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Prevention of Glutamate Neurotoxicity in Cultured Neurons by 3,4-Dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2*H*)-benzopyran (CR-6), A Scavenger of Nitric Oxide

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ABSTRACT. Glutamate neurotoxicity in cerebellar neurons in culture is mediated by excessive production of nitric oxide (NO). We anticipated that 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2*H*)-benzopyran (CR-6) could act as a scavenger of NO since it contains a position (C-5) highly activated towards nitration reaction. The aim of this work was to assess whether CR-6 acts as an NO scavenger and prevents glutamate neurotoxicity in cultures of cerebellar neurons. It was shown that CR-6 reduced, in a dose-dependent manner, glutamate-induced formation of cGMP ($EC_{50} \approx 15$ μM) and prevented glutamate neurotoxicity. The protection was ≈50% at 3–10 μM and nearly complete at 100 μM. CR-6 did not prevent glutamate-induced activation of NO synthase, but interfered with the glutamate-NO-cGMP pathway at a later step. CR-6 reduced the formation of cGMP induced by *S*-nitroso-*N*-acetylpenicillamine (SNAP), an NO-generating agent, indicating that CR-6 acts as a scavenger of NO in cultured neurons. This was further supported by experiments showing that in neurons treated with CR-6 and glutamate, the 5-nitro derivative of CR-6 was formed, as determined by GC-MS analyses. Moreover, *in vitro* incubation of CR-6 with SNAP also produced the 5-nitroderivative, thus confirming that CR-6 directly reacts with NO. The results reported indicate that CR-6 acts as an NO scavenger in neurons and prevents glutamate neurotoxicity. BIOCHEM PHARMACOL **58**;2:255–261, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. glutamate neurotoxicity; nitric oxide; scavenger; protective effect; cGMP; NMDA receptor

Glutamate is the main excitatory neurotransmitter in mammals. However, persistent stimulation of glutamate receptors causes neuronal degeneration and death. Excitatory amino acid neurotoxicity has been proposed to contribute to the pathogenesis of different neurodegenerative situations, including ischemia, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease. In many systems, including cerebellar neurons, glutamate neurotoxicity is mainly mediated by excessive activation of the NMDA§ type of glutamate receptors [1–3]. Disruption of intracellular Ca^{2+} homeostasis is an essential event in the process of glutamate neurotoxicity [1, 4]. However, the subsequent events are not clearly understood. Activation of NMDA receptors leads to increased intracellular Ca^{2+} which, after binding to calmodulin, activates NOS, leading to increased production of NO, which in turn activates guanylate cyclase, resulting in an increased concentration of cGMP. This glutamate-NO-cGMP pathway plays important roles in the modulation of intracellular events and intercellular communication.

In several types of neurons in culture, one of the steps in the neurotoxic process is the glutamate-induced formation of NO. This is supported by experiments showing that glutamate neurotoxicity is prevented by inhibitors of NOS such as nitroarginine [5–8]. Activation of NMDA receptors also leads to increased production of superoxide radical, which has also been proposed to play a role in the excitotoxic process [9, 10]. Superoxide and NO react to produce peroxynitrite ion, a highly reactive species capable of generating hydroxyl radicals and of promoting the nitration of constituents of macromolecules, i.e. tyrosine in proteins [11]. Peroxynitrite has also been proposed as a mediator of glutamate and NO neurotoxicity [12, 13]. NO is a radical species. Therefore, compounds with antioxidant activity, particularly if they exert this action through a

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§ *Abbreviations:* CR-6,3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2*H*)-benzopyran; N-CR-6, 5-nitro derivative of CR-6: 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-5-nitro-1(2*H*)benzopyran; EBM, Eagles's basal medium; NO, nitric oxide; NOS, nitric oxide synthase; NMDA, *N*-methyl-D-aspartate; and SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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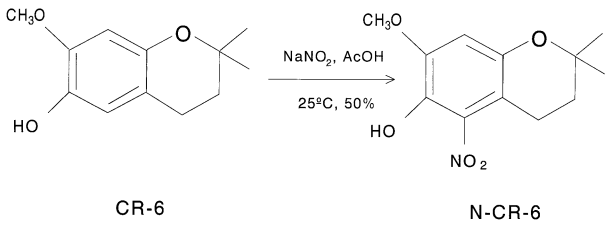

SCHEME 1. Formation of the 5-nitro derivative of CR-6.

radical scavenger mechanism, can reduce the neurotoxic effects of these compounds.

A simple tocopherol analogue, 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2*H*)-benzopyran (CR-6, Scheme 1), has been found to exhibit potent inhibitory activity of the lipid peroxidation induced on rat liver microsomes. This activity was comparable or higher than that elicited by 2,6-di-*tert*-butyl-4-methylphenol or α-tocopherol under the conditions assayed [14, 15]. Furthermore, the low toxicity shown by CR-6 along with its ability to be embedded into liposomes led us to explore the possible use of this antioxidant to reduce the undesirable side effects produced by the administration of liposome-encapsulated antitumoral drugs in cancer therapy. Clinical trials along this line are in progress [16].

On the other hand, the fact that CR-6 contains two non-substituted and highly activated aromatic positions led us to anticipate that this compound could also be considered as a γ-tocopherol analogue, particularly in regard to its potential capacity to react with nitrating species such as NO or $ONOO^-$. If this were the case, its value as antioxidant would be significantly expanded, since compounds exhibiting both protecting activities are scarce. Christen *et al.* [17] have recently shown the efficacy of γ-tocopherol, a minor component of vitamin E complex, in preventing the potential toxicity of peroxynitrite. Thus, in addition to its antioxidant activity, γ-tocopherol is a molecule susceptible of nitration by peroxynitrite [18], thus acting as a peroxynitrite scavenger. We anticipated that CR-6 could also act as a scavenger of NO and of peroxynitrite since it contains a position (C-5) highly activated towards nitration reaction.

The aim of this work was to assess whether CR-6 is able to act as an NO scavenger and to prevent glutamate neurotoxicity in primary cultures of cerebellar neurons. It is shown that CR-6 reduced glutamate-induced formation of cGMP and prevented glutamate neurotoxicity. CR-6 did not prevent glutamate-induced activation of NOS but did interfere with the glutamate-NO-cGMP pathway at a later step. Moreover, CR-6 reduced the formation of cGMP induced by SNAP, an NO-generating agent. *In vitro* incubation of SNAP with CR-6 led to the formation of the expected nitroderivative (N-CR-6). In neurons treated with CR-6 and glutamate, the 5-nitro derivative of CR-6 was also formed, indicating that CR-6 can act as a scavenger of NO in cultured neurons.

MATERIALS AND METHODS

Products

The CR-6 was prepared as previously described [14]. The synthesis of the 5-nitro derivative (N-CR-6) was carried out according to the method involving the nitrous acid-induced nitration of phenols [17], with minor modifications. Briefly, glacial acetic (2 mL) was added to a solution of CR-6 (0.2 g, 0.96 mmol) in absolute ethanol (50 mL), followed by addition of 2% $NaNO_2$ solution (30 mL). After 2 min at 25°, the orange reaction mixture was neutralized with 20% KOH and diluted with 100 mL of water. The product was extracted into hexane (3 × 100 mL) and washed with water, brine, and dried over $MgSO_4$. The residue obtained from solvent evaporation was purified by preparative TLC on silica gel using 3:1 hexane:ethyl acetate eluent mixture to give pure N-CR-6 (retention factor: r_f = 0.56) (0.120 g, 50% yield) as a bright yellow solid, i.r. (CCl_4): n (wave number) = 3554, 2979, 2937, 1732, 1544, 1444, 1272; ^{1}H NMR ($CDCl_3$): δ = 1.33 (s [singlet], 6 H, CH_3), 1.77 (t [triplet], J = 6.7 Hz, 2 H, H-3), 2.88 (t, J = 6.7 Hz, 2 H, H-4), 3.88 (s, 3 H, CH_3O), 6.61 (s, 1 H, H-8), 8.95 (s, 1 H, OH); ^{13}C NMR ($CDCl_3$): δ = 20.32 (C-4), 26.29 (CH_3), 32.21 (C-3), 56.38 (CH_3O), 74.12 (C-2), 106.8, 106.9 (C-4a, C-8), 135.8, 138.7 (C-5, C-6), 146.7, 147.8 (C-7, C-8a); MS (70 eV): m/z: (%): 253 (93) [M^+], 235 (16), 220 (100), 203 (47), 204 (40), 198 (62), 180 (52). $C_{12}H_{15}NO_5$. (253.3): calcd. C 56.91, H 5.97, N, 5.53; found C 56.58, H 6.12, N 5.13. The infrared (i.r.) spectra were registered with a Bomen MB120 spectrophotometer. The NMR spectra were registered with a Varian Unity 300 (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively). For ^{1}H NMR, $CDCl_3$ was used as solvent and tetramethylsilane as internal standard; for ^{13}C NMR, $CDCl_3$ was used as solvent and internal standard. The elemental analyses were performed with a 1108 Carlo Erba (Microanalysis Service, CID).

Detection of the 5-Nitro Derivative of CR-6 (N-CR-6)

Treatment of CR-6 with sodium nitrite in acetic acid led to the regioselective formation of the nitro derivative N-CR-6 in 50% yield (Scheme 1). The presence of the nitro group in N-CR-6 was evidenced by spectral and analytical data, and NOE (nuclear Overhauser effect) experiments confirmed that nitration occurred regioselectively at C-5. The gas GC-MS (electron impact) analyses were performed with a Fisons GC 8000 gas chromatograph fitted with a 2-μm BPX5, 25 m × 0.25 mm capillary column coupled to a Fisons MD 800 mass spectrometer. Standards and samples were injected in the splitless mode and the hot needle technique was used. The ion source and the injector were heated to 200 and 250°, respectively, and the program of injection was 130° (1 min), 7°/min. Under these conditions, CR-6 had a retention time of 8.67 min, while the 5-nitro derivative N-CR-6 eluted at 13.8 min. Samples were analyzed using the single ion monitoring technique.

For this purpose, peaks at m/z 208 (M^+) and 153 ($M^+ - 33$) were monitored for CR-6 and 253 (M^+) and 220 ($M^+ - 33$) for N-CR-6. The HPLC analyses were carried out using an Applied Biosystems 400 solvent delivery system, a Merck LiChrocart analytical column (5 μm LiChrospher 100 RP-18, 12.5 × 0.4 cm), and an Applied Biosystems 100 S Diode Array Detector. Samples were eluted with a mobile phase consisting of 55:45 methanol:water at a flow rate of 1 mL per min and detection was set at 285 nm. Under these conditions, CR-6 and N-CR-6 eluted at 5.90 and 9.05 min, respectively.

For detection of CR-6 and N-CR-6 in cultured neurons, cells were treated as described below for determination of cGMP. Neurons were incubated with 100 μM CR-6 for 10 min, then 1 mM glutamate was added to some plates and the incubation continued for 5 min. The neurons were washed, resuspended in distilled water, and immediately lyophilized. At the time of analysis, lyophilized neurons were resuspended in 350 μL of 0.4% perchloric acid, sonicated with a LABSONIC U (B. Brawn- Biotech S.A) apparatus for 30 sec at 4°, and extracted into methyl *tert*-butyl ether (2 × 500 μL). The solvent was evaporated under nitrogen, the extracts were redissolved in 10 μL of ethyl acetate, and 2-μL aliquots were used for the GC-MS analyses (assays in duplicate). The remaining solution was evaporated under nitrogen and redissolved in methanol (20 μL), and 10-μL aliquots were injected into the HPLC system. The presence of CR-6 and N-CR-6 in the corresponding extracts was examined by GC-MS using the single ion monitoring technique to improve the sensitivity and specificity of the analysis. Figure 4 shows the results obtained.

Reaction of CR-6 with SNAP

Assays were carried out in 2 mL of methanol:0.05 M potassium phosphate/acetic acid solution containing 0.5 mM CR-6 and 1 mM SNAP. The reaction mixture was stirred at room temperature for 3 hr, extracted with hexane (3 × 0.7 mL), evaporated, redissolved in methanol, and analyzed by HPLC. The HPLC analyses were carried out as indicated above. In other experiments, the incubation of SNAP with CR-6 was carried out at neutral pH (7.4) in Locke's solution (see below) or in 50 mM phosphate buffer, pH 7.4. Extraction and analysis of the products was carried out as above.

Primary Neuronal Cultures

Preparation of primary cultures of cerebellar neurons from 7- to 8-day-old Wistar rats was based on a procedure previously reported [19] and modified by Miñana *et al.* [20] to minimize mechanical damage and avoid undesirable cell clumping. Cells were plated onto polylysine-coated plates (312,000 cells/cm^2; 2 mL for plates 35 mm in diameter) and, after 15 min at 37°, medium containing unattached cells was removed and fresh medium added. The cells were grown at 37° in a 5% CO_2 atmosphere. To prevent proliferation of non-neuronal cells, 10 μM cytosine arabinoside was added 24 hr after plating. Glucose, 5.6 mM final concentration, was added to the culture medium twice a week. Under these culture conditions, more than 95% of the cells in the culture are neurons.

Assay of Glutamate Neurotoxicity and of Its Prevention

Glutamate toxicity in cerebellar neurons was assayed after 11–16 days of culture. Briefly, culture medium was removed and kept at 37° (conditioned medium). Cells were washed and incubated at 37° for 15 min with Locke's solution (in mM: NaCl, 154; KCl, 5.6; $NaHCO_3$, 3.6; $CaCl_2$, 2.3; glucose, 5.6; HEPES, 5; pH 7.4), containing 10 μM glycine. Then, this solution was removed and cells were incubated in Locke's solution without glycine for 3.5 hr at 37° in the presence of 1 mM glutamate. To test the protective effect of CR-6, it was added 10 min before addition of glutamate. Cells were washed with Locke's solution without glycine and the conditioned medium previously removed was added again. Cell viability was measured 24 hr later by staining with fluorescein diacetate (15 μg/mL) and propidium iodide (4.6 μg/mL), as previously described [19]. The percentage of surviving neurons was calculated by assessing the ratio of fluorescein diacetate/propidium iodide (green/red) staining directly under the microscope. At least 1,200 cells were counted for each point.

Determination of cGMP in Cultured Neurons

Primary cultured neurons were used 11–15 days after seeding. Monolayers in tissue culture dishes (35-mm diameter) were washed three times with prewarmed Locke's solution without magnesium. The assays of glutamate- or SNAP-induced formation of cGMP were carried out in Locke's solution without magnesium. 3-Isobutylmethylxanthine (100 μM) was added 5 min before addition of the compounds to be tested. Treatments with glutamate (1 mM) or SNAP (0.1 mM) were carried out for 5 min at 37°. CR-6 was added 10 min before addition of glutamate or SNAP. cGMP was determined using the BIOTRAK cGMP enzyme immunoassay kit from Amersham. After treatments, Locke's solution was removed and cGMP in neurons was measured. Values are given as the means ± standard deviations of at least six experiments with five different cultures. For each experiment, samples were measured in duplicate, deviations within samples in the same experiment always being less than 10% of the value.

Determination of NOS Activity in Intact Cerebellar Neurons

Eleven to fourteen days after culture, monolayers in tissue culture dishes were washed twice with prewarmed Locke's solution without magnesium. For the assay of NOS activity in intact neurons, the conversion of [^{14}C]arginine to [^{14}C]citrulline was determined as described by Kiedrowski *et al.* [21]. [^{14}C]arginine (1.7 μM, 0.25 μCi) was first added

to the cultures followed by glutamate (1 mM) 15 min later, and the incubation was continued for a further 5 min. The medium was removed and the neurons were washed three times with 2 mL of cold Locke's solution without magnesium and resuspended in 1 mL of 0.3 M $HClO_4$. After centrifugation, [^{14}C]citrulline was determined in the supernatant and protein in the pellet. Before liquid scintillation counting, [^{14}C]citrulline was separated from [^{14}C]arginine by chromatography through a Dowex AG50WX-8 (Na^+ form) column. For each sample, a blank treated with 100 μM nitroarginine to inhibit NOS was carried out. NOS activity was expressed as the difference between the [^{14}C]citrulline formed in the absence and the presence of nitroarginine. For neurons treated with glutamate, nitroarginine inhibited [^{14}C]citrulline formation by 82 ± 6%. CR-6 was added 10 min before glutamate. For high concentrations of CR-6, the solvent (dimethylsulfoxide) interfered with the inhibition of NOS by nitroarginine, resulting in a higher formation of [^{14}C]citrulline than in samples without dimethylsulfoxide. However, the formation of [^{14}C]citrulline in neurons treated or not with glutamate was the same in the presence or absence of CR-6 when nitroarginine was not present. Values were calculated as the means ± standard deviations of three experiments using three different cultures. For each experiment, samples were measured in duplicate, deviations within samples in the same experiment always being less that 9% of the value.

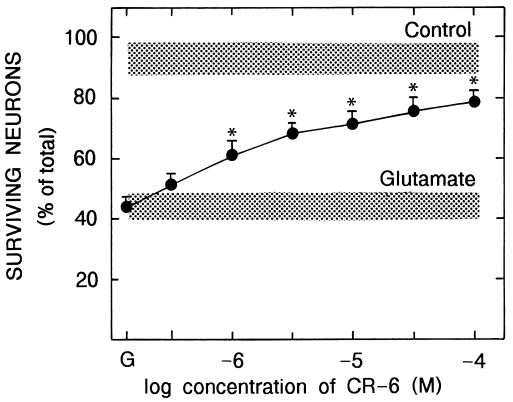

FIG. 1. CR-6 prevents glutamate neurotoxicity in primary cultures of cerebellar neurons. Neuronal cultures were used 11–16 days after seeding. Cells were washed with Locke's solution without magnesium as described in Methods and the indicated concentrations of CR-6 were added. After 10 min, glutamate (1 mM final) was added and incubation was continued for 3.5 hr. Cell survival was determined as indicated in the Methods section. Values are the means ± standard deviations of duplicate samples from four different cultures. At least 1200 neurons were counted for each sample. Dotted areas indicate the survival of control neurons and of neurons treated only with glutamate, in the absence of CR-6. G indicates the value for samples treated only with glutamate, in the absence of CR-6. Asterisks indicate the points for which CR-6 afforded significant protection against glutamate neurotoxicity.

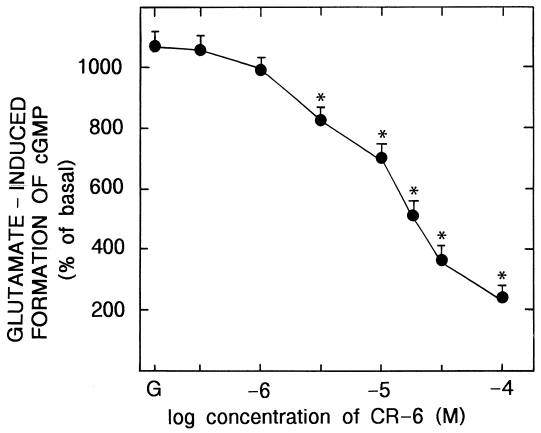

FIG. 2. CR-6 reduces glutamate-induced formation of cGMP. Primary cultures of cerebellar neurons were incubated with the indicated concentrations of CR-6 for 10 min in Locke's solution. Then, 1 mM glutamate was added and the incubation continued for 5 min. Basal and glutamate-induced formation of cGMP were measured as indicated in Methods. The basal values of cGMP in control neurons varied somewhat (between 0.6 and 1.2 pmol/mg protein) for different experiments, but the effects of glutamate and CR-6 were highly reproducible. For each experiment, the basal concentration of cGMP in control neurons was considered as 100%. Values for cGMP in neurons treated with CR-6 and glutamate are presented as percentages of basal cGMP in control neurons. Values which were significantly different ($P < 0.001$) from control neurons treated with glutamate are indicated by asterisks. G indicates the value for samples treated only with glutamate, in the absence of CR-6.

RESULTS

Treatment of primary cultures of cerebellar neurons with 1 mM glutamate induced neuronal death, with only 43% of the neurons surviving. However, when the neurons were preincubated with CR-6, glutamate neurotoxicity was significantly prevented in a dose-dependent manner: 61% of the neurons preincubated with 1 μM CR-6 survived and the protective effect was maximum (79% neuron survival) at 100 μM CR-6 (Fig. 1).

We tested whether CR-6 interferes with the glutamate-NO-cGMP pathway. Addition of 1 mM glutamate increased cGMP content into the neurons by 10.7 ± 1.2-fold. As shown in Fig. 2, CR-6 reduced the formation of cGMP induced by glutamate in a dose-dependent manner. The increase in cGMP induced by glutamate was only 7.5-, 5-, 4-, and 2.6-fold for neurons preincubated with 10, 20, 30, and 100 μM CR-6, respectively.

The above results indicate that CR-6 interferes with the glutamate-NO-cGMP pathway. To clarify at which step CR-6 was acting, we tested whether it prevents glutamate-induced activation of NOS. As shown in Table 1, CR-6 did not affect glutamate-induced activation of NOS in intact neurons, thus indicating that it interferes at a later step.

We then assessed whether CR-6 acts as a scavenger of NO. We tested whether CR-6 prevents the formation of cGMP induced by SNAP, an NO-generating agent. As shown in Fig. 3, addition of 0.1 mM SNAP to the intact

TABLE 1. Effect of addition of CR-6 to primary cultures of cerebellar neurons on NOS activity

CR-6	Glutamate	NOS activity (pmol/mg protein)
No	No	3.1 ± 1.5
No	Yes	97 ± 17
Yes	No	2.8 ± 1.2
Yes	Yes	113 ± 11

Primary cultures of cerebellar neurons were prepared from 7- to 8-day-old rats. Cultures were used 11–15 days after seeding. Basal and glutamate activation of NOS was measured as indicated in Methods. Values are the means ± standard deviations of duplicate samples from three different experiments using three different cultures. No significant effect of CR-6 on NOS activity was found.

neurons in culture induced the formation of cGMP, leading to a 11.3 ± 0.8-fold increase in cGMP concentration. Addition of CR-6 significantly reduced SNAP-induced formation of cGMP. The increase in cGMP induced by SNAP was reduced to 9.5-, 7.6-, and 3.6-fold in neurons preincubated with 20, 30, and 40 μM CR-6, respectively. This supports the idea that CR-6 acts as a scavenger of NO.

To confirm this possibility, neurons were incubated with CR-6 and it was tested whether this incubation leads to the formation of the 5-nitro derivative of CR-6. As shown in Fig. 4b, the neurons incubated with CR-6 but in the absence of glutamate did show the presence of CR-6, but no trace of the 5-nitro derivative N-CR-6 was detected.

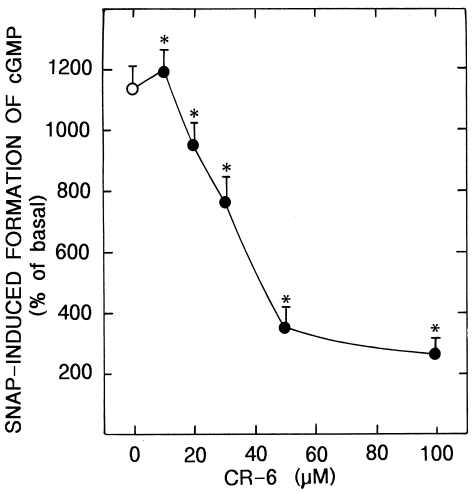

FIG. 3. CR-6 reduces SNAP-induced formation of cGMP. Primary cultures of cerebellar neurons were incubated with the indicated concentrations of CR-6 for 10 min in Locke's solution. Then, 0.1 mM SNAP was added and the incubation continued for 5 min. Basal and SNAP-induced formation of cGMP were measured as indicated in Methods. The basal values of cGMP in control neurons varied somewhat (between 0.9 and 1.5 pmol/mg protein) for different experiments, but the effects of SNAP and CR-6 were highly reproducible. For each experiment, the basal concentration of cGMP in control neurons was considered as 100%. Values for cGMP in neurons treated with CR-6 and SNAP are presented as percentages of basal cGMP in control neurons. Values which were significantly different ($P < 0.001$) from control neurons treated with glutamate are indicated by asterisks.

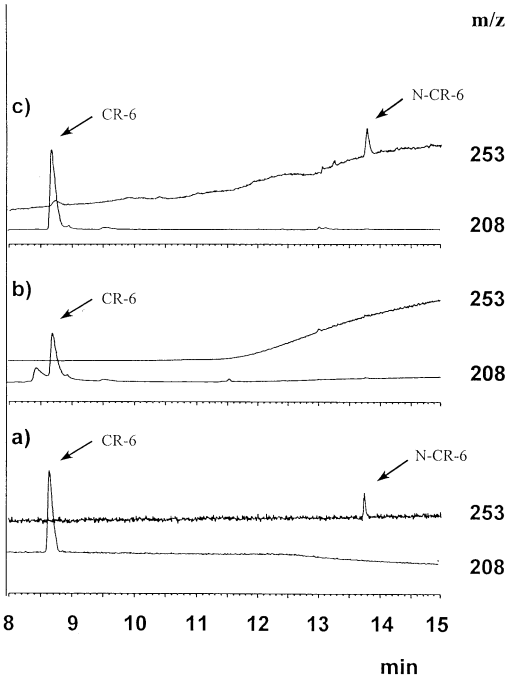

FIG. 4. Addition of glutamate induces the formation of the 5-nitro derivative of CR-6 in intact neurons. Selected ion mass chromatograms (electron impact) of gas chromatography eluates of: (a) standard sample of CR-6 (m/z = 208, M^+) and its 5-nitro derivative (m/z = 253, M^+); (b) extracts of neurons incubated with 100 μM CR-6 in the absence of glutamate; and (c) extracts of neurons incubated with 100 μM CR-6 and 1 mM glutamate. For detection of CR-6 and of N-CR-6 in cultured neurons, the neurons were incubated with 100 CR-6 for 10 min. Then, 1 mM glutamate was added to some plates (c) but not others (b) and the incubation was continued for 5 min. The neurons were washed, resuspended in distilled water, and immediately lyophilized. CR-6 and N-CR-6 were detected as indicated in Methods. It can be seen that neurons treated only with CR-6 (b) show only the peak corresponding to this antioxidant, while those treated with CR-6 and glutamate show both the CR-6 peak and that corresponding to the 5-nitro derivative.

Conversely, in the neurons incubated in the presence of both CR-6 and glutamate, the GC-MS profile showed, in addition to the peak of the former compound, the peak for the 5-nitro derivative N-CR-6, which appeared at the retention time expected for this compound (Fig. 4c). The identification of N-CR-6 in this sample was also confirmed by reversed phase HPLC. It is worth noting that the detection of N-CR-6 can only be assessed qualitatively, since in our experience this nitro derivative is unstable and undergoes an easy oxidation to give an *ortho*-quinone derivative as major compound. These results indicate that the NO synthesized as a consequence of the activation of glutamate receptors leads to the formation of the 5-nitro derivative of CR-6.

To confirm that CR-6 can actually react directly with NO, we tested whether *in vitro* incubation of pure CR-6 with pure SNAP, which generates NO, leads to the formation of the expected 5-nitro derivative. At neutral pH, incubation of 0.1 or 1 mM SNAP with CR-6 produced

the 5-nitro derivative N-CR-6 (although it was not the main derivative formed), while the 5-nitroso derivative was not detected. The formation of the 5-nitro derivative under these conditions was confirmed by reversed phase HPLC (coincidence in retention time and UV spectra with those of the authentic standard), thus confirming that CR-6 can react directly with NO.

DISCUSSION

The results reported herein show that CR-6 completely prevents glutamate neurotoxicity in primary cultures of cerebellar neurons. We had previously shown that, in the primary cultures of rat cerebellar neurons used in this work, glutamate neurotoxicity was completely prevented by NOS inhibitors such as nitroarginine [7], indicating that NO plays an essential role in the mediation of glutamate neurotoxicity in these cells. The protective effect of CR-6 might be due to its role as an NO scavenger. CR-6 also acts as an antioxidant and a scavenger of reactive oxygen species (ROS). It has been reported that in primary cultures of mice cerebellar neurons, ROS, especially superoxide, are more implicated than NO in the mediation of NMDA neurotoxicity [9]. As mentioned above, in primary cultures of rat cerebellar neurons, inhibition of NOS by nitroarginine completely prevents glutamate-induced neuronal death [7], thereby suggesting that scavenging of NO could be sufficient in itself to explain the protective effect of CR-6 in these neurons. However, a contribution of scavenging ROS to the protective effect of CR-6 cannot be ruled out. The use of a bifunctional molecule such as CR-6 to trap NO and ROS could provide a more beneficial effect than species scavenging alone on one of these toxic species.

Figure 2 shows that CR-6 reduces the formation of cGMP induced by glutamate. The glutamate-NO-cGMP pathway involves activation of glutamate receptors, leading to increased intracellular Ca^{2+}, which, after binding to calmodulin, activates NOS, leading to increased levels of NO. NO activates guanylate cyclase, leading to an increased concentration of cGMP. The reduction by CR-6 of the formation of cGMP induced by glutamate indicates that it interferes with some step of the glutamate-NO-cGMP pathway. As CR-6 did not reduce glutamate-induced activation of NOS, this indicates that it interferes at a later step in the pathway.

CR-6 reduced the formation of cGMP induced by an NO-generating agent (SNAP), indicating that it acts as a scavenger of NO or prevents NO-induced activation of guanylate cyclase. As shown in Fig. 4, neurons treated with glutamate and CR-6 contain the 5-nitro derivative of CR-6, while those treated only with CR-6 did not show this derivative. This indicates that CR-6 reacts with the NO formed in response to activation of glutamate receptors, thus acting as an NO scavenger and avoiding activation of guanylate cyclase by NO. As mentioned in the Results section, CR-6 can react directly with nitric oxide. CR-6 would therefore reduce the concentration of free NO by reacting with it and forming the 5-nitro derivative of CR-6.

These results indicate that CR-6 may be useful as an experimental tool for research on the role of NO in the modulation of different signal transduction pathways under physiological or pathological conditions. Moreover, CR-6 could also have potential therapeutic utility in neuropathological situations associated with glutamate or NO neurotoxicity.

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