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Research Report

The effect of nNOS inhibitors on toxin-induced cell death in dopaminergic cell lines depends on the extent of enzyme expression

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ABSTRACT

Nitric oxide is linked with neurodegeneration in Parkinson's disease (PD) through the involvement of both inducible (iNOS) and neuronal nitric oxide synthase (nNOS). While non-selective NOS inhibitors are neuroprotective, the role of nNOS has not been determined using selective NOS inhibitors. The present study investigated the neuroprotective effect of selective iNOS and nNOS inhibitors on MPP^+- and MG-132-induced cell death in cell lines with differing levels of nNOS expression. Inhibition of endogenously expressed nNOS by 7-NI and ARR17477 enhanced the toxicity of MPP^+ and MG-132 in N1E-115 cells, whereas in transfected SH-SY5Y cells overexpressing nNOS, ARR17477 and 7-NI protected against MPP^+- and MG-132-induced cell death. In contrast, inhibition of iNOS by 1400W was ineffective in preventing MPP^+ and MG-132 toxicity in these cell lines. These results suggest a dual role for NOS in dopaminergic cell viability. nNOS is protective against toxic insult when produced endogenously. When nNOS is overexpressed, it becomes neurotoxic to cells suggesting that inhibition of nNOS may be a promising strategy to prevent cell death in PD.

1. Introduction

Oxidative and nitrative stress have been proposed as key components of the pathogenic process that underlies the loss of nigral dopaminergic neurones in Parkinson's disease (PD) (Jenner, 2003). In brain, NO production can occur as a result of activity of either inducible nitric oxide synthase (iNOS) or neuronal nitric oxide (nNOS) and both isoforms have been linked to cell death in PD (Bolanos et al., 1997; Gatto et al., 2000). iNOS is found in activated glial cells in PD, most notably reactive microglia, and contributes to the inflammatory changes that characterise the pathological process (Hunot et al., 1996; Iravani et al., 2002). nNOS is constitutive and it is present in intrinsic striatal GABAergic neurones and in diffuse terminal fields in the substantia nigra (Egberongbe et al., 1994; Vincent and Kimura, 1992). The role of iNOS in neuronal loss in PD has been extensively studied but there has been relatively little investigation of the involvement of nNOS in recent years.

In PD, there is increased expression of nNOS mRNA in the globus pallidus and subthalamic nucleus in PD (Eve et al., 1998).

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In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, nNOS expression is transiently increased in the substantia nigra (Muramatsu et al., 2003) and nNOS inhibitors, such as 7-nitroindazole (7-NI) or S-methylthiocitrulline, protect against striatal dopamine depletion and loss of tyrosine hydroxylase positive neurones in the substantia nigra (Matthews et al., 1997; Muramatsu et al., 2002; Schulz et al., 1995; Watanabe et al., 2004). Similar protective effects are observed against MPTP-induced cell loss in the striatum of baboons (Hantraye et al., 1996) and against intranigral MPP^+- and 6-OHDA-induced toxicity in rats (Di Matteo et al., 2006; Gomes et al., 2008). Furthermore, nNOS knockout mice are less susceptible to MPTP neurotoxicity than their wild type littermates (Przedborski et al., 1996).

Despite this range of evidence for a role of nNOS in nigral dopaminergic cell loss and the protective effect of nNOS inhibitors, a number of *in vitro* studies contradict this conclusion. For example, SH-SY5Y cells transfected with overexpressing different levels of nNOS showed that highly overexpressing nNOS cells were more resistant to apoptosis induced by serum deprivation compared to cells with lower protein expression levels of nNOS (Ciani et al., 2002). In another study, nNOS overexpressing SH-SY5Y cells were resistant to MPP^+-induced cell death and inhibition of nNOS resulted in increased susceptibility to MPP^+ (Shang et al., 2005). Also, NO may have a dual role in cell death at least in the context of glutamatergic excitoxicity in cell culture exerting both apoptotic and necrotic effects (Ankarcrona et al., 1995) but this concept has not been investigated for its relationship to nNOS. In addition, some of the controversy over its role arises from indirect nature of the evidence and the lack of measurement of nNOS expression at baseline and following toxin exposure. In addition, the NOS inhibitors used in previous studies were not selective for nNOS and the extent of inhibition of the enzyme was not assessed. For this reason, we have investigated the relationship between nNOS expression, the susceptibility to toxin-induced cell death and the ability of selective NOS inhibitors to protect against toxin action using a range of cell lines that express different levels of the enzyme. N1E-115 cells that express nNOS endogenously were compared with SH-SY5Y cells that lack the nNOS protein. Transfected SH-SY5Y cells overexpressing nNOS were also compared to the wild type cells. Cell lines were exposed to either MPP^+ or to the proteasome inhibitor, MG-132 and cell loss correlated to nNOS expression. In addition, the ability of nNOS inhibitors to protect the cell lines from MPP^+- or MG-132-induced cell death was investigated in relation to nNOS expression levels.

2. Results

2.1. Expression of dopaminergic markers in cell lines

N1E-115, wt SH-SY5Y and SH-SY5Y-nNOS$^+$ cells were immunoreactive for the dopaminergic markers TH, DAT, VMAT and AADC (Fig. 1). TH and DAT were strongly expressed in the cytosol of N1E-115 cells, whereas only weak expression was observed in wt SH-SY5Y and SH-SY5Y-nNOS$^+$ cells. All cell lines showed strong immunoreactivity for VMAT-2, which was predominantly expressed in the cytoplasma. By contrast, all cell lines stained weakly for AADC which was localised to the cytosol.

2.2. Determination of nitrergic phenotype

NOS protein expression in the different cell lines was assessed by Western blot analysis (Fig. 2). Expression of nNOS protein as a 160 kDA band was detected in N1E-115 cell lysates (Fig. 2A). By contrast, nNOS was not detected in wt SH-SY5Y cells but markedly expressed in SH-SY5Y-nNOS$^+$ cells (Fig. 2A).

Expression of nNOS protein correlated with nNOS enzymatic activity as assessed by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline (Fig. 2B). Moderate NOS activity was detected in N1E-115 cells but was absent in wt SH-SY5Y cells. SH-SY5Y-nNOS$^+$ cells showed marked enzymatic activity. Expression of eNOS and iNOS protein was not detected in any cell line using Western blot analysis (data not shown).

2.3. Role of nNOS in toxin-induced LDH release

To investigate whether the extent of nNOS expression affects toxin-induced cell death, N1E-115, wt SH-SY5Y and SH-SY5Y-nNOS$^+$ cells were treated with MPP^+ (10^{-6}–10^{-2} M) or MG-132 (10^{-9}–10^{-4} M) for 24 h and cell death was assessed by lactate dehydrogenase (LDH) release (Fig. 3). MPP^+ (10^{-6}–10^{-2} M) or MG-132 (10^{-9}–10^{-4} M) induced a concentration-dependent LDH release in all cell lines. Basal release of LDH was significantly higher in N1E-115 cells compared to wt SH-SY5Y, and SH-SY5Y-nNOS$^+$ cells (Fig. 3A). There was no significant difference in EC_{50} values for MPP^+-induced LDH release between N1E-115 cells (3±0.6 mM), wt SH-SY5Y (5±0.4 mM) and SH-SY5Y-nNOS$^+$ cells (5±0.7 mM; Fig. 3).

Following MG-132 treatment, the EC_{50} values for LDH release were significantly higher in N1E-115 cells (40±8 μM) compared to wt SH-SY5Y and SH-SY5Y-nNOS$^+$ cells (Fig. 3). No significant difference in EC_{50} values for the effect of MG-132 on LDH release was detected between wt SH-SY5Y (0.3±0.1 μM) and SH-SY5Y-nNOS$^+$ cells (2±0.7 μM) respectively (Fig. 3C).

2.4. Involvement of nNOS in toxin-induced alterations in caspase-3 activity

To study the effect on endogenous NOS activity on MPP^+- or MG-132-induced apoptotic cell death N1E-115, wt SH-SY5Y and SH-SY5Y-nNOS$^+$ cells were exposed to MPP^+ (10^{-6}–10^{-2} M) and MG-132 (10^{-8}–10^{-4} M). Apoptosis was assessed by measurement of caspase-3 activity (Fig. 4). MPP^+ and MG-132 induced concentration-dependent changes in caspase-3 activity in all cell lines.

In N1E-115 cells MPP^+ (10^{-6}–10^{-2} M) caused a small increase in caspase-3 activity, that was maximal at MPP^+ (10^{-5} M) followed by a decline to below control levels (Fig. 4A). Wt SH-SY5Y cells showed a concentration-dependent increase in caspase-3 activity up to MPP^+ (10^{-3} M) but this declined at the highest MPP^+ (10^{-2} M) concentration (Fig. 4B). In SH-SY5Y-nNOS$^+$ cells there was a concentration dependent increase in caspase-3 activity over the entire concentration range reaching its maximum effect at MPP^+ (10^{-2} M; Fig. 4A).

In N1E-115 cells, MG-132 treatment (10^{-8}–10^{-5} M) induced a significantly lower activity in caspase-3 compared to the other cell lines, with a small but significant increase up to MG-132 (10^{-6} M) which declined at higher MG-132 concentration (Fig. 4B). By contrast in wt SH-SY5Y and SH-SY5Y-nNOS$^+$