50 mM Tris-Cl (pH 7.5). Assays were carried out in the presence of saturating levels of all NOS-2 cofactors (NADPH, FMN, FAD, BH_4), 10 μM L-arginine, and 2 mM EGTA to inhibit any calcium dependent activity.

Immunoblot Analysis. After overnight incubation with LPS plus IDA, astrocytes were collected in PBS, and total cellular protein prepared by sonication in 8 M Urea. Samples were diluted into 2% SDS, 62 mM Tris-Cl (pH 6.8), 10% 2-mercaptoethanol, and 25% glycerol, boiled, and aliquots (containing 20 μg protein) separated through 7.5% polyacrylamide gels. The proteins were electrotransferred to polyvinylidene difluoride membranes, blocked with 5% dry milk, incubated overnight at 4°C with primary antibody (1:2000 dilution of rabbit anti-NOS-2 antibody, Chemicon), washed, horseradish peroxidase-labeled anti-rabbit IgG added, and after washing bands visualized with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

RNA Analysis. Total cytoplasmic RNA was prepared from C6 cells with TRI Reagent (Sigma Chemicals, St. Louis, MO) and levels of NOS-2 mRNA determined by RT-PCR assay as described (Galea et al. 1998). In brief, 1 μg of RNA was converted to cDNA using random hexamer primers and reverse transcriptase, and then an aliquot corresponding to 50 ng starting RNA amplified by PCR for levels of NOS-2 cDNA. The primers used for NOS-2 detection were 1704F (5′ CTGCATGGAACAGTATAAGGCAAAC-3′), corresponding to bases 1704–1728; and 1933R (5′ CAGACAGTTTCTGGTCGATGTCATGA-3′), complementary to bases 1908–1933 of rat NOS-2 cDNA sequence (Galea et al. 1994). PCR conditions were 35 cycles of denaturation at 93°C for 35 s, annealing at 63°C for 45 s, and extension at 72°C for 45 s; followed by 10 min at 72°C.

CAT Assays. After 18–24 h incubation in the presence of NOS-2 inducers, cells were washed twice in cold PBS, and 500 μl lysis buffer (Promega) added. Cell extracts were heated at 60°C to inactivate endogenous CAT activities. A 10–50-μl aliquot of cell lysate (containing 20–100 μg protein) was incubated at 37°C for 1–2 h in the presence of 12 μM [^{14}C]chloramphenicol (Amersham, 54 mCi/mmol; 25 μCi/μl) and 0.2 mg/ml *n*-butyryl CoA. The reaction was halted by addition of 300 μl of mixed xylenes, centrifuged, the organic phase back-extracted once with 300 μl of 250 mM Tris-Cl (pH 8.0), and the amount of radiolabeled product recovered in the organic phase determined by liquid scintillation counting. Activity is presented as nmol of acetylated chloramphenicol produced per h per mg of cell lysate protein.

Data Analysis. All experiments were done at least in triplicate, and mean ± S.E.M. determined. Statistical significance was assessed by one-way analysis of variance followed by Fisher's post hoc comparison tests, and P values $< .05$ were considered significant.

Results

Incubation of primary astrocyte cultures with bacterial endotoxin LPS plus a CM induces maximal expression of NOS-2 (Galea et al., 1992; Hewett et al., 1993; Lee et al., 1993; Simmons and Murphy, 1993; Feinstein et al., 1994). When cells were incubated with IDA, the NOS-2 activity, as assessed by nitrite accumulation in the culture media, was dose-dependently inhibited (Fig. 1A). Longer preincubation times with IDA before the addition of NOS-2 inducers resulted in a greater attenuation of nitrite accumulation. When present 30 min before NOS-2 inducers, significant inhibition was only observed at 100 μM IDA. When present for 4 h before NOS-2 inducers, both 25 and 100 μM IDA reduced NOS-2 expression by >50%. Complete inhibition of NOS-2 activity was attained when cells were preincubated for 8 h with 100 μM IDA. The IC_{50} for IDA was ~25 μM when either a 4 or 8 h preincubation was used. Twenty four hour incuba-

tion with up to 100 μM IDA, in the presence or absence of LPS plus CM, did not increase extracellular LDH levels, consistent with previous observations (Regunathan and Reis, 1996), thus ruling out the possibility that decreased nitrite accumulation was due to cytotoxic effects on the cells.

Incubation with IDA also blocked LPS plus IFN-dependent NOS-2 induction in rat C6 glioma cells, which express the same NOS-2 as primary astrocyte cultures (Feinstein et al., 1994) (Fig. 1B). IDA appeared to be more potent in blocking C6 cell than primary astrocyte NOS-2 expression, because a 30-min preincubation with 25 μM IDA significantly reduced C6, but not astrocyte NOS-2 expression. Increasing the preincubation time of C6 cells with IDA to 4 h afforded only small increases in the potency of inhibition, although a slight, but not significant effect was observed as low as 10 μM IDA. Inhibition of nitrite accumulation by IDA was not cell specific, because IDA at comparable concentrations significantly blocked nitrite production in LPS stimulated RAW 264.7 macrophage cells (to 58 ± 3% and 23 ± 5% of control values at 10 and 50 μM, respectively, $n = 3$, $P < .05$ versus no IDA).

To determine whether decreased nitrite levels were directly a consequence of decreased NOS-2 protein levels, and not to reduced NOS-2 substrate or cofactor levels, or decreased NO breakdown, we directly measured NOS-2 activity. Whole cell lysates prepared from astrocytes incubated

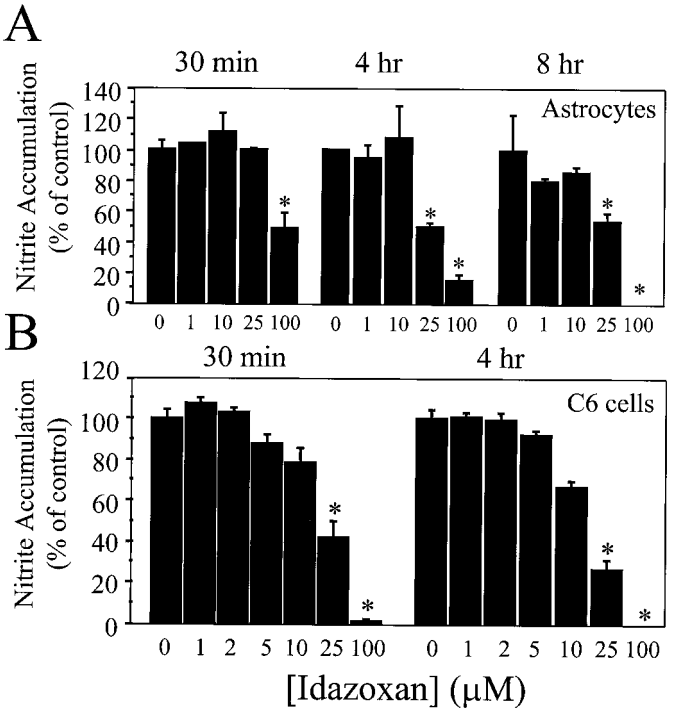

Fig. 1. Effect of IDA on nitrite accumulation in glial cells. Primary astrocytes (A) or C6 glioma cells (B) were incubated with the indicated concentrations of IDA in DMEM for either 30 min, 4 h, or 8 h (astrocytes only). At that time, NOS-2 expression was induced by the addition of LPS plus CM (astrocytes) or LPS plus IFN (C6 cells) and expression was assessed indirectly by the measurement of nitrite in the cell culture media 18–24 h after. Data are presented as relative nitrite levels measured in the presence of IDA compared with that in obtained in the absence of IDA (control cells) and is mean ± S.E.M. of three to six independent experiments. *$P < .05$ versus control cells. NOS-2 activity in control astrocytes was 64 ± 9 nmol nitrite produced per 24 h/mg of protein; in C6 cells it was 80 ± 10 nmol nitrite produced/24 h/mg of protein.

18 h with LPS plus CM carried out conversion of L-arginine to L-citrulline (940 ± 50 pmol per mg protein per h). In lysates prepared from cells incubated overnight with LPS plus CM and 100 μM IDA, the conversion of L-arginine to L-citrulline was reduced >6-fold (160 ± 25 pmol per mg protein per h). The presence of IDA (100 μM) in the L-citrulline assay had no effect on NOS-2 activity. Immunoblot analysis of whole cell lysates prepared from similarly treated cells revealed the presence of NOS-2 protein in LPS plus CM-treated astrocytes, and a reduced amount present in 100 μM IDA treated cells (Fig. 2). These results demonstrate that IDA reduced the overall amount of NOS-2 protein and functional activity present in astrocytes.

To test whether the continuous presence of IDA was needed to observe inhibition, we carried out pulse experiments in C6 cells (Fig. 3). Whereas the continuous presence of 100 μM IDA caused over 70% inhibition of NOS-2 expression, a 1-h pulse with IDA caused only slight (20%) decrease. This is in contrast to the effects of NE on NOS-2 expression, in which a 1 h pulse of NE blocked over 70% of subsequent NOS-2 expression, slightly less than over 90% inhibition due to continuous incubation with NE.

To determine whether IDA reduced NOS-2 mRNA levels, we carried out RT-PCR analysis of RNA isolated from C6 cells incubated with LPS plus IFN and either 0, 10, or 100 μM IDA (Fig. 4). Low levels of NOS-2 mRNA were detected in control C6 cell RNA, and were increased >10-fold by 4 h incubation with LPS plus IFN. IDA (10 μM) reduced NOS-2 mRNA levels to 60% ($n = 2$) of control values, whereas 100 μM IDA reduced levels to ~50%. These results indicate that IDA reduces the steady state levels of NOS-2 mRNA.

To determine whether IDA influenced the transcriptional activity of the NOS-2 promoter, we tested the effects of IDA in C6 cells (C6-p1 cells) stably transfected with a 1588-bp mouse NOS-2 promoter driving expression of the bacterial CAT reporter gene (Xie et al., 1993; Feinstein, 1998). This cell line has previously been used to assess drug effects on the activity at the NOS-2 promoter, and maintains regulatory features of the endogenous NOS-2 gene. Overnight incubation of C6-p1 cells with LPS plus IFN led to appearance of CAT activity (203 ± 8 pmol acetylated CoA/h/mg/protein, $n = 3$). When coincubated with 100 μM IDA, subsequent CAT

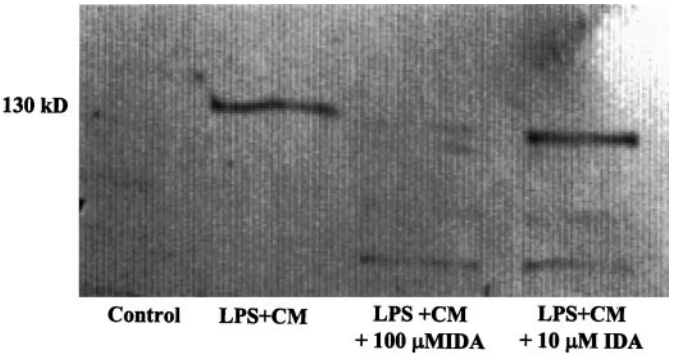

Fig. 2. Effect of IDA on NOS-2 protein expression. NOS-2 expression was induced in astrocytes by overnight incubation with LPS plus CM, and either 0, 10 μM, or 100 μM IDA. The next day total cell lysates were prepared and analyzed by immunoblotting for the presence of NOS-2 protein with a specific antibody against NOS-2 protein. Control cell lysates were prepared from astrocytes incubated overnight without LPS or CM. The experiment shown was repeated two other times with comparable results.

activity was reduced ~5-fold (45 ± 20 pmol acetylated CoA/h/mg protein, $n = 3$, $P < .05$ versus no IDA). These results suggest that IDA influences transcriptional events occurring at the NOS-2 promoter.

Discussion

In this report we demonstrate that incubation of brain-derived glial cells with the imidazoline IDA significantly reduces subsequent NOS-2 activity, protein levels, and promoter activity. We have shown that incubation of astrocytes with 100 μM IDA caused ~50% decrease in nitrite accumulation, presumably as a consequence of reduced NOS-2 expression. Consistent with this finding, we have demonstrated that intracellular NOS-2 activity, as measured by conversion of L-arginine to L-citrulline by whole cell lysates, was reduced ~6-fold when cells were incubated with IDA. This decrease in NOS-2 activity was paralleled by a decrease in NOS-2 protein content, as determined by Western blot analysis, and lower steady-state NOS-2 mRNA levels induced by LPS plus IFN as assessed by RT-PCR, demonstrating that IDA decreased overall NOS-2 protein and mRNA levels. Finally, we have also shown that in stably transfected C6 cells expressing the bacterial CAT gene under control of the mouse NOS-2 promoter, incubation with IDA reduced the LPS plus CM induced promoter activity by 5-fold. Together, these results demonstrate that IDA interferes with overall NOS-2 expression, and suggests that this inhibition is occurring at the level of gene transcription.

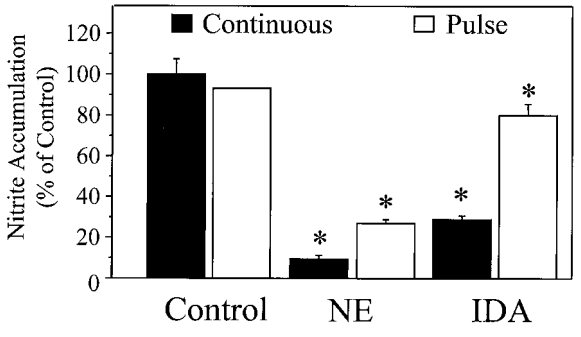

Fig. 3. Effect of incubation time with IDA on nitrite production. C6 cells were incubated for 1 h in DMEM containing NE or IDA (both at 100 μM). At that time, LPS plus IFN was added directly to one set of cells ("continuous") or to a second set from which the IDA or NE had first been washed out ("pulse"). Data are presented as relative nitrite levels measured in the presence of the inhibitor compared with that in obtained in the absence of inhibitor, and is mean ± S.E.M. of two to three independent experiments. *$P < .05$ versus control cells.

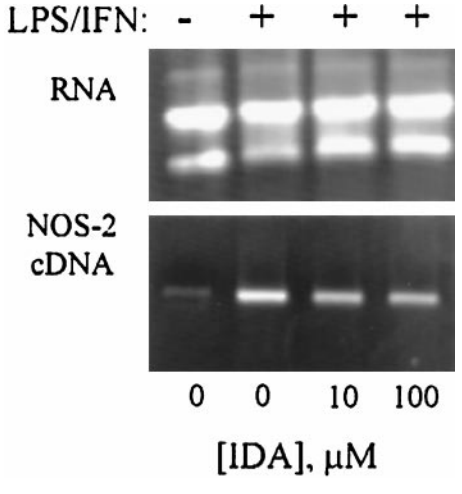

Fig. 4. Effect of IDA on steady-state NOS-2 mRNA levels. C6 cells were incubated with LPS plus IFN, and either 0, 10, or 100 μM IDA. After 4 h, cytosolic RNA was prepared, converted to cDNA, and aliquots assayed for NOS-2 mRNA levels by reverse transcriptase–polymerase chain reaction. Aliquots of the cytosolic RNA (1 μg) were electrophoresed through 1% agarose gel and stained with ethidium bromide to assess RNA quality and amount. The PCR results shown are representative of two separate experiments with comparable results.

The fact that 100 μM IDA only reduced astroglial nitrite production by 50%, despite the large decrease observed in protein levels, may be due to the fact that nitrite determinations measure the accumulation of nitrites over the course of the incubation period (up to 24 h) so that even low levels of residual NOS-2 protein could account for the nitrites produced. Consistent with the large decrease of NOS-2 protein detected by western analysis are the observations that L-citrulline synthesis in astrocyte cell lysates was reduced ~6-fold by IDA, whereas NOS-2 promoter activity in stably transfected C6 cells was reduced ~5-fold. Thus, measurements of accumulated nitrite production overestimate actual NOS-2 expression levels.

In primary astrocytes, but not C6 glioma cells, the inhibition of nitrite accumulation due to IDA was greatly increased after longer (up to 8 h) preincubation times. Because IDA and related imidazolines are highly lipophilic, entrance into the cell is via passive diffusion through cell membranes. Thus, prolonged preincubation times may be necessary to allow sufficient IDA concentrations to become established within astrocytes to mediate intracellular effects. The fact that increasing preincubation times from 30 min to 4 h had little effect on the inhibition of C6 cell NOS-2 expression suggest that IDA more readily enters this cell type. Alternatively, lengthy preincubation times in astrocytes but not C6 cells could be an indication that the intracellular signaling pathways responsible for IDA's effects are more quickly activated in C6 cells than in astrocytes. For example, increased mitochondrial content of C6 glioma cells compared with primary astrocytes could provide a larger number of target sites for the actions of IDA.

The intracellular mechanism by which IDA acts to reduce NOS-2 expression is as yet unknown. However, several observations suggest that it differs from the mechanism responsible for inhibition of NOS-2 expression by NE. Whereas both IDA and NE reduce NOS-2 expression in astrocytes, NE only minimally suppresses RAW cell NOS-2 expression (Feinstein et al., 1993). Secondly, whereas a short incubation with NE is sufficient to reduce subsequent induction of NOS-2 (Feinstein et al., 1993), removing IDA from the incubation media greatly diminished its inhibitory action. That a short pulse of NE is inhibitory could mean that an early event in NOS-2 induction pathway is perturbed by NE. In contrast, the requirement for continuous IDA presence suggests that a later event in NOS-2 induction is perturbed. Although NOS-2 promoter activity was also blocked by IDA incubation, IDA could also interfere with the sustained transcription of the NOS-2 gene. Finally, whereas inhibition by NE is known to be mediated by increased intracellular cAMP levels (Feinstein, 1998), we have shown that IDA does not

modify the intracellular cAMP levels in astrocytes (Regunathan and Reis, 1996). However, because the exact mechanisms of IDA's effects are not yet known, it remains possible that inhibition by IDA and by NE shares common features.

The knowledge that incubation with IDA increases astroglial expression of the intermediate filament protein GFAP (Regunathan et al., 1993), a marker of astroglial differentiation, suggested to us that agents that potentiate the maturation and/or differentiation of glial cells could also exert anti-inflammatory effects on cells. This hypothesis is supported by observations that incubation of astrocytes with NE, which via elevation of intracellular cAMP levels increases GFAP expression (Goldman and Chiu, 1984), also reduces NOS-2 expression in response to cytokines and/or LPS (Feinstein et al., 1994; Feinstein, 1998). These observations suggest that as a consequence of cellular maturation and/or differentiation, cells are rendered refractory to inflammatory activation of, at least, NOS-2 gene expression. We also observed that IDA blocked NOS-2 expression in RAW cells; however whether IDA modifies cellular maturation or differentiation of this cell type as well is not known.

Whether the effect of IDA is mediated by binding to IRs or *alpha*-2 ARs is not known. However, whereas IDA potently inhibits the induction of NOS-2 in astrocytes and C6 cells, only astrocytes express I_2-receptors (Regunathan et al., 1993), suggesting that IDA may be acting by a nonreceptor-mediated mechanism. This is supported by the observations that IDA also reduced NOS-2 expression in the RAW 267.4 macrophage cell line that does not express binding sites for IDA (our unpublished observations). IDA may therefore prove valuable as a non-cell-type selective inhibitor of NOS-2 expression.

To conclude, we have shown that IDA causes inhibition of the induction of NOS-2 expression in response to inflammatory stimuli and thus may offer a novel therapeutic approach for conditions associated with increased NO production. The expression of the NOS-2 protein in brain glial cells has been implicated in the pathogenesis of a variety of neurologic diseases including multiple sclerosis, Alzheimer's disease, cerebral ischemia, and AIDS dementia. Agents that can reduce NOS-2 expression and activity will therefore be of value in the therapeutic treatment or prevention of these diseases. The previous findings that treatment of rats with IDA reduced the damage due to focal brain ischemia (Gustafson et al., 1990; Maiese et al., 1992), in which a role for NOS-2 expression has been described (Iadecola, 1997; Galea et al., 1998), may therefore be related to its ability to block NOS-2 levels.

Acknowledgments

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