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BRAIN RESEARCH

Research Report

Resistance to kynurenic acid of the NMDA receptor-dependent toxicity of 3-nitropropionic acid and cyanide in cerebellar granule neurons

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ABSTRACT

During cerebral hypoxia or ischaemia, mitochondrial dysfunction is induced which can lead to free radical production and cell death. This phenomenon is mimicked by the acute administration of mitochondrial poisons such as 3-nitropropionic acid (3-NPA) and potassium cyanide (KCN), with the production of reactive molecular species secondary to the activation of glutamate receptors. Also during ischaemia, the kynurenine pathway of tryptophan metabolism is activated, leading to the production of quinolinic acid and kynurenic acid which can modulate *N*-methyl-D-aspartate (NMDA) receptors as agonist and antagonist respectively. Since kynurenic acid is known to be neuroprotective, we have now examined its ability to prevent the neurotoxic effects of mitochondrial dysfunction in primary cultures of postnatal rat cerebellar granule neurons. Viability was quantified using the Alamar Blue (AB) assay and by direct morphological examination. Both 3-NPA and KCN (10 μM–1 mM) reduced neuronal viability in a concentration-dependent manner. The NMDA receptor antagonists 2-amino-5-phosphonopentanoic acid (D-AP5) at a concentration of 50 μM, and a 10 μM dose of (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate (MK-801) prevented cell death, although the non-NMDA receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at a concentration of 10 μM did not. The antioxidant enzymes catalase and superoxide dismutase, and the nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) afforded partial protection. Kynurenic acid, a glutamate antagonist with preference for the glycine site of the NMDA receptors, had no protective effect at all against 3-NPA or KCN toxicity at concentrations up to 1 mM. Although these data confirm a major role for NMDA receptors and oxidative stress in the neurotoxic effects of mitochondrial inhibitors, they reveal a resistance to kynurenic acid which suggests a non-classical activation of NMDA receptors by mitochondrial inhibitors that is independent of the glycine site or which occurs distal to the site of action of kynurenic acid.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AB, Alamar Blue; CAT, Catalase; CGN, Cerebellar granule neuron; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, 2-amino-5-phosphonopentanoic acid; KCN, potassium cyanide; L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; MK-801, (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; 3-NPA, 3-nitropropionic acid; SOD, superoxide dismutase

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1. Introduction

One major consequence of cerebral hypoxia or ischaemia is a loss of mitochondrial function leading to opening of the mitochondrial permeability transition pore and the triggering of cell death programmes. Ischaemic brain damage can be reduced by blockers of the transition pore (Korde et al., 2007; Muramatsu et al., 2007). Substances which interfere with mitochondrial function therefore represent important tools for understanding the mechanisms of neuronal damage. Two such compounds are 3-nitropropionic acid (3-NPA) and potassium cyanide (KCN), which inhibit succinate dehydrogenase (respiratory chain complex II) in the mitochondrial inner membrane (Alexi et al., 1998) and cytochrome C oxidase (complex IV) respectively. These effects result in an excessive calcium influx, which leads to the generation of reactive oxygen and nitrogen species (Schulz et al., 1996; Gunasekar et al., 1996; Fontaine et al., 2000) and oxidative and nitrosative stress-mediated cell death (Coyle and Puttfarcken, 1993). There is also a loss of mitochondrial membrane potential which can be prevented by inhibition of the mitochondrial permeability transition pore (Leventhal et al., 2000), and an associated redistribution of cytochrome C (Bizat et al., 2003). Our recent finding that an inhibition of the permeability pore by cyclosporin A attenuates glutamate toxicity is consistent with this chain of events (Fatokun et al., 2008).

There is good evidence that these effects of 3-NPA and KCN involve activation of glutamate receptors. However, during periods of cerebral ischaemia there is also an activation of the kynurenine pathway of tryptophan metabolism, which generates the NMDA receptor glycine site antagonist kynurenic acid (Perkins and Stone, 1982; Stone and Darlington, 2002). This compound should, therefore, limit neuronal damage and raising its brain concentration by inhibiting kynurenine-3-monoxygenase might represent a method of reducing the impact of mitochondrial disruption. This approach may be especially relevant to some neurodegenerative disorders in which mitochondrial dysfunction has been implicated, such as Huntington's disease (Smith and Ord, 1983). We have now tested kynurenic acid against damage induced by 3-NPA and KCN in cerebellar granule neuron (CGN) cultures.

2. Results

Cultures of CGNs were exposed to the two mitochondrial toxins at concentrations of 10, 100 and 1000 μM for periods of 1 or 6 h. Following this treatment and recovery for 18–24 h in normal medium, cell viability was reduced in a concentration-dependent manner, compared to untreated controls, the toxic effects being significant at 100 μM or above for 6 h exposure (Fig. 1). The combination of 1 mM applied for 6 h was selected for further work since it produced approximately 50% reduction of cell viability (Fig. 1). Cell death was completely prevented by the inclusion of either the NMDA channel blocker MK-801 (10 μM), or the competitive NMDA antagonist D-AP5 (50 μM), which maintained levels of cell viability at control levels (Fig. 2A). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), however, failed to modify the toxicity of either 3-NPA or KCN ($n=7$) (data not shown).

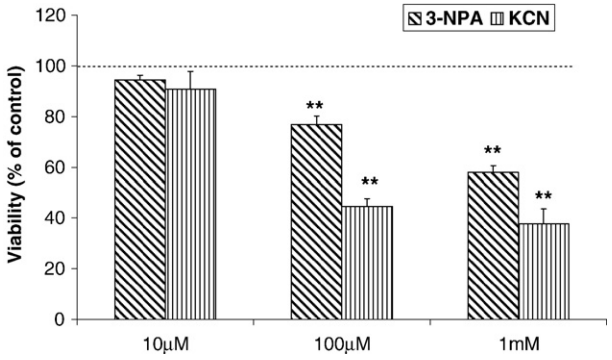

Fig. 1 – Concentration-dependent neurotoxic effects of the mitochondrial poisons 3-nitropropionic acid (3-NPA) and KCN, on cerebellar granule neurons (CGN) following 6 h exposure. Each column represents the mean ± S.E.M. for $n=4$ cultures in the 3-NPA data and $n=10$ for KCN. $^{**}P<0.01$ compared to the respective untreated control cultures.

Since over-activation of glutamate receptors causes the generation of reactive oxygen and nitrogen species, we examined the possible protective effects of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) (each at 100U/ml), and the nitric oxide synthase inhibitor, L-nitroarginine methyl ester (L-NAME) at 1 mM, on the toxicity of the mitochondrial poisons. 3-NPA reduced CGN viability to 48.90% ± 3.89 of the control ($P<0.001$), but CAT reduced this damage, with viability at 80.37% ± 8.73 of the control value ($P<0.01$) and SOD also protected to a lesser but still significant extent (viability of 68.39% ± 8.34 of the control, $P<0.05$) (Fig. 2B).

On the other hand, KCN lowered viability to 43.27% ± 0.74 ($P<0.001$), an effect that was ameliorated very significantly by either CAT (to 59.34% ± 4.86 of the control, $P<0.01$), or by SOD (to 62.27% ± 2.42 of the control, $P<0.001$, $n=5$). L-NAME elicited slight but significant protection, raising viability after exposure to 3-NPA from 55.35% ± 4.44 ($P<0.001$ compared to control) to 70.13% ± 6.01 ($P<0.05$), and viability after exposure to KCN from 37.93% ± 5.99 ($P<0.001$ compared to control) to 56.23% ± 6.73 of the control ($P<0.05$; $n=5$) (Fig. 2C).

Kynurenic acid is known to block both NMDA and non-NMDA receptors (Perkins and Stone, 1982), though with a greater potency at the former. Blockade of NMDA receptors is largely the result of preventing activation of the strychnine-resistant glycine co-agonist site on the receptor (Birch et al. 1988). In a previous study we have shown that kynurenate can block the toxic effects of glutamate or NMDA on CGNs at a concentration of 100 μM (Fatokun et al., 2008). In the present experiments, kynurenate was also tested at 100 μM against the toxic effects of both 3-NPA and KCN and failed to produce any protection (Fig. 3). Even at higher levels up to 1 mM kynurenate did not attenuate the loss in neuronal viability resulting from the exposure to the mitochondrial poisons (data not shown).

Morphological observations of cultures treated with 1 mM 3-NPA or 1 mM KCN, and the protection afforded by MK-801 but not kynurenate were consistent with the viability assay data (Fig. 4). Untreated control cultures contain numerous