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Effects of kainic acid lesioning on poly(ADP-ribose) polymerase (PARP) activity in the rat striatum *in vivo*

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Summary. Poly(ADP-ribose) polymerase (PARP) is activated in glutamate-induced toxicity of neurons in culture (Cosi et al., 1994). Since injection of the excitatory amino acid, kainic acid (KA) into the rat striatum induces a delayed neuronal death, the effects of this *in vivo* excitotoxin lesioning procedure on striatal PARP activity was investigated. PARP activity was measured in striatal extracts both in the absence ("endogenous" activity) and presence ("total" activity) of exogenously-added fragmented DNA. KA (5 nmols/1 μl) produced significant and time-dependent changes in striatal PARP activity, compared to saline-injected control animals: no changes at 6 h after intrastriatal KA, a 68% and 48% decrease in endogenous and total PARP activity respectively at 12 h, a doubling in endogenous PARP activity at 24 h, and a 382% and 60% increase in endogenous and total activities at 1 week after KA. PARP cleavage was not detected at any time point. These results suggest a participation of PARP in KA-induced toxicity in the brain *in vivo*.

Keywords: Amino acids – Kainic acid – Neurodegeneration – Excitotoxicity – Poly(ADP-ribose) polymerase – PARP, *In vivo*

Introduction

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is a DNA binding protein which uses NAD^+ as a substrate. PARP regulates the activity of proteins which are generally involved in DNA plasticity-related phenomena, including histones, topoisomerases, DNA and RNA polymerases, DNA ligases and Ca^{2+}/Mg^{2+}-dependent endonucleases. PARP is activated by nicks in DNA induced by different damaging agents including free radicals. PARP appears to be especially involved in DNA single strand base excision-repair in response to free radical attack (for a review, see De Murcia and Ménissier-de Murcia, 1994). When fully activated, PARP can deplete NAD^+ and

consequently ATP energy stores within a matter of minutes, to an extent which would prime the cell to death (Gaal et al., 1987).

The early stages of glutamate-induced neuronal death appear to be correlated to PARP activation, both *in vitro* and *in vivo*. It has been reported that PARP is activated during the early stages of glutamate-induced neurotoxicity in cerebellar granule neurons in primary culture and that it actively contributes to the subsequent cell death 24 h later (Cosi et al., 1994). More recently, it has been proposed that PARP mediates ischemic/reperfusion brain injury *in vivo* (Eliasson et al., 1997; Endres et al., 1997), since PARP knockout mice or normal mice pretreated with the PARP inhibitor 3-aminobenzamide presented a reduced infarct size 24 h after filamentous middle cerebral artery occlusion, and PARP activation was observed only at the earliest times (5 min) of the reperfusion period immediately following two hours of ischemia (Eliasson et al., 1997; Endres et al., 1997).

Activation of ionotropic glutamate receptors may be an important mediator of neuronal death in a variety of pathological conditions. Ionotropic glutamate receptors are classified, according to their selective ligand agonists, as N-methyl-D-aspartate (NMDA), kainic acid (KA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Evidence suggests that activation of the non-NMDA glutamate receptors, KA and AMPA, may contribute to neuronal death in acute pathological situations such as ischemia and trauma, or progressive chronic disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's and Alzheimer's diseases. For example, non-NMDA receptor antagonists reduce tissue loss and functional impairment after spinal cord trauma in the rat (Wrathall et al., 1994) and are neuroprotective in hypoxic-ischemic injury *in vivo* (Sheardown et al., 1990). The cycad plant-derived excitatory amino acid, β-N-methylamino-L-alanine, implicated in the ALS-Parkinsonism-dementia complex of Guam (Spencer et al., 1987), preferentially activates non-NMDA receptors at low doses. In addition, basal forebrain cholinergic neurons which degenerate in Alzheimer's disease and spinal motor neurons which degenerate in ALS, appear selectively vulnerable to AMPA/KA receptor-mediated neurotoxicity *in vitro* (Weiss et al., 1994).

Evidence for the involvement of free radicals in non-NMDA receptor-mediated neurotoxicity includes the accumulation of lipid peroxidation products that occurs concurrently with KA-induced neurotoxicity *in vivo* and *in vitro*, and the attenuation of both lipid peroxidation and neurodegeneration by treatments with antioxidants (Puttfarcken et al., 1993; Sun et al., 1992). Sun and coworkers (1992) have detected free radicals *in vivo* in the brain after systemic administration of KA by using spin-trapping techniques. Furthermore, the neurotoxic effects of intracerebrally (intrastriatal) administrated KA or quisqualic acid (an AMPA receptor agonist), but not NMDA, are blocked by a centrally active antioxidant (Miyamoto and Coyle, 1990).

Despite the growing evidence that non-NMDA receptor activation may contribute to neuronal death in both acute and chronic neurological diseases, the intracellular processes that are initiated by this subtype of glutamate receptor and that mediate neurodegeneration are still poorly understood, and

might involve a participation of PARP. The present study investigated the time-course of effects of KA lesioning on PARP activity in the rat striatum *in vivo*.

Material and methods

Animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and the European Directive N° 86/609, and the experimental protocol was carried out in compliance with local ethical committee guidelines for animal research. Male Sprague-Dawley rats (Ico: OFA-SD[IOPS.Caw], IFFA CREDO, Domaine des Oncins, France; 280–300 g) were anesthetized with Nembutal (60 mg/kg) and placed on a thermostated heating pad (37.5°C) in a stereotaxic apparatus (Kopf model 900, incisor bar fixed at −3.3 mm). One microliter of KA (5 nmols/μL) or vehicle (0.9% NaCl) was infused (0.2 μL/min for 5 min) through a 26 g cannula into one of the 2 striata, using the following stereotaxic coordinates (Paxinos and Watson, 1986): 0.5 mm anterior to bregma, 3.0 mm lateral to bregma, and 6.0 mm ventral from the skull surface. The concentration of KA used here has been shown to produce both apoptosis and necrosis as indicated by morphological changes and DNA fragmentation (Portera-Cailliau et al., 1997). Rats were killed by decapitation at 6 h, 12 h, 24 h and one week after the intrastriatal infusions. Brains were dissected on an ice-cold surface and the striata frozen on dry ice and stored at −80°C.

PARP activity was measured according to Berton et al. (1991). Briefly, striata were gently homogenized in 50 mM Tris HCl, pH 8, 4°C, containing 0.1% NP-40, 200 mM KCl, 2 mM $MgCl_2$, 50 μM $ZnCl_2$, 2 mM DTT, and protease inhibitors (1 mM PMSF, 5 γ/ml leupeptina and antipaina). Samples were then centrifuged and 10 μl of each supernatant were incubated for 5 min at 25°C with 2 μl of [^{3}H]NAD^+ (specific activity 25 Ci/nmol) in 50 mM Tris HCl, pH 8, containing 20 mM $MgCl_2$, 1 mM DTT and 20 μM NAD^+, 100 μl final volume ("endogenous" activity). The incubation was also made in the presence of calf thymus DNA fragmented by sonication ("total" activity). The reaction was stopped by the addition of 5% trichloroacetic acid. Samples were filtered and the radioactivity in the acid-insoluble fraction was counted by a Beckman LS1801 liquid scintillation spectrometer. Since PARP is activated exclusively by breaks in the DNA molecule, the total activity, measured in absence of PARP inhibitors, gives an estimation of the amount of the enzyme present in the tissue sample (Benjamin and Gill, 1980).

For Western blot analysis, a volume of supernatant from each sample (containing 40 μg protein) was denatured in a solution of 0.0625 M Tris HCl (pH 6.8), 2% sodium dodecylsulphate (SDS), 5% 2-mercaptoethanol, 5% glycerol and 0.002% bromophenol blue. Proteins were separated on a 7.5% SDS-PAGE gel and transferred on a Millipore-PVDF membrane. The nonspecific binding on the PVDF membrane was blocked by 5% (W/V) nonfat milk and 0.1% Tween 20 in Tris-buffered saline (TBS = 10 mM Tris-HCl, pH 7.5, 100 mM NaCl) for 1 h at room temperature. The membrane was then incubated with polyclonal anti-human PARP antibody at 1:500 dilution in TBS containing 5% nonfat milk and 0.1% Tween 20 for 1 h at room temperature. The membrane was washed 3 times for 10 min each with TBS containing 0.1% Tween-20 and incubated for 1 h at room temperature with horseradish peroxide linked secondary antibody (1:500 dilution in 5% nonfat milk and 0.1% Tween 20 in TBS) followed by washing 3 times in TBS containing 0.1% Tween 20 for 10 min each. The bound antibody on the PVDF membrane was detected by an enhanced chemiluminescence method (ECL Amersham Life Science) according to the manufacturers' instructions. Polyclonal anti-PARP antibody was a kind gift of Dr. Masutani (National Cancer Center Research Institute, Tokyo). This antibody was raised against human recombinant C-terminal 99 kDa part. It recognizes human, mouse and rat PARP. It recognizes both 85 and short 28 kDa apoptotic cleavage product (Ohta et al., 1997).

Results

PARP activity was measured in striatal homogenates in the absence ("endogenous") or in the presence ("total") of exogenously added fragmented DNA. As shown in Fig. 1, at 6h after intrastriatal infusion of KA, no significant changes were detected in either endogenous or total striatal PARP activity, in comparison to saline-infused (control) striata. At 12h after KA, both endogenous and total striatal PARP activities were decreased by 68% and 48% relative to the 6h KA group. At 24h, endogenous striatal PARP activity was increased by 197% relative to saline controls, while total PARP activity was not changed. One week after KA, endogenous and total PARP activities were increased by 382% and 60%, respectively, compared to the saline controls. Total PARP activity was also increased by

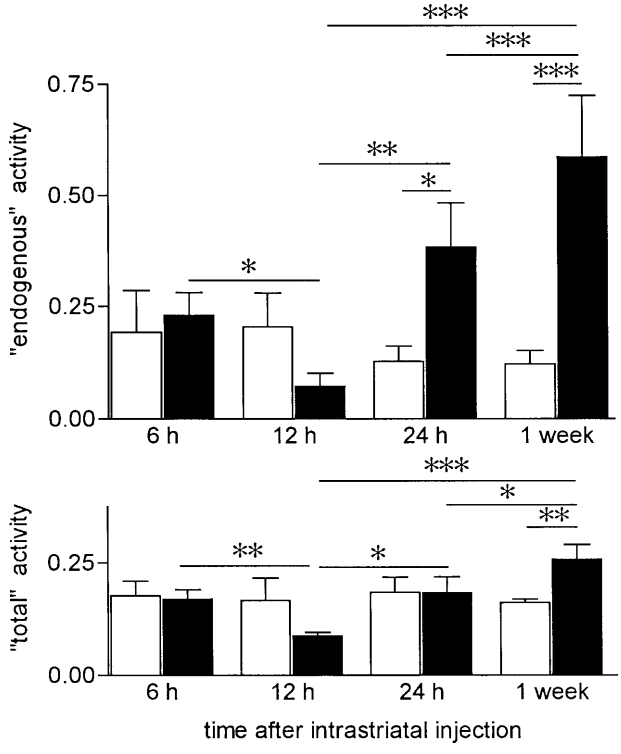

Fig. 1. Time course of "endogenous" (upper panel) and "total" (lower panel) PARP activities after infusion of 5nmols kainic acid into the rat striatum. "Endogenous" and "total" PARP activities were determined by the incorporation of [^{3}H]NAD$^+$ into the acid-insoluble fraction in the absence (upper panel) or presence (lower panel) of exogenously-added fragmented DNA. Values are expressed as the ratios between striatal PARP activities and bull testicle PARP activity, the latter included within each assay as an internal control. <<Endogenous>> striatal PARP activities ranged between 0 (non-detectable) and 13pmols/min/mg protein. "Total" striatal PARP activities ranged between 0.15 and 21pmols/min/mg protein. *Open bars*: saline injected striata. *Filled bars*: kainic acid injected striata. *Statistics*: Kruskal-Wallis ANOVA followed by Mann-Whitney U-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 7–11 rats per group

41% relative to the 24 h KA group. Neither the endogenous nor the total activities of PARP in saline-infused control striata were significantly altered at the time points examined in this study.

Endogenous PARP activity in the intact striata (i.e. in the non-injected striata, contralateral to the saline- or KA-injected side) did not present any statistically significant changes over time, although a tendency to increase at 24 h was noticed in the KA injected animals (data not shown). At 24 h, total PARP activity in the intact striata of both saline and KA injected animals were increased by 78% and 145%, respectively, in comparison to the activity measured at 12 h (data not shown).

To examine further the reason for the decrease in both the endogenous (−68%) and total (−48%) striatal PARP activities in the injected striata at 12 h after KA (Fig. 1), the possibility of an inactivation of PARP by caspases-mediated cleavage induced by KA was investigated. The polyclonal anti-PARP antibody recognized a single protein band at 116 kDa at both the 6 and 12 h time points, (Fig. 2A and 2B, respectively) suggesting a lack of PARP cleavage in KA lesioned striata at these post-injection intervals. No PARP cleavage was observed either at 24 h or one week after KA treatment (data not shown).

Discussion

PARP activation plays an important causative role in glutamate (Cosi et al., 1994) and NMDA (Zhang et al., 1994) mediated cell death in primary neuronal cultures. Glutamate induced-neurotoxicity is mediated also by non-NMDA receptor subtypes, such as KA and AMPA receptors, and the mechanisms involved in these toxicities appear to be specific to the receptor subtype. For example, KA-induced neurotoxicity in certain *in vivo* models seems to be correlated to free radical formation while NMDA-induced toxicity is not (Miyamoto and Coyle, 1990). Consistent with this notion is the finding that antioxidants protect against neuronal degeneration caused by intrastriatal injections of KA and quisqualic acid but not by the NMDA receptor agonist, quinolinic acid (Miyamoto and Coyle, 1990). Since PARP appears to be specifically activated by free radical-induced DNA damage, the present study extends previous work on the role of PARP in excitotoxicity *in vitro*, to a glutamate-receptor subtype other than NMDA, and to an *in vivo* model of neurotoxicity were free radicals have been implicated.

It has been previously reported that PARP participates in the early events of glutamate-mediated death of neurons in culture (Cosi et al., 1994) and to early stages of ischemia-induced brain damage *in vivo* (Eliasson et al., 1997; Endres et al., 1997). The present study indicates that PARP is not only involved in the early stages of KA-induced excitotoxicity but also participates at later times. Intrastriatal injection of KA resulted in an initial drop in endogenous PARP activity, which then rose dramatically over the following week, whilst total activity (as an index of enzyme protein levels) also fell at first and rose only after one week. The decrease in PARP activity at 12 h after

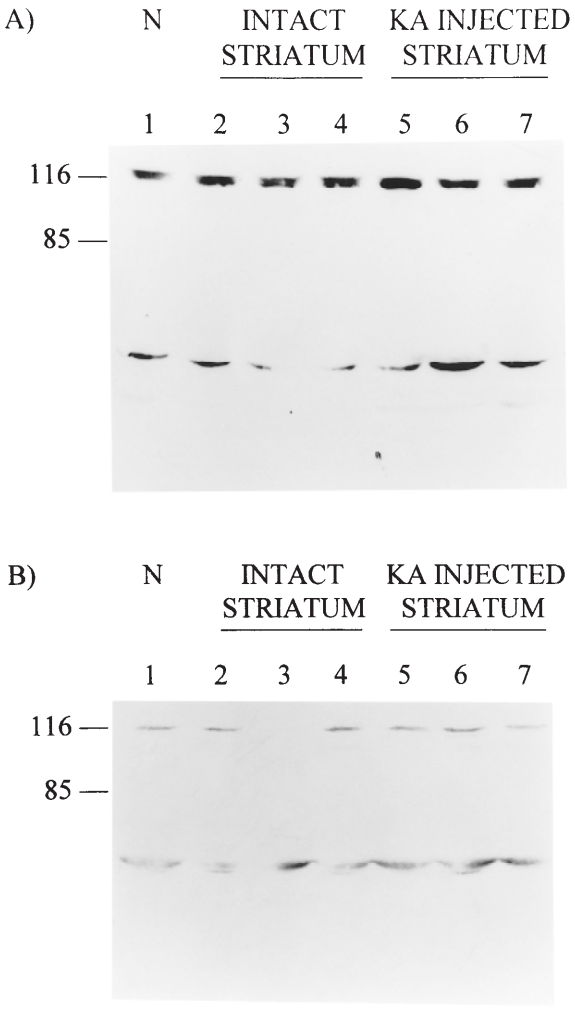

Fig. 2. Lack of PARP cleavage in KA lesioned striata. Each lane was loaded with 40 μg of total protein of single striata from different rats. Proteins were separated on 7.5% SDS-PAGE and blotted to a PVDF membrane as described under Material and Methods using polyclonal anti-PARP antibodies. A single band in each sample was detected at the position of 116 KDa, corresponding to that of intact PARP. No band at 85 KDa was observed in any sample. Immunoblotting of striata of naïve animals (N) (lane *1*), and intact (lanes *2–4*) and KA injected (lanes *5–7*) striata of KA lesioned rats sacrificed at 6 h (Panel A) and at 12 h (Panel B) after KA administration, are presented. A ~60 KDa band, observed in all samples, was considered as a non-specific band, since no difference was observed among samples

KA injection appeared not to be related to an inactivation of the enzyme by cleavage, since no evidence for PARP cleavage products such as the 85 kDa fragment was detected by Western blotting either at 6 h or at 12 h (Fig. 2), nor at any of the other time points (data not shown). Data from our laboratory confirmed that the polyclonal anti-PARP antibody used in the present study was able to recognize an 85 kDa fragment in extracts from

HL60 cells treated with etoposide. This *in vivo* evidence seem consistent with previous *in vitro* findings showing that PARP can contribute to cell death in the absence of cleavage of the enzyme by CPP32, in cultured neuroblastoma cells treated with hydrogen peroxide or peroxynitrite (Cookson et al., 1998). Other factors such as a decrease in the synthesis or an inhibition of the enzyme, by an endogenous factor or a change in DNA integrity might be responsible for the changes observed at 12 h. The increase in endogenous PARP activity at 24 h after KA injection was likely due to an increase in ADP-ribosylation and not to an enhancement of enzyme levels, since total PARP activity in saline-injected and KA injected striata at 24 h were unchanged.

PARP is activated by nicks and breaks in the DNA molecule. Interestingly, it has been reported that the fragmentation of DNA into internucleosomes induced by intrastriatal injection of KA increased with time, being barely visible at 6 h and being followed by a peak at 24 h (Portera-Cailliau et al., 1997); this time-related change parallels the lack of PARP activation at 6 h and the increase in PARP activity observed at 24 h in the present study. On the contrary, while Portera-Cailliau et al. (1997) did not observe DNA fragmentation at seven days after injection of KA, in the present study both endogenous and total PARP activities of the KA injected striata were increased at this time, suggesting the existence of other mechanisms underlying KA-induced PARP activation at these later times. Increases in PARP activities have been reported during proliferation and differentiation of astrocytes in culture (Chambert et al., 1992) and in interferon γ-activated human monocyte-derived macrophages (Berton et al., 1991). The increase in both activities in the KA injected striata at one week is not likely to be a direct action of KA on neurons, given the long delay after the intracerebral injection and the elimination of the toxin after this time, but possibly by the presence of hypertrophic and/or proliferating glia cells in response to cellular necrosis (reactive gliosis) (see Altar and Baudry, 1990, and references therein).

Despite the likelihood of some minor local damage induced by the intrastriatal injection procedure, the intrastriatal injection of saline did not appear to trigger any detectable increase in striatal PARP activity.

In conclusion, these results indicate that an excitotoxic concentration of KA can produce significant and time-dependent changes in both striatal endogenous PARP activity and enzyme levels *in vivo*, and suggest a participation of PARP in KA-induced toxicity in the rat brain.

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