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Nicotinamide and 1-methylnicotinamide reduce homocysteine neurotoxicity in primary cultures of rat cerebellar granule cells

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Nicotinamide is an important cofactor in many metabolic pathways and a known neuroprotective substance, while its methylated product, 1-methylnicotinamide, is a suspected neurotoxin. Homocysteine is a risk factor in Alzheimer's disease and neurodegeneration, causing inhibition of methylation processes and inducing excitotoxicity. In this study, using primary cultures of rat cerebellar granule cells and propidium iodide staining, we investigated the neurotoxicity of nicotinamide and 1-methylnicotinamide, and their neuroprotective potential in acute and sub-acute homocysteine neurotoxicity. Our results demonstrated that nicotinamide and 1-methylnicotinamide applied for 24 h to cultures at concentrations of up to 25 mM had no effect on neuronal viability. Moreover, nicotinamide at concentrations of 5–20 mM and 1-methylnicotinamide at 1–10 mM applied to cells 24 h before, and for 24 h after an acute 30 min application of 25 mM D,L homocysteine, reduced neuronal damage. 1-Methylnicotinamide at concentrations of 250 and 500 μM showed neuroprotective activity during a sub-acute 24-h exposure to 2.5 mM D,L-homocysteine, while 5 and 25 mM nicotinamide also evoked neuroprotection. These findings do not support suggestions that 1-methylnicotinamide may act as an endogenous neurotoxic agent; rather, they indicate the neuroprotective ability of nicotinamide and 1-methylnicotinamide in homocysteine neurotoxicity. The exact mechanisms of this neuroprotection are unclear and require further investigation.

Key words: cerebellar granule cells, excitotoxicity, homocysteine, 1-methylnicotinamide, nicotinamide, neuroprotection

INTRODUCTION

Nicotinamide (NAM), the amide form of niacin (vitamin B_3), is an essential precursor of NAD^+ required for cellular energy metabolism. A number of recent studies have demonstrated neuroprotective effects of NAM, not only in *in vitro* models of cytotoxicity, but also *in vivo* in different forms of brain ischemia (Mokudai et al. 2000, Chong et al. 2002, 2004, Bedalov and Simon 2004, Tam et al. 2005, Feng et al. 2006, Lee et al. 2006). Proposed mechanisms of NAM-evoked neuroprotection include stabilization of the mitochondrial membrane potential, reduction of caspase activation and inhibition of PARP (for review see Maiese and Chong 2003).

1-Methylnicotinamide (MNA) is the endogenous product of NAM methylation in many tissues including brain: a reaction catalyzed by nicotinamide N-methyl transferase (NNMT, EC2.1.1.1) using S-adenosyl methionine as the methyl donor (Fukushima et al. 2002, Parsons et al. 2002). MNA is a recognized anti-inflammatory agent (Gebicki et al. 2003); however, it has been suggested that MNA may be neurotoxic and might play a role in the pathogenesis of Parkinson's disease (Fukushima et al. 1995, 2002, Ogata et al. 2000). There are epidemiological data indirectly supporting this idea (Williams and Ramsden 2005, Williams et al. 2005). In addition, an MPP^+-mimicking effect of infrastriatal MNA injections has been described (Fukushima et al. 2002). It is not clear whether MNA might induce neurodegeneration in non-dopaminergic neurons, like glutamatergic cerebellar granule cells in culture, or if this compound shares the neuroprotective properties of its precursor NAM.

Given the availability of an adequate *in vitro* model for studying the putative neuroprotective effects of NAM and MNA, the impact of homocysteine (Hcy)

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neurotoxicity should also be taken into account. There is a reciprocal causal relationship between accumulation of Hcy in body fluids and perturbations in methylation processes, caused by strong inhibition of methyltransferases by S-adenosyl homocysteine (Selhub and Miller 1992, Selhub 1999). Thus, an excess of Hcy may inhibit methylation of NAM to MNA. Inhibition of DNA methylation has been suggested as an important factor in the mechanism of indirect Hcy neurotoxicity (Mattson and Shea 2003). The results of previous studies indicate that NMDA receptors and group I metabotropic glutamate receptors mediate Hcy-induced excitotoxic neuronal damage (Kim and Pae 1996, Lipton et al. 1997, Zieminska et al. 2003, 2006, Zieminska and Lazarewicz 2006). The *in vitro* models of Hcy neurotoxicity have important clinical relevance, since elevated Hcy concentration in human blood (hyperhomocysteinemia) has been recognized as a risk factor, not only in cardiovascular diseases, but also in neurodegenerative diseases (Clarke et al. 1998, Hankey and Eikelboom 1999, Morris 2003, Urbanska et al. 2006).

The aim of the present study, using primary cultures of rat cerebellar granule cells (CGC), was to evaluate the neurotoxicity of MNA in comparison with NAM, and to characterize the neuroprotective potentials of NAM and MNA in acute and sub-acute Hcy-mediated excitotoxicity.

METHODS

D,L-homocysteine (Hcy), propidium iodide (PI) dye and materials for cell culture were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nicotinamide and 1-methylnicotinamide were obtained from the Institute of Applied Radiation Chemistry, Technical University of Lodz, Poland (courtesy of Professors Jerzy Gebicki and Jan Adamus). Other chemicals were of analytical grade. Primary cultures of granule neurons were prepared from cerebella of 7-day-old rats bred in the Animal Colony of the Medical Research Centre, Polish Academy of Sciences in Warsaw. The rat pups were treated in accordance with Polish and the European Community regulations concerning experiments on animals. The procedure was approved by the First Local Ethical Committee in Warsaw. Granule neurons were prepared by the method of Schousboe and coauthors (1985), and cultivated in modified BME medium containing 25 mM KCl, exactly as described previously (Zieminska et al. 2003, 2006, Zieminska and Lazarewicz 2006).

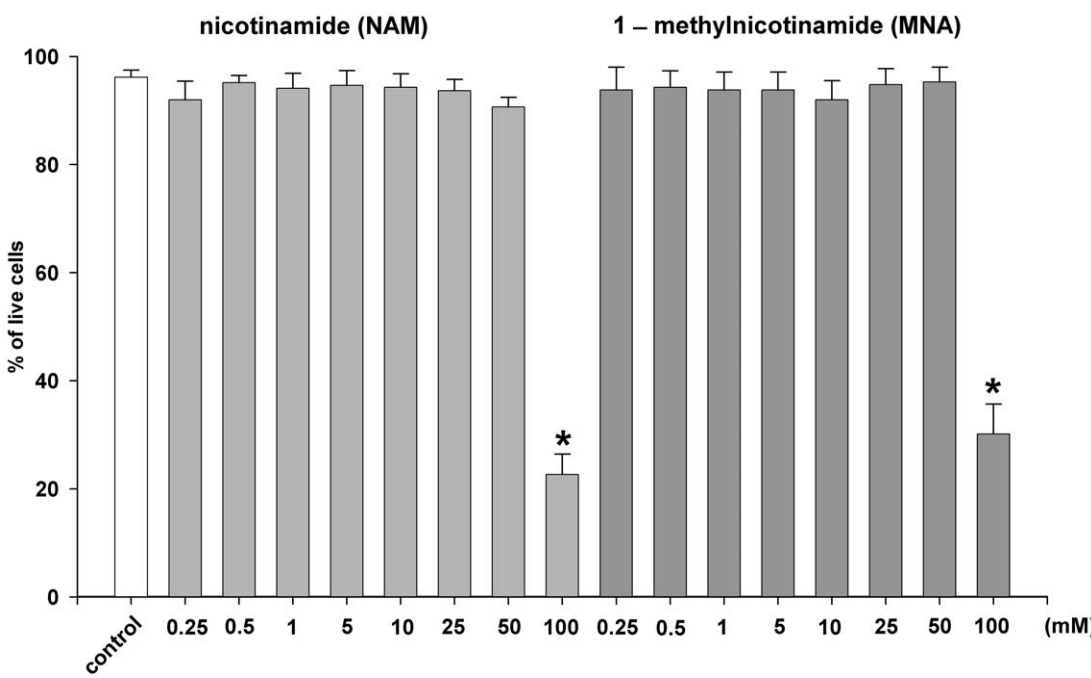

Fig. 1. Concentration-dependent sub-acute neurotoxicity of nicotinamide and 1-methylnicotinamide in cultured cerebellar granule cells. Cells were incubated for 24 h in the presence of nicotinamide (NAM) or 1-methylnicotinamide (MNA) as indicated. The percentage of live cells was determined by staining with propidium iodide. Results are means ± SD, (n=6). *Means significantly different from the control (P<0.05).

The cells were used for experiments on the 6th to 8th day *in vitro* (DIV). To verify the intrinsic neurotoxicity of NAM and MNA, on DIV 6 or 7 these substances were added directly to the cell growth medium and culturing was continued for 24 h under standard conditions. The acute neurotoxicity of D,L-homocysteine was induced at DIV 7 by replacing the BME growth medium with Locke 25 buffer containing aliquots of freshly prepared D,L-homocysteine alone or with NAM or MNA as required. After 30 min, the Locke medium was replaced with the original growth medium and the cultures continued for 24 h. In other experiments, NAM or MNA were present in the growth medium for 24 h before and after acute exposure to Hcy. To examine sub-acute Hcy neurotoxicity, this amino acid was applied at different concentrations directly to cultures for 24 h. In these experiments NAM or MNA, if present, were added 24 h before Hcy.

To evaluate neurotoxicity, the cells were fixed with 80% methanol, stained with 0.5 μg/ml propidium iodide and viable and dead cells were counted using a Zeiss Axiovert fluorescence microscope by an investigator unaware of the exact experimental conditions, as described previously (Zieminska et al. 2003, 2006). Results were expressed as the percentage of live cells. The number of repetitions within each trial was n=6 and experiments were repeated at least 3 times with the same qualitative results. The results of representative experiments are presented as means ± standard deviation (SD). For comparisons between groups, one-way analysis of variance (ANOVA) followed by Dunnett's test was used, with differences considered significant at P<0.05.

RESULTS

To characterize the intrinsic neurotoxicity of NAM and MNA we studied the effects on cell viability of supplementing CGC culture media for 24 h with these substances at concentrations ranging between 250 μM and 100 mM. The results presented in Fig. 1 demonstrate that NAM and MNA at concentrations of up to 25 mM were completely free of any neurotoxic effects, at 50 mM a tendency towards decreased viability was seen in cells incubated with NAM, and at 100 mM, both substances induced pronounced neurotoxicity.

Further investigations aimed at evaluating the neuroprotection afforded by NAM and MNA against Hcy neurotoxicity focused on acute exposure to these compounds. Our previous studies (Zieminska et al. 2003, 2006) demonstrated that in CGC, significant neuronal damage may be observed after acute exposure of the cells to Hcy at high millimolar concentrations. As shown in Fig. 2A, application of 25 mM Hcy for 30 min resulted in a drastic decrease in the number of living cells after a further 24 h in culture, from the control value of about 92% viability down to 50–55%. Co-application of NAM or MNA at concentrations from 1–25 mM together with Hcy had no neuroprotective effect. Pre-incubation of the cells for 24 h with 1–25 mM NAM or MNA before exposure to Hcy followed by 24 h in culture resulted in different patterns of modest concentra-

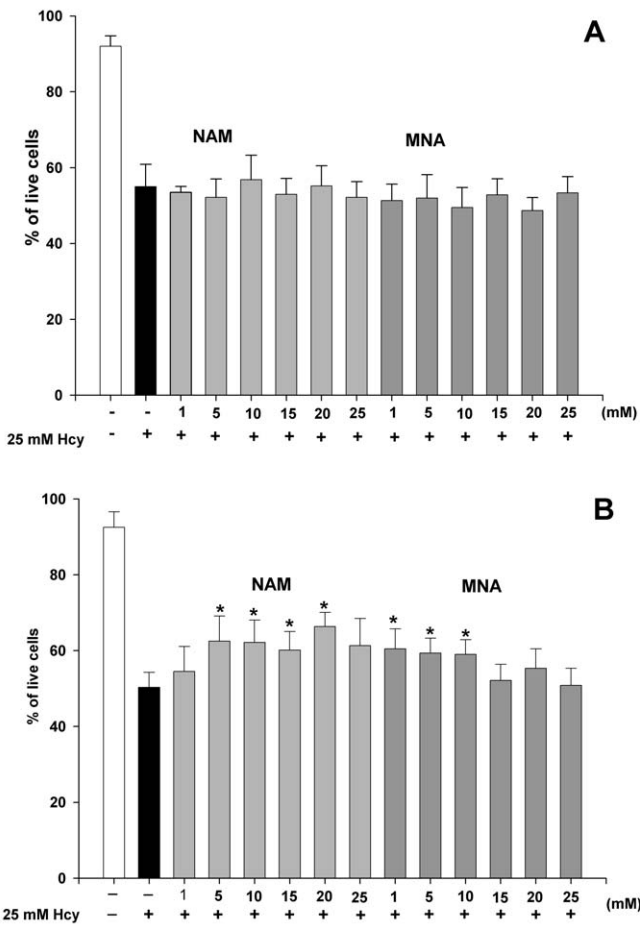

Fig. 2. Effects of nicotinamide and 1-methylnicotinamide on acute homocysteine-evoked neurotoxicity induced by 30 min incubation of cerebellar granule cells with 25 mM D,L-homocysteine. The number of live cells was evaluated 24 h after the insult. (A) Nicotinamide (NAM) or 1-methylnicotinamide (MNA) were applied for 30 min together with D,L-homocysteine (Hcy); (B) NAM or MNA were applied for 24 h before and after exposure to Hcy. Results are means ± SD, (n=6). *Means significantly different from the effect of 25 mM Hcy alone (P<0.05).

tion-dependent neuroprotection. The percentage of living cells increased from 50% to 60–67% (Fig. 2B). A statistically significant neuroprotective effect was observed in cells treated with 5–20 mM NAM and 1–10 mM MNA. At higher concentrations, MNA lost its neuroprotective potential.

We next examined the neuroprotective potentials of NAM and MNA against sub-acute Hcy neurotoxicity. As shown in Fig. 3A, incubation of cultured CGC for 24 h with Hcy present at millimolar concentrations resulted in concentration-dependent neuronal loss, with an EC_{50} of about 3 mM. In further experiments we applied 2.5 mM Hcy to CGC for 24 h to induce a sub-acute lesion. When the neuroprotective potential of NAM and MNA was assessed, both compounds were applied at different concentrations during a 24 h preincubation and then were present together with Hcy for the subsequent 24 h in culture. As shown in Fig. 3B, relatively slight but statistically significant neuroprotection was induced by NAM at 5 and 25 mM, whereas

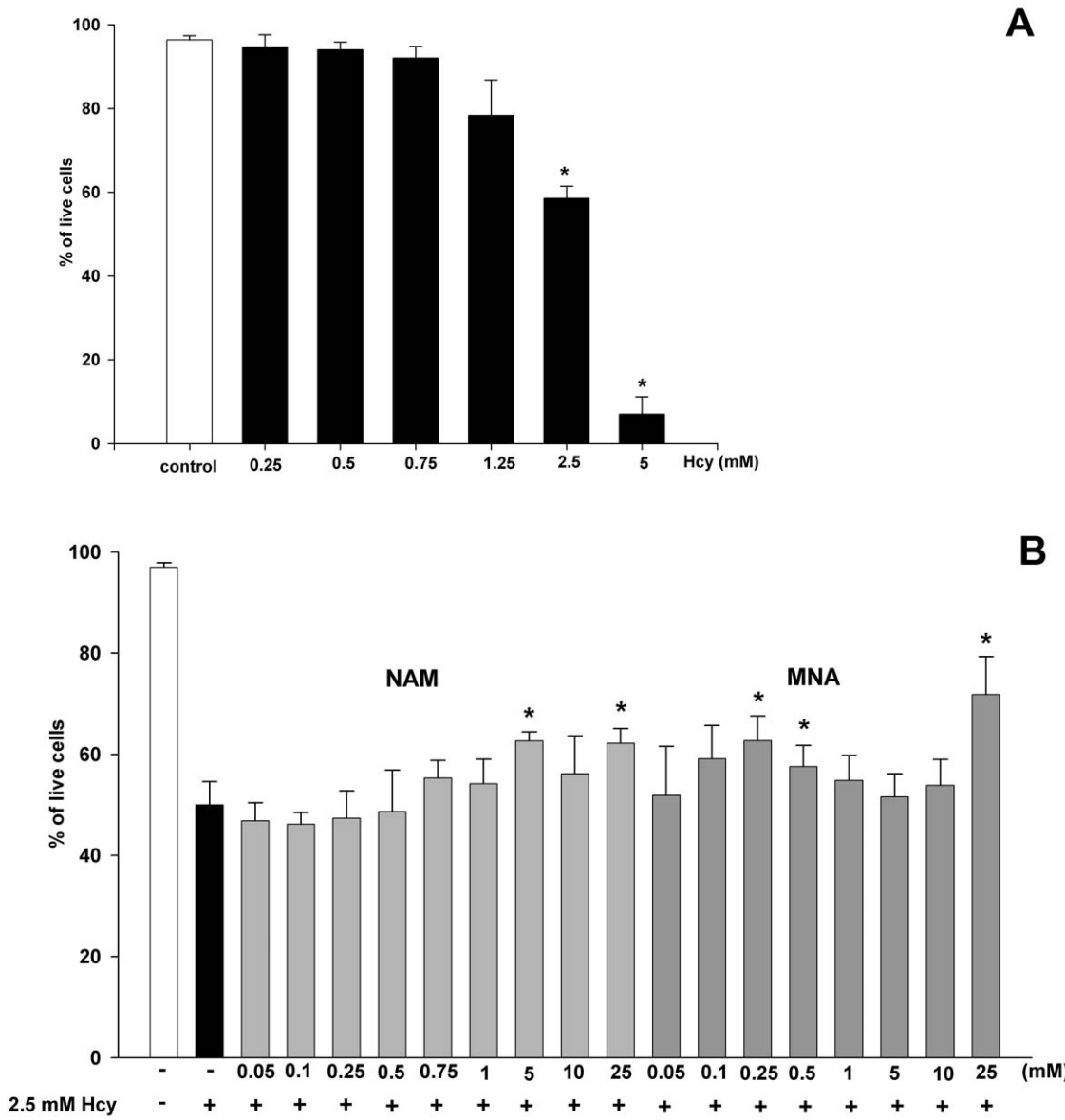

Fig. 3. Effects of nicotinamide and 1-methylnicotinamide on sub-acute homocysteine neurotoxicity. (A) Concentration-dependent sub-acute Hcy neurotoxicity in cultured cerebellar granule cells; (B) Effect of different concentrations of nicotinamide (NAM) and 1-methylnicotinamide (MNA) applied for 24 h together with 2.5 mM Hcy. Results are means ± SD, (n=6). *Means significantly different from the effect of 2.5 mM Hcy alone (P<0.05).

MNA-evoked neuroprotection was already observed at concentrations of 250 and 500 μM, and then at 25 mM.

DISCUSSION

The results of this study demonstrated that NAM and MNA at reasonable concentrations are practically nontoxic to primary cultures of CGC. Moreover, NAM applied at millimolar concentrations and MNA at even lower concentrations, provide protection to CGC against acute (30 min) and sub-acute (24 h) exposure to Hcy, provided these compounds are present in the culture media for 24 h before and during or after treatment with Hcy.

The primary cultures of CGC used in these experiments (after approximately 7 days of *in vitro* growth) are particularly useful for neurotoxicological studies on excitotoxicity, since after this period in culture they reach maximal cell density and the glutamate receptors have matured (Schousboe et al. 1985, Gallo et al. 1987, Marini et al. 1999). However, these cells undergo apoptosis starting at 8–9 days *in vitro*, which complicates experiments investigating their prolonged exposure to neurotoxins. Therefore, in this study we used models of acute and sub-acute neurotoxicity. In the former protocol, CGC in culture were exposed to high millimolar concentrations of Hcy for 30 min and neuronal viability was evaluated 24 h later (Zieminska et al. 2003, 2006). Alternatively, they were cultured for 24 h in the presence of the potentially neurotoxic substances (Hcy, but also NAM or MNA were tested). In the latter protocol, Hcy neurotoxicity was induced with low millimolar concentrations of this amino acid.

Both NAM and its methylated derivative MNA applied to neuronal cultures for 24 h at concentrations of ≤25 mM induced no visible decrease in cell viability. This finding concerning NAM does not differ significantly from the observations of Chong and coworkers (2002), who described toxic effects of NAM, applied to cerebral vascular endothelial cells in culture, only at high concentrations exceeding 25 mM. Our results indicate that the neurotoxic potentials of NAM and MNA are equally low and do not support suggestions that MNA, a methylated derivative of nicotinamide, may induce neurotoxicity *via* a mechanism similar to MPP+, i.e. by inhibition of mitochondrial complex 1 in the electron transport chain and production of oxygen free radicals (Fukushima et al. 2002). Additional data (not shown), in agreement with previously published results (for review see Gonzales-Polo et al. 2004), demonstrated that CGC are susceptible to MPP+, which induces pronounced neurotoxic effects at low micromolar concentrations when applied for 24 h. Moreover, it appears that CGC are able to synthesize MNA from NAM, since the presence of NNMT protein has been demonstrated in human cerebellar granule cells (Parsons et al. 2002). Therefore, our results from experiments on cultured rat CGC do not validate the hypothesis that nicotinamide may act as a protoxin substance that is converted in neurons into toxic MNA (Fukushima et al. 1995, 2002, Williams and Ramsden 2005, Williams et al. 2005). However, these data should be considered in light of comparative information concerning the uptake of MNA and NAM vs. MPP+ by rat CGC, the activity of NNMT forming MNA in these neurons, and interference of MNA with respiratory activity and oxidative phosphorylation in isolated brain mitochondria. As the knowledge required to clarify this situation is currently lacking, appropriate experiments are in progress in our laboratory to provide the necessary data.

A major concern in studies on Hcy neurotoxicity is the disparity between the relatively high Hcy concentrations needed to induce neuronal damage *in vitro* and the much lower micromolar levels observed in hyperhomocysteinemic patients. Moreover, only the unstable free reduced L form of Hcy seems to be biologically active. These points were addressed in our previous paper (Zieminska and Lazarewicz 2006) and will not be discussed here.

Several studies examining Hcy toxicity in cultured brain neurons indicate that the mechanism of this phenomenon is complex and still poorly understood (for recent review, see Obeid and Herrmann 2006). It has long been known that an elevated concentration of Hcy induces disturbances in cellular methylation and remethylation processes, which is caused by the accumulation of S-adenosylhomocysteine, a competitive inhibitor of many methyltransferases (Yudkoff 1999). This results in inhibition of the repair of damaged DNA and the involvement of PARP leading to apoptosis (Kruman et al. 2000, 2002, Duan et al. 2002, Baydas et al. 2005). Furthermore, recent data have linked disturbed methylation in hyperhomocysteinemia with amyloid production and tau hyperphosphorylation characteristic of Alzheimer's disease (Fuso et al. 2005, Obeid et al. 2007, Sontag et al. 2007). Another hypothesis explains Hcy neurotoxicity by excitotoxic mechanisms

involving NMDA receptors (Kim and Pae 1996, Lipton et al. 1997) in conjunction with group I mGlu receptors (Zieminska et al. 2003, 2006, Zieminska and Lazarewicz 2006). We have observed Hcy-evoked mitochondrial dysfunction, cytochrome c release and activation of caspases without visible symptoms of neuronal calcium overload or oxidative stress (Zieminska et al. 2003, 2006). This might suggest a specific form of Hcy excitotoxicity, distinct from the traditional glutamate or NMDA-induced, calcium-mediated mechanism (Choi 1985, Ankarcrona et al. 1995, Kristal and Dubinsky 1997). The other possible mechanisms of the homocysteine-induced neurotoxicity (at higher, mM concentrations) may be the reduced availability of kynurenic acid, which is an endogenous antagonist of glutamate receptors (Luchowska et al. 2005, Stazka et al. 2005). It seems especially interesting providing that nicotinamide and 1-methylnicotinamide are metabolites of tryptophan along kynurenine pathway, and that kynurenic acid is also a product of this metabolic route. The precise cellular and molecular mechanisms of Hcy-induced excitotoxicity remain unclear.

The results of this study demonstrate that both MNA and NAM, applied at millimolar concentrations 24 h before homocysteine, provide neuroprotection against Hcy neurotoxicity. It has been reported that NAM exhibits pronounced neuroprotective potential *in vitro* and also *in vivo* in different models of brain ischemia (Mokudai et al. 2000, Frantseva et al. 2001, Maiese and Chong 2003, Tam et al. 2005, Feng et al. 2006, Lee et al. 2006). This phenomenon may be attributed to both indirect and direct effects of NAM. NAM is a precursor of NAD(H) and NADP(H), two critical cofactors in many metabolic processes. NAD(H) is mainly involved in energy production and ATP formation, stabilizing complex 1 in the mitochondrial electron transport chain impaired in MPP+ toxicity (Nicklas et al. 1985). NADP(H) is connected with the regulation of redox processes and anti-radical protection (Klaidman et al. 2001). However, a number of neuroprotective mechanisms have also been ascribed directly to nicotinamide, including effects on poly (ADP-ribose) polymerase (PARP) and kinase Akt, stabilization of membrane potential in mitochondria, inhibition of cytochrome c release, suppression of iNOS expression and inhibition of caspases and proinflammatory cytokines (for review see Fujimura et al. 1997, Chong et al. 2002, Maiese and Chong 2003, Ungerstedt et al. 2003). Many of these potential mechanisms of neuroprotection by NAM could be instrumental in protection against Hcy-induced pathology.

There are similarities and differences between our recent results and previous data concerning NAM-evoked neuroprotection. In earlier *in vitro* studies, the neuroprotective effects of NAM were observed at relatively high concentrations, from around 12.5 mM (Li et al. 2004) up to 50 mM (Shen et al. 2004). Our results confirm this observation: we found that NAM was neuroprotective when applied at concentrations of 5–25 mM. However, in our present experiments, NAM and MNA failed to induce neuroprotection in acute Hcy toxicity unless CGC were preincubated with these substances for 24 h before the insult. This is in contrast to the findings of previous *in vitro* studies where such a prerequisite for neuroprotection by NAM was not seen (Shen et al. 2004). In studies using cultured cerebral endothelial cells exposed to NO, post-treatment with NAM at 2, 4 and even 6 h after the insult significantly prevented cytotoxicity (Chong et al. 2002). *In vivo* studies have also demonstrated beneficial effects of NAM administered after ischemic insult (Mokudai et al. 2000). At present we cannot explain these discrepancies in the data. It is also difficult to identify the mechanism(s) of neuroprotection evoked by MNA, especially since this compound exhibited its neuroprotective activity at significantly lower concentrations than NAM, even at 250 μM. However, this result suggests that endogenously produced MNA might be instrumental in the neuroprotection afforded by the addition of NAM, while this additional mechanism may be obstructed by excessive Hcy concentrations that could inhibit NNMT. Our reservations discussed above, concerning MNA transport and accumulation within CGC and their impact on its neurotoxicity, also apply here. On the other hand, the structural similarity between NAM and its methylated derivative may suggest similar mechanisms of interference with metabolic processes. Taken together, our results demonstrate that NAM and its methylated derivative MNA provide neuroprotection in Hcy-induced neurotoxicity.

CONCLUSIONS

The results of this *in vitro* study demonstrated that nicotinamide and 1-methylnicotinamide applied for 24 h to primary cultures of cerebellar granule cells at concentrations of up to 25 mM are non toxic. Moreover, both these substances, when applied to primary cere-

bellar granule cell cultures for 24 h before the insult, provided significant protection against neurodegeneration evoked by acute (30 min) and sub-acute (24 h) exposure to D,L-homocysteine. Surprisingly, we found that 1-methylnicotinamide gave neuroprotection against homocysteine-induced toxicity at lower concentrations compared with its precursor substance nicotinamide, a known neuroprotective agent.

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