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Neuroprotective and antioxidant activities of HU-211, a novel NMDA receptor antagonist

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Abstract

This study examines the ability of (+)-(3*S*,4*S*)-7-hydroxy-Δ^6-tetrahydrocannabinol-1,1-dimethylheptyl (HU-211), a non-competitive NMDA receptor antagonist to: (1) rescue neurons in culture from injury evoked by sodium nitroprusside, hydrogen peroxide (H_2O_2) and oxygen glucose deprivation; and (2) scavenge reactive oxygen species in vitro. Qualitative and quantitative assessments of cell survival have indicated that: (1) Neuronal cell injury produced following deprivation of oxygen and glucose was significantly attenuated by 5 μM HU-211. (2) Glial and neuronal cell damage induced by sodium nitroprusside was markedly ameliorated by 10 μM HU-211. (3) HU-211 reduced protein oxidation initiated by gamma irradiation, and scavenged peroxyl radicals. (4) HU-211 carries an oxidation potential of 550 mV. These findings suggest that HU-211 holds a unique position among putative neuroprotectant agents in that it combines NMDA receptor antagonistic activity and free radical scavenging abilities in a single molecule.

Keywords: Toxicity; Antioxidant; Free radical; NMDA receptor; Cortical culture

1. Introduction

Accumulating evidence suggests that multiple processes are linked to central neuronal degeneration following hypoxic-ischemic insult and head trauma or various neurodegenerative disorders. These are associated with excessive activation of excitatory amino acid receptors, in particular those of the NMDA receptor subtype (Choi, 1988; Meldrum and Garthwaite, 1990; Rothman and Olney, 1986, Lipton, 1993), and with the formation of reactive oxygen species which subsequently affect different biological molecules, such as lipids, proteins and nucleic acids (Braughler and Hall, 1989; Coyle and Puttfarcken, 1993; Werns and Lucchesi, 1990; Traystman et al., 1993). Selective NMDA receptor antagonists and free radical scavengers have gained tremendous interest in recent years because of their potential use as neuroprotectant agents in the clinic. NMDA receptor antagonists were shown to decrease cortical neuronal cell loss evoked by hypoxia in culture (Goldberg et al., 1987, 1988; Weiss et al., 1986), and to diminish focal ischemic brain injury in vivo (McIntosh et al., 1989; Scatton et al., 1991). Antioxidants were found to improve reactive oxygen species-induced brain damage produced in different experimental models (Clements et al., 1993; Uyama et al., 1990; Watanabe et al., 1994). Attempts to develop new drugs that may combine NMDA receptor antagonistic activity as well as antioxidant activity are in process. A possible candidate is the compound, HU-211, which is the (+)-(3*S*,4*S*) enantiomer of the synthetic cannabinoid 7-hydroxy-Δ^6-tetrahydrocannabinol (THC-DMH). Although HU-211 is structurally related to cannabinoids, it does not possess a cannabinoid pharmacological profile. HU-211 was shown to display a very low affinity to the cannabinoid receptors located within the central nervous system (CNS) and was inactive as a cannabimimetic in vivo (Howlett et al., 1990; Mechoulam et al., 1988). On the other hand, pharmacological studies carried out in vivo and in vitro indicate that HU-211 antagonizes glutamatergic neuro-

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transmission in the brain and describe HU-211 as an NMDA non-competitive antagonist (Feigenbaum et al., 1989). HU-211 was recently shown to block the activation of the NMDA preferring glutamate receptors via specific interactions with sites located within the receptor ionophore (Eshhar et al., 1993), thereby inhibiting the NMDA-stimulated influx of calcium ions into neurons (Nadler et al., 1993b). Recent investigations have suggested HU-211 as a neuroprotectant agent. These studies have demonstrated the ability of HU-211 to rescue cultured rat embryonal cortical neurons from injury evoked by exposure of cells to NMDA (Eshhar et al., 1993; Nadler et al., 1993a), and to reduce brain damage produced in ischemic and head trauma in vivo models (Bar-Joseph et al., 1994; Shohami et al., 1993). The ability of HU-211 to inhibit metabolic events which cause neuronal degeneration remains to be further clarified. The main goal of the current work therefore was to examine potential protective properties of HU-211 against oxygen-glucose deprivation insult and reactive oxygen species-induced neuronal degeneration in rat cortical cell cultures, and to provide a better understanding of the mechanism(s) underlying HU-211 neuroprotective activity.

2. Materials and methods

2.1. Cell culture preparation

Mixed cortical cell cultures consisting of neurons plated at various densities (from 100 000 cells/well up to 500 000 cells/well) over a confluent glial-cell feeder layer, were prepared from 18-to 20-day-old rat fetuses by mechanoenzymatic dissociation according to a procedure described previously (Eshhar et al., 1993). In general, a lawn of glial cells was generated by plating the dissociated cortical cells, at a very low density, onto polylysine-coated tissue culture plates. Upon reaching confluency, glial cell proliferation was inhibited by the addition of 5-fluoro-2′-deoxyuridine (FUDR)/uridine mixture to the cells, and neurons were plated over glial cells 3–5 days later. Growth conditions and medium used for both glial cells and for mixed culture maintenance were according to the protocol described previously (Eshhar et al., 1993). After 10 days in culture cells were subjected to toxicity experiments and subsequently processed for both qualitative and quantitative assessments of cell survival.

2.2. Oxygen-glucose deprivation

Immediately prior to oxygen-glucose deprivation, the maintenance culture medium was replaced by deoxygenated, glucose-free neuronal (MEM/N2) medium. Deoxygenated medium was prepared by flushing the medium for 5 min with an anaerobic gas mixture composed of 95% N_2/5% CO_2. Deprivation of oxygen was obtained by placing the cultures in an anaerobic chamber containing the same gas mixture as specified above for a 1 h period in a humidified 37°C incubator. Oxygen-glucose deprivation was terminated by replacing the medium with oxygenated regular neuronal cell maintenance medium, supplemented with 0.6% glucose. Cultures were returned to normoxic conditions (5% CO_2/95% O_2 incubator) for an additional 24 h prior to final assessments of neuronal cell survival. Protective effects of HU-211 against neuronal cell loss produced following deprivation of oxygen and glucose were determined by the addition of 5 and 10 μM HU-211 (Pharmos Corp.) to the bath medium present during and/or following exposure to hypoxic-hypoglycemic environment. HU-211 solution was prepared at a 100 μM concentration in 10% β-hydroxypropyl cyclodextrin solution and diluted in culture media prior to its use. The neuroprotective activity of HU-211 was compared to that elicited by 30 μM MK-801 (RBI, MA, USA), which was added to cells of sister cultures in parallel.

2.3. Exposure to sodium nitroprusside

Cells were exposed to various concentrations of sodium nitroprusside (300–5000 μM), either alone or in concert with 10 μM HU-211 or 30 μM MK-801. All exposures were carried out at 37°C for 20–24 h in a 5% CO_2 humidified atmosphere before determining the extent of sodium nitroprusside-induced cell injury. Cells of sister cultures exposed to the vehicle only were referred to as control.

2.4. Nitrite assay

Nitrite was used as an indicator for the formation of NO in cultures exposed to sodium nitroprusside. The extent of nitrite produced was determined according to a procedure described by Gross and Levi (1992). In brief, NO formation in culture was quantified colorimetrically at 540 nM following the addition of 50 μl of Greiss reagent (1% sulfanilamide and 0.1% naphthalendiamine in 5% *O*-phosphoric acid) to an equal volume of sample.

2.5. Hydrogen peroxide (H_2O_2) exposure

Cell cultures were exposed to several concentrations of H_2O_2 (from 100 μM up to 1000 μM) either alone or in the presence of 10 μM HU-211 (prepared as described above) or 30 μM MK-801 for 24 h prior to cell survival evaluation. Incubation conditions were identical to those used for cells exposed to sodium nitroprusside.

2.6. Evaluation of neuronal survival by morphological criteria

Cell viability was observed morphologically under phase-contrast optics at 100–200 × following enzyme-linked immunostaining of neurons for the neuronal marker enolase using the ABC biotin-avidin complex method according to a method previously described (Eshhar et al., 1993).

2.7. Quantitative measurements of neuronal survival

The proportion of cells surviving oxygen-glucose deprivation, or SNP and H_2O_2-evoked toxicity, was determined by measuring the extent of mitochondrial activity in living cells using the XTT (2,3-bis[2-methoxy-4-nitro-5-solfophenyl]-2*H*-tetrazolium-5-caroxanilide inner salt)-based assay (Tox-2, Sigma), as previously described (Eshhar et al., 1993). Mitochondrial activity was expressed as the optical density (O.D.) values of the colored XTT-reduced formazan products. The intensity of color formation stands in direct correlation to the number of live cells. The percent increase in cell viability following exposure of cells to medium lacking oxygen and glucose, in conjunction with HU-211 or MK-801, was calculated according to the formula: (O.D. treated cells/O.D. vehicle − 1) × 100.

2.8. Oxidation potential assessment

The oxidation potential displayed by 100 μM HU-211 was determined by using the Bioanalytical Systems (BAS) model CV-1B cyclic voltameter apparatus (West Lafayette, IN, USA). All cyclic voltammograms were conducted between O and + 2.0 V using the three-electrode system. This system included a glassy carbon disk 3.2 mm in diameter (BAS MF-2012) as the working electrode, a platinum wire as the counter electrode, and a Ag/AgCl (BAS) as the reference electrode. HU-211 applied to the system was prepared in a mixture composed of phosphate buffer saline (PBS) pH 7.2 and ethanol. The ratio (by volume) of the respective components in the mixture was 9:1.

2.9. Generation of peroxyl radicals and lipid oxidation measurements

Lipid-soluble peroxyl radicals were induced by the thermal decomposition of the azo compound AMVN (2,2′-azobis (2,4-dimethylvaleronitronitrile, purchased from Polysciences, Warrington, PA, USA) according to a procedure described by Yamamoto et al. (1984). The rate of peroxyl-radical generation from the initiator AMVN is constant at a given temperature and once produced can initiate free radical chain oxidation of lipids. Lipid oxidation of the compound methyl linoleate was determined. The oxidation of 100 mM methyl linoleate was induced by 100 mM AMVN, either alone or in the presence of various concentrations of HU-211. The rate of oxidation was followed by monitoring of oxygen consumption, as measured by the use of YSI oxygen monitor equipped with a Clark oxygen electrode. A decrease in the slopes of oxygen consumption versus time in samples containing HU-211 would reflect the ability of HU-211 to inhibit lipid peroxidation by scavenging peroxyl radicals.

2.10. Radical-induced oxidation of proteins

Protein oxidation was performed by exposure of a bovine serum albumine solution (0.5 mg/ml) containing 10 mM H_2O_2 and 2% alcohol to reactive oxygen species (hydroxyl and superoxide anion radicals) generated radiolytically following gamma irradiation of the protein. Irradiation was carried out using a ^{60}Co source at a dose rate of 10 Gy/min for the duration of 4 h. Following irradiation, 2 ml of 50 mM Hepes buffer pH 7.2 were added to the samples, and the extent of protein oxidation was determined by fluorescence analysis using a Jasco spectrofluorometer model FP-770 (λ_{ex} = 280 nm, λ_{em} = 345 nm). A decrease in fluorescence intensity in protein samples following irradiation is attributed to the oxidation of tryptophan. The influence of HU-211 on protein oxidation was determined in protein samples containing various concentrations of HU-211. An increase of fluorescence in samples containing HU-211 would indicate that HU-211 is able to prevent tryptophan oxidation in the bovine serum albumin.

2.11. Measurements of hydrogen peroxide (H_2O_2) breakdown

Levels of H_2O_2 were determined using two distinct methods. The first assay was carried out according to the procedure published by Thurman et al. (1972). In this system, the influence of HU-211 on H_2O_2 decomposition was determined by incubating various concentrations of HU-211 with 200 μM of H_2O_2 for 15 min at 37°C. The reaction was terminated by the addition of 200 μl of a solution containing 30% TCA, 0.55 mM ferric ammonium sulfate (NH_4 $Fe(SO_4)_2$ $12 \times H_2O$), and 246 mM sodium thiocyanate (NaSCN). Following an additional 10-min incubation period, the samples were centrifuged at room temperature for particle removal. Hydrogen peroxide decomposition was followed by the loss of its light absorbance at 480 nm. Optical density values are proportional to the H_2O_2 levels in samples. In the second method applied, the absorption of 2.3 mM H_2O_2, either alone or in conjunction with 50 μM HU-211, was recorded at 240 nm. In both methods, a reduction in the absorption in the presence

of HU-211 would indicate HU-211 ability in decomposing H_2O_2.

2.12. Generation and measurements of superoxide radicals

Superoxide radicals (O_2^-) were formed by incubating xanthine oxidase (0.02 U/ml) and hypoxanthine (1.2 mM) in 0.1 M phosphate buffer pH 7.8 containing 100 μM Detapac (diethylenetriamine-pentaacetic acid). Determination of the amount of radicals produced was carried out by monitoring the reduction of cytochrome *c* by superoxide radicals at 550 nm, as described by McCord and Fridovich (1969). The reaction was carried out in spectrophotometer cuvettes for a duration of 6 min. The influence of HU-211 on superoxide radicals was analyzed by measuring the extent of cytochrome *c* reduction in the presence of various concentrations of HU-211. A decrease in cytochrome *c* reduction in samples containing HU-211 would indicate HU-211 capacity to scavenge superoxide radicals.

2.13. Formation and detection of NO radicals in vitro

NO radicals were generated from the compound sodium nitroprusside and when converted to nitrite in the presence of oxygen were identified by the addition of Griess reagent. Various concentrations of sodium nitroprusside (up to 8 mM) were incubated for 2 h at 37°C, either alone or concurrently with 25 μM HU-211. The color intensity of the azo compound formed was assessed at 548 nm and is proportional to the extent of radical formation. A decrease in color formation in the presence of HU-211 would reflect the ability of HU-211 to scavenge NO radicals.

3. Results

3.1. Toxic effects of oxygen-glucose deprivation

Deprivation of oxygen and glucose for 1 h produced a widespread acute neuronal swelling. Neuronal morphology began to deteriorate 2–4 h following oxygen-glucose deprivation, and was finally associated with extensive degeneration of cell bodies and processes. In contrast, the underlying glial-cell monolayer remained intact following oxygen-glucose deprivation. Deprivation of oxygen and glucose was shown to produce extensive loss of neuron-specific enolase (NSE) (Fig. 1, panel A), as compared to NSE staining intensity within cells cultured in normoxic condition in a medium containing normal concentrations of glucose (Fig. 3, panel A).

3.2. Protective effects of HU-211 against oxygen glucose deprivation evoked neurotoxicity

Addition of 5 and 10 μM HU-211 to cultures during/following oxygen-glucose deprivation resulted in a marked reduction in neuronal injury. NSE immunostaining revealed that HU-211 at 10 μM concentration blocked morphological evidence of oxygen-glucose de-

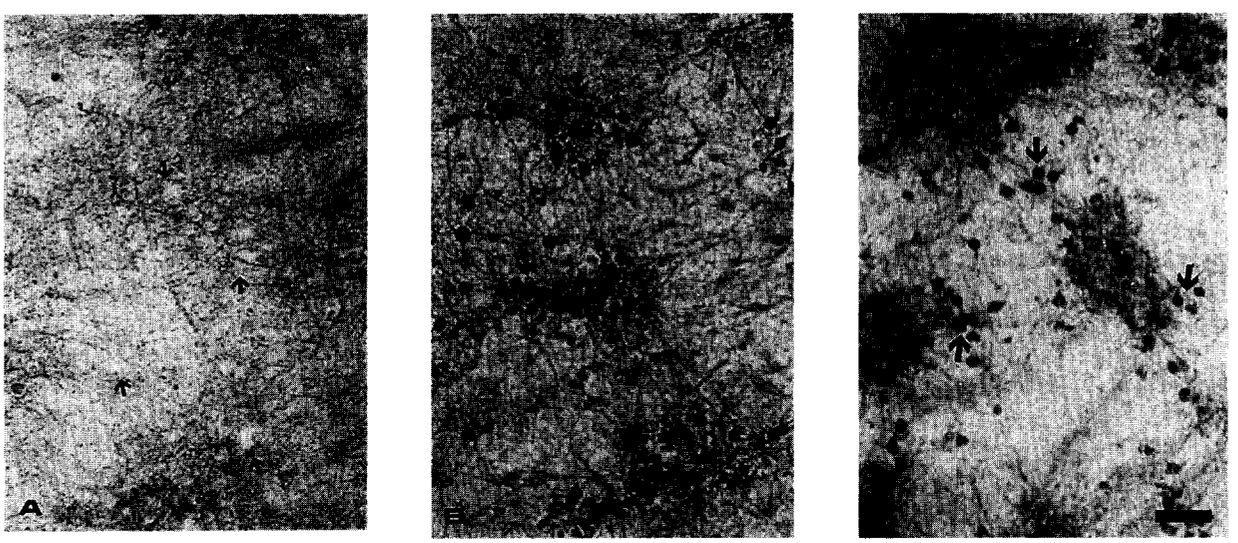

Fig. 1. Immunocytochemical illustration of HU-211 and MK-801 neuroprotective activity against oxygen-glucose deprivation-evoked toxicity. Phase-contrast micrographs show representative fields in cultures 24 h following 1 h of oxygen-glucose deprivation either in the absence (A) or in the presence of 10 μM HU-211 during and following the insult (B), or 30 μM MK-801 (C). Immunocytochemistry documents the expression of NSE in neurons (125 000 cells/well/24-well tissue culture plate). Note dense staining in cell soma (large arrows) of neurons following exposure to oxygen and glucose deprivation in the presence of HU-211 (B) or MK-801 (C). Cell bodies of injured neurons (small arrows) appear larger and are devoid of staining (A). One hour of oxygen-glucose deprivation results in neuronal cell damage, while glial cells remain intact. Line scale, 40 μm.

privation evoked neuronal injury (Fig. 1, panel B). To ascertain if oxygen-glucose deprivation evoked toxicity is mediated through the activation of NMDA receptors, the influence of MK-801 on cells was examined in parallel. Morphological observations and quantitative determination of cell viability have shown that MK-801 at 30 μM concentration and HU-211 at 10 and 5 μM concentrations have both reduced morphological and metabolic evidence of neuronal injury (Fig. 1, panel C and Fig. 2). As deduced from Fig. 2, the presence of 5 μM HU-211 or 30 μM MK-801 during and following deprivation of oxygen and glucose increased the metabolic activity in cultures by 33 and 36% respectively, compared to vehicle treated cultures.

3.3. Neurotoxic effects of sodium nitroprusside

Exposure of cortical derived cell cultures for 24 h to sodium nitroprusside at concentrations below 500 μM did not evoke either neuronal or glial cell loss. However, incubation of cells with increasing concentrations of sodium nitroprusside (500–5000 μM) resulted in a massive neuronal and glial cell injury which was detected 15–20 h following drug exposure. Morphology of cells exposed to 500 μM sodium nitroprusside is illustrated in Fig. 3, panel B. Immunostaining with antibodies to NSE revealed that sodium nitroprusside-elicited toxicity is associated with neuronal cell body 'ballooning', accompanied by degeneration of axonal and dendritic processes. Intensity of NSE staining in sodium nitroprusside injured neurons appeared lower than that expressed in cells of sister cultures treated with vehicle only (Fig. 3, panel A). Phase contrast optics indicated extensive destruction of the glial cell feeder layer supporting the neurons (Fig. 3, panels B and D). Quantitative assessments of overall cell mortality revealed that sodium nitroprusside, at concentrations up to 5000 μM, produced approximately 66–86% cell death. The extent of cell survival in cultures exposed to 500 and 2000 μM is illustrated in Fig. 4 and Fig. 5. Optical density values measured in cultures exposed to sodium nitroprusside, at either concentration, were significantly lower than those measured in cells of sister cultures treated with vehicle only. The application of the Greiss reagent (Gross and Levi, 1992) indicated that NO was, indeed, formed in the system. However, the extent of nitrite generated in culture was not dependent on the concentration of sodium nitroprusside within the range used in these experiments.

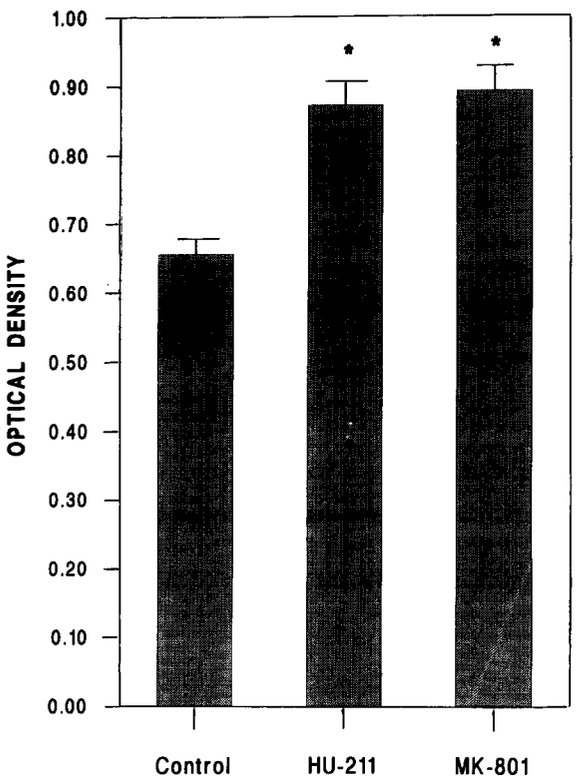

Fig. 2. Neuroprotective potency of HU-211 and MK-801 against injury produced following deprivation of oxygen and glucose. Cultures were deprived of oxygen and glucose for 1 h either alone or in the presence of 5 μM HU-211 or 30 μM MK-801. Cells were incubated for an additional 24 h in oxygenated high glucose medium containing HU-211 and MK-801, at indicated concentrations. Quantitative determinations of cell viability were performed using the XTT-based assay. The extent of overall neuronal cell viability stands in direct correlation with O.D. values. Results are mean ± S.E.M. of 3 experiments performed in sextuplicate. *HU-211 and MK-801 significantly differ from control ($P < 0.01$, by ANOVA followed by Fischer's protected least significant difference test).

3.4. HU-211 protective effects against sodium nitroprusside-evoked toxicity

As deduced from morphological and biochemical assays, sodium nitroprusside-mediated toxicity (up to 2000 μM sodium nitroprusside) was markedly attenuated by the presence of 10 μM HU-211. Neuroprotective efficiency of 10 μM HU-211 was directly related to the concentration of sodium nitroprusside applied. Co-administration of 10 μM HU-211 with 500 μM sodium nitroprusside increased neuronal survival from 40% to 95% approximately (Fig. 4), and from 20% to 65% when added to cells exposed to 2000 μM sodium nitroprusside (Fig. 5). The percentage of live cells in cultures following exposure to sodium nitroprusside, either in the absence or presence of HU-211 and MK-801, was calculated using the formula: (O.D. treated cells/O.D. control) × 100. The morphological features of HU-211 treated cells (Fig. 3, panel C) appeared almost identical to those of control cells (Fig. 3, panel A). NSE immunostaining revealed that 10 μM HU-211 substantially reduced the 'ballooning' of the neuronal cell bodies, and salvaged the axonal and dendritic network from degeneration produced by

sodium nitroprusside (Fig. 3, panel C). In order to verify whether nitroprusside toxicity is associated with NMDA receptor activation, the effects of MK-801 on cells exposed to sodium nitroprusside were examined. Co-application of 30 μM MK-801, with either of the concentrations of sodium nitroprusside used, failed to

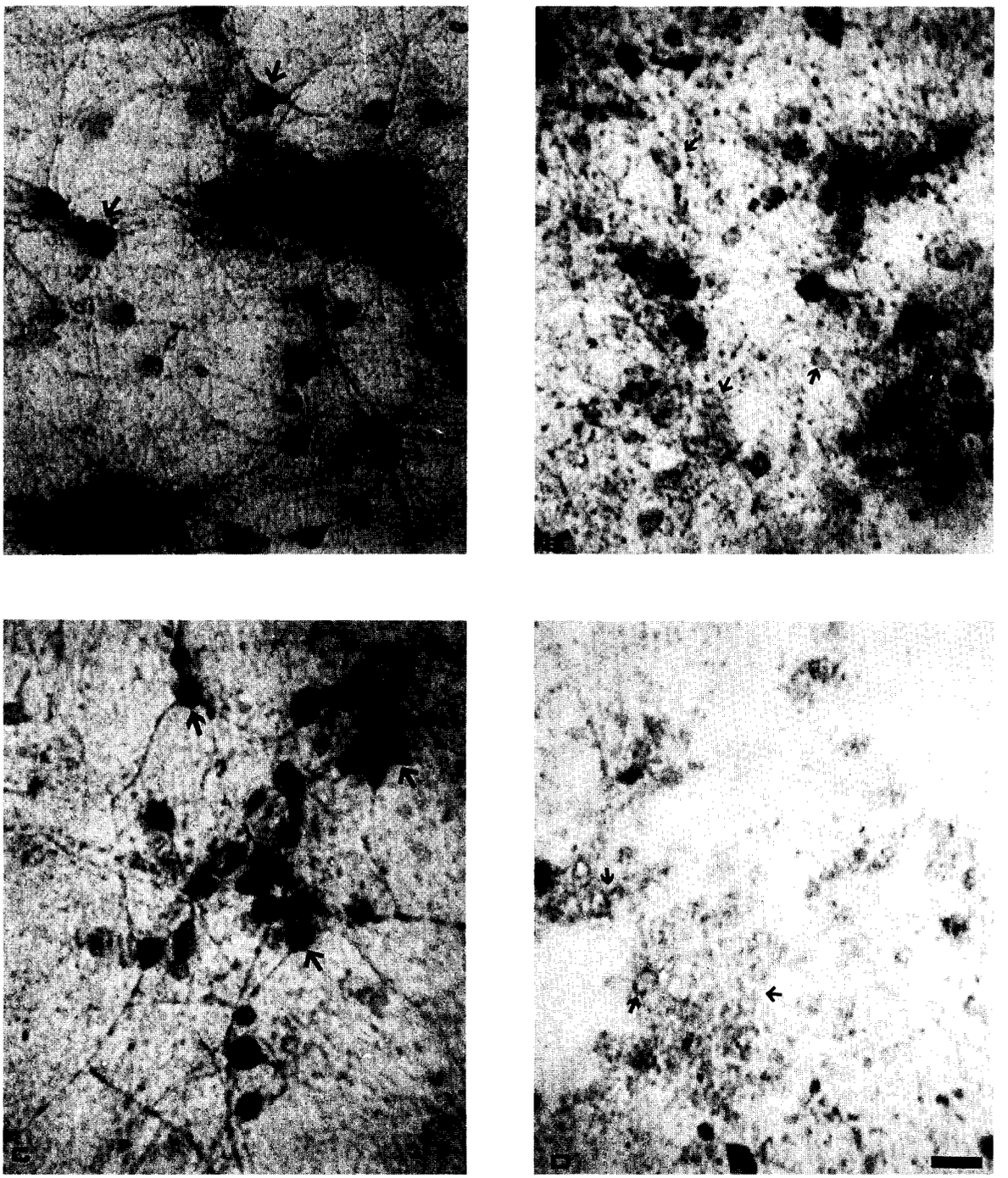

Fig. 3. HU-211 blocks morphological changes following sodium nitroprusside (SNP)-induced cell injury. Phase-contrast micrographs show representative fields in cultures immunostained with antibodies against enolase following a 24 h exposure to 500 μM sodium nitroprusside, either alone (B) or in concert with 10 μM HU-211 (C), or 30 μM MK-801 (D). Micrograph A represents morphology of cells in sister cultures exposed to the vehicle only. Sodium nitroprusside-evoked toxicity results in a massive injury of neurons and glial cells (B). Immunocytochemistry reveals dense staining in neuronal cell soma of controls (A), and in neurons exposed to sodium nitroprusside in the presence of HU-211 (large arrows). NSE expression in cultures exposed to sodium nitroprusside (B) or to sodium nitroprusside in the presence of MK-801 (D) is very low or absent (small arrows). Neuronal density in cultures is: 125 000 cells/well/24-well tissue culture plate. Line scale, 20 μm.

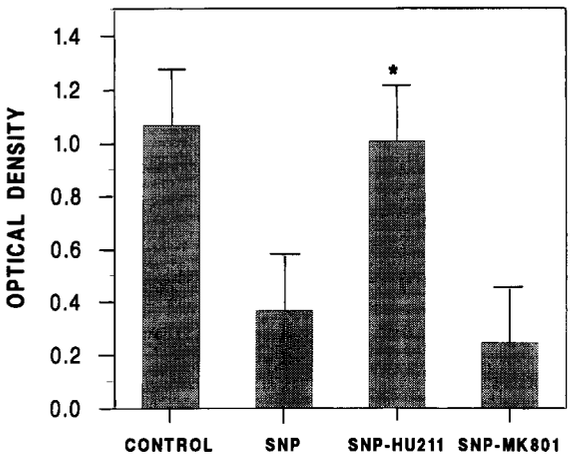

Fig. 4. Sodium nitroprusside (SNP)-induced toxicity is markedly prevented by HU-211 but not affected by MK-801. Cells were exposed to 500 μM sodium nitroprusside either alone or in the presence of 10 μM HU-211 or 30 μM MK-801. Results are expressed as O.D. values (measured by an ELISA reader) and stand in direct correlation with the extent of cell viability. Results are mean ± S.E.M. of 3 experiments performed in sextuplicate. * Significantly different from sodium nitroprusside and from sodium nitroprusside with MK-801 but not from controls ($P < 0.01$, by ANOVA followed by Fischer's protected least significant difference test).

antagonize sodium nitroprusside-evoked toxicity. The lack of MK-801 effect on cell survival during exposure to 500 μM sodium nitroprusside is illustrated in Fig. 3 and Fig. 4. Morphological observations of neurons in cultures treated with MK-801 indicated that it was unable to ameliorate sodium nitroprusside-evoked toxicity to any extent (Fig. 3, panel D). NSE staining in cells exposed to sodium nitroprusside in the presence of MK-801 appeared weak (Fig. 4, panel D) and was similar to that of cells exposed to sodium nitroprusside alone (Fig. 4, panel B).

3.5. H_2O_2 toxicity

Application of H_2O_2 to cells at concentrations between 100 μM and 300 μM had no toxic effects on glial or neuronal cells. However, exposure of cells to higher concentrations of H_2O_2 (from 400 μM up to 1000 μM) revealed a dose-dependent cell injury which was associated with glial, as well as neuronal cell loss. While exposure to 400 μM H_2O_2 damaged only about 12% of cells in culture, a 92% cell loss was measured in cultures exposed to 1000 μM H_2O_2. Co-application of 10 μM HU-211 or 30 μM MK-801, with either of the toxic concentrations of H_2O_2, failed to reduce H_2O_2-mediated toxicity in culture (data not shown).

3.6. HU-211 acts as a reducing agent

The oxidation potential of HU-211, recorded under the experimental conditions described in the Materials and methods section, was found to be $E_{1/2} = 550$ mV. These data indicate that HU-211 is an efficient reducing compound capable of donating electrons to an electron acceptor, and suggest HU-211 as a potential antioxidant (Kohen et al., 1988).

3.7. HU-211 as an antioxidant

The antioxidant activity of HU-211 was measured by recording its capacity to prevent lipid peroxidation initiated by peroxyl radicals, to prevent protein oxidation induced by gamma irradiation, to scavenge superoxide and NO radicals, and to decompose hydrogen peroxide. HU-211 inhibited the oxidation of methyl linoleate induced by peroxyl radicals generated from AMVN, and attenuated protein oxidation following gamma irradiation. As shown in Fig. 6, oxygen consumption in control samples containing methyl linoleate and AMVN only (controls) was found to be 15.5 μM O_2/min. Addition of increasing amounts of HU-211 to the reaction mixture resulted in a significant decrease

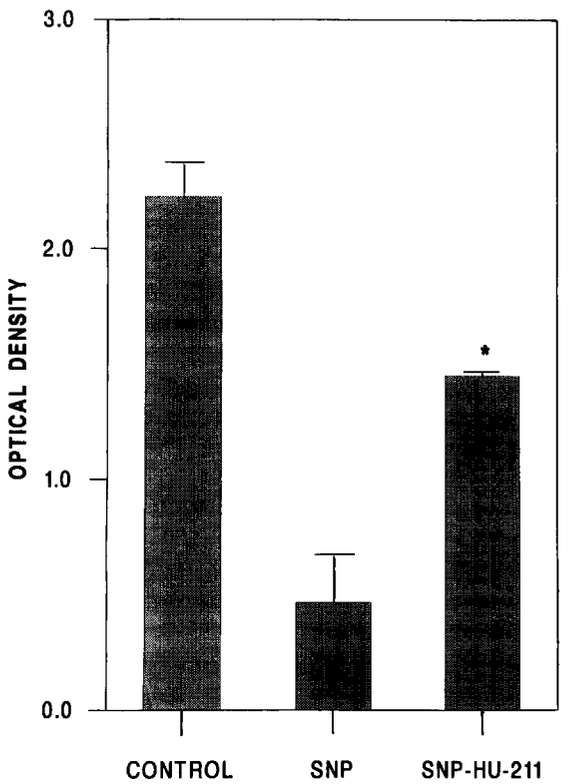

Fig. 5. HU-211 increases cortical cell survival in cultures exposed to sodium nitroprusside (SNP) (2000 μM). Cells were exposed to 2000 μM sodium nitroprusside for 24 h, either alone or in the presence of 10 μM HU-211. Quantitative assessments of cell survival were performed using the XTT-based assay. Results are expressed as O.D. values (measured by ELISA reader) and stand in direct correlation with the extent of cell viability. Results are mean ± S.E.M. of 3 experiments performed in sextuplicate. * Significantly different from sodium nitroprusside ($P < 0.01$, by ANOVA followed by Fischer's protected least significant difference test).

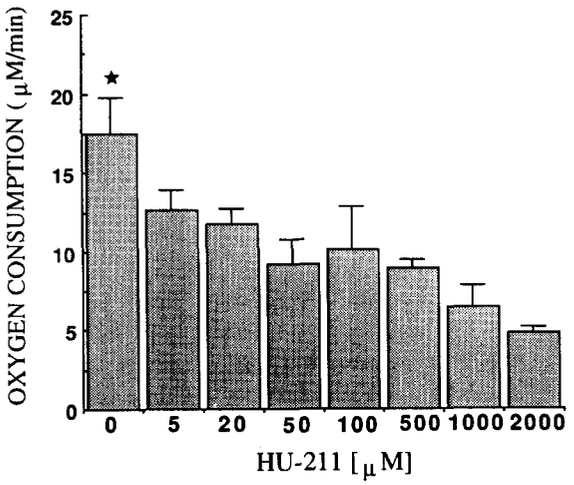

Fig. 6. Dose-dependent scavenging of peroxyl radicals by HU-211. Lipid peroxidation of 100 mM of methyl linoleate was induced by 100 mM of the azo compound AMVN used as the initiator of peroxyl radicals. The rate of lipid oxidation was determined by monitoring oxygen consumption. The presence of HU-211 at various concentrations in the system induced a significant decrease in the slope of oxygen consumption. The results are mean ± S.E.M of experiments performed a minimum of 3 times. *Significantly different from HU-211 inclusions, ($P < 0.001$) by Mann-Whitney test.

in the consumption of oxygen. Inclusion of 5 μM HU-211 resulted in an 18.7% reduction of oxygen consumed, while application of 50 μM HU-211 induced a 40% reduction. At the highest concentration of HU-211 examined (2 mM), a 69% reduction in oxygen consumption was recorded. As deduced from fluorescence analysis of protein samples subjected to gamma irradiation, protein oxidation was accompanied by a massive loss in tryptophan content, and was prevented by HU-211 in a concentration-dependent manner. Fluorescence was found to increase by 17% in the presence of 65 μM HU-211 and by 25% when irradiated in the presence of 130 μM HU-211 (fluorescence intensity values are shown in Table 1). HU-211 failed to induce the breakdown of hydrogen peroxide, and was unable to scavenge superoxide and NO radicals (data not shown).

Table 1
Fluorescence intensity of bovine serum albumin following gamma irradiation either alone or in the presence of HU-211

Treatment	Fluorescence intensity[a]
Control[b]	0.22, 0.22
	0.06, 0.06
Irradiation + 65 μM HU-211	0.086, 0.086
Irradiation + 130 μM HU-211	0.10, 0.095

Irradiation of 0.5 mg bovine serum albumin was carried out using a ^{60}Co source for 4 h. Fluorescence intensity was measured using a Jasco spectrophotometer model FP-770 ($\lambda_{ax} = 280$ nm, $\lambda_{em} = 345$ nm). [a]Duplicate samples were assayed and both values are shown. [b]Non-irradiated bovine serum albumin.

4. Discussion

Previous reports have demonstrated the ability of HU-211, a non-psychotropic cannabinoid and a non-competitive NMDA receptor antagonist, to block NMDA-induced toxicity in cultured cortical neurons (Eshhar et al., 1993; Nadler et al., 1993a). In this study we have performed a series of in-vitro experiments in order to further characterize its neuroprotective effects and mechanism of action. Neuroprotective activity was demonstrated in rat cortical cell cultures deprived of oxygen and glucose or in cultures exposed to free radical generating systems. Neuroprotective effects of HU-211 were compared to those elicited by MK-801. Results from these experiments show that HU-211: (a) increases the level of resistance of cultured cortical neurons to both oxygen-glucose deprivation insult and sodium nitroprusside-induced injury; (b) is a potent reducing agent capable of donating electrons; (c) prevents lipid peroxidation triggered by peroxyl radicals; and (d) inhibits protein oxidation (as determined by monitoring tryptophan oxidation) initiated by gamma irradiation.

Qualitative and quantitative assessments of cell survival have clearly revealed that HU-211 possesses an overall broader spectrum of neuron protective activity, as compared to that exhibited by MK-801. MK-801 was neuron protective in cells deprived of oxygen and glucose but ineffective during sodium nitroprusside exposure. The latter observation is in agreement with a report demonstrating that NMDA toxicity in hippocampal slices is not mimicked by sodium nitroprusside, and that MK-801 is ineffective in preventing sodium nitroprusside toxicity (Izumi et al., 1993). Neuron-protective activity of HU-211 and MK-801 against oxygen-glucose deprivation-induced neuronal damage confirms and extends previous observations indicating that this injury is indeed mediated by NMDA receptors (Rothman and Olney, 1986; Goldberg et al., 1987). However, the inability of MK-801 to influence sodium nitroprusside toxicity provides evidence that injury produced in cells exposed to sodium nitroprusside is not associated with NMDA receptor activation, but is rather mediated through other receptor systems and/or is associated with different metabolic processes. Such a conclusion gains additional support from morphological observations made in this study, which have shown that oxygen-glucose deprivation and application of sodium nitroprusside or H_2O_2 produced a different pattern of cell degeneration. While oxygen-glucose deprivation produced a selective destruction of neurons (results which are in accordance with morphological

observations, as shown also by Goldberg and Choi, 1993), sodium nitroprusside and H_2O_2-induced toxicity resulted in a substantial loss of both glial and neuronal cells. In contrast to previous reports showing that HU-211 blocking effects against NMDA activities are exerted in a non-competitive manner, e.g., HU-211 effects depend on its concentration and not on NMDA concentrations (Eshhar et al., 1993; Nadler et al., 1993b), results of the present study indicated that protective potency of HU-211 against sodium nitroprusside toxicity does depend on sodium nitroprusside concentrations. It is therefore probable that HU-211 does not block sodium nitroprusside effects in a non-competitive manner, but via a different mode of action. The capability of HU-211 to rescue glial cells of mixed cell cultures from sodium nitroprusside toxicity although glial cells have been shown to lack NMDA receptors (Teichberg, 1991), further suggests that HU-211 exerts its protective activity against sodium nitroprusside toxicity through an additional mechanism of action, possibly related to reactive oxygen species decomposition. The mechanism by which sodium nitroprusside elicits cell death is not entirely clear (Bates et al., 1991; Dawson et al., 1993; Izumi et al., 1993). Several of the above mentioned reports and others (Loiacono and Beart, 1992; Lustig et al., 1992; Maiese et al., 1993) have suggested that sodium nitroprusside-evoked toxicity is triggered by NO, which is spontaneously liberated from the nitroprusside anion when present in aqueous environment. Although NO is considered an important messenger molecule in the CNS, the exact role of NO in the CNS is still controversial. While some studies have suggested that NO is neurotoxic and mediates tissue damage in cerebral ischemia and in hypoxia-related neurotoxicity (Cazevielle et al., 1993; Faraci and Brian, 1994; Maiese et al., 1994; Nowicki et al., 1991), others have shown opposite results providing evidence that NO can function as a protective agent (Buisson et al., 1992; Wink et al., 1993; Zhang and Iadecola, 1993). Several studies have proposed that NO-mediated toxicity is engendered by its reaction with the superoxide anion radical (O_2^-), to yield cytotoxic reactive oxygen intermediates (peroxinitrite leading to the production of hydroxyl free radicals) which subsequently initiate lipid peroxidation (Beckman et al., 1990; Radi et al., 1991), protein oxidation (Neuzil et al., 1993) and DNA breaking (Nguyen et al., 1992). The inability of HU-211 to scavenge NO radicals clearly indicates that its protective activity is not associated with directly removing NO from the system. A plausible explanation is the ability of HU-211 to significantly antagonize oxidative damage, while it cannot be ruled out that yet another neuroprotective mechanism of action is operative. The present study has provided evidence that HU-211 functions to protect against oxidative mechanisms involved in cell destruction. This was first suggested by cyclic voltammetry, a method for quantitation of the oxidation potentials which indicate the ability of a compound to act as an electron(s) donor (Kohen et al., 1992); HU-211 displays an oxidation potential of 550 mV, a value within the range found for efficient reducing compounds (Kohen et al., 1992). Other antioxidants have been shown to possess oxidation potential values in the same range (as measured under similar conditions). This includes molecules such as carnosine, which was found to act as a potent antioxidant in muscle and brain (oxidation potential value of 750 mV, Kohen et al., 1988) and α-tocopherol; the major member of the vitamin E family (oxidation potential is around 750 mV, Kohen, unpublished data) defined as a 'chain-breaking antioxidant' (McCay et al., 1978). Further experiments have demonstrated a significant reduction in the extent of oxygen consumption during methyl linoleate peroxidation initiated by peroxyl free radicals, and in the rate of oxidative damage caused to bovine serum albumin, triggered by ionizing irradiation. The reduction in lipid peroxidation in the presence of HU-211 can be attributed to its ability to interact with peroxyl free radicals and thereby blocking the peroxidation process. HU-211 exhibits a concentration-dependent antioxidant activity, while α-tocopherol effects were shown to be concentration-independent (Liebler, 1993). It is therefore possible that the efficiency of antioxidant activity of these two compounds differs due to the stabilizing effects of the radicals that are produced following the interaction with peroxyl radicals. Inhibition of protein oxidation by HU-211 is most probably attributable to its ability to scavenge hydroxyl free radicals (which are generated radiolytically under the experimental conditions), thereby preventing their interaction with the protein. Protein oxidation is only partially inhibited by HU-211. It is possible that higher concentrations of HU-211 (above 130 μM) are required in the system in order to elicit greater efficiency of antioxidant activity; but these cannot be achieved due to solubility problems. The latter explanation may as well stand for the inability of HU-211 to protect cells of H_2O_2-mediated toxicity in culture.

In summary, these experiments have proved that HU-211 belongs to the class of compounds which are able to prevent lipid peroxidation and protein oxidation triggered by peroxyl and hydroxyl free radicals, the main radicals responsible for oxidative processes involved in postischemic tissue injury and brain trauma (Siesjö, 1992; McIntosch, 1993). The findings of this study strongly suggest that HU-211 holds a unique position among putative neuroprotectant agents in that it combines NMDA receptor blocking activity and free radical scavenging abilities in a single molecule. Multi-protective mechanisms displayed by HU-211 suggest that HU-211 may prevent brain damage associated

with stroke, cardiac arrest and head trauma. This study therefore supports the development of HU-211 as a new treatment approach for brain damage of various etiologies.

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