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Effects of the 3-hydroxyanthranilic acid analogue NCR-631 on anoxia-, IL-1β- and LPS-induced hippocampal pyramidal cell loss *in vitro*

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Summary. The kynurenine pathway intermediate 3-hydroxyanthranilic acid (3-HANA) is converted by 3-HANA 3,4-dioxygenase (3-HAO) to the putative neuropathogen quinolinic acid (QUIN). In the present study, the neuroprotective effects of the 3-HANA analogue and 3-HAO inhibitor NCR-631 was investigated using organotypic cultures of rat hippocampus. An anoxic lesion was induced by exposing the cultures to 100% N_2 for 150 min, resulting in a pronounced loss of pyramidal neurons, as identified using NMDA-R1 receptor subunit immunohistochemistry. NCR-631 provided a concentration-dependent protective effect against the anoxia. NCR-631 was also found to counteract the loss of pyramidal neurons in two models of neuroinflammatory-related damage; incubation with either LPS (10 ng/ml) or IL-1β (10 IU/ml). The findings suggest that NCR-631 has neuroprotective properties and that it may be a useful tool to study the role of kynurenines in neurodegeneration.

Keywords: Kynurenine pathway – 3-Hydroxyanthranilic acid – Neurodegeneration – Anoxia – Neuroinflammation – Interleukin-1

Abbreviations: EAA, excitatory amino acid; 3-HANA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilic acid 3,4-dioxygenase; IL-1β, interleukin-1β; KYNA, kynurenic acid; LPS, lipopolysaccaride; NCR-631, 4,6-dibromo-3-hydroxyanthranilic acid; NMDA, N-methyl-D-aspartate; QUIN, quinolinic acid

Introduction

The major route of L-tryptophan metabolism in mammals is through the kynurenine pathway. Essential products of this pathway are nicotinamide and its nucleotide conjugates, but also several intermediates may have important biological actions. There are compelling evidence that two kynurenine pathway intermediates, quinolinic acid (QUIN) and kynurenic acid (KYNA), are

able to interact with excitatory amino acid (EAA) receptors of the brain. The dicarboxylic acid QUIN is an agonist at N-methyl-D-aspartate (NMDA) receptors that also may act as a lipid peroxidant (Rios and Santamaria, 1991), whereas KYNA is a broad-spectrum antagonist of ionotropic EAA receptors (see Stone, 1993). It has been shown that intracerebral administration of QUIN causes convulsions (Lapin, 1978) and neurodegeneration (Schwarcz et al., 1983). Moreover increased cerebral or cerebrospinal fluid levels of QUIN have been found in several conditions, such as after ischemic insults, brain injury and encephalitis, as well as following systemic immune stimulation and liver dysfunction (Heyes et al., 1992; see Stone, 1993).

The precursor of QUIN is 3-hydroxyanthranilic acid (3-HANA). 3-HANA is first converted by the enzyme 3-HANA 3,4-dioxygenase (3-HAO; Malherbe et al., 1994) to an unstable product that is transformed to QUIN non-enzymatically (see Stone, 1993). QUIN is formed in the normal rodent brain (Foster et al., 1986), while 3-HAO activity and gene expression have been found to increase in neuropathological states (e.g. Heyes et al., 1992; Nakagawa et al., 1995).

On basis of the assumption that inhibition of 3-HAO may lead to reduced brain levels of QUIN and neuroprotective effects, NCR-631, an inhibitor of the 3-HANA class (Todd et al., 1989), has been developed. While this compound has been used as a tool to study the kynurenine pathway of the normal rat brain (Luthman et al., 1996), its neuroprotective potential has not been reported previously. In the present study NCR-631 was evaluated for its ability to provide neuroprotective actions using *in vitro* models of anoxia and neuroinflammatory-mediated loss of hippocampal pyramidal neurons.

Materials and methods

Organotypic cultures

Organotypic cultures were prepared essentially according to Studer et al. (1994). The brains of three days postnatal Sprague Dawley rat pups were removed and hippocampus dissected out and cut into 250 μm thick slices. The slices were placed on glass coverslips, and embedded by means of reconstituted chicken plasma (Sigma; P-3266) and thrombin solution (T-4648; Sigma). The coverslips were transferred into sterile plastic tubes (No. F-2095; Falcon) containing 1.1 ml of medium. The medium consisted of 55.0 ml Dulbecco's modified Eagle's medium with glutamine (No. 22320; Gibco), 32.5 ml Hank's balanced salt solution (No. 24020; Gibco), 1.5 ml of 20% glucose solution (No. 19002; Gibco), 1.0 ml of HEPES solution (2.39 g HEPES; Kebo; No. 1.4286 + 10 ml destilled water, No. 15230; Gibco), and 10 ml heat inactivated fetal calf serum (No. 10108; Gibco). 1% antibiotic-antimycotic solution was also added (No. 15240; Gibco) to the medium the first days. The resulting concentration of QUIN in the final incubation medium was around 400–500 nM, as determined by gas-chromathography coupled to a mass spectrometer (GCMS; see Luthman et al., 1996). The test tubes were placed in a roller drum (Bellco), tilted 5° to the horizontal axis and rotating at 60 rph. The incubation was performed at 37°C at an humidity of approximately 95–98%, with an initial CO_2 concentration of 5%. The medium was changed three times a week. The cultures were grown for 21 days before initiation of the experiments.

In the anoxia experiment, NCR-631 (batch 205/94, Astra Arcus AB) was dissolved in N_2 saturated medium and immediately added to the cultures. Fifteen minutes later the

cultures were exposed to 100% N_2 for 150 minutes. At 24 h after the anoxic insult, fresh medium without NCR-631 was added to the cultures. In the neuroinflammation experiments, NCR-631 was dissolved in the medium together with lipopolysaccaride (LPS 10 ng/ml; Salmonella abortus equi, Sigma, Lot: 69F4003) or IL-1β (10 IU/ml, recombinant mouse expressed in E. Coli, Sigma) and added to the cultures. Fresh medium containing the compounds was added every second day.

Histology

Four days after the anoxic insult or seven days after the LPS or IL-1β exposure the cultures were fixed in 0.4% formaldehyde and 0.125% glutaraldehyde in 0.1 M PBS. After rinsing, the cultures were incubated with a mouse monoclonal primary antibody against the glutamate NMDA-R1 receptor (diluted 1:800; batch MO16228; AMS Biotechnology, USA) for three days at 4°C in 0.1 M PBS containing 0.3% Triton-X100 and 1.5% normal horse serum. The cultures were thereafter washed, and incubated with a biotinylated secondary antibody for 30 minutes in the PBS buffer. After washing, the cultures were incubated for one hour with an avidin-biotin complex (ABC-Elite kit; Vectastain) in PBS buffer. The cultures were thereafter washed, and chromogen reaction was performed by incubation in 0.1 M Tris-HCl buffer with diaminobenzidine tetrahydrochloride (DAB) at a concentration of 1 mg/ml and 0.02% H_2O_2. The cultures were dehydrated, followed by xylene and mounted on glass slides.

The cultures were studied using light microscopy and all NMDA-R1 immunoreactive cells showing neuronal pyramidal morphology were counted in each culture. The results were presented as mean value ± standard error of the mean (SEM). The effects of the treatments were analyzed with Kruskal-Wallis test and subsequent *post hoc* comparisons employed Mann-Whitney U test. The differences between the mean values were defined as significant when $p < 0.05$.

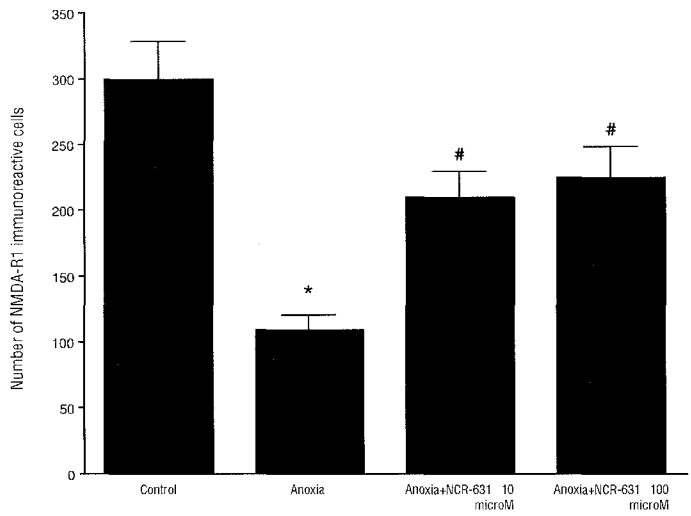

Fig. 1. Effect of NCR-631 on anoxia-induced (150 minutes) loss of hippocampal pyramidal neurons *in vitro*, when analyzed at four days after the insult. NCR-631 was administered 15 minutes before the insult at concentrations of 10 or 100 μM and remained in the medium until the following day (24 h). The data are presented as number of pyramidal neurons identified using immunohistochemical staining of the NMDA-R1 receptor subunit. * $p < 0.05$, compared to control group; # $p < 0.05$, compared to the anoxia group

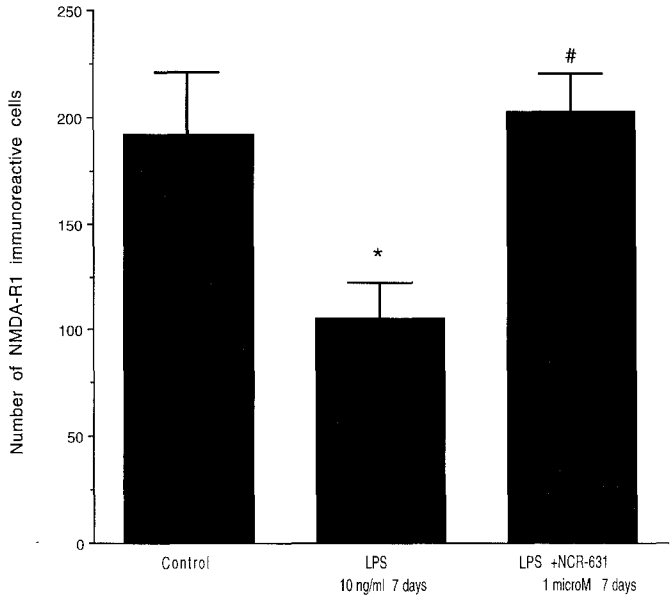

Fig. 2. Effect of NCR-631 on LPS-mediated (10 ng/ml) loss of hippocampal pyramidal neurons *in vitro*, when analyzed after seven days of exposure. NCR-631, at a concentration of 1 μM, was present in the medium during the entire exposure period. The data are presented as number of pyramidal neurons identified using immunohistochemical staining of the NMDA-R1 receptor subunit. *$p < 0.05$, compared to control group; #$p < 0.05$, compared to the LPS group

Results

The hippocampal slices developed into thin monolayered structures when studied after 21 days in culture. The cultures showed a structure resembling the hippocampal formation, but with a loss of distinct cell-layers. Patches of neuronal cells were occasionally found outside the proper hippocampal structure. In control cultures, the NMDA-R1 immunoreactive cells showed a pyramidal morphology and an extensive branching.

The number of NMDA-R1 immunoreactive cells decreased to 50–75% after the anoxic insult as compared to control cultures. In an initial experiment, the anoxia-induced cell loss (Control 398 ± 80; Anoxia 191 ± 15 NMDA-R1 cells/culture) was not affected by NCR-631 at a concentration of 3 μM (203 ± 18 cells/culture), while 10 μM (305 ± 25 cells/culture) and 30 μM (290 ± 23 cells/culture) provided significant ($p < 0.05$; anoxia vs anoxia+NCR-631), but not complete, protection. In a second experiment, significant protective effects were found after incubation in 10 μM or 100 μM NCR-631, still not completely reaching control levels (Fig. 1.). When added to control cultures for 24 h, NCR-631 did not affect the number of NMDA-R1 cells at concentrations of 10 or 30 μM (data not shown).

Exposure to LPS for seven days reduced the number of NMDA-R1 cells in the hippocampal cultures by 40–50%, while co-incubation with 1 μM NCR-631 completely counteracted the toxic effect of LPS (Fig. 2). Likewise, NCR-

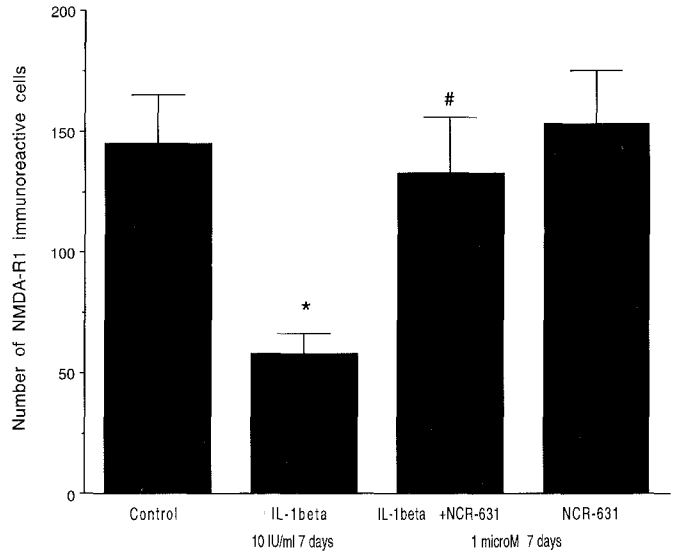

Fig. 3. Effect of NCR-631 on IL-1β-mediated (10 IU/ml) loss of hippocampal pyramidal neurons *in vitro*, when analyzed after seven days of exposure. NCR-631, at a concentration of 1 μM, was present in the medium during the entire exposure period. The data are presented as number of pyramidal neurons identified using immunohistochemical staining of the NMDA-R1 receptor subunit. *$p < 0.05$, compared to control group; #$p < 0.05$, compared to the IL-1β group

631 provided potent neuroprotective effects against the neurotoxicity of IL-1β (Fig. 3). NCR-631 did not in itself affect the number of NMDA-R1 cells in control tissue, when added to the culture medium for seven days at the neuroprotective concentration (see Fig. 3).

Discussion

The results show that NCR-631 provided protection against anoxia-induced loss of pyramidal neurons in rat organotypic cultures of hippocampus, when present during the insult. In the same culture system, NCR-631 also counteracted LPS- and IL-1β-mediated neurotoxicity.

NCR-631 acts as an inhibitor of 3-HAO, preventing the formation of QUIN following intracerebral administration of 3-HANA (Luthman et al., 1996). One may therefore speculate that the neuroprotective effect observed is related to the ability of NCR-631 to inhibit 3-HAO, and thereby reduce QUIN levels. It has previously been shown that QUIN-mediated neurodegeneration can occur *in vitro*. For example, QUIN is neurotoxic after acute exposure to low μM concentrations in primary cultures (Giulian et al., 1993), or following chronic exposure to sub-μM concentrations in organotypic cultures of rat brain (Whetsell and Schwarcz, 1989). On the other hand, *in vitro* systems present conditions that may limit studies on the role of kynurenines in neurodegenerative processes. Most importantly, plasma levels of QUIN are generally high compared to levels measured in brain tissue (see Luthman et

al., 1996). Therefore, when using serum in the culture medium the neuronal tissue is exposed to substantially higher concentrations of QUIN than under normal *in vivo* conditions. The functional state of certain kynurenine pathway enzymes as well as EAA receptors, in particular the NMDA receptor, could therefore differ significantly in cultured tissue as compared to in situ tissue. The exogenous supply of QUIN (approximately 0.5 μM) via the medium may also have a major impact on the possibility of 3-HAO inhibition to decrease QUIN levels in the cultured tissue. Hence, it remains to be established whether the observed effects of NCR-631 are related to 3-HAO inhibition and a reduction of QUIN in the tissue.

Major increases in cerebral QUIN levels have been shown to occur following experimental ischemia (Saito et al., 1993). Moreover QUIN appears to potentiate the neurotoxic effects of glutamate agonists in hippocampal slices exposed to hypoxia (Schurr and Rigor, 1993). It is therefore possible that QUIN is implicated in the anoxia-induced damage *in vitro*. While the neuroprotective effects of NCR-631, and other 3-HANA analogues, have to be further characterized, the present findings suggest that these compounds have neuroprotective actions *in vitro*; an effect that has been reported previously in an *in vivo* model of brain injury (Blight et al., 1995).

Kynurenines, and QUIN in particular, have been implicated in various neuroinflammatory conditions (Heyes et al., 1992, 1993). For example, after systemic LPS or pokeweed mitogen administration in mice increases in cerebral QUIN are seen that can be counteracted by 4-Cl-3-HANA treatment (Heyes et al., 1989; Saito et al., 1994). The immune-stimulated increase in QUIN seems to be critically linked to activation of macrophages or microglial cells, since the kynurenine pathway can be induced in both cell types following LPS or cytokine exposure (Heyes et al., 1993; Alberati-Giani et al., 1996). Furthermore, 4-Cl-3-HANA has been shown to inhibit the *in vitro* production of QUIN in macrophages (Heyes et al., 1993). It is therefore possible that LPS- or IL-1β-mediated neurotoxicity *in vitro* (Rothwell, 1991) could involve stimulation of the kynurenine pathway and QUIN production by microglial cells in the cultures. The neuroprotective effect of NCR-631 in both the LPS and IL-1β models may therefore be related to effects on kynurenine synthesis.

It can be concluded that a neuroprotective effect of NCR-631 was found in different *in vitro* models of anoxic damage or neuroinflammation, spurring further studies on the neuroprotective potential and mechanism of action of 3-HANA analogues.

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