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# Cytochrome *c* Is Released from Mitochondria in a Reactive Oxygen Species (ROS)-dependent Fashion and Can Operate as a ROS Scavenger and as a Respiratory Substrate in Cerebellar Neurons Undergoing Excitotoxic Death*

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**In rat cerebellar granule cells both reactive oxygen species production and release of cytochrome *c* take place during glutamate toxicity. This investigation was aimed (*i*) to ascertain whether and how these two processes are related and (*ii*) to gain insight into the role played by the released cytochrome *c* in the onset of neurotoxicity. Cytochrome *c* release takes place owing to the generation of reactive oxygen species both in glutamate-treated cerebellar granule cells and in sister control cultures incubated in the presence of the reactive oxygen species-generating system consisting of xanthine plus xanthine oxidase. In the early phase of neurotoxicity (30-min glutamate exposure) about 40% of the maximum (as measured at 3 h of glutamate exposure) cytochrome *c* release was found to occur in cerebellar granule cells from mitochondria that were essentially coupled and intact and that had a negligible production of oxygen free radicals. Contrarily, mitochondria from cells treated with glutamate for 3 h were mostly uncoupled and produced reactive oxygen species at a high rate. The cytosolic fraction containing the released cytochrome *c* was able to transfer electrons from superoxide anion to molecular oxygen via the respiratory chain and was found to partially prevent glutamate toxicity when added externally to cerebellar neurons undergoing necrosis. In the light of these findings, we propose that in the early phase of neurotoxicity, cytochrome *c* release can be part of a cellular and mitochondrial defense mechanism against oxidative stress.**

An excessive and prolonged glutamate release has been found to play a major role in the death of neurons in many forms of acute and chronic diseases (1, 2). Moreover *in vitro* cultured cerebellar granule neurons undergo death via necrosis when exposed to excessive glutamate (1, 2). However the events that underlie necrosis as well as the participation of the different cell components to the biochemical processes that lead the cell to death have not been yet established in detail. As far as mitochondria are concerned, their major role in the glutamate-dependent necrosis is now commonly accepted as follows. Dying neurons have been shown to lose their mitochondrial membrane potential and energy charge (3), and a key role in $Ca^{2+}$ homeostasis (4–6) was proposed for mitochondria that were also shown to undergo $Ca^{2+}$-dependent uncoupling (7). An early and progressive mitochondrial dysfunction (5, 8) and oxidative stress (9–11) have been shown to occur under glutamate neurotoxicity (GNT).[1] Consistently, an increase of glucose uptake by cerebellar granule cells (CGCs) was shown accounting for the higher rate of anaerobic glycolysis (12). We have recently reported that during necrotic death caused by GNT, cytochrome *c* (cyt *c*) release from mitochondria of CGCs occurs. This release was found to not impair mitochondrial respiration as measured in cell homogenate, at least in the early phase of glutamate exposure, but it was suggested to allow for cell defense against the onset of glucose uptake deficiency (13). On the other hand, it should be noted that cyt *c* release has been already reported to occur during apoptosis (14–19); cyt *c* release was found to be associated with permeability transition pore opening (20, 21) or occurring via the voltage dependent anion channel (22). Cyt *c* release has also been found to occur before mitochondrial membrane potential collapse (14) and to actively participate in the cascade of events leading to caspase activation (15, 23). During GNT, how cyt *c* release occurs as well as its function (see 13) outside the mitochondria remain to be established. Since reactive oxygen species (ROS) production, mostly due to xanthine oxidase (XOD) activity (11), occurs together with cyt *c* release during GNT (13), we carried out experimental work aimed at ascertaining whether and how ROS could cause the release of cyt *c* and whether this cyt *c* could take part in the mitochondrial defense mechanism against cell oxidative stress.

## EXPERIMENTAL PROCEDURES

*Reagents*—Tissue culture medium (basal medium Eagle's) and fetal calf serum were purchased from Life Technologies, Inc., tissue culture

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[1] The abbreviations used are: GNT, glutamate neurotoxicity; CGCs, cerebellar granule cells; C-CGC, control CGCs; GLU-CGC, glutamate-treated CGCs; C-CGC-M, mitochondria isolated from control cells; GLU-CGC-M, mitochondria isolated from glutamate-treated cells; cyt c, cytochrome *c*; ROS, reactive oxygen species; XOD, xanthine oxidase; XX, xanthine; PBS, phosphate-buffered saline; CCCF, cytosolic fraction containing cyt *c*; SOD, superoxide dismutase; e.u., enzymatic unit.

dishes were purchased from NUNC (Denmark), and enzymes and biochemicals were purchased from Sigma. Anti-cytochrome *c* antibodies (7H8.2C12) were purchased from Pharmingen (San Diego, CA). Anti-glutamate dehydrogenase antibodies were kindly supplied by Dr. F. Rothe (Institut fuer Medizinische Neurobiologie, University of Magdeburg, Germany). Anti-cytochrome *c* oxidase (subunit IV) antibodies (20E8-C12) were purchased from Molecular Probes (Eugene, OR). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies and Enhanced Chemiluminescence Western blotting reagents were purchased from Amersham Pharmacia Biotech.

*Cell Cultures*—Primary cultures of cerebellar granule neurons were obtained as described in Levi *et al.* (24). Before each experiment, the culture medium was removed, and the plated cerebellar granule cells were washed with phosphate-buffered saline medium (PBS), pH 7.4, containing NaCl (138 mM), KCl (2.7 mM), $Na_2HPO_4$ (8 mM), and $KH_2PO_4$ (15 mM) and then collected by gentle scraping in a final volume of 4 ml of PBS/9-cm dish. Suspended granule cells showed full viability, even though they lacked the morphological organization present in culture dishes such as cell-cell and cell-substrate contacts as well as neuritis. Cell integrity, which remains rather constant for 3–5 h, was quantitatively assessed by checking the inability of cells to oxidize externally added succinate, which cannot enter intact cells (25), by checking the ability of ouabain to block glucose transport in cells (8), and by counting dead cells, identified as large phase-bright cell bodies, as in Volontè *et al.* (26). The final cell suspension contained routinely 85–95% intact cells and was prepared after 7–8 days *in vitro*.

*Cell Toxicity Studies*—Glutamate exposure was performed 7 days after plating. Primary cultures were exposed usually for 30 min to 100 μM glutamate at 25 °C in Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 10 mM Hepes, pH 7.4) in the presence of 1 μM glycine, added to fully activate *N*-methyl-D-aspartate-sensitive glutamate receptors (27). Cells were then replenished with basal medium Eagle's containing 25 mM KCl, 2 mM glutamine, and gentamicin (100 μg/ml) and put in the incubator. For the quantitative assessment of GNT, cell integrity and count were measured as described above after 12–24 h. Neurotoxicity was expressed as the percentage of intact cells with respect to control cells kept under the same experimental conditions without the glutamate addition. In control experiments 95–97% integrity was found in 24 h.

*Cell Homogenate, Mitochondria, and Cytosolic Fraction Preparation*—Cell homogenate from a cell suspension was obtained by 10 strokes with a Dounce potter at room temperature. With this procedure, lactate dehydrogenase was released, and subsequent treatment with Triton X-100 did not cause further release.

Mitochondria were isolated from cell homogenates, essentially as reported in Almeida and Medina (28). Briefly: the homogenate was centrifuged at 1500 × *g* for 10 min at 4 °C, and the supernatant was kept on ice. The pellet was re-homogenized with a further 3 ml of isolation buffer consisting of 320 mM sucrose, 1 mM $K^+$-EDTA, 10 mM Tris-HCl, pH 7.4, and the homogenate was centrifuged at 1500 × *g* for 5 min at 4 °C. The two supernatants were pooled and centrifuged at 1500 × *g* for 10 min at 4 °C. Supernatant was further centrifuged at 17,000 × *g* for 11 min at 4 °C. The pellet, *i.e.* the mitochondrial fraction, was then resuspended in 200 μl of isolation buffer to obtain about 4 mg of mitochondrial protein/ml. These mitochondria, incubated in PBS, were checked for their intactness and coupling by measuring the activities of both adenylate kinase (EC 2.7.4.3) and glutamate dehydrogenase (EC 1.4.1.3) (see below), which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively. The percentage of damaged mitochondria ranged between 0.5 and 1.5%. Mitochondrial coupling was checked by measuring the respiratory control ratio, *i.e.* (oxygen uptake rate after ADP addition)/(oxygen uptake rate before ADP addition), which reflects the ability of mitochondria to produce ATP; in both cases succinate was used as a respiratory substrate. As expected, both the inhibitors of electron flow and atractyloside, a powerful inhibitor of the ADP/ATP translocator (29), blocked ADP-stimulated oxygen uptake. Inhibition was also caused by oligomycin, which can inhibit ATP synthase (30) and is rapidly reversed by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which stimulates the rate of oxygen consumption.

Cytosolic fractions of both control CGCs (C-CGCs) and glutamate-treated CGCs (GLU-CGCs) (about $30 \times 10^6$ cells in 1 ml) were obtained after homogenization in PBS and centrifugation at 15,000 × *g* for 15 min. The supernatant was concentrated to 0.2 ml by using the Savant Speed Vac concentrator and indicated as cytosolic fraction containing cyt *c* (CCCF). Both the protein assay of cell and mitochondria were determined according to Waddel and Hill (31), with bovine serum albumin used as a standard.

*Oxygen Uptake Studies*—$O_2$ consumption was measured polarographically by means of a Gilson 5/6 oxygraph using a Clark electrode, as in Atlante *et al.* (8). Either the cell homogenate or mitochondria suspension in PBS (about 0.2 mg of protein) was incubated in a thermostatted (25 °C) water-jacketed glass vessel (final volume equal to 1.5 ml). Instrument sensitivity was set to a value that allowed the monitoring of rates of $O_2$ uptake as low as 0.5 nanoatoms $min^{-1}mg^{-1}$ of protein.

*Immunoblot Analysis*—Immunoblot analysis was performed on cytosolic extracts from control and glutamate-treated cultures essentially as in Bobba *et al.* (18). In both cases cells were washed once with PBS and collected by centrifugation at 2000 × *g* for 5 min at 4 °C. The cell pellet was resuspended in 500 μl of extraction buffer containing 250 mM sucrose, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM 1,10-phenantroline, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. The cells were homogenized in a Teflon glass homogenizer (10 strokes), and after 5 min on ice, the suspension was centrifuged at 15,000 × *g* for 15 min. The supernatants, *i.e.* cytosolic fractions, were removed and stored at −80 °C until analyzed by gel electrophoresis. 10 μg of cytosolic proteins were loaded onto a 15% SDS-polyacrylamide gel, separated, and transferred to a polyvinylidene difluoride membrane with immunoblot analysis performed as described in Bobba *et al.* (18). Relative optical densities and areas of bands were quantified using the GS-700 imaging densitometer implemented with the Molecular Analyst Software (Bio-Rad).

*Cytochrome c Assay*—Cyt *c* was assayed as in Errede *et al.* (32) by using the Shimadzu double-wavelength (548 minus 540 nm) spectrophotometer model UV3000. Either the cell homogenate or the resulting supernatant (obtained by centrifuging homogenate at 15,000 × *g* for 15 min), in the presence of 3 μM rotenone plus 0.8 μM antimycin A and 6 μM myxothiazole, were first incubated with potassium ferricyanide (0.1 mM) to oxidize the reduced cyt *c,* then potassium cyanide (1 mM) was added to inhibit cyt *c* oxidase. The reduction of cytochrome *c* ($\epsilon_{550}$ = 21 $mM^{-1}$ $cm^{-1}$) was then obtained by adding a few grains of sodium dithionite as in Atlante *et al.* (13) and measured as an increase of absorbance.

*Superoxide Anion Detection in CGCs*—$O_2^-$ was detected as in Atlante *et al.* (11) and Atlante and Passarella (33), according to either the $Fe^{3+}$-cyt *c* (34) or the adrenochrom assay (35). The newly formed $O_2^-$ gives an increase of absorbance at 550 and 480 nm for $Fe^{2+}$-cyt *c* and adrenochrom formation, respectively, measured using a PerkinElmer lambda-5 spectrophotometer equipped with a thermostatted holder. A calibration curve is made by using an $O_2^-$-producing system, *i.e.* XX + XOD, and an $O_2^-$ detection system, *i.e.* epinephrine/$Fe^{3+}$-cyt *c* that, in the presence of $O_2^-$, gives adrenochrom/$Fe^{2+}$-cyt *c* with a 1:1 stoichiometry.

*Detection of Superoxide Anion Produced by Mitochondria*—To detect mitochondrial superoxide anion production, mitochondria (about 0.2 mg of protein) were incubated in 2 ml of PBS in the presence of $Fe^{3+}$-cyt *c* (10 μM). The reduction of $Fe^{3+}$-cyt *c* that is a result of superoxide formation was then obtained by adding a mitochondrial respiratory substrate such as succinate (5 mM). The increase of absorbance at 550 nm, *i.e.* $Fe^{2+}$-cyt *c* formation ($\epsilon_{550}$ = 31.7 $mM^{-1}$ $cm^{-1}$), is a measure of $O_2^-$ production by mitochondria.

## RESULTS

*In Situ Generated ROS Can Cause Cyt c Release in Cerebellar Granule Cells*—To assess whether cyt *c* release takes place due to reactive oxygen species, produced either during GNT or in the presence of a ROS-generating system, two cell culture systems were employed: 1) GLU-CGCs and 2) CGCs, incubated in the presence of the ROS-producing system consisting of xanthine plus xanthine oxidase (ROS-CGCs), previously reported to cause about 60% ROS production under neurotoxicity (11).

In both cases cyt *c* release was measured by two independent procedures: namely, Western blot analysis and polarographic measurement of the activation of cyt *c*-dependent ascorbate oxidation in cell homogenate (13, 18). This last measurement, at variance with Western blot assessments, allows for the evaluation of whether the released cyt *c* is still functionally active, a prerequisite for any hypothesis about the possible functional significance of its cytoplasmic location. In the same experiment, the ongoing $O_2^-$ production was also monitored (Figs. 1 and 2).

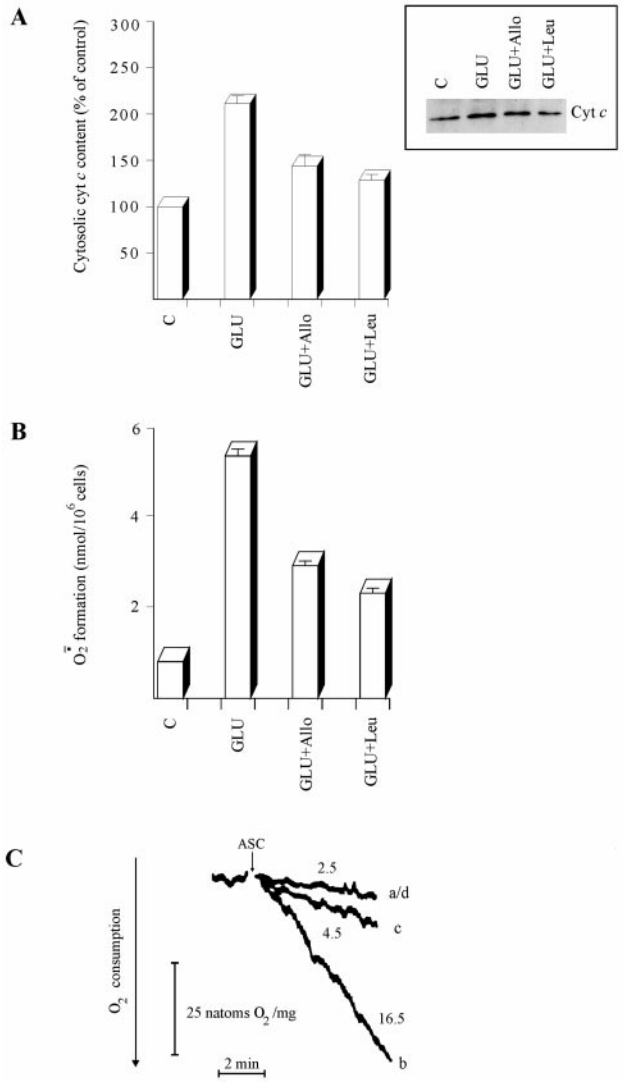


FIG. 1. **Cytochrome *c* release from mitochondria and ROS production under glutamate neurotoxicity.** Rat CGCs, cultured for 7 days *in vitro*, were incubated in Locke's solution in the presence of 100 $\mu$M glutamate (*GLU*) or the same volume of Locke's solution alone (*C*) for 30 min in the absence or presence of either 10 $\mu$M allopurinol (*Allo*) or 1 $\mu$M leupeptin (*Leu*). *A*, Western blot analysis of cytochrome *c*. Cytosolic fractions from either control or glutamate-treated cells were analyzed by Western blotting as described under "Experimental Procedures." The cytosolic content of cytochrome *c* was measured by densitometric scanning of the film and expressed as a percentage of control cells (*C*), to which a value of 100 has been given. *B*, superoxide anion production. Control as well as glutamate treatment were made in the presence of 10 $\mu$M $Fe^{3+}$-cyt c. Aliquots (1 ml) of sister culture solutions were taken, and the absorbance of $Fe^{2+}$-cyt c was measured at 550 nm (for details, see "Experimental Procedures."). The experimental data are reported as nmol of $O_2^{\cdot-}$ formed/$10^6$ cells ± S.E. values. *C*, oxygen consumption caused by externally added ascorbate. Homogenates (about 0.2 mg of protein) from either control (*a*) or GLU-CGCs (*b*) were incubated at 25 °C in 1.5 ml of PBS in the presence of rotenone (3 $\mu$M), antimycin (0.8 $\mu$M), and myxothiazole (6 $\mu$M). Oxygen consumption was started by adding 5 mM ascorbate (*ASC*). *Numbers* along the traces are rates of oxygen uptake expressed as nanoatoms of $O_2$ $min^{-1}$ $mg^{-1}$ cell of protein. In *c* and *d* glutamate treatment was made in the presence of allopurinol and leupeptin, respectively. In *A*, *B*, and *C*, results are the means ± S.E. of triplicate measurements and are representative of at least six different experiments carried out with different cell preparations obtained from different groups of animals.

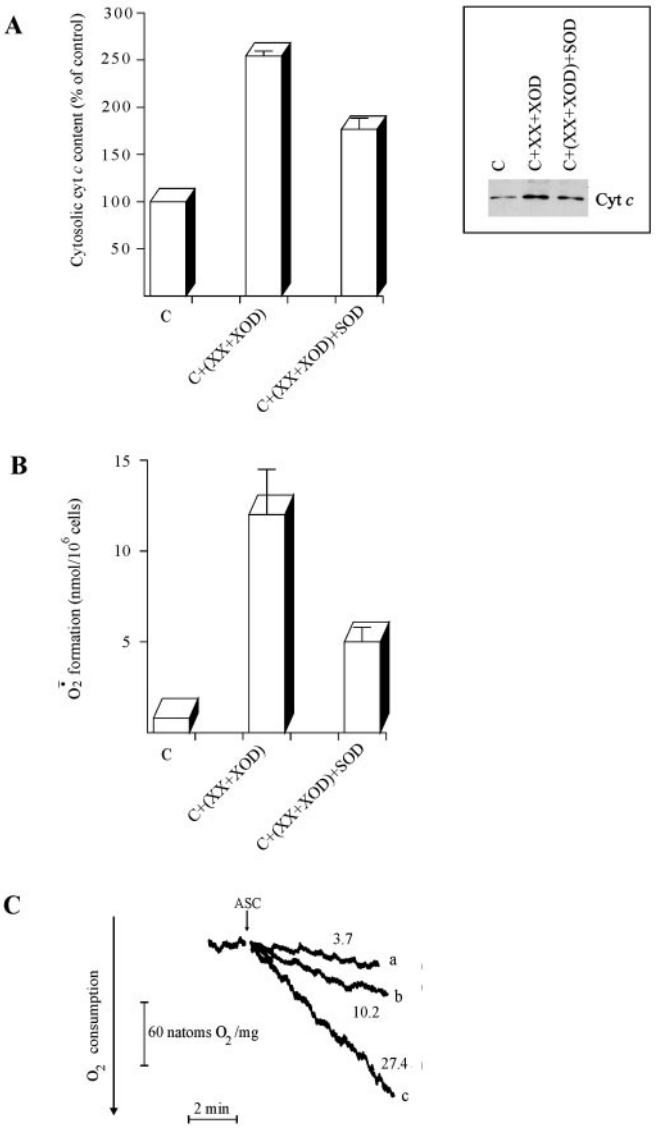


FIG. 2. **ROS production due to externally added xanthine/xanthine oxidase system causes cytochrome *c* release from mitochondria of control cells.** Rat CGCs cultured for 7 days *in vitro* were incubated in Locke's solution in the absence (*C*) or presence of 10 $\mu$M xanthine plus 1 e.u./ml xanthine oxidase (*C+XX/XOD*) for 30 min. 5 e.u./ml SOD was present where indicated. *A*, Western blot analysis of cytochrome *c*. The analysis was carried out as reported in the legend of Fig. 1*A*. *B*, superoxide anion production. The measurements were carried out as reported in the legend of Fig. 1*B*. *C*, oxygen consumption caused by externally added ascorbate. The measurements were carried out as described in the legend of Fig. 1*C* except that neither allopurinol or leupeptin was used. *Numbers* along the traces are rates of oxygen uptake expressed as nanoatoms of $O_2$ $min^{-1}$ $mg^{-1}$ of cell protein. In *b*, ROS treatment was made in the presence of SOD. In *A*, *B*, and *C*, results are the means ± S.E. of triplicate measurements and are representative of at least five different experiments carried out with different cell preparations prepared from different groups of animals. Variations up to 5% were found.

As a result of 30-min incubation of CGCs with 100 $\mu$M glutamate, a marked increase of cyt *c* released in the extramitochondrial phase was found with respect to the control, 210 ± 10% as evaluated by Western blot analysis (Fig. 1*A*), and with respect to the cell homogenate capability to oxidize externally added ascorbate at more than 6-fold increase in the rate of oxygen uptake was measured in GLU-CGC homogenate (Fig. 1*C*, *trace b*) as compared with C-CGCs (Fig. 1*C*, *trace a*). These data confirm the marked release of cyt *c* in the extramitochondrial phase (see Ref. 13) and that such a pool is functionally active. Under the same experimental conditions, GLU-CGCs had a superoxide anion production, as detected with the ferricytochrome *c* method, 5–6-fold higher than controls (Fig. 1*B*).

As expected, the specific inhibitor of XOD activity allopurinol

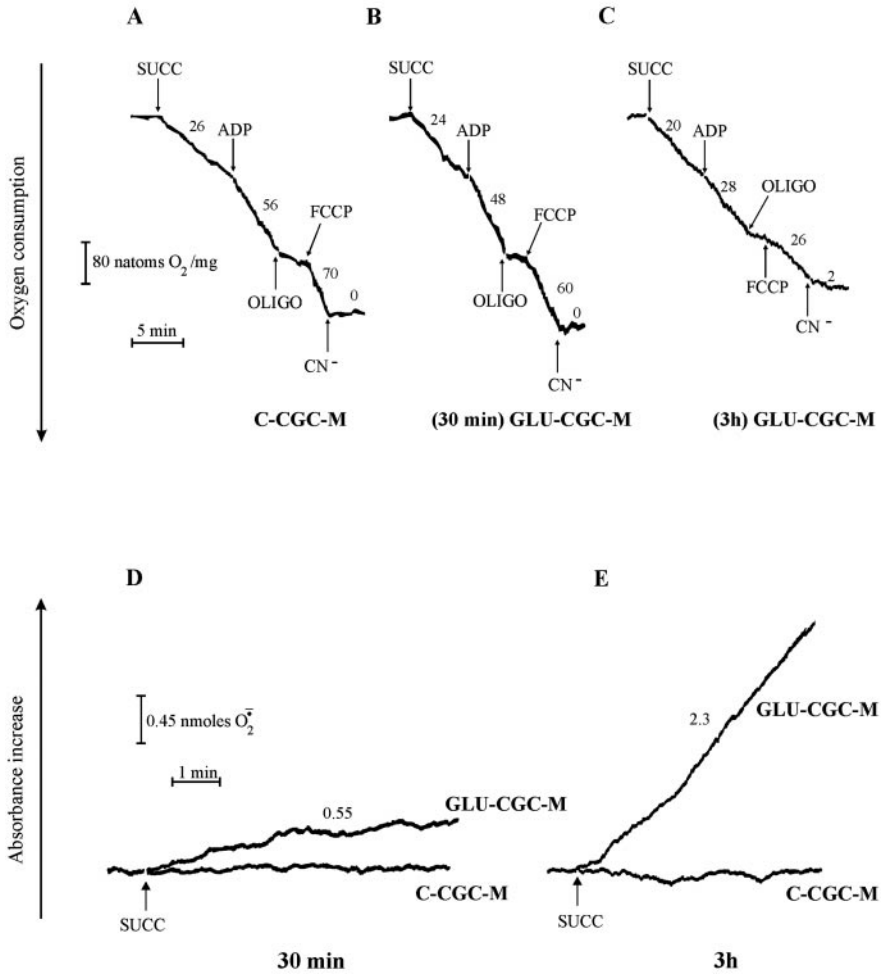


FIG. 3. **The oxygen uptake and ROS production by either C-CGC-M and GLU-CGC-M added with succinate.** Mitochondria (0.2 mg of protein) isolated from rat CGCs cultured for 7 days *in vitro* and incubated in Locke's solution in the presence of 100 $\mu$M glutamate (*GLU-CGC-M*) or the same volume of Locke's solution alone (*C-CGC-M*) for either 30 min or 3 h were incubated in 1.5 ml of PBS. *A–C*, oxygen consumption due to succinate addition to mitochondria. At the *arrows*, the following additions were made: 5 mM succinate (*SUCC*), 1 mM ADP, 0.1 $\mu$g/mg oligomycin (*OLIGO*), 1 $\mu$M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*), 1 mM cyanide ($CN^-$). The rate of oxygen uptake is expressed as nanoatoms of $O_2$/min × mg of protein. *D–E*, ROS production due to succinate addition to mitochondria. 5 mM succinate (*SUCC*) was added to mitochondria incubated with 10 $\mu$M $Fe^{3+}$-cyt *c*. The rate of $Fe^{2+}$-cyt *c* production, *i.e.* superoxide anion formation, was measured as the absorbance increase at 550 nm and expressed as nmol of $O_2^-$ formed/min × mg of protein.

(36) was found to reduce cyt *c* levels (Fig. 1*A*), ROS production (Fig. 1*B*), and oxygen consumption rate (Fig. 1*C*, *trace c*). Consistently, leupeptin, which is known to specifically inhibit $Ca^{2+}$-dependent proteases (37, 38) and, consequently, XOD formation (11), strongly prevented both $O_2^-$ production and cyt *c* release (Fig. 1, *A*, *B*, and *C*, *trace d*).

Cyt *c* release was fully prevented by (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK801), a specific *N*-methyl-D-aspartate receptor antagonist, and largely prevented by externally added superoxide dismutase (SOD) (not shown). Controls confirmed that all the compounds failed to influence the assay conditions as shown in an *in vitro* system in the absence of CGCs.

Interestingly, artificial ROS generation with the xanthine (XX) plus XOD system (Fig. 2*B*) stimulated cyt *c* release in C-CGCs, as detected by both Western analysis (254 ± 5%) in the extramitochondrial phase (Fig. 2*A*) and polarographic measurement (Fig. 2*C*), in good agreement with the results reported in Fig. 1. In both the assays, externally added SOD largely prevented both cyt *c* release and ROS production (Fig. 2, *A*, *B*, *C*, *trace b*).

It should be noted that in the experiments reported in the Figs. 1 and 2 and in the following experiments, the presence of mitochondria in the cytosolic fraction as well as mitochondrial damage due to cell manipulations must be carefully controlled. This was done on the basis of controls performed with filters probed with a monoclonal antibody against cyt *c* oxidase (subunit IV) and a polyclonal antibody against glutamate dehydrogenase. Moreover, the intactness of the mitochondrial membranes was checked by assaying both adenylate kinase and glutamate dehydrogenase, marker enzymes of the intermembrane and matrix space, respectively, in supernatants of control, ROS, and GLU-CGC homogenates at different times after glutamate exposure. In all cases the activity was negligible (0.5–1%) with respect to that measured in the whole homogenates treated with 0.5% Triton X-100 used to dissolve mitochondria and was always of the same extent within the time of cell harvesting after the glutamate pulse (0–300 min). On the other hand, the possibility that mitochondria can swell in the incubation medium due to the occurrence of $Na^+/H^+$ exchange (39), with cyt *c* release caused by ROS in only swollen organelles, was also considered. Therefore, in another experiment, the $Na^+$-containing medium was substituted with mannitol medium (consisting of 210 mM mannitol, 60 mM sucrose, 10 mM KCl, 10 mM Hepes, pH 7.5) with no significant change in the experimental findings (not shown).

*ROS Generation and Cyt c Release Do Not Affect Mitochondrial Coupling*—We have previously shown that in the early phase of neurotoxicity, mitochondrial coupling in control and GLU-CGCs do not significantly differ from each other when measured in cell homogenates in which cyt *c* release has already occurred (13). Another set of experiments was carried out to rule out that the ROS-dependent cyt *c* release is accompanied by mitochondrial uncoupling, as measured with isolated organelles, and, more importantly, to ascertain whether mitochondria can produce ROS under conditions in which cyt *c* is released.

In a typical experiment, mitochondria isolated from both control cells (C-CGC-M) (Fig. 3*A*) and 30-min glutamate-treated cells (GLU-CGC-M) (Fig. 3*B*) and 3-h glutamate-

treated cells (Fig. 3*C*) were found to oxidize succinate at a rate equal to 26, 24, and 20 nanoatoms of $O_2$/min × mg of mitochondrial protein, respectively. The addition of 1 mM ADP increased these rates to 56, 48, and 28 nanoatoms of $O_2$/min × mg of mitochondrial protein with a respiratory control ratio equal to 2.2, 2.0, and 1.4, respectively. It is worth noting that no significant difference in mitochondrial coupling was found between control and 30-min glutamate-treated mitochondria ($n = 4$). That these two treatments do not differ from each other was also shown by the capability of oligomycin (0.1 μg/mg), which inhibits ATP synthase, to decrease with the same efficiency the rate of oxygen uptake in a way reversed by adding the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1 μM). The same mitochondria were analyzed for their capability to produce ROS (Fig. 3, *D–E*), as detected by the $Fe^{3+}$-cyt *c* method. Negligible ROS production was found in the control (C-CGC-M), whereas in mitochondria from 30 min glutamate-treated cells, ROS production increased to 3 nmol of $O_2^{\cdot-}$ formed/min × mg of protein (Fig. 3*D*) and further increased to 12 nmol of $O_2^{\cdot-}$ formed/min × mg of protein in organelles isolated from cells exposed to glutamate for 3 h (Fig. 3*E*). In this latter case, large uncoupling and a dramatic decrease in the rate of oxygen uptake takes place (Fig. 3*C*) (see also Ref. 8). Control for the release of cyt *c* was made in the postmitochondrial supernatants and in parallel cell preparations; a significant release of cyt *c* was found only in the extramitochondrial phase of GLU-CGC organelles.

*The Fraction Containing the Released Cyt c Can Function as a ROS Scavenger and Electron Donor to Oxygen via the Respiratory Chain*—A set of experiments was carried out to gain some insights into the possible role played by the cyt *c* released in the extramitochondrial phase. To achieve this, GLU-CGC homogenate was centrifuged, and the capability of the obtained cytosolic fraction (see "Experimental Procedures"), containing cyt *c*, to function as an electron acceptor from ROS, *i.e.* as a ROS scavenger, and as an electron donor in the respiratory chain, was determined.

The capability of the cytosolic fractions containing cyt *c* (CCCF) to oxidize the superoxide anion to oxygen was analyzed by monitoring cyt *c* reduction photometrically as an increase of absorbance at 550 nm (Fig. 4). First, to check the existence of oxidized cyt *c* in the extract, cyt *c* reduction via dithionite, consistent with Atlante *et al.* (13), was also verified (see Fig, 4, *inset*). CCCF preparations, obtained by CGCs treated with glutamate for times ranging from 2 to 300 min, were added to the ROS-producing system, XX + XOD. Cyt *c* reduction increased with increasing time of exposure of CGCs to glutamate. The CCCF can, therefore, operate as an electron acceptor from the superoxide anion. In another control, the cytosolic fraction obtained from GLU-CGCs incubated in the presence of either SOD or MK801 gave no absorbance increase (not shown).

To ascertain whether cyt *c* reduced as a consequence of superoxide anion formation can function as an electron donor to molecular oxygen, mitochondria were added with XX plus XOD (Fig. 5), and oxygen consumption was measured at a rate equal to 22 nanoatoms of $O_2$/min × mg of cell protein (Fig. 5*A*). As a result of CCCF addition, an increase of the rate of oxygen uptake was found to occur (46 nanoatoms of $O_2$/min × mg of cell protein). The separate addition of either cyanide or allopurinol only partially inhibited the rate of oxygen uptake, which was completely blocked only in the presence of both the inhibitors. In the experiment shown in Fig. 5*B*, no change in oxygen level occurred after CCCF addition to mitochondria; however, as soon as the ROS-producing system was added, oxygen consumption started with a rate equal to 47 nanoatoms of $O_2$/min × mg of cell protein. Externally added allopurinol was

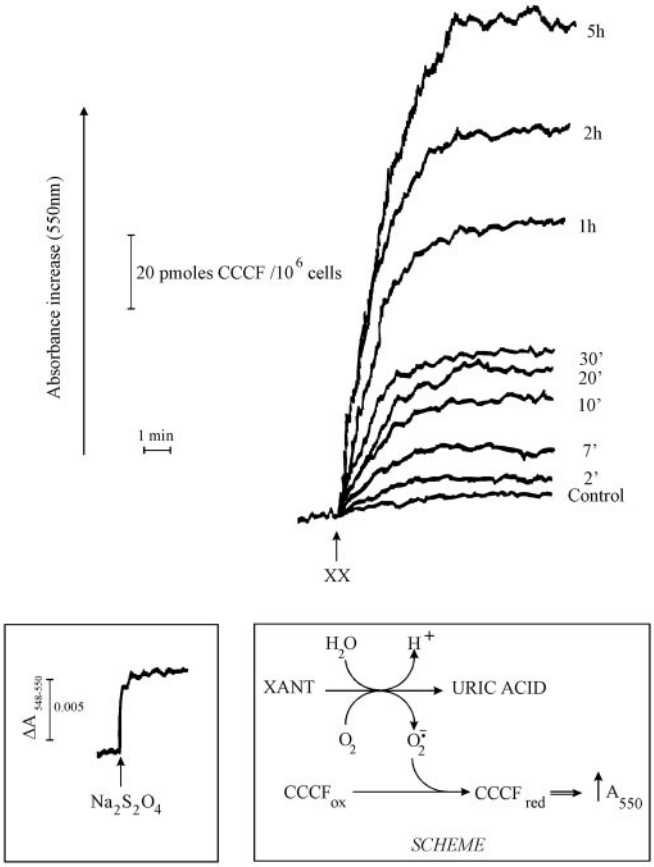


FIG. 4. **The released cyt *c* can work as a ROS scavenger.** CCCF (200 μl) (prepared as described under "Experimental Procedures") from either control or GLU-CGCs with different treatment times was incubated in 1.5 ml of PBS in the presence of xanthine oxidase (1 e.u./ml). The CCCF reduction at 550 nm was started with 10 μM XX addition. Triplicate measurements, representative of at least five different experiments, were carried out with different cell preparations prepared from different groups of animals. Variations up to 5% were found. In the *inset*, the increase of absorbance at 548–540 nm due to the dithionite ($Na_2S_2O_4$)-dependent reduction of oxidized c-cyt *c* is reported. XANT, xanthine.

found to decrease this rate of oxygen uptake to 17 nanoatoms of $O_2$/min × mg of protein, with a complete inhibition of oxygen uptake when cyanide ($CN^-$) was added. These findings suggest that the XX + XOD-dependent oxygen consumption is dependent both on the $O_2$ consumption due to superoxide formation (see the scheme in Fig. 4) and on the presence of cyt *c* released as a result of ROS formation. Consistent with this hypothesis, when $CN^-$ was added to block cyt *c* oxidase, the rate of oxygen uptake was found to decrease from 22 ± 1.4 to 18 ± 0.8 nanoatoms of $O_2$/min × mg of cell protein, *i.e.* about 20% of oxygen consumption is due to cytochrome *c* oxidase activity (not shown). Thus, as far as CCCF is concerned, the findings reported in Fig. 5 are consistent, as will be discussed, with a two-phase process characterized first by reduction of cyt *c* present in the cytosolic fraction followed by the oxidation of the reduced cyt *c* acting as an electron donor for cyt *c* oxidase.

In another experiment, commercial cyt *c* was added to the C-CGC-M in the presence of XX plus XOD, and the rate of oxygen uptake was measured in the absence or presence of increasing cyt *c* amount (see inset Fig. 5). Oxygen uptake rate was found to depend on the added cyt *c* in a roughly linear manner. Comparison made between this experiment and Fig. 5*A* indicates that CCCF-stimulated rate of oxygen uptake is similar to that found in the presence of 1.5 ± 0.1 μM cyt *c* ($n = 3$). It should be noted that the intercept to the ordinate axis of

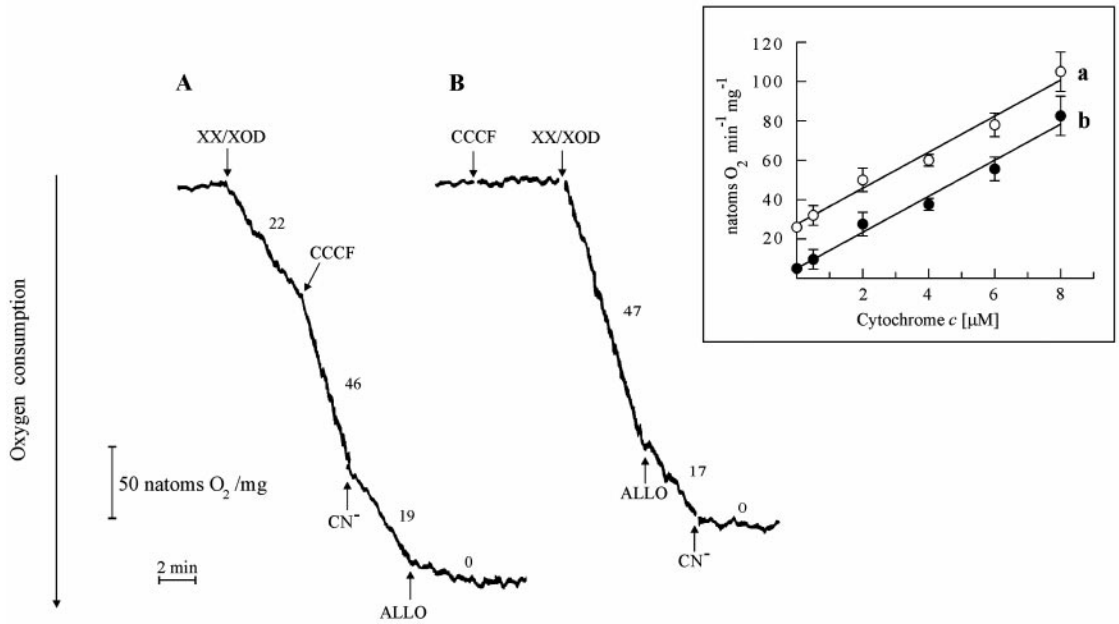


FIG. 5. **The released cyt *c* can work as a respiratory substrate.** Mitochondria (0.2 mg of protein) isolated from rat CGCs cultured for 7 days *in vitro* were incubated in a polarographic vessel at 25 °C in 1.5 ml of PBS. At the *arrows*, the following addition were made: 10 $\mu$M XX plus 1 e.u./ml XOD, 200 $\mu$l of CCCF, 10 $\mu$M allopurinol (*ALLO*), 1 mM cyanide ($CN^-$). The rate of oxygen uptake is expressed as nanoatoms (*natoms*) of $O_2$/min × mg of mitochondrial protein. In the *inset*, commercial cyt *c* amount *versus* oxygen consumption rate is reported: *A*, the rate of $O_2$ consumption due to cyt c added to XX + XOD system; *B*, the rate of $O_2$ consumption due to cyt *c* added to the XX + XOD system, from which the rates due only to XX + XOD before and after $CN^-$ addition have been subtracted (see "Results"). The experiment reported is representative of at least five experiments carried out with different cell preparations obtained from different groups of animals. Variations up to 5–10% were found.

the line fitted on the measurements carried out in the presence of added cyt *c* coincides with the control experiment in which oxygen uptake is measured in the absence of externally added cyt *c*.

*The Fraction Containing the Released Cyt c Can Scavenge Both ROS Production and Prevent Glutamate Neurotoxicity*—The capability of CCCF to be a ROS scavenger in glutamate-treated CGCs raises the question as to whether the released cyt *c* could play a role in the cell defense against necrosis. This was investigated by checking ROS production in these cells plus or minus added CCCF (Fig. 6*A*). In fairly good agreement with Atlante *et al.* (11), the cultured cells produced $O_2^-$ at a rate of 4.6 ± 0.51 nmol/$10^6$ cells measured in a medium used to monitor superoxide anion production that contains epinephrine (1 mM) (35); externally added CCCF caused partial prevention of epinephrine reduction. This prevention depended on the time of glutamate treatment before CCCF preparation: 26, 33, and 42% prevention was found after 30-, 90-, and 180-min glutamate treatment, respectively. ROS production did not occur in control cultures and was strongly reduced in CGCs that were exposed to glutamate in the presence of SOD (5 e.u./ml) (50%), leupeptin (1 $\mu$M) (55%), or allopurinol (10 $\mu$M) (60%), in good accordance with Fig. 1 and Atlante *et al.* (11). Glutamate treatment in the presence of MK801, a classical inhibitor of glutamate receptor *N*-methyl-D-aspartate, was found to reduce the extent of $O_2^-$ after glutamate pulse (97%) to control levels.

Since CCCF can prevent ROS production in GLU-CGCs, its capability to exert an anti-toxic action comparable with that of antioxidants added to neurons undergoing excitotoxic death (11) is expected. Thus, we analyzed the possibility that CCCF could prevent cell death. Cytosolic fractions (obtained at different times of glutamate treatment), were added to sister cultures of CGCs. These cultures were then subjected to 30-min glutamate pulse, and 24 h later, the extent of cell death was measured (Fig. 6*B*). As can be seen, the extent of cell death decreased significantly from 95% to 70, 58, and 40 in cells incubated with fractions prepared after 30-, 90-, and 180-min glutamate exposure, respectively. In the presence of MK801, cell death was almost completely abolished. In another set of experiments, we compared the CCCF antitoxic action with SOD, leupeptin, or allopurinol and found that these substances prevent cell death by 55, 50, and 40%, respectively, in agreement with Atlante *et al.* (11).

## DISCUSSION

In previous papers dealing with glutamate neurotoxicity in rat CGCs, we have shown partial inhibition of both cell death and the parallel production of reactive oxygen species by incubation with allopurinol, leupeptin, antioxidants, reducing agents, and superoxide dismutase (11) as well as cyt *c* release (see 13) under conditions in which mitochondrial coupling was not significantly impaired (8, 13). This prompted us to investigate the possible interrelationship between ROS production and cyt *c* release and the role of the released cyt *c* in the cytosol. In this paper, we show that ROS generated outside mitochondria can cause cyt *c* release from intact and respiring mitochondria and that the fraction containing the released cyt *c* can transfer electrons from superoxide anion to molecular oxygen via the respiratory chain. Furthermore, CCCF proved to reduce ROS production in GGCs and to play a significant role in protecting CGCs from glutamate-dependent neurotoxicity, thus suggesting a role for the released cyt *c* (either alone or together with other cytosolic components) in those processes that occur in glutamate-treated CGCs in the early phase of neurotoxicity. In this respect, cyt *c* is assumed to play two roles, namely to contribute to energy supply as already reported (13) and to function as a ROS scavenger.

Experiments reported in Figs. 1 and 2 demonstrate that cyt *c* release is driven by ROS produced by glutamate exposure or by exogenous production via the XX + XOD system. This conclusion was supported by the partial prevention of cyt *c* release found after SOD addition, which removes superoxide anion, and by specific XOD inhibitors, which prevent ROS formation by impairing XOD activity.

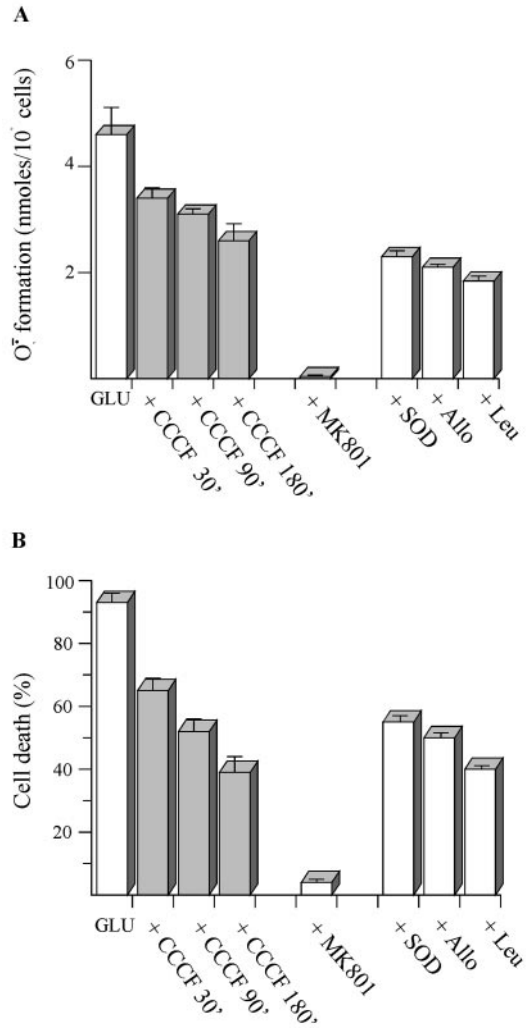


FIG. 6. **The CCCF effect on both ROS production and cell necrosis.** Rat CGCs cultured for 7 days *in vitro* were incubated in Locke's solution added with 100 $\mu$M glutamate (*GLU*) for 30 min in the absence or presence of CCCF (200 $\mu$l) (prepared as described under "Experimental Procedures") from GLU-CGCs at different treatment times. MK801 (1 $\mu$M), SOD (5 e.u./ml), allopurinol (*ALLO*, 10 $\mu$M), and leupeptin (*LEU*, 1 $\mu$M) separately added. *A*, superoxide anion production. Control as well as glutamate treatment was made in the presence of 1 mM epinephrine. Aliquots (1 ml) of sister culture solutions were taken, and the absorbance of adrenochrom was measured at 480 nm (for details, see "Experimental Procedures"). The experimental data are reported as nmol of $O_2^-$ formed/$10^6$ cells ± S.E. values. *B*, cell necrosis. The extent of cell death was evaluated by counting viable neurons and is expressed as the percentage of necrotic cells *versus* total viable cells 24 h after the exposure for 30 min to 100 $\mu$M glutamate. Obtained values are within 10% variations in three different experiments.

The identity of the reactive oxygen species responsible for the observed cyt *c* release remains to be fully clarified. Indeed, externally added SOD was found to only partially prevent cyt c release (Fig. 2) as well as death necrosis (Fig. 6). It should be noted that SOD has been reported to block cyt c release during apoptosis (40). Since SOD removed superoxide anion while still permitting $H_2O_2$ formation that produces OH·, the possibility that this OH· formation can contribute to the cell/mitochondria damage must be taken into consideration. These results together with those showing a low mitochondrial ROS production during cytochrome *c* release demonstrate that cyt *c* release is mostly dependent on cytosolic ROS production rather than from the mitochondrial impairment that is accompanied by ROS generation (Fig. 3, *C* and *D*). Consistent with this hypothesis, mitochondrial function measured in both cell homogenates (see Ref. 8) and in isolated mitochondria (Fig. 3) was not significantly affected in the time intervals in which cyt *c* release had already occurred.

One of the most critical points to be taken in consideration when discussing *in vitro* experiments regards the assumption that the observed findings depend on biological processes and are not a result of cell manipulation. Therefore, when discussing the results shown in Figs. 1 and 2, where we show ROS-dependent cyt *c* release, one could argue that cyt *c* release takes place due to mitochondrial outer membrane damage perhaps enhanced by its greater susceptibility to mechanical disruption during homogenization and centrifugation rather than being due to whatever ROS was produced. Our findings rule out such a possibility, since cyt *c* release occurred under conditions in which neither glutamate dehydrogenase nor, more importantly, adenylate kinase were significantly released. Thus, mitochondria damage as a cause of cyt *c* release must be excluded, at least at the beginning of neurotoxicity. On the other hand, the constantly low levels of leaked adenylate kinase and glutamate dehydrogenase in CCCF observed after different glutamate exposure times (ranging between 0–2 h) and the finding that mitochondria, both isolated and when studied in cell homogenate, are coupled and can synthesize ATP under conditions in which cyt *c* is already mostly released, definitely confirms that cyt *c* release is a physiological occurrence and not the result of laboratory manipulation. We further rule out that the cyt *c* release is caused by the interaction of swollen mitochondria with ROS given that cyt *c* release occurs also in a $Na^+$-free medium in which no mitochondrial swelling takes place. Differently from apoptosis, in GNT the involvement of the mitochondrial transition pore (41, 42) in the release of cyt *c* can be ruled out in light of the lack of adenylate kinase leakage that is expected to occur if cyt *c* release was dependent on the pore.

Although at present the exact amount of cyt *c* release cannot be evaluated with certainty, the calibration curve made with commercial cyt *c*, added to isolated C-CGC-M in the presence of XX + XOD, suggests CCCF contained about 1.5 $\mu$M cyt *c* (see Fig. 5). This is in a fairly good agreement with measurements already reported (13) of the mitochondrial cyt *c* variation in neurotoxicity.

The results of Fig. 4, in which the capability of CCCF to work as a ROS scavenger is directly shown, raises the question as to its mechanism. Indeed, we observed a large prevention in the ROS production when CCCF was added outside the CGCs, whereas *in vivo* the scavenger role of the released cyt *c* should occur in the cytosol with higher efficiency. Whether other compound(s) different from cyt *c* are also present in the extract as a potential ROS scavenger(s) must be taken into consideration. Nonetheless, although we cannot exclude the presence of another electron carrier(s) in the cytosolic fraction released from mitochondria, there is no doubt that cyt *c* is at least one of them. However, since externally added cyt *c* is reduced similarly in extracts from control cells, we are in favor of the possibility of a direct, unique electron transfer from superoxide anion to cyt *c*. Such a proposal is consistent with the calibration curve reported in the inset of Fig. 5 in which the coincidence of the intercept with the experimental point obtained in the absence of externally added cyt *c* could rule out the involvement of other unknown electron carriers. Future studies by immunodepletion experiments could shed light on this problem. Interestingly, while this work was in progress, the antioxidant function of cyt *c* was shown in the light of the capability of externally added cyt *c* to prevent ROS production in rat heart mitochondria (43).

We demonstrate in this paper that the reduced cyt *c* released from mitochondria causes oxygen consumption in isolated mi-

tochondria (Fig. 5), *i.e.* it functions as a respiratory substrate. The $CN^-$ inhibition demonstrates that this $O_2$ reduction takes place via cyt *c* oxidase; thus, we are forced to conclude that CCCF can be oxidized via this enzyme. How this occurs remains to be established in detail, since the direct oxidation via cyt *c* oxidase at mitochondrial contact sites, as suggested (44), is difficult to explain considering both the structure of the cytochrome oxidase complex and its apparent inability to span the two mitochondrial membranes.

The capability of the cytosolic fraction containing cyt *c* to partially prevent both ROS formation and cell death (Fig. 6) is consistent with the above discussed results. Indeed, we found a high efficiency in prevention of both ROS detection and death by adding the cytosolic fraction obtained by neurons exposed to glutamate to cell cultures ongoing neurotoxicity. Surprisingly, even though the cyt *c* assayed in the cytosolic fractions obtained from 1- and 3-h-treated neurons is more than 2-fold greater than controls (see Ref. 13 and Figs. 1 and 2), the death prevention was not consistently higher. At present, this point must be only a matter of speculation; we suggest the possibility that during neurotoxicity other processes at present unknown are evoked that contribute to cell death, thus reducing the ability of cyt *c* to protect. Nonetheless, we propose that the cyt *c* release from mitochondria owing to ROS production in a feedback-like process can protect mitochondria both from mitochondrial ROS as well as from ROS generated by other cellular constituents, including the XX plus XOD system, as previously suggested (13, 33). On the other hand, long glutamate treatment results in a permanent damage of mitochondria and large uncoupling that occurs simultaneously with high mitochondrial ROS production (see Fig. 3).

Future studies should be aimed at assessing whether the role suggested for cyt *c* in this paper in connection with excitotoxic pathways may also hold in neuronal apoptotic death, where a role for the released cyt *c* only in the caspase cascade activation (14–23) and in the maintenance of adequate cell ATP (18) has to our knowledge has been attributed.

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REFERENCES

1. Choi, D. W. (1988) *Neuron* **1,** 623–634
2. Choi, D. W. (1994) *Prog. Brain Res.* **100,** 47–51
3. Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995) *Neuron* **15,** 961–973
4. Budd, S. L., and Nicholls, D. G. (1996) *J. Neurochem.* **67,** 2282–2291
5. Schinder, A. F., Olson, E. C., Spitzer, N. C., and Montal, M. (1996) *J. Neurosci.* **1,** 6125–6133
6. Nicholls, D. G., and Budd S. L. (1998) *Biochim. Biophys. Acta* **1366,** 97–112
7. Dugan, L. L., Sensi, S. L., Canzoniero, L. M. T., Handran, S. D., Rothman, S. M., Lin, T.-S., Goldberg, M. P., and Choi, D. W. (1995) *J. Neurosci.* **15,** 6377–6388
8. Atlante, A., Gagliardi, S., Minervini, G. M., Marra, E., Passarella, S., and Calissano, P. (1996) *Neuroreport* **7,** 2519–2523
9. Coyle, J. T., and Puttfarcken, P. (1993) *Science* **262,** 689–695
10. Lafon-Cazal, M., Pietri, S., Culcasi, M., and Bockaert, J. (1993) *Nature* **364,** 535–537
11. Atlante, A., Gagliardi, S., Minervini, G. M., Ciotti, M. T., Marra, E., and Calissano, P. (1997) *J. Neurochem.* **68,** 2038–2045
12. Minervini, G. M., Atlante, A., Gagliardi, S., Ciotti, T. M., Marra, E., and Calissano, P. (1997) *Brain Res.* **768,** 57–62
13. Atlante, A., Gagliardi, S., Marra, E., Calissano, P., and Passarella, S. (1999) *J. Neurochem.* **73,** 237–246
14. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136
15. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) *EMBO J.* **17,** 37–49
16. Du, Y., Dodel, R. C., Bales, K. R., Jemmerson R., Hamilton-Byrd, E., and Paul, S. M. (1997) *J. Neurochem.* **69,** 1382–1388
17. Gormal, A. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., and Ceccatelli, S. (1999) *Eur. J. Neurosci.* **11,** 1067–1072
18. Bobba, A., Atlante, A., Giannattasio, S., Sgaramella, G., Calissano, P., and Marra, E. (1999) *FEBS Lett.* **457,** 126–130
19. Jürgensmejer, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4997–5002
20. Kantrow, S. P., and Piantadosi, C. A. (1997) *Biochem. Biophys. Res. Commun.* **232,** 669–671
21. Petit, P. X, Goubern, M., Diolez, P., Susin, S. A., Zamzami, N., and Kroemer, G. (1998) *FEBS Lett.* **426,** 111–116
22. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) *Nature* **399,** 483–487
23. Li, P., Nijhawan, D., Budihardajo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
24. Levi, G., Aloisi, F., Ciotti, M. T., and Gallo, V. (1984) *Brain Res.* **290,** 77–86
25. Berry, M. N., Edwards, A. M., and Barritt, G. J. (1991) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Burdon, R. H. and Van Knippenberg, P. H., eds) pp. 83–98, Elsevier Science Publishing Co., Inc., New York
26. Volontè, C., Ciotti, M. T., and Battistini, L. (1994) *Cytometry* **17,** 274–276
27. Johnson, J. W., and Ascher, P. (1987) *Nature* **325,** 529–531
28. Almeida, A., and Medina, J. M. (1998) *Brain Res. Protocols* **2,** 209–214
29. LaNoue, F. K., and Schoolwerth, A. C. (1984) in *Bioenergetics* (Ernster, L., ed) pp. 221–261, Elsevier Science Publishing Co., Inc., New York
30. Nicholls, D. G. (1982) *Bioenergetics*, Academic Press, Inc., London
31. Waddel, W. J., and Hill, C. (1956) *J. Lab. Clin. Med.* **48,** 311–314
32. Errede, B., Kamen, M. D., and Hatefi, Y. (1978) *Methods Enzymol.* **53,** 40–47
33. Atlante, A., and Passarella, S. (1999) *Brain Res. Protocols* **4,** 266–270
34. Forman, H. J., and Fridovich, I. (1973) *Arch. Biochem. Biophys.* **158,** 396–400
35. Misra, H. P., and Fridovich, I. (1972) *J. Biol. Chem.* **217,** 3170–3175
36. Flower, R. J., Moncada, S., and Vanet, J. R. (1985) *The Pharmacological Bases of Therapeutics* (Goodman, A. G., Gilman, L. S., Rall, T. W., and Murad, F., eds) pp. 674–715, Macmillian Publishing Co, New York,
37. Arai, A., Kessler, M., Lee, K., and Lynch, G. (1990) *Brain Res.* **532,** 63–68
38. Rami, A., and Krieglstein, J. (1993) *Brain Res.* **609,** 67–70
39. Numata, M., Petrecca, K., Lake, N., and Orlowski, J. (1998) *J. Biol. Chem.* **273,** 6951–6959
40. Fujimura, M., Morita-Fujimura, Y., Noshita, N., Sugawara, T., Kawase, M., and Chan, P. H. (2000) *J. Neurosci.* **20,** 2817–2824
41. Single, B., Leist, M., and Nicotera, P. (1998) *Cell Death Differ.* **5,** 1001–1003
42. Bernardi, P. (1999) *Physiol. Rev.* **79,** 1127–1155
43. Korshunov, S. S., Krasnikov, B. F., Pereverzev, M. O., and Skulacev, V. P. (1999) *FEBS Lett.* **462,** 192–198
44. Ardail, D., Privat, J-P., Egret-Charlier, M., Levrat, C., Lerme, F., and Louisott, P. (1990) *J. Biol. Chem.* **265,** 18797–18802

# Cytochrome *c* Is Released from Mitochondria in a Reactive Oxygen Species (ROS)-dependent Fashion and Can Operate as a ROS Scavenger and as a Respiratory Substrate in Cerebellar Neurons Undergoing Excitotoxic Death

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