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Mitochondrial Free Radical Production Induced by Glucose Deprivation in Cerebellar Granule Neurons

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Abstract—Using a fluorescent probe for superoxide, hydroethidine, we have demonstrated that glucose deprivation (GD) activates production of reactive oxygen species (ROS) in cultured cerebellar granule neurons. ROS production was insensitive to the blockade of ionotropic glutamate channels by MK-801 (10 μM) and NBQX (10 μM). Inhibitors of mitochondrial electron transport, i.e. rotenone (complex I), antimycin A (complex III), or sodium azide (complex IV), an inhibitor of mitochondrial ATP synthase—oligomycin, an uncoupler of oxidative phosphorylation—CCCP, a chelator of intracellular Ca^{2+}—BAPTA, an inhibitor of electrogenic mitochondrial Ca^{2+} transport—ruthenium red, as well as pyruvate significantly decreased neuronal ROS production induced by GD. GD was accompanied by a progressive decrease in the mitochondrial membrane potential and an increase in free cytosolic calcium ions, $[Ca^{2+}]_i$. Pyruvate, BAPTA, and ruthenium red lowered the GD-induced calcium overload, while pyruvate and ruthenium red also prevented mitochondrial membrane potential changes induced by GD. We conclude that GD-induced ROS production in neurons is related to potential-dependent mitochondrial Ca^{2+} overload. GD-induced mitochondrial Ca^{2+} overload in neurons in combination with depletion of energy substrates may result in the decrease of the membrane potential in these organelles.

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Key words: hypoglycemia, reactive oxygen species, free radicals, mitochondria, calcium, cerebellar granule neuron, cell culture

Reactive oxygen species (ROS) are not only important factors contributing to neuronal death induced by various pathologies including aging [1-4], they are also important signal molecules involved into synaptic transmission during normal cell metabolism [5, 6]. Excessive ROS production and activation of oxidative processes in brain cells may be associated not only with increased ROS production but also with the decrease in activity of antioxidant systems; such decrease may be caused by intracellular deficit of glucose induced by hypoglycemia [7]. There is evidence that mitochondria, the intracellular organelles producing the largest amount of ROS in the cell, play a major role in the development of oxidative stress under both physiological and pathological conditions [8-10]. Most brain diseases can result in impaired mitochondrial functions and regulation of oxidative metabolism in neurons [11-14]. However, the role of mitochondria in ROS production under conditions of deficit of substrate for oxidative metabolism is not completely understood. Using fluorescent probes for superoxide, calcium ions, and mitochondrial membrane potential, we have investigated in this study the effect of glucose deprivation (GD) on superoxide production by neurons.

Abbreviations: BAPTA-AM) 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate-acetoxymethyl ester; BSS) balanced salt solution; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; CGN) cultured granular neurons; DCF-DA) dichlorofluorescin diacetate; GD) glucose deprivation; MK-801) (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate; NBQX) 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide; NMDA) N-methyl-D-aspartate; ROS) reactive oxygen species; TMRE) tetramethylrhodamine ester.

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In particular, we have explored the effect of mitochondrial electron transport inhibitors on these parameters.

MATERIALS AND METHODS

All culture media and additions employed for cultivation of cell cultures were purchased from Biochrom KG Berlin (Germany). Fluo-3 AM, hydroethidine, and tetramethylrhodamine ester (TMRE) were obtained from Molecular Probes (USA). Other reagents were purchased from Sigma Chemicals (Germany).

Primary cerebellar cultures. The study was carried out using 7-9-day-old cultures of cerebellar granule neurons obtained from brain of 8-day-old Wistar rats by enzymatic mechanical dissociation. Brain tissue was dissociated as follows: isolated cerebella were transferred to a plastic Petri dish filled with phosphate buffer lacking calcium and magnesium ions. Tissue fragments were incubated at 37°C for 15 min in phosphate buffer containing 0.05% trypsin and 0.02% EDTA. After the incubation, the tissue was washed twice with phosphate buffer and once with the cultivation medium and then subjected to mechanical dissociation in the cultivation medium containing 10% fetal calf serum, 2 mM glutamine, and 10 mM Hepes buffer, pH 7.2-7.4. Cell suspension was centrifuged 1 min at 1000 rpm, supernatant was discarded, and the pellet was resuspended in the cultivation medium. Cells were cultivated in polylysine coated 96-well plastic plates. (Each well contained 0.1 ml of the cell suspension.) The cell cultures were maintained in a CO_2 incubator at 35.5°C and 98% relative humidity.

On the second day, the culture medium was replaced by a new one containing 25 mM KCl, and cells were cultivated in this medium up to 7-8 days. Proliferation of non-neuronal cells was suppressed by adding 1 μM cytosine arabinoside.

All experimental procedures were carried out in accordance with International Guiding Principles for Biomedical Research.

Glucose deprivation. For initiation of GD, the cultivated cells were washed twice with balanced salt solution (BSS) containing 154 mM NaCl, 25 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 0.35 mM Na_2HPO_4, 10 mM Hepes, pH 7.2-7.4; then the cells were incubated in the same solution for 15-60 min. Control cells were incubated in BSS containing glucose (1 g/liter).

Intracellular assay of ROS, calcium ions, and mitochondrial membrane potential. For determination of superoxide production, cells were incubated with 1 μM hydroethidine for 40 min. Fluorescence of the hydroethidine oxidation product was measured using excitation at 530 nm and emission at 640 nm. For analysis of changes in mitochondrial membrane potential cells were incubated with 0.1 μM TMRE (excitation at 530 nm, emission at 640 nm) for 15-30 min, and for measurements of changes of free cytosolic calcium ions, $[Ca^{2+}]_i$, cells were incubated with 10 μM Fluo-3 AM (excitation at 485 nm, emission at 530 nm) for 60 min. All incubations with the probes were carried out at 36.5 ± 0.5°C followed by triple wash with BSS.

Free radical production, membrane potential, and $[Ca^{2+}]_i$ changes were measured using a fluorescence reader CytoFluor II (PerSeptive Biosystems, USA).

Fluorescence microscopy. In all experiments, cell fluorescence was monitored with an inverted fluorescence microscope (Leica, Geosystems, AG, Switzerland) using filters of 530/640 nm for TMRE and the hydroethidine oxidation product and 485/>530 nm for Fluo-3.

Statistical analysis. Data were treated using the ANOVA test followed by Bonferroni or Dunnett posttests; differences were considered as statistically significant at $p < 0.05$. Data obtained using at least 12 cultures from three independent experiments were expressed as mean ± SEM.

RESULTS

Glucose deprivation causes increase in ROS production in cerebellar granule neurons. We have used hydroethidine as an indicator of ROS in cultured granular neurons (CGN). Incubation of CGN in glucose-free BSS caused intensive production of ROS (Fig. 1). Hypoglycemia induces accumulation of endogenous glutamate in neuronal cultures [15, 16]. Glutamate activates the ionotropic glutamate receptors and induces ROS production in neurons [3, 4, 17]. For evaluation of possible involvement of the ionotropic glutamate receptors in ROS production, we supplemented the incubation medium with antagonists of these receptors ((+)-5-methyl-

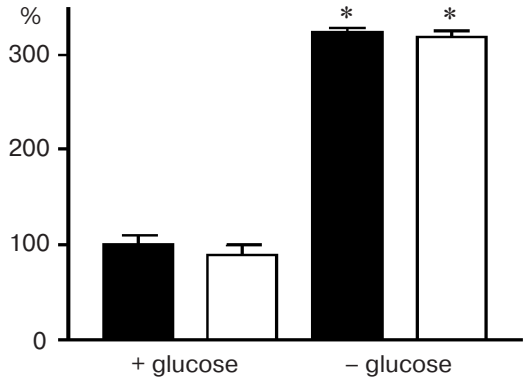

Fig. 1. Glucose deprivation stimulates production of superoxide anion in cerebellar granule neurons that is insensitive to antagonists of ionotropic glutamate receptors (10 μM MK-801 and 10 μM NBQX). Relative superoxide anion content in cell cultures is expressed as percent of hydroethidine fluorescence in control cells (incubated in BSS in the presence of glucose and in the absence of MK-801 and NBQX). Empty and filled columns indicate BSS and BSS with MK-801 and NBQX, respectively; * $p < 0.001$ compared with control.

10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX)). The results demonstrated that under conditions of GD the blockade of ionotropic glutamate receptors insignificantly influences ROS production in CGN (Fig. 1).

Glucose deprivation modulates intracellular calcium ion level and mitochondrial membrane potential. Using Fluo-3, a fluorescent probe for $[Ca^{2+}]_i$, we have shown that incubation of CGN under glucose-free conditions in the presence of antagonists of ionotropic glutamate receptors (MK-801 and NBQX) for 60 min results in the gradual increase in $[Ca^{2+}]_i$ (Fig. 2). Mitochondrial membrane potential was registered in the sister cultures by fluorescent probe TMRE. The experiments showed a decrease of the mitochondrial membrane potential in cells exposed to GD for 60 min (Fig. 2).

Mitochondrial inhibitors decrease ROS production but increase the level of intracellular calcium ions under GD. Mitochondria are the principal organelles generating ROS in the cell thus playing a major role in the incidence of oxidative stress [10, 18-20]. Taking into consideration these data, we hypothesized that mitochondria are responsible for ROS overproduction in CGN under conditions of GD. For experimental testing of this hypothesis we supplemented the system with respiratory chain inhibitors rotenone, antimycin A, and sodium azide affecting complexes I, III, and IV, respectively, and oligomycin (ATP synthase inhibitor) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (uncoupler). Use of these compounds significantly decreased GD-induced production of ROS, but increased calcium overload of CGN cytosol (Table 1).

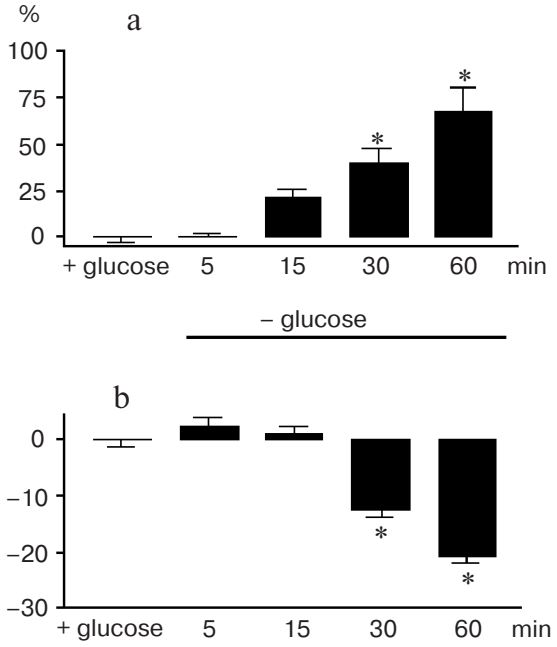

Fig. 2. Time-course of $[Ca^{2+}]_i$ changes in CGN (assayed by Fluo-3 fluorescence) (a) and mitochondrial membrane potential (evaluated by TMRE fluorescence) (b). CGNs were incubated in BSS with or without glucose with additions of antagonists of ionotropic glutamate receptors (10 μM MK-801 and 10 μM NBQX). Changes in Fluo-3 or TMRE fluorescence were calculated as $(F - F_0)/F_0 \times 100\%$, where F_0 and F are fluorescence of the dye in cell cultures incubated in BSS with and without glucose, respectively; * $p < 0.01$. Abscissa shows time of GD.

Table 1. Effect of mitochondrial inhibitors on hydroethidine oxidation and intracellular level of calcium ions in CGN under glucose deprivation

Treatment	Fluorescence of hydroethidine oxidation product (% of GD)	Fluo-3 fluorescence (% of GD)
– Glucose	100 ± 4.5*	100 ± 0.6*
+ Glucose	32 ± 3.6	42 ± 1
– Glucose + 2 μM rotenone	54 ± 3.5**	328 ± 34**
– Glucose + 0.054 μg/ml antimycin A	39 ± 2.7**	324 ± 32**
– Glucose + 5 mM sodium azide	52 ± 4.4**	117 ± 3.8**
– Glucose + 10 μM CCCP	51 ± 3**	347 ± 29**
– Glucose + 4 μg/ml oligomycin	39 ± 2.7**	155 ± 9**

Note: CGN were incubated in BSS for 60 min with or without glucose in the presence of the glutamate receptor antagonists MK-801 (10 μM) and NBQX (10 μM). Mitochondrial inhibitors were in the incubation solution during the whole incubation period. Data are expressed as mean ± SEM.
* $p < 0.001$ compared with (+glucose).
** $p < 0.001$ compared with (–glucose).

Relationship between ROS production and calcium overload under GD. Results of numerous studies have demonstrated that neuronal mitochondrial ROS production is related to cytosolic calcium overload. For experimental testing of a possibility that ROS production in CGN is related to calcium overload, glucose-free incubation medium was supplemented with the calcium ion chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate-acetoxymethyl ester (BAPTA) or ruthenium red, an inhibitor of potential dependent transport of calcium ions into mitochondria. The results of these experiments demonstrates that both BAPTA and ruthenium red significantly decreases ROS production in CGN under conditions of GD. Pyruvate (a substrate for aerobic energy production) supplemented to the glucose-free incubation medium also prevents the increase of ROS production in CGN (Table 2).

Table 2. Effect of BAPTA, ruthenium red, and pyruvate on hydroethidine oxidation and intracellular calcium ion level measured in CGN under conditions of GD

Treatment	Fluorescence of hydroethidine oxidation product (% of GD)	Fluo-3 fluorescence (% of GD)
+ Glucose	29 ± 1.5	43 ± 1
– Glucose	100 ± 1.3*	100 ± 0.5*
– Glucose + 0.05 mM BAPTA	36 ± 2.6**	69 ± 2.3**
– Glucose + 20 μg/ml ruthenium red	34 ± 6.7**	58 ± 2.2**
– Glucose + 10 mM pyruvate	25 ± 2.2**	44 ± 2.6**

Note: CGN were incubated in BSS for 60 min with or without glucose in the presence of the glutamate receptor antagonists MK-801 (10 μM) and NBQX (10 μM). Mitochondrial inhibitors were in the incubation solution during the whole incubation period. Data are expressed as mean ± SEM.
* $p < 0.001$ compared with (+glucose).
** $p < 0.001$ compared with (–glucose).

Measurements of relative $[Ca^{2+}]_i$ content demonstrated that BAPTA and ruthenium red as well as pyruvate significantly decreased GD-induced calcium overload of neurons (Table 2).

Effect of ruthenium red and pyruvate on mitochondrial membrane potential in cerebellar granule neurons under GD. The measurements of mitochondrial membrane potential of neurons by the fluorescent probe for mitochondrial membrane potential, TMRE, demonstrated that pyruvate and ruthenium red supplemented to the incubation solution in the beginning of GD significantly prevented the decrease of rhodamine fluorescence in neuronal mitochondria. This suggests maintenance of membrane potential of these organelles. Thus, these results demonstrate that pyruvate and ruthenium red prevent GD-induced mitochondrial de-energization of the neurons (Fig. 3).

DISCUSSION

This study has been carried out using dissociation cell cultures prepared from cerebella of 7-8-day-old rats. In such cultures the neuronal population is morphologically and neurochemically identical and homogenous; it consists mainly (by 95%) of granule neurons [21, 22], which are glutamate and aspartate receptive and glutamatergic cells [23-25]. Morphological and neurochemical homogeneity of the cultured neurons makes this neuronal culture a convenient model for studies of the mechanism responsible for the development of various pathological processes in neurons, including pathological processes associated with impairments of energy metabolism. Cultured cerebellar granule neurons have already

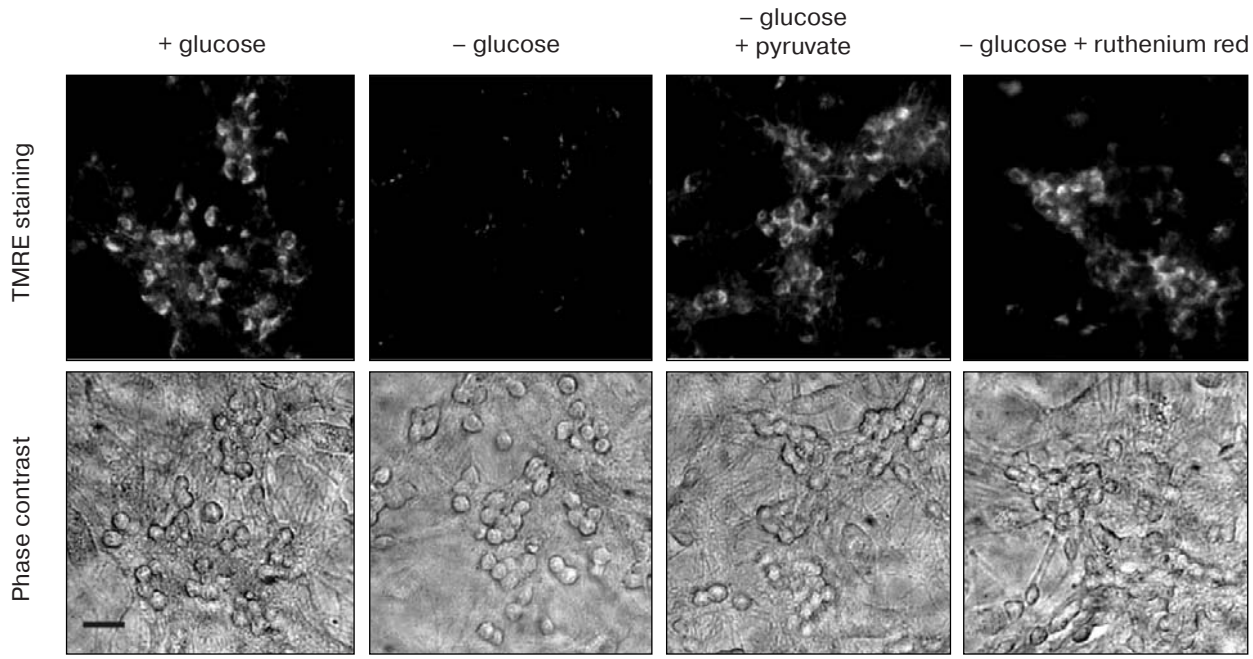

Fig. 3. Ruthenium red and pyruvate prevent the decrease of the mitochondrial membrane potential of CGN under conditions of GD. Staining with TMRE. CGN were incubated in BSS for 60 min with or without glucose in the presence of the glutamate receptor antagonists MK-801 (10 μM) and NBQX (10 μM). Ruthenium red and pyruvate were in the incubation solution during the whole incubation period. Scale, 10 μm.

been used for studies of mechanisms underlying anoxia [26-28], glutamate toxicity [29-33], oxygen-glucose deprivation [34], and hypoglycemia [35, 36]. Cultivation of CGN and experiments were carried out in media containing 25 mM KCl [35, 36] since a decrease in K^+ to 3-5 mM caused a decrease in physiological $[Ca^{2+}]_i$ below normal levels and initiation of apoptosis [37].

Hypoglycemia of cells of central and peripheral nervous systems under normal oxygen tension and its extreme manifestation, glucose deprivation, causes special interest since in the central nervous system glucose molecules are not only the major energy source required for its functioning—they may also prevent glutathione oxidation and mitochondrial damage after glutamate induced neurotoxicity [38]. Such dangerous pathologies of nervous tissue as ischemia, hypoglycemia, and Alzheimer's disease [7, 39, 40] share one common sign—they are related to intracellular glucose deficit, which may occur due to a decrease in glucose concentration in the intracellular space or due to impaired transport of glucose into cells. Transport and metabolism of glucose are important regulatory sites of the apoptotic cascade [41]. Decrease of glucose level in the neuron cultivation medium is accompanied by increased activity of caspase-3 in neurons and increase in cell sensitivity to the toxic effect of glutamate [42]. Glucose starvation of brain cells is accompanied by the development of oxidative stress [43] and increased mitochondrial production of ROS [44]. Similar results were obtained during GD of PC12 cells [45] or chemically induced hypoglycemia (inhibition of glycolysis by iodoacetate) in retinal cells [46], in which these pathological treatments induced high production of ROS. These authors used dichlorofluorescin diacetate (DCF-DA) and dihydrorhodamine-123 for detection of ROS production. It should be noted that fluorescence intensity of the product of DCF-DA is pH-dependent [4] and the product of dihydrorhodamine-123 oxidation, rhodamine-123, is a probe for mitochondrial membrane potential.

In this study using hydroethidine for monitoring of intracellular ROS production, we explored mechanisms responsible for changes in ROS production in cultured neurons under conditions of GD. Some authors do not recommend hydroethidine for quantitative evaluation of ROS [47, 48]. However, in spite of some defects of this probe many researchers still use hydroethidine for detection of intracellular ROS production [33, 49-52], since in contrast to other probes (DCF-DA, dihydrorhodamine-123) hydroethidine interacts directly with superoxide [53]. Detection of superoxide is especially important during studies of mechanisms of hypoglycemia, because this pathological process is accompanied by a decrease in activity of antioxidant systems of the cell [7], with superoxide being the main primary radical formed in mitochondria.

We found that incubation of CGN cultures in glucose-free media is accompanied by intensive hydroethidine oxidation corresponding to increased ROS production in these neurons [54]. In our experiments, GD was accompanied by an increase in $[Ca^{2+}]_i$ and decrease in mitochondrial membrane potential; the same changes were earlier reported on GD of hippocampal neurons [55]. It should be noted that during the first minutes of GD there was a small increase in mitochondrial membrane potential. This phenomenon may be attributed to the calcium-dependent stimulation of key enzymes of Krebs cycle observed during entry of moderate number of calcium ions into mitochondria [56]. Antagonists of the ionotropic glutamate receptors did not abolish GD-induced production of ROS, the increase in $[Ca^{2+}]_i$ and the decrease in mitochondrial membrane potential of neurons. These results demonstrate that the increase in ROS production of neurons under conditions of GD is not associated with activation of the ionotropic glutamate receptors. This is consistent with results obtained by Monyer and Choi [57] who demonstrated that GD of neurons lacking glutamine (precursor of glutamate synthesis) increases neuronal damage and decreases protective effect of NMDA (N-methyl-D-aspartate) receptor antagonists. Zhang et al. [58] demonstrated that activation of NMDA receptors has insignificant contribution to the development of hypoglycemic depolarization of neurons. The increase in intracellular calcium observed under conditions of hypoglycemia may occur not only due to entry of this ion via NMDA-channels and its efflux from endoplasmic reticulum [59], but also due to decreased calcium efflux by Ca^{2+}-ATPase. It should be noted that KCl-induced depolarization induces opening of voltage-dependent calcium channels, and this may also contribute to the calcium overload observed in CGN during glucose deprivation.

Some authors have demonstrated that neuronal mitochondria are involved in the mechanism of ROS production under GD [7, 44]. Our results are consistent with these data, because inhibitors of mitochondrial respiratory chain decrease GD-induced production of ROS. An ATP-synthase inhibitor, oligomycin, also significantly decreased ROS production under conditions of GD. Good experimental evidence has been accumulated to explain this effect of oligomycin. There are several reports on protective properties of this compound. Using hippocampal neurons isolated from CA1, it has been shown that under conditions of GD oligomycin can prevent the decrease of the mitochondrial membrane potential in these neurons [60]. Chavez et al. [61] demonstrated that oligomycin can decrease calcium overload and the calcium overload related swelling of isolated kidney cortex mitochondria. Novgorodov et al. [62] reported that oligomycin prevented nonspecific mitochondrial permeability; later this was confirmed by others [63]. It has also been demonstrated that in HeLa cells oligomycin inhibits cytochrome *c* release from mitochondria and apoptosis induced by tumor necrosis factor [64].

The decrease in ROS production induced by respiratory chain inhibitors cannot be explained by the decrease

in cell respiration because the stimulator of mitochondrial respiration, the uncoupler CCCP, also decreased ROS production under glucose deprivation.

Experiments on cells and isolated mitochondria have provided convincing evidence that calcium overload of these organelles causes overproduction of ROS [65]. This suggests that mitochondrial calcium overload associated with GD of neurons is involved in the mechanism of ROS production [14]. This suggestion is supported by the fact that all mitochondrial inhibitors used in this study decreased ROS production under GD but significantly increased $[Ca^{2+}]_i$. This increase may be associated with inhibition of electrogenic Ca^{2+} uptake by mitochondria and efflux of accumulated calcium from mitochondrial matrix. An inhibitor of mitochondrial electrogenic Ca^{2+} influx (ruthenium red) not only decreased ROS production but also prevented the increase in $[Ca^{2+}]_i$. It seems plausible that its effect was determined by blockade of Ca^{2+} entry into cells. Interrelation between ROS production and calcium ions under conditions of GD is also supported by the fact that BAPTA (a chelator of intracellular Ca^{2+}) prevented ROS production.

However, in experiments with human cancer cells it was shown that inhibitors of the mitochondrial electron transport chain increased ROS production induced by GD [66]. Unfortunately, in that study the authors did not investigate the relationship between ROS production and changes in $[Ca^{2+}]_i$. It is possible that in the case of GD neurons and cancer cells employ different mechanisms of mitochondrial ROS production, since these cells are characterized by huge differences in expression of receptors and antiapoptotic proteins. The most probable thing is that in the case of GD-related mechanisms of ROS production in CGN are similar to the mechanisms of glutamate-induced ROS production [67]. As in the case of GD, glutamate toxicity is accompanied by calcium overload of neurons and decrease in mitochondrial membrane potential and ATP content, and mitochondrial respiratory chain inhibitors decrease glutamate-induced ROS production [17, 68, 69]. It is also possible that in our experiments other bivalent ions (in addition to calcium) are involved in stimulation of mitochondrial ROS production. One such ion could be zinc [70, 71]. Zinc accumulation in neurons of some brain regions was found after transitory decrease in glucose [72].

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