Plasticity of the central nervous system (CNS) following perinatal asphyxia: Does nicotinamide provide neuroprotection?

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Summary. We have investigated the idea that nicotinamide, a non-selective inhibitor of the sentinel enzyme Poly(ADP-ribose) polymerase-I (PARP-1), provides neuroprotection against the long-term neurological changes induced by perinatal asphyxia. Perinatal asphyxia was induced in vivo by immersing foetuses-containing uterine horns removed from ready-to-deliver rats into a water bath for 20 min. Sibling caesarean-delivered pups were used as controls. The effect of perinatal asphyxia on neurocircuitry development was studied in vitro with organotypic cultures from substantia nigra, neostriatum and neocortex, platted on a coverslip 3 days after birth. After approximately one month in vitro (*DIV 25*), the cultures were treated for immunocytochemistry to characterise neuronal phenotype with markers against the N-methyl-D-aspartate receptor subunit 1 (NR1), the dopamine pacemaker enzyme tyrosine hydroxylase (TH), and nitric oxide synthase (NOS), the enzyme regulating the bioavailability of NO. Nicotinamide (0.8 mmol/kg, i.p.) or saline was administered to asphyctic and caesarean-delivered pups 24, 48 and 72 h after birth.

It was found that nicotinamide treatment prevented the effect of perinatal asphyxia on several neuronal parameters, including TH- and NOS-positive neurite atrophy and NOS-positive neuronal loss; supporting the idea that nicotinamide constitutes a therapeutic alternative for the effects produced by sustained energy-failure conditions, as occurring during perinatal asphyxia.

Keywords: Perinatal asphyxia – Basal ganglia – Nicotinamide – Poly (ADP-ribose) polymerase-1 (PARP-1) – Organotypic cultures – Neuroprotection – Rat

Introduction

Asphyxia is characterised by hypoxia and reduced pH, and is the most common clinical condition at birth causing brain injury, or subtle perturbations affecting the development of the CNS (Thompson, 1994; Simon, 1999; Maneru et al., 2001), including the neurocircuitries and neurotransmission systems of the basal ganglia (Pasternak et al., 1991).

Asphyxia alters: (i) oxygen availability; (ii) glycolysis (Lubec et al., 2002; Seidl et al., 2000); (iii) membrane conductance (Numagani et al., 1997); (iv) calcium transport (Akhter et al., 2000); and (v) DNA integrity (Kihara et al., 1994; Akhter et al., 2001). DNA damage triggers a cascade of events for buffering the menaces to the stability of the genome, notably the immediate activation of PARP-1 and PARP-2, which are members of a large family of Poly(ADP-ribose) polymerases (Amé et al., 2004).

PARP-1 catalyzes the attachment of chains of poly (ADP-ribose) (PAR), by reaction with NAD^+, to a variety of nuclear proteins, including PARP-1 itself. When DNA damage is mild, PARP-1 is involved in the maintenance of chromatin integrity, by signalling cell-cycle arrest and by reacting with DNA repairing enzymes, such as X-Ray Cross Complementing Factor 1 (XRCC1) and DNA-dependent protein kinase (De Murcia and Menissier de Murcia, 1994). Excessive activation of PARP-1 leads, however, to NAD^+ exhaustion and energy crisis (Berger, 1985), and to a caspase-independent apoptosis, via translocation of the mitochondrial pro-apoptotic protein, Apoptosis-Inducing Factor (AIF), to the nucleus, initiating nuclear condensation (Jiang et al., 1996; Yu et al., 2002; Hong et al., 2004). Furthermore, PARP-1 is involved in the regulation of cell proliferation and differentiation, and may modulate the transcription of several inflammatory signals, including NF-κB (Hassa and Hottingert, 1999). It has been suggested that PARP-1 modulates nitric oxide (NO) synthesis, via inducible NO synthase (iNOS), but even via the neuronal isoform of NOS (nNOS) (Hwang et al., 2002; Mishra et al., 2003; Hortobagyi et al., 2003).

Severe perinatal asphyxia can produce DNA breaks (Kihara et al., 1994). With the same experimental model

used in this study, it has been shown that perinatal asphyxia increases the expression of the XRCC1 and Excision Repair Cross-Complementing Rodent Repair Group 2 (ERCC2) genes (Chiappe-Gutierrez et al., 1998). XRCC1 is involved in repairing single and double strand breaks and recombination repair (Green et al., 1992), while ERCC2, which encodes an ATP-dependent DNA $5'$-$3'$ helicase activity, is involved in repairing DNA damage by nucleotide excision (Sung et al., 1993). When DNA is damaged, PARP-1 is over-activated, resulting in massive NAD^+ consumption, and decreased glycolysis, electron transport and ATP levels (Ying et al., 2005). Hence, PARP-1 inhibition has emerged as a main target for neuroprotection following hypoxic/ischemic insults. The strongest evidence for this hypothesis is from studies showing that ischemic injuries are markedly decreased in PARP $(-/-)$ mice (Eliasson et al., 1997a), supporting previous evidence that PARP inhibitors, with increasing degrees of potency, decrease brain damage and improve the neurological outcome of perinatal brain injury (Zhang et al., 1995; Ducrocq et al., 2000; Sakakibara et al., 2000; Virag and Szabo, 2002).

The idea that PARP activation is beneficial has also been explored, because PARP activity may have either a protective or a detrimental effect depending upon the level of cellular NAD^+ contents. While PARP inhibitors offer remarkable protection under conditions associated to depletion of NAD^+ and ATP, inhibition of PARP sensitizes cells to DNA damage, and subsequently increase cell death, in the presence of NAD^+ (Nagayama et al., 2000). Furthermore, it has been reported that inhibition of PARP-1 by nicotinamide can induce apoptosis in rapidly dividing cells (Saldeen and Welsh, 1998), perhaps by blocking the access of DNA to replication or repair enzymes, promoting G2 arrest followed by p53 independent apoptosis (Saldeen et al., 2003), thus resulting in inhibition of cell proliferation.

Nicotinic acid and nicotinamide have been proposed to protect against oxidative stress (Yan et al., 1999; Wan et al., 1999), ischemic injury (Sakakibara et al., 2000) and inflammation (Ducrocq et al., 2000) in neonatal rat brain, by replacing $NADH/NAD^+$ (Zhang et al., 1995), or by inhibiting PARP-1 overactivation. Interestingly, we have reported (Bustamante et al., 2003) that nicotinamide prevents several of the changes induced by perinatal asphyxia on monoamines, even if the treatment is delayed by 24 h, suggesting a clinically relevant therapeutic window. In a recent study (Bustamante et al., in preparation), we have confirmed previous observations regarding a decrease of extracelullar levels of striatal dopamine in adult rats exposed to perinatal asphyxia, monitored under basal, d-amphetamine-stimulated and K^+-depolarising conditions. These effects were restored to control levels when the pups were treated with nicotinamide, confirming previous observations (Bustamante et al., 2003), and supporting the idea that nicotinamide can constitute a therapeutic strategy against the long-term deleterious consequences of perinatal asphyxia, as already proposed for several pathophysiological conditions (Virag and Szabo, 2002). The use of nicotinamide has, however, been challenged because of its low potency, limited cell uptake and short cell viability, stimulating the investigation for more specific compounds inhibiting PARP-1 overactivation. Several more selective compounds have been investigated and developed, including (i) 3-aminobenzamide (Ducrocq et al., 2000; Hortobagyi et al., 2003; Koh et al., 2004); (ii) 3,4-dihydro-5-[4-(1-piperidynyl)butoxy]-1(2H)-isoquinoline (DPQ) (Takahashi et al., 1999); (iii) PJ34 (Abdelkarim et al., 2001); (iv) N-3-(4-Oxo-3,4-dihydrophthalazin-1-yl) phenyl]-4-(morpholin-4-yl) butanaide metane sulfanate monohydrate (ONO-1924H) (Kamanaka et al., 2004); (v) 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H)-pyridinyl) propyl]-4(3H)-quinazolinone (FR247304) (Iwashita et al., 2004); and (vi) 2-methyl-3,5,7,8-tetrahydrothiopyranol [4,3-d]pyrimidine-4-one] (DR2313) (Nakajima et al., 2005).

Nevertheless, nicotinamide is still an interesting molecule, since its low potency can be an advantage when used in developing animals, because the drug will only antagonise the effect elicited by PARP-1 overactivation, without impairing DNA repair, or cell proliferation.

In this paper, we focus on the effect of nicotinamide on basal ganglia neurocircuitry studied with organotypic cultures, a model originally developed by Gähwiler and colaborators (1981), but largely validated by Plenz and Kitai (1996a, b) and Plenz et al. (1998), for reconstructing the neurocircuitries of the basal ganglia (Gomez-Urquijo et al., 1999). The neuronal phenotype was assessed with markers against: (i) the N-methyl-D-aspartate receptor subunit 1 (NR1); (ii) the dopamine pacemaker enzyme tyrosine hydroxylase (TH); and (iii) the neuronal isoform of NOS.

As previously reported (Morales et al., 2003; Klawitter et al., 2005), perinatal asphyxia produced a regionally specific neuronal decrease and neurite atrophy in studies with organotypic cultures of the basal ganglia. We further report here that several of these changes can be reversed by treating the animals with nicotinamide before the explanting procedure.

Materials and methods

Perinatal asphyxia

Pregnant Wistar rats (UChA, bred at a local colony) within the last day of gestation (G22) were anaesthetised, sacrificed by neck dislocation and hysterectomized. One or two pups were removed immediately and used as non-asphyxiated caesarean-delivered controls, and the uterine horns containing the remaining foetuses were immersed in a water bath at $37\,^\circ\text{C}$ for 20 min. Following asphyxia, the uterine horns were incised and the pups were removed, and stimulated to breathe. After a 60 min observation period, the pups were given to surrogate dams for nursing, pending further experiments. Three days after birth (P3), the pups were used for preparing organotypic cultures (Morales et al., 2003; Klawitter et al., 2005).

Nicotinamide treatment

Nicotinamide (0.8 mmol/kg, i.p. [100 mg/kg i.p.]; Niacinamide, Sigma-Aldrich AB, Stockholm, Sweden) or the corresponding vehicle (0.9% NaCl) was administered to asphyxia-exposed (>20 min) or caesarean-delivered pups, 24, 48 and 72 h after birth. The last dose was given 2 h before preparing the organotypic cultures.

Organotypic cultures

All procedures including medium and drug preparation were performed within the arena of a Laminal Flow Cabinet equipped with UV antibacterial light (Factomet VR24242, Filtro Met Ltda. Santiago, Chile). Following decapitation of asphyctic and control pups, the brain was rapidly removed under sterile conditions and stored in a Petri dish containing a Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Täby, Sweden). Coronal sections were cut at mesencephalic (300 μm thick) and telencephalic (350 μm thick) levels and stored in cold DMEM. Samples from substantia nigra (SN), neostriatum (Str) and frontoparietal cortex (Cx) were dissected using the atlas by G. A. Foster as a reference (1998). The dissected tissue was plated on a coverslip (Nunc Thermanox Coverslips; Nunc, Naperville, IL, USA) containing a spread layer of chicken plasma (25 μl), and further coagulated by bovine thrombin (20 μl, 1000 NIH units in 0.28 ml DMEM; Sigma-Aldrich). Then, the coverslips were transferred to sterile (Nunc), containing an un-buffered culture medium (50% Basal Medium Eagle; 25% Hanks Balanced Salt Solution, and 25% horse serum [Gibco BRL], 0.5% glucose, 0.5 mM of L-glutamine [Sigma-Aldrich AB], and 0.1% antibiotic/antimycotic [Gibco BRL]).

The cultures were grown at $35\,^\circ\text{C}$, 10% CO_2 in a Cell Incubator (Model TC2323, ShelLab, USA), with a Roller device exposing the cultures to gaseous or water phases every minute. At DIV 3, 10 μl of a mitosis inhibitor cocktail (4.4 mM cytosine-β-D-arabinofuranoside, 4.4 mM uridine and 4.4 mM 5-fluoro-2′-deoxyuridine; all from Sigma-Aldrich AB) was added for 24 h for decreasing glial proliferation. The medium was changed every 3–4 days.

The experimental protocol was approved by a Local National Committee for Ethic Experiments with Laboratory Animals (Protocol CBA#, FMUCH).

Immunocytochemistry

The cultures were fixed in a formalin solution at ~DIV 25. After rinsing cycles, endogenous peroxidase activity was blocked with 1% H_2O_2. After rinsing with PBS, the tissue was preincubated with 2% of bovine serum albumin (BSA) (Calbiochem, CA, USA), 0.3% triton X-100, in PBS, for 1 h at $37\,^\circ\text{C}$. The slices were then incubated for 72 h with a mouse monoclonal antibody against the subunit 1 of the NMDA receptor (NR1), for characterizing a general neuronal phenotype (Petralia et al., 1994)

(dilution1:600, 2% BSA, 0.3% triton X-100, in PBS) (Pharmingen, San Diego, CA, USA). When treated for tyrosine hydroxylase (TH, the dopamine rate-limiting synthesis enzyme), the slices were incubated overnight with mouse monoclonal antibody (1:300, 2% BSA, 0.3% triton X-100, in PBS) (Diasorin, Stillwater, MN, USA). When treated for nNOS, the slices were incubated for 24 h with a sheep monoclonal antibody (1:500, 1% BSA, PBS/0.6% triton X-100) (generously donated by Prof. P. Emson, Babraham Institute, Cambridge, UK). After rinsing, the slices were treated with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), according to the instructions of the manufacturer, and/or biotinilated anti-mouse IgG (1:500 in PBS) for 1 h, followed by a further incubation with a streptavidin phosphatase complex for 1 h, rinsed and incubated with a levamisole solution (Vector Laboratories) for 15 min, to inhibit the endogenous alkaline phosphatases. The biotinilated anti-mouse IgG reaction was visualized with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Vector Laboratories). When using the Vectastain Elite ABC kit (containing the biotinilated antibody and the peroxydase), the reaction was visualized with Vector Nova Red (containing the substrate for the reaction) (Vector Laboratories). Some slices were also counterstained with Vector Hematoxylin Qs (Vector Laboratories) for labelling cell nucleus. Sections were dehydrated through graded alcohols, cleared in xylene and coverslipped in entellan mounting medium (Merck, Darmstadt, Germany).

Quantification

Cell quantification was performed directly on the stage of a microscope using appropriate objectives and filters for the corresponding markers (NR1, TH or NOS), or on pictures taken at $20\times$ as primary magnification. The microphotographs were digitally stored, composed and analysed with Adobe Photoshop® 7.0. Positive cells were quantified in 1 mm^2 samples for each region, selecting three (3) areas from the core of each culture showing the largest number of positive cells. Three parameters were quantified: (i) number of positive cells/mm^2; (ii) soma size (diameter) (μm); and (iii) neurite length (maximum length) (μm). The same parameters were measured pair wise by an investigator blinded to the codes of the corresponding culture, plated from any of the following experimental groups: (i) caesarean-delivered, saline-(CS), and (ii) nicotinamide (CN)-treated animals, or (iii) asphyxia-exposed, saline-(AS), and (iv) nicotinamide (AN)-treated animals. The values were transferred to an EXCEL matrix for the corresponding quantitative analysis. All data are expressed as means ± S.E.M. Comparisons were tested with Mann-Whitney test with GraphPad Prism (version 4.0, 2003; S. Diego, CA, USA), using a level of $p < 0.05$ as a limit for statistical significance.

Results

Some cultures were routinely kept for determination of cell viability at 27 DIV, using calcein-acetoxymethyl ester (AM) and ethidium homodimer-1 (EthD-1) for labelling alive (AM-Calcein, green fluorescence) and dying (EthD-1, red fluorescence) cells (Molecular Probes L3224; Eugene, OR, USA) (Klawitter et al., 2005). Since only few dying cells were observed in cultures from both control and asphyxia-exposed rats, a full quantification of dying cells, on the stage of a microscope equipped with epi-fluorescence, or on digitalized pictures, was easy.

The results shown here are from cultures fixed at 25 DIV. While a full account is reported somewhere else

(Klawitter et al., in preparation), we focus on the quantitative analysis of several morphological parameters, including: (A) number of neurons/mm^2; (B) size of soma (μm); and (C) maximal length (μm) of neurites labelled

Table 1. Effect of perinatal asphyxia on cells with neuronal phenotype labelled with an antibody against the NMDA receptor subunit NR1 in organotypic cultures (DIV 25) from caesarean-delivered, saline (CS)- and nicotinamide (CN)-treated animals and asphyxia-exposed, saline (AS)- and nicotinamide (AN)-treated animals

Group	N	Substantia nigra	Neostriatum	Neocortex
A Number of NR1 positive neurons/mm^2				
CS	10–15	228 ± 54	319 ± 70	250 ± 49
CN	6–14	189 ± 48	204 ± 30	*123 ± 6*[a]
AS	15–17	*98 ± 12*[a]	218 ± 26	*134 ± 19*[a]
AN	9–14	*80 ± 16*[a]	179 ± 43	163 ± 23
B Size of soma (μm) of NR1 positive neurons				
CS	10–15	17 ± 2	12 ± 1	26 ± 2
CN	6–14	16 ± 2	13 ± 1	25 ± 3
AS	15–17	22 ± 2	14 ± 1	28 ± 2
AN	9–14	19 ± 3	12 ± 1	26 ± 2
C Maximum length (μm) of NR1 positive neurites				
CS	10–15	156 ± 30	188 ± 25	144 ± 29
CN	6–14	150 ± 28	117 ± 24	131 ± 25
AS	15–17	145 ± 30	97 ± 21	145 ± 24
AN	9–14	159 ± 27	122 ± 24	148 ± 31

[a] $p < 0.05$ (italics), compared to the CS group

Table 2. Effect of perinatal asphyxia on cells labelled with an antibody against tyrosine hydroxylase (TH), the dopamine synthesis enzyme, in organotypic cultures (DIV 25) from caesarean-delivered, saline (CS)- and nicotinamide (CN)-treated animals and asphyxia-exposed, saline (AS)- and nicotinamide (AN)-treated animals

Group	N	Substantia nigra
A Number of TH positive neurons/mm^2		
CS	16–17	26 ± 14
CN	8–12	13 ± 6
AS	17	*9 ± 4*[a]
AN	12–13	*10 ± 5*[a]
B Size of soma (μm) of TH positive neurons		
CS	16–17	19 ± 2
CN	8–12	24 ± 2
AS	17	21 ± 3
AN	12–13	22 ± 3
C Maximum length (μm) of TH positive neurites		
CS	16–17	484 ± 53
CN	8–12	478 ± 76
AS	17	*271 ± 47*[a]
AN	12–13	*471 ± 59*[b]

[a] $p < 0.05$ (italics), compared to the CS group. [b] $p < 0.05$, compared to the AS group

Table 3. Effect of perinatal asphyxia on cells labelled with an antibody against nitric oxide synthase (NOS), the NO synthesis enzyme, in organotypic cultures (DIV 25) from caesarean-delivered, saline (CS)- and nicotinamide (CN)-treated animals and asphyxia-exposed, saline (AS)- and nicotinamide (AN)-treated animals

Group	N	Substantia nigra	Neostriatum	Neocortex
A Number of NOS positive neurons/mm^2				
CS	11–15	6 ± 2	*39 ± 7*[b]	*54 ± 11*[b]
CN	6–7	8 ± 2	58 ± 20	47 ± 16
AS	6–9	*42 ± 17*[a]	*10 ± 5*[a]	28 ± 10
AN	9	*33 ± 12*[a]	*48 ± 15*[c]	39 ± 11
B Size of soma (μm) of NOS-positive neurons				
CS	11–15	28 ± 2	26 ± 2	36 ± 1
CN	6–7	27 ± 1	24 ± 1	34 ± 2
AS	6–9	28 ± 3	20 ± 2	33 ± 1
AN	9	21 ± 3	21 ± 3	34 ± 1
C Maximum length (μm) of NOS-positive neurites				
CS	11–15	140 ± 22	147 ± 22	122 ± 18
CN	6–7	144 ± 19	140 ± 22	121 ± 14
AS	6–9	130 ± 22	*91 ± 17*[a]	97 ± 9
AN	9	175 ± 33	*175 ± 32*[c]	121 ± 14

[a] $p < 0.05$ (italics), compared to the CS group; [b] $p < 0.05$, compared to the substantia nigra of the same group. [c] $p < 0.05$, compared to the AS group

with antibody against NR1 (Table 1), TH (Table 2) and/or NOS (Table 3), in SN, Str and Cx of cultures from CS, CN, AS and AN-treated animals.

As shown in Table 1, there was a decrease in the number of NR1 positive neurons/mm^2 in cultures from AS and AN-treated animals, as compared to CS or CN-treated control animals, reaching statistically significant levels when cell number was compared in the SN (A). In Cx, there was a decrease in the number of NR1-positive neurons in AS versus CS group, but also when CN was compared to CS group. There is no explanation for the effect of nicotinamide on Cx of caesarean-delivered non-asphyctic animals. No statistically significant differences were observed regarding soma size (B) or neurite length (C) of NR1 positive neurons, among any of the experimental groups. No significant differences were observed, when the parameters were compared among regions from control (CS) cultures, although more NR1-positive neurons/mm^2 were estimated in Str compared to that observed in other regions of CS cultures (SN, ∼200 neurons/mm^2; Str, >300 neurons/mm^2; Cx, ∼200 neurons/mm^2).

While TH-positive plexuses and fibers were observed in all regions, TH-positive soma was only observed in the SN, with bipolar or multipolar features, and variably developed dendrite trees. A crude estimation revealed that TH-positive cells represented approximately 5% of

the number of NR1-positive neurons/mm^2 in the SN of cultures from control animals, a figure similar to that reported in vivo, if NR1 is considered as a universal neuronal marker (Petralia et al., 1994). The estimation of neurite length was restricted to that observed in the SN, where a one-to-one characterization was easily done regarding individual neurons. As shown in Table 2, there was a decrease in the number of TH-positive neurons/mm^2, in both AS and AN, compared to that in CS cultures (A). No differences were observed in soma size (B), but the length of TH positive neurites was decreased in AS cultures, compared to controls (C). The length of TH-positive neurites observed in AN cultures was similar to that observed in the controls.

NOS positive soma, plexuses and fibers were observed in all regions. NOS positive cells showed bipolar or multipolar features. As shown in Table 3, under control conditions, Str and Cx regions contained more NOS positive cells than the SN. In the SN, NOS-positive neurons represented less than 5% of the number of the observed NR1-positive cells/mm^2. In AS animals, the number of NOS positive neurons/mm^2 was larger ($>$6-fold) in SN, but lower in the Str, when compared to the controls. No significant differences were observed in Cx, despite an apparent decrease in NOS-positive neurons/mm^2, when comparing AS to that observed in CS cultures. While the amount of NOS-positive neurons/mm^2 was still increased in SN from AN animals, the amount of NOS positive neurons/mm^2 observed in Str from AN animals was similar to that observed in the controls (A). No differences were observed regarding soma size (B), but there was a significant decrease in neurite length of NOS positive neurons in Str from AS, compared to the controls (C). That effect was also reversed by nicotinamide treatment.

Discussion

The main result shown here is that nicotinamide treatment reversed the effect of perinatal asphyxia on several neuronal parameters assessed with organotypic cultures, including neurite atrophy and neuronal loss, alterations observed by several in vivo (Chen et al., 1997a, b; Kohlhauser et al., 1999a, b) and in vitro (Morales et al., 2003; Klawitter et al., 2005) studies, mainly affecting dopaminergic systems.

Nicotinamide showed no effect on NR1- and TH-positive cell number, but appeared to promote recovery of TH-positive neurites. In Str, nicotinamide reversed the effect on NO systems, which have also been shown to be vulnerable to perinatal asphyxia (Jiang et al., 1997; Capani et al.,

1997; Loidl et al., 1997; Bolanos et al., 1998; Lubec et al., 1999), although with rather controversial results. Jiang et al. (1997) reported significantly reduced NOS activity in neostriatum and other areas, but not in neocortex, in agreement with that observed in the present study. In contrast, Peci-Saavedra and co-workers (Capani et al., 1997; Loidl et al., 1997), using nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) as a histochemical marker for NO synthesis in neurons (Hope et al., 1991), reported that striatal NADPH-d neurons showed a significant increment in soma size and dendrite length following subsevere and severe asphyxia (Loidl et al., 1997), as well as an increase of NADPH-d reactivity in blood vessels of striatal and cortical regions (Caponi et al., 1997). Investigating the effect of perinatal asphyxia on nNOS mRNA (by Northern and dot blot analysis), immunoreactive protein (by Western blot analysis) and NOS activity (by electron paramagnetic resonance spectroscopy) in total brain homogenates, we found that NOS mRNA and NO generation were unaffected, whereas the NOS-immunoreactive protein of 150,000 mol. Wt. was decreased, and that of 136,000 mol. Wt. was increased with the length of the asphyctic period (Lubec et al., 1999). Although a large series of different mRNAs for nNOS have been described, the 136,000 mol. Wt. form accounts for the majority of the catalytic NOS activity in the brain (Eliasson et al., 1997b). Interestingly, it was shown by a gene hunting study in brain of rat pups exposed to perinatal asphyxia (Labudova et al., 1999), that NOS was strongly up regulated.

Capani et al. (1997) reported that the NOS positive staining is, under control conditions, largely restricted to neuronal soma, but NOS positive processes were only seen when studied in tissue from asphyctic animals. However, the features of NOS positive neurons are greatly magnified in organotypic cultures, making possible to observe in detail the soma and neurites, identifying an intriguing point-to-point synaptic interaction between NOS- and TH-positive neurons and processes, in SN, Str and Cx (Gomez-Urquijo et al., 1999; Herrera-Marschitz et al., 2000), providing a morphological support to the idea that NO modulates dopamine release (Zhu and Luo, 1992; West et al., 2002), but also dopamine reciprocally modulates NO synthesis.

In this study, we found a decrease in the number of NR1 positive neurons/mm^2 in SN of cultures from AS or AN, as compared to that in CS or CN animals. However, the number of NOS-positive neurons/mm^2, but not the length of NOS-positive neurites, was increased in SN. In contrast, the number of NOS-positive neurons/mm^2 was strongly

decreased in Str of cultures from AS animals, accompanied by a decrease of the length of NOS-positive neurites. In Str, both effects were significantly reversed by the nicotinamide treatment, but not that observed in the SN. The number of TH-positive neurons/mm^2 in the SN was decreased by perinatal asphyxia, accompanied by a decrease of TH-positive neurite length. Nicotinamide reversed the effect of perinatal asphyxia on neurite length, but not that on the number of neurons/mm^2. In contrast to that reported by Loidl et al. (1997), no effects were observed on size soma by any of the treatments.

The present study supports the idea that nicotinamide constitutes a therapeutic alternative for the effects produced by sustained energy-failure conditions, like that occurring during perinatal asphyxia. Nicotinamide can be neuroprotective by directly replacing NADH/NAD$^+$ depletion (Maynard, 2002), or by inhibiting over-activated PARP-1 (Zhang et al., 1995). Although the use of nicotinamide has been challenged because of its low potency, limited cell uptake and short cell viability (Virag and Szabo, 2002), the present results support a previous report (Bustamante et al., 2003), showing that nicotinamide prevents the long-term outcome on monoamine transmission induced by perinatal asphyxia, even if the treatment is delayed for 24 h, suggesting a clinically relevant therapeutic window. The low potency of nicotinamide on PARP-1 inhibition may provide an advantage when used in developing animals, because the drug will only antagonise the effect of PARP-1 over activation, without impairing DNA repair and proliferation. Furthermore, nicotinamide may also exert non-specific antioxidant effects, which is an attractive feature for treatments against the long-term consequences of anoxic/ischemic insults.

Acknowledgements

References

Abdelkarim GE, Gertz K, Harms C, Katchanov J, Dimagl U, Szabo C, Endres M (2001) Protective effects of PJ34, a novel, potent inhibitor of poly(ADP-ribose) polymerase (PARP) in vitro and in vivo models of stroke. Int J Mol Med 7: 255–260

Akhter W, Ashraf QM, Zanelli SA, Mishra OP, Delivoria-Papadopoulus M (2001) Effect of graded hypoxia on cerebral cortical genomic DNA fragmentation in newborn piglets. Biol Neonate 79: 187–193

Akhter W, Zanelli SA, Mishra OP, Gavini G, Delivoria-Papadopoulus M (2000) Effect of graded hypoxia on neuronal nuclear calcium influx in newborn piglets. Pediatr Res 47: 2266

Amé J-C, Spenlehauer C, de Murcia G (2004) The PARP superfamily. BioEssays 26: 882–893

Berger NA (1985) Poly (ADP-ribose) in the cellular response to DNA damage. Radiat Res 1001: 4–15

Bolanos JP, Almeida A, Medina JM (1998) Nitric oxide mediates brain mitochondrial damage during perinatal anoxia. Brain Res 787: 117–122

Bustamante D, Goiny M, Åström G, Gross J, Andersson K, Herrera-Marschitz M (2003) Nicotinamide prevents the long-term effects of perinatal asphyxia on basal ganglia monoamine systems in the rat. Exp Brain Res 148: 227–232

Capani F, Loidl F, Lopez-Costa JJ, Selvin-Test A, Saavedra JP (1997) Ultrastructural changes in nitric oxide synthase immunoreactivity in the brain of rats subjected to perinatal asphyxia: neuroprotective effects of cold treatment. Brain Res 775: 11–23

Chen Y, Engidawork E, Loidl F, Dell'Anna E, Goiny M, Lubec G, Andersson K, Herrera-Marschitz M (1997a) Short- and long-term effects of perinatal asphyxia on monoamine, amino acid and glycolysis product levels measured in the basal ganglia of the rat. Dev Brain Res 104: 19–30

Chen Y, Herrera-Marschitz M, Bjelke B, Blum M, Gross J, Andersson K (1997b) Perinatal asphyxia-induced changes in rat brain tyrosine-hydroxylase-immunoreactive cell body number: effects of nicotine treatment. Neurosci Lett 221: 77–80

Chiappe-Gutierrez M, Kitzmueller E, Labudova O, Fuerst G, Hoeger H, Hardmeier R, Nohl H, Gille L, Lubec B (1998) mRNA levels of the hypoxia inducible factor (HIF-1) and DNA repair genes in perinatal asphyxia of the rat. Life Sci 63: 1157–1167

De Murcia G, Menissier de Murcia J (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biol Sci 19: 172–176

Ducrocq S, Benjelloun N, Plotkine M, Ben-Ari Y, Charriaut-Marlangue C (2000) Poly(ADP-ribose) synthase inhibition reduces ischemic injury and inflammation in neonatal rat brain. J Neurochem 74: 2504–2511

Eliasson MJ, Sampei K, Madier AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH (1997a) Poly(ADP-Ribose) Polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3: 1089–1095

Eliasson MJL, Blackshaw S, Schell MJ, Snyder SH (1997b) Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. Proc Natl Acad Sci USA 94: 3396–3401

Forster GA (1998) Chemical neuroanatomy of the prenatal rat brain. Oxford University Press, New York

Gähwiler BH (1981) Organotypic monolayer cultures of nervous tissue. J Neurosci Methods 4: 329–342

Gomez-Urquijo S, Hökfelt T, Ubink R, Lubec G, Herrera-Marschitz M (1999) Neurocircuitries of the basal ganglia studied in organotypic cultures: focus on tyrosine hydroxylase, nitric oxide synthase and neuropeptide immunocytochemistry. Neuroscience 94: 1133–1151

Green A, Prager A, Stoudt PM, Murray D (1992) Relationships between DNA damage and the survival of radiosensitive mutant Chinese hamster cell lines exposed to gamma-radiation. Part 1: intrinsic radiosensitivity. Int J Radiat Biol 61: 465–472

Hassa PO, Hottinger MO (1999) A role of poly(AFP-ribose) polymerase in NF-κB transcriptional activation. Biol Chem 380: 953–959

Herrera-Marschitz M, Kohlhauser C, Gomez-Urquijo S, Ubink R, Goiny M, Hökfelt T (2000) Excitatory amino acids, monoamine, and nitric synthase systems in organotypic cultures: biochemical and immunohistochemical analysis. Amino Acids 19: 33–43

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signalling. Trends Pharmacol Sci 25: 259–264

Hope B, Michael G, Knigge K, Vincent S (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88: 2811–2814

Hortobagyi T, Görlach C, Benyo ZL, Lacza Z, Hortobagyl S, Wahl M, Harkany T (2003) Inhibition of neuronal nitric oxide synthase-mediated activation of poly(ADP-ribose) polymerase in traumatic brain injury: neuroprotection by 3-aminobenzamide. Neuroscience 121: 983–990

Hwang J-J, Choi S-Y, Koh J-Y (2002) The role of NADPH oxidase, neuronal nitric oxide synthase and poly(ADP-ribose) polymerase in oxidative neuronal death induced in cortical cultures by brain-derived neurotrophic factor and neurotrophin-4/5. J Neurochem 82: 894–902

Iwashita A, Tojo N, Matsuura S, Yamazaki S, Kamijo K, Ishida J, Yamamoto H, Hattori K, Matsuoka N, Mutoh S (2004) A novel and potent Poly(ADP-Ribose) Polymerase-1 inhibitor, FR247304 (5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H0-pyridinyl)propyl]-4(3H)-quinazolinone) attenuates neuronal damage in vitro and in vivo models of cerebral ischemia. J Pharmacol Exp Ther 310: 425–436

Jiang B-H, Rue E, Wang GL, Roe R, Semenza GL (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 271: 17771–17778

Jiang K, Kim S, Murphy S, Song D, Pastuszko A (1997) Effect of hypoxia and reoxygenation on regional activity of nitric oxide synthase in brain of newborn piglets. Neurosci Lett 206: 199–203

Kamanaka Y, Kondo K, Ikeda Y, Kamoshima W, Kitajima T, Suzuki Y, Nakamura Y, Umemura K (2004) Neuroprotective effects of ONO-1924H, an inhibitor of poly ADP-ribose polymerase (PARP), on cytotoxicity of PC12 cells and ischemic cerebral damage. Life Sci 76: 151–162

Kihara S, Shiraishi T, Nakagawa S, Toda K, Tabuchi K (1994) Visualization of DNA double strand breaks in the gerbil hippocampal CA1 following transient ischemia. Neurosci Lett 175: 133–136

Klawitter V, Morales P, Johansson S, Bustamante D, Goiny M, Gross J, Luthman J, Herrera-Marschitz M (2005) Effect of perinatal asphyxia on cell survival, neuronal phenotype and neurite growth evaluated with organotypic triple cultures. Amino Acids 28: 149–155

Koh S-H, Park Y, Song CW, Kim JG, Kim K, Kim J, Kim M-H, Lee SR, Kim DW, Yu H-J, Chang D, Hwang SJ, Kim SH (2004) The effect of PARP inhibitor on ischemic cell death, its related inflammation and survival signals. Eur J Neurosci 20: 1461–1472

Kohlhauser C, Kaehler S, Mosgoeller W, Singewald N, Kouvelas D, Prast H, Hoeger H, Lubec B (1999b) Histological changes and neurotransmitter levels three months following perinatal asphyxia in the rat. Life Sci 64: 2109–2124

Kohlhauser C, Mosgoeller W, Hoeger H, Lubec G, Lubec B (1999a) Cholinergic, monoaminergic and glutamatergic changes following perinatal asphyxia in the rat. Cell Mol Life Sci 55: 1491–1501

Labudova O, Schüller E, Yeghiazarjan K, Kitzmueller E, Hoeger H, Lubec G, Lubec B (1999) Genes involved in the patophysiology of perinatal asphyxia. Life Sci 64: 1831–1838

Loidl CF, Capani F, Lopez-Costa JJ, Selvin-Testa A, Lopez EM, Goldstein J, Pecci-Saavedra J (1997) Short-term changes in NADPH-diaphorase reactivity in rat brain following perinatal asphyxia. Mol Chem Neuropathol 31: 301–316

Lubec B, Kozlov AV, Krapbenbauer K, Berger A, Hoeger H, Herrera-Marschitz M, Nohl H, Koeck T, Lubec G (1999) Nitric oxide and nitric oxide synthase in the early phase of perinatal asphyxia of the rat. Neuroscience 93: 1017–1023

Lubec B, Labudova O, Hoeger H, Kirchner L, Lubec G (2002) Expression of transcription factors in the brain of rats with perinatal asphyxia. Biol Neonate 81: 266–278

Maneru C, Junque C, Botet F, Tallada M, Guardia J (2001) Neuropsychological long-term sequelae of perinatal asphyxia. Brain Int 15: 1029–1039

Maynard KI (2002) Natural neuroprotectans after stroke. Sci Med Sep/Oct: 260–269

Mishra OP, Akter W, Ashraf QM, Delivoria-Papadopoulus M (2003) Hypoxia-induced modification of poly (ADP-Ribose) Polymerase and DNA polymerase β activity in cerebral cortical nuclei of newborn piglets: role of nitric oxide. Neuroscience 119: 1023–1032

Morales P, Klawitter V, Johansson S, Huaiquin P, Barros VG, Avalos AM, Fiedler J, Bustamante D, Gomez-Urquijo S, Goiny M, Herrera-Marschitz M (2003) Perinatal asphyxia impairs connectivity and dopamine neurite branching in organotypic triple culture from rat substantia nigra. Neurosci Lett 348: 175–179

Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA, Chen J (2000) Activation of Poly(ADP-Ribose) Polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. J Neurochem 74: 1636–1645

Nakajima H, Kakui N, Ohkuma K, Ishikawa M, Hasegawa T (2005) A newly synthesized Poly(ADP-Ribose) Polymerase inhibitor, DR2313[2-methyl-3,5,7,8-tetrahydrothiopyranol[4,3-d]-pyrimidine-4-one]: pharmacological profiles, neuroprotective effects and therapeutic time window in cerebral ischemia in rats. J Pharmacol Exp Ther 312: 472–481

Numagani Y, Zubrow AB, Mishra OP, Delivoria-Papadopoulus M (1997) Lipid free radical generation and brain cell membrane alteration following nitric oxide synthase inhibition during cerebral hypoxia in the newborn piglet. J Neurochem 69: 1542–1547

Pasternak JF, Predey TA, Mikhael MA (1991) Neonatal asphyxia: vulnerability of basal ganglia, thalamus and brain stem. Pediatr Neurol 7: 147–149

Petralia RS, Yokotani N, Wenthold RJ (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J Neurosci 14: 667–696

Plenz D, Herrera-Marschitz M, Kitai ST (1998) Morphological organization of the subthalamic nucleus-globus pallidus system studied in organotypic cultures. J Comp Neurol 397: 437–457

Plenz D, Kitai ST (1996a) Organotypic cortex-striatum-mesencephalon cultures: the nigrostriatal pathway. Neurosci Lett 209: 177–180

Plenz D, Kitai ST (1996b) Generation of high frequency oscillations in cortical circuits of somatosensory cortex cultures. J Neurophysiol 76: 4001–4005

Sakakibara Y, Mitha AP, Ogilvy CS, Maynard KI (2000) Post-treatment with nicotinamide (vitamin B(3)) reduces the infarct volume following permanent focal cerebral ischemia in female Sprague-Dawley and Wistar rats. Neurosci Lett 281:111–114

Saldeen J, Tillmar L, Karlsson E, Welsh N (2003) Nicotinamide- and caspase-mediated inhibition of poly(ADP-ribose) polymerase are associated with p53-independent cell cycle (G2) arrest and apoptosis. Mol Cell Biochem 243: 113–122

Saldeen J, Welsh N (1998) Nicotinamide-induced apoptosis in insulin producing cells is associated with cleavage of poly(ADP-ribose) polymerase. Mol Cell Endocrinol 139: 99–107

Seidl R, Stoeckler-Ipsiroglu S, Rolinski B, Kohlhauser C, Herkner KR, Lubec B, Lubec G (2000) Energy metabolism in graded perinatal asphyxia of the rat. Life Sci 67: 421–435

Simon NP (1999) Long-term neurodevelopmental outcome of asphyxiated newborns. Clin Perinatol 26: 767–778

Sung P, Bailly V, Weber C, Thompson LH, Prakash L, Prakash S (1993) Human xeroderma pigmentosum group D gene encodes a DNA helicase. Nature 365: 852–855

Takahashi K, Pieper AA, Croul SE, Zhang J, Snyder SH, Greenberg JH (1999) Post-treatment with an inhibitor of poly(ADP-ribose) polymerase attenuates cerebral damage in focal ischemia. Brain Res 829: 46–54

Thompson DG (1994) Consequences of perinatal asphyxia. Clin Issues Crit Care Nurs 5: 242–245

Virag L, Szabo C (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol Rev 54: 375–429

Wan FJ, Lin HC, Kang BH, Tseng CJ, Tung CS (1999) D-amphetamine-induced depletion of energy and dopamine in the rat striatum is attenuated by nicotinamide pretreatment. Brain Res Bull 50: 167–171

West AR, Galloway MP, Grace AA (2002) Regulation of striatal dopamine neurotransmission by nitric oxide: effector pathways and signalling mechanisms. Synapse 44: 227–245

Yan Q, Briehl M, Crowley CL, Payne CM, Bernstein H, Bernstein C (1999) The NAD$^+$ precursors, nicotinic acid and nicotinamide upregulate glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase mRNA in Jurkat cells. Biochem Biophys Res Commun 255: 133–136

Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD$^+$ as a metabolic link between DNA damage and cell death. J Neurosci Res 79: 216–223

Yu SW, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GC, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1 dependent cell death by apoptosis-inducing factor. Science 297: 250–263

Zhang J, Pieper A, Snyder SH (1995) Poly(ADP-ribose) synthase activation: an early indicator of neurotoxic DNA damage. J Neurochem 65: 1411–1414

Zhu X, Luo L (1992) Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. J Neurochem 59: 932–935

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