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Original Contribution

INFLUENCE OF THE ANTIOXIDANTS VITAMIN E AND IDEBENONE ON RETINAL CELL INJURY MEDIATED BY CHEMICAL ISCHEMIA, HYPOGLYCEMIA, OR OXIDATIVE STRESS

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Abstract—A role for the antioxidants vitamin E and idebenone in decreasing retinal cell injury, after metabolic inhibition induced by chemical ischemia and hypoglycemia, was investigated and compared with oxidative stress conditions. Preincubation of the antioxidants, vitamin E (20 μM) and idebenone (10 μM), effectively protected from retinal cell injury after oxidative stress or hypoglycemia, whereas the protection afforded after postincubation of both antioxidants was decreased. Delayed retinal cell damage, mediated by chemical ischemia, was attenuated at 10 or 12 h postischemia, only after exposure to the antioxidants during all the experimental procedure. An antagonist of the *N*-methyl-D-aspartate (NMDA) receptors, an inhibitor of nitric oxide synthase (NOS) or a blocker of L-type Ca^{2+} channels were ineffective in reducing cell injury induced by chemical ischemia, hypoglycemia or oxidative stress. Oxidative stress and hypoglycemia increased (about 1.2-fold) significantly the fluorescence of the probe $DCFH_2$-DA, that is indicative of intracellular ROS formation. Free radical generation detected with the probe dihydrorhodamine 123 (DHR 123) was enhanced after oxidative stress, chemical ischemia or hypoglycemia (about 2-fold). Nevertheless, the antioxidants vitamin E or idebenone were ineffective against intracellular ROS generation. Cellular energy charge decreased greatly after chemical ischemia, was moderately affected after hypoglycemia, but no significant changes were observed after oxidative stress. Preincubation with vitamin E prevented the changes in energy charge upon 6 h posthypoglycemia. We can conclude that irreversible changes occurring during chemical ischemia mainly reflect the alterations taking place at the ischemic core, whereas hypoglycemia situations may reflect changes occurring at the penumbra area, whereby vitamin E or idebenone may help to increase cell survival, exerting a beneficial neuroprotective effect.

Keywords—Hypoglycemia, Idebenone, Ischemia, Oxidative stress, Retinal cell death, ROS, Vitamin E, Free radicals

INTRODUCTION

During ischemia, a massive depletion of glucose and oxygen occurs, inducing the formation of a severe ischemic region characterized by irreversible neuronal damage, the ischemic core. The pathogenesis of cerebral ischemia has been associated with depletion of cellular energy sources, a massive release of excitatory amino acids, mitochondrial dysfunction and formation of reactive oxygen species, that contribute to the oxidative damage [1]. The brain area surrounding the ischemic core, the penumbra area, is characterized by a decrement in glucose supply and further neurodegeneration resulting from hypoglycemia. The study of neurochemical changes derived from hypoglycemia are extremely important because cell brain injury may not be as irreversible as in the ischemic core. Under this perspective, Nehlig [2] reviewed that only severe hypoglycemia decreased cerebral glucose utilization. During chronic hypoglycemia, the brain "adapted" through a normal cerebral function [3] and the maintenance of glucose utilization at normal levels [2].

Many studies have determined the neuronal damage occurring after a short or acute situation of hypoglycemia/ischemia. Hypoxia and glucose deprivation, resembling changes that occur during ischemia, reduced the ATP levels and induced the release of [^{3}H]dopamine by a Ca^{2+}-independent manner [4]. Chemical ischemia also

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increased the release of endogenous amino acids and highly reduced the energy levels in retinal cells in culture, used as neuronal models [5–7]. However, the recovering processes or the continual exposure to hypoglycemia situations have been less investigated. Goldberg and Choi [8] determined the involvement of *N*-methyl-D-aspartate (NMDA) receptors activation in both the acute and delayed neuronal degeneration, although the way the cells died differed on the dependence of extracellular Ca^{2+}. It would be of interest to examine the progression of neuronal injury that occurred after a brief interruption of blood flow to the brain and define the changes associated with cerebral ischemia and reperfusion, to identify capable therapies that would modify the extent of neuronal damage. Such an investigation seems to be highly important in the clinic, because if the flow interruption is not reversed during the first hour, the cells within the most severely ischemic region will inevitably die [1]. Because there is a narrow therapeutical window after cerebral ischemia to reverse or interfere with the progression of neuronal damage, the search for effective agents capable of reducing the progression of neuronal injury occurring after ischemia further supports the research. According to this purpose, the present study was undertaken to determine the effectiveness of several agents, including antioxidants, in alleviating cell injury that followed the withdrawal of metabolic inhibitors, to mimic neuronal reperfusion after situations of hypoxia, hypoglycemia or ischemia.

Thus, we determined the contribution of ROS generation to cell injury after the depletion of cellular energy sources in the presence of metabolic inhibitors, by using retinal cells in culture as a neuronal model. We evaluated retinal cell injury induced by oxidative stress, chemical hypoxia, hypoglycemia or ischemia and analyzed the formation of endogenous peroxides and other ROS. The lypophilic antioxidants, vitamin E and idebenone, a noncompetitive antagonist of the NMDA receptors, MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate], a blocker of L-type Ca^{2+} channels, nitrendipine and an inhibitor of NOS, nitro^{G}-w-arginine, were tested in their capacity to reduce cell injury induced by energy depleting conditions, after chemical ischemia or hypoglycemia, and further compared to oxidative stress, induced with ascorbate/Fe^{2+}. The present work seems to have a fundamental importance in providing evidence that increased oxidative membrane damage upon metabolic dysfunction can contribute to decrease neuronal recovery, that may be reverted in the presence of antioxidants. Vitamin E and idebenone seem to be particularly important in reducing cell injury induced by moderate energy depleting conditions associated with ROS formation, under hypoglycemia situations.

MATERIALS AND METHODS

Materials

Basal Medium of Eagle (Earle's salts-BME) was purchased from Sigma (St. Louis, MO, USA), trypsin from GIBCO (UK) and fetal calf serum from BioChrom KG (Berlin, Germany). The diacetate form of $DCFH_2$, 2′,7′-dichlorodihydrofluorescein ($DCFH_2$-DA) and dihydrorhodamine 123 (DHR 123) were obtained from Molecular Probes Inc. (Eugene, OR, USA). $DCFH_2$-DA was dissolved in methanol at 10 mM and kept under nitrogen, in the dark, at −20°C until use. DHR 123 stock (5 mM) was made in DMSO and stored at −20°C. Vitamin E (D-α-tocopherol) succinate was obtained from Sigma Chemical Co., MO, USA. Idebenone was a kind gift of Seber, Portugal. MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate) was a kind gift of Merck Sharp and Dohme (West Point, PA, USA). Nitrendipine was obtained from Sandoz Laboratories, Switzerland. All other reagents were of analytical grade.

Culture of chick retinal cells

Retinal cells were prepared from 8-day-old chick embryos, as described previously [5,6,9,10]. The retinas were dissociated with trypsin in a Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution for 15 min, at 37°C. The digested tissue was centrifuged and the pellet was resuspended in BME medium, buffered with 25 mM Hepes and 25 mM $NaHCO_3$, and supplemented with 5% fetal calf serum (heat inactivated), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were plated at a density of 0.76×10^6 cell/cm^2 on poly-L-lysine (0.1 mg/ml) coated glass coverslips for fluorescence measurements or at a density of 2.09×10^6 cells/cm^2 on Costar 12-multiwell plates for other measurements. Cells were cultured for 6 days at 37°C in an atmosphere of 95% air and 5% CO_2. The culture medium of cells plated at a higher density was changed every two days. Similar cultures of retinal cells were previously described to be highly enriched in amacrine neuron-like cells [11,12]. These cultures also contain neurons resembling bipolar cells [9] and glial cells.

Induction of oxidative stress or metabolic inhibition

The reaction of retinal cells with inducers of oxidative stress or metabolic dysfunction was performed similarly as described previously [5,6], in a Na^+ saline solution, containing (in mM): NaCl 140.0, KCl 5.0, $CaCl_2$ 1.5,

$MgCl_2$ 1.0, NaH_2PO_4 1.0, glucose 5.6 and Hepes 20.0, at pH 7.4. Briefly, the BME medium was removed, the cells were washed and allowed to equilibrate in the saline solution for 10–15 min. Then, they were incubated, for 15 min (37°C), with 5 mM ascorbate/100 μM Fe^{2+} (pH 6.5) to induce oxidative stress, with 5 μg/ml oligomycin (OL) and 2 mM sodium cyanide (CN) to induce hypoxia (inhibition of mitochondrial function), with 0.5 mM iodoacetic acid (IAA) to induce hypoglycemia (inhibition of glycolysis), or with both hypoxia and hypoglycemia inducers (OL + CN + IAA) to induce chemical ischemia. Control conditions were carried out in Na^+ saline solution, in the absence of the chemicals, at pH 7.4, for 15 min. After rinsing the cells with Na^+ solution, the experiment was followed by a postincubation of retinal cells in serum-free BME medium buffered with 25 mM Hepes and 25 mM $NaHCO_3$, at neutral pH, up to 24 h, at 37°C, in an atmosphere of 5% CO_2 and 95% air. The chemical inducers were only present during the 15 min-reaction. The withdrawal of metabolic inhibitors was used as an experimental approach to mimic reperfusion after situations of hypoxia, hypoglycemia or ischemia, and compared with similar procedure after cellular oxidation.

In some experiments the cells were supplemented with 20 μM vitamin E succinate or 10 μM idebenone, for 20 h, added to the BME culture medium containing fetal calf serum, before the reaction with the chemical inducers (preincubation). In other experiments, the antioxidants (vitamin E or idebenone) were incubated after the 15 min-reaction, in BME medium without serum, for up to 12 h (postincubation). When analyzing the effect of pre- or postincubation of the vitamin E or idebenone, the induction of oxidative stress or hypoglycemia-like conditions was carried out in the absence of the antioxidants. The antioxidants were also incubated before, during and after the induction of chemical ischemia (pre/postincubation). When present, 1 μM MK-801, 2 μM nitrendipine or 500 μM nitro^G-w-arginine were present during the induction of oxidative stress and metabolic inhibition and were present during the postincubation period, up to 12 h. Under certain conditions, MK-801 (1 μM) was also incubated in Na^+ saline solution without Mg^{2+}, for up to 12 h postincubation.

Analysis of cell injury

Retinal cell injury was quantitatively assessed by measuring lactate dehydrogenase (LDH) released from damaged cells into the extracellular medium during the postincubation, monitored from samples recovered from 1 h and up to 12 or 24 h, after induction of oxidative stress, hypoglycemia or ischemialike conditions. Assessment to intracellular LDH was performed after freezing the cells, at −20°C. Cellular debris in both aliquots (intra and extracellular) were removed by centrifugation at 15,200 × *g*, for 2 min. Lactate dehydrogenase activity was determined spectrophotometrically, by following the rate of conversion of NADH to NAD^+ at 340 nm, according to Bergmeyer and Brent [13]. Lactate dehydrogenase leakage was expressed as a % of total activity (% of LDH released = extracellular LDH/extracellular LDH + intracellular LDH).

Monitoring ROS generation

Reactive oxygen species (ROS) were measured by following the oxidation of $DCFH_2$ to the fluorescent DCF, that detects the formation of intracellular peroxides, as described previously [14–16]. Free radical generation was also monitored by following the oxidation of DHR 123, an oxidation-sensitive indicator that, when oxidized to the positively charged fluorescent derivative rhodamine 123 (RH 123), it moves to the inside-negative mitochondrial environment, being suggested as a probe to measure mitochondrial ROS production [17], namely superoxide [18], hydrogen peroxide [15] and peroxinitrite [19].

After the reaction for 15 min and postincubation for 2 or 6 h in BME without serum, the retinal cells were washed twice and loaded, in the dark, with 20 μM $DCFH_2$-DA in Na^+ saline solution, at pH 7.4, for 20 min, at 37°C. Loading of the probe was renewed for each experiment. After rinsing, cell fluorescence was measured in Na^+ medium, at pH 7.4 (37°C), with excitation at 502 nm and emission at 550 nm, using a SPEX Fluorolog spectrometer equipped with a thermostated waterbath. The increments of fluorescence, due to the oxidation of $DCFH_2$ to DCF, were recorded for 15 min, and expressed as arbitrary units above the initial values.

Similarly, treated retinal cells, post-incubated for 2 or 6 h in serum free BME, were washed with Na^+ saline solution and loaded with 5 μM DHR 123 in Na^+ saline solution, pH 7.4. Fluorescence was recorded for 60 min, at 37°C, to allow the probe to equilibrate between the intracellular and extracellular compartments and to maintain a steady-state intracellular concentration of DHR 123. Cell fluorescence was measured with excitation at 500 nm and emission at 536 nm, by using a SPEX Fluorolog spectrometer. Rhodamine fluorescence increments were expressed as arbitrary units above the initial values.

Determination of cellular energy charge

The adenine nucleotides (ATP, ADP and AMP) were measured after removal of the incubation medium and cell extraction with 0.3 M perchloric acid (0–4°C).

Then, the cells were recovered and centrifuged at 15,800 × *g*, for 5 min. The pellets were used to determine the protein content by the Sedmak method [20], whereas the supernatants were neutralized with 10 M KOH/5 M Tris and centrifuged at 15,800 × *g* for 10 min. The resulting supernatants were analyzed by separation in a reverse-phase HPLC, as described by Stocchi et al. [21]. The chromatographic apparatus used was a Beckman-System Gold, consisting of a 126 Binary Pump Model, a 166 Variable UV detector and a Lichrospher 100 RP-18 (5 μm) column, from Merck (Darmstadt, Germany). The samples were eluted with 100 mM KH_2PO_4 buffer, at pH 7.4, and 1% methanol, at a flow rate of 1.2 ml/min. ATP, ADP and AMP were detected at 254 nm, for 6 min. The energy charge (EC) of retinal cells was calculated according to Atkinson [22], as follows: EC = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Statistical analysis

Data were expressed as the means ± SEM of the indicated number of experiments. The measurements were statistically analyzed using the unpaired two-tailed Student's *t*-test for the comparison of the means of two Gaussian populations or the one-way ANOVA Tukey post-test for multiple comparisons ($p < .05$ was considered significant).

RESULTS

Time-dependent changes in retinal cells viability after oxidative stress, chemical hypoxia, hypoglycemia or ischemia

Figure 1 shows the time-dependent changes in retinal cells viability induced by oxidative stress, chemical hypoxia, hypoglycemia or ischemia, as determined by the release of LDH. Chemical hypoxia, induced by inhibiting both ATP synthase and cytochrome c oxidase, did not significantly affect cell viability up to 24 h postincubation, in comparison with the controls (Fig. 1). Oxidative stress, previously shown to exacerbate the oxidation of retinal cells [10,23], increased significantly LDH leakage 1 h after the reaction with ascorbate/Fe^{2+} (3.44 ± 0.28%, $p < .01$, as compared to the controls). Loss of cell viability induced by cellular oxidation stabilized after approximately 6 h (10.34 ± 0.82% LDH released, Figs. 1 and 2). Significative changes in retinal cell viability evoked by hypoglycemia-like conditions, that evolve the inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, occurred later as compared to oxidized conditions, upon 4 h (4.74 ± 0.72% LDH, $p < .05$), as shown in Figs. 1 and 3. A major reduction in cell viability was observed 10 h after

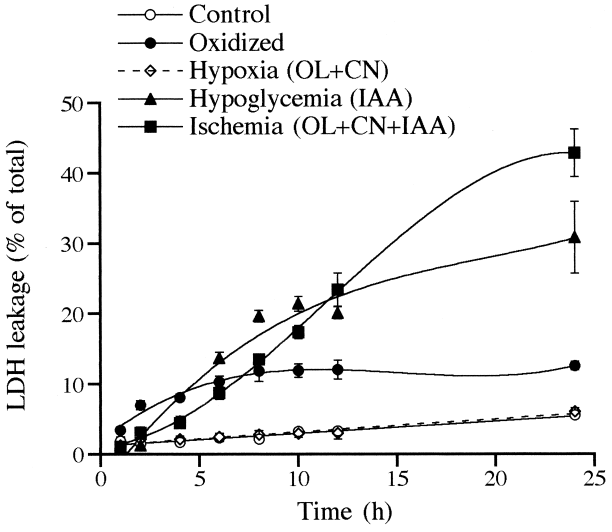

Fig. 1. Comparison of retinal cell injury after oxidative stress, hypoxia, hypoglycemia or ischemialike conditions. Oxidative stress, hypoxia, hypoglycemia or ischemia were induced for 15 min, at 37°C, in the presence of the 5 mM ascorbate/100 μM Fe^{2+}, pH 6.5 (Oxidized), 5 μg/ml OL + 2 mM CN, 0.5 mM IAA, or 5 μg/ml OL + 2 mM CN + 0.5 mM IAA, respectively. Control cells were incubated in Na^+ saline solution without chemical inducers, for 15 min. After rinsing, the retinal cells were incubated with BME without serum, up to 24 h, at 37°C. The extracellular medium was recovered after 1, 2, 4, 6, 8, 10, 12 and 24 h to determine the activity of LDH. Retinal cell injury was evaluated as a function of the increase in LDH leakage. Data are the means ± SEM of triplicates from 7–11 experiments.

exposure to IAA (21.42 ± 1.05% of LDH leakage). Retinal cell injury induced by chemical ischemia, in the presence of both hypoxia and hypoglycemia inducers, occurred upon 2 h (3.07 ± 0.79%, $p < .05$), after oxidative stress and before hypoglycemia increments in LDH leakage (Figs. 1 and 4). Although the release of LDH upon ischemia occurred at a slower rate as compared to hypoglycemia, up to about 10 h, ischemia induced a major decrement in cell viability, from 12 h and up to 24 h (Fig. 1). Upon 24 h, chemical ischemia induced the release of 42.83 ± 3.39% LDH, indicative of toxic effects induced by the metabolic inhibitors. After 15 min exposure to inducers of oxidative stress, chemical hypoxia or ischemia, no significant changes in LDH leakage were observed, as compared to non-treated (control) retinal cells [5].

Effect of MK-801, nitrendipine and nitro^G-w-arginine on retinal cell viability after chemical ischemia, hypoglycemia or oxidative stress

Ischemia situations and metabolic dysfunction have also been associated with a massive release of excitatory amino acids, an increase in intracellular Ca^{2+} concentration and the activation of NOS [5,24–26]. Thus, we analyzed whether the antagonist of the NMDA glutamate

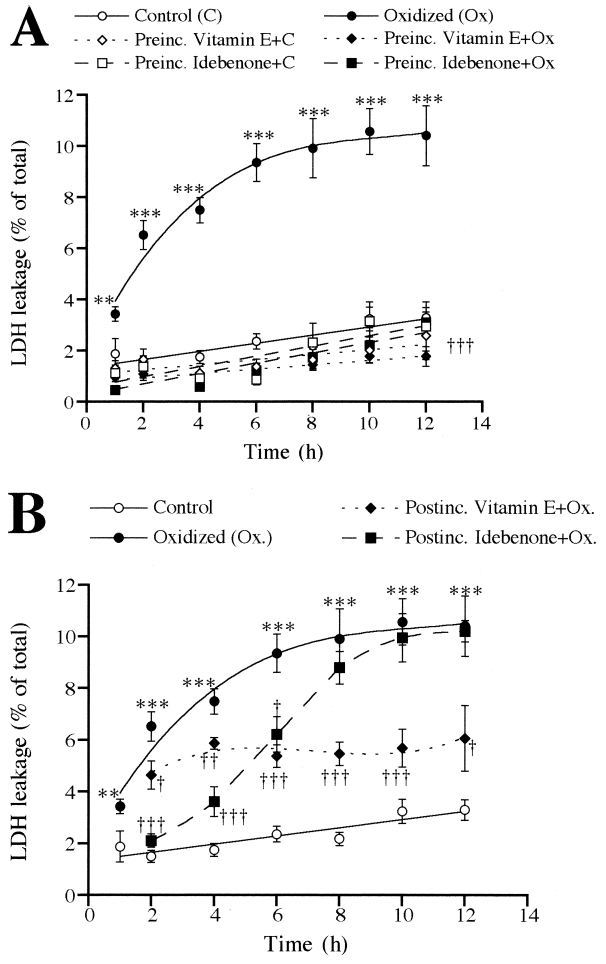

Fig. 2. Oxidative stress-induced retinal cell injury. Influence of vitamin E or idebenone. Retinal cells were incubated in the presence of 5 mM ascorbate/100 μM Fe^{2+}, at pH 6.5, whereas the controls were incubated in the absence of the oxidant pair, at pH 7.4, for 15 min. Analysis of LDH leakage after oxidative stress was performed during the postincubation, up to 12 h. Vitamin E (20 μM) or idebenone (10 μM) were preincubated (Preinc.) for 20 h as a supplement to the culture medium (A) or were postincubated (Postinc.) in BME medium without serum up to 12 h (B). Data are the means ± SEM from 6–11 experiments run in triplicates. Statistical significance: $^{**}p < .01$ or $^{***}p < .001$ as compared to the control; $\dagger p < .05$, $\dagger\dagger p < .01$ or $\dagger\dagger\dagger p < .001$ as compared to oxidative stress conditions in the absence of the antioxidants.

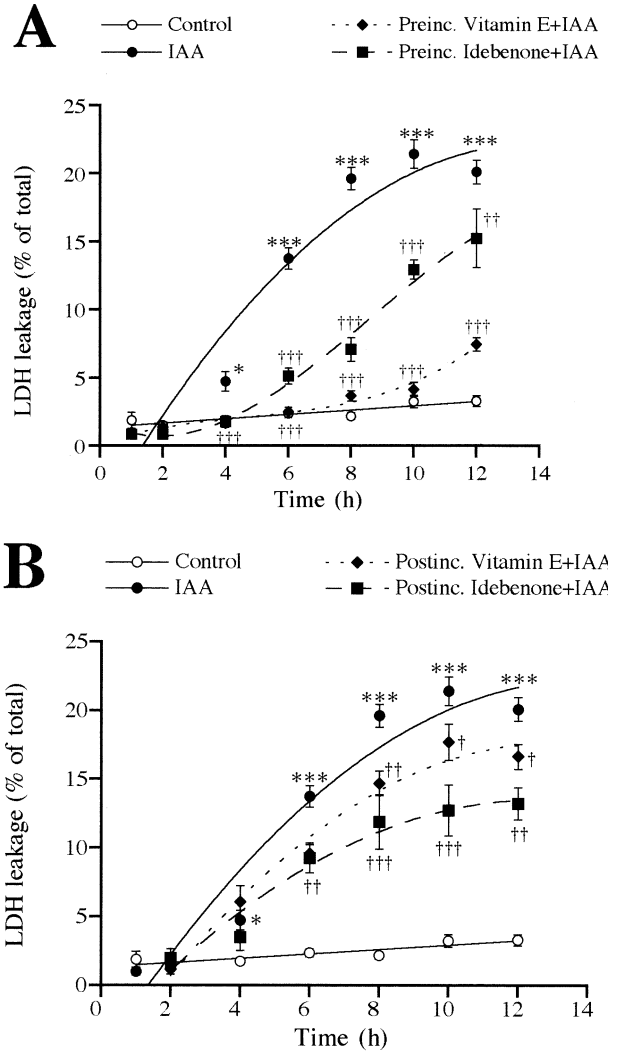

Fig. 3. Hypoglycemia-induced retinal cell injury. Influence of vitamin E or idebenone. The cells were incubated in the presence of 0.5 mM IAA, for 15 min. The controls were incubated in the absence of IAA. The analysis of LDH leakage was performed during the postincubation, up to 12 h. Vitamin E (20 μM) or idebenone (10 μM) were preincubated (Preinc.) for 20 h as a supplement to the culture medium (A) or were postincubated (Postinc.) in BME medium without serum, up to 12 h (B). Data are the means ± SEM of triplicates from six to seven experiments. Statistical significance: $^{***}p < .001$ as compared to the control; $\dagger p < .05$, $\dagger\dagger p < .01$ or $\dagger\dagger\dagger p < .001$ as compared to hypoglycemia (IAA)-like conditions in the absence of the antioxidants.

receptors, MK-801 (1 μM), a blocker of L-type Ca^{2+} channels, nitrendipine (2 μM), or an inhibitor of NOS activity, nitro^G-w-arginine (500 μM), caused significative alterations in retinal cell injury induced by oxidative stress, chemical ischemia or hypoglycemia. MK-801 was previously shown to decrease the response to the activation of the NMDA receptors in retinal cells in culture [9]. By using nitrendipine $[Ca^{2+}]_i$ changes, in response to activation of ionotropic glutamate receptors [27] or upon K^+-depolarization [28], were previously shown to involve the L-type Ca^{2+} channels in retinal cells in culture. Furthermore, we showed that nitro^G-w-arginine decreased the activity of NOS in retinal cells in culture [29] by decreasing the conversion of citrulline from arginine. Nevertheless, retinal cell injury induced by oxidative stress, chemical ischemia or hypoglycemia was not significantly affected after incubation with MK-801, nitrendipine or nitro^G-w-arginine (Table 1). Significative protective data were only observed at 4 h posthypoglycemia in the presence of MK-801 or nitrendipine ($p < .05$) or at 4 h postischemia in the presence of MK-801 ($p < .05$), as shown in Table 1. Incubation with MK-801 (1 μM) in

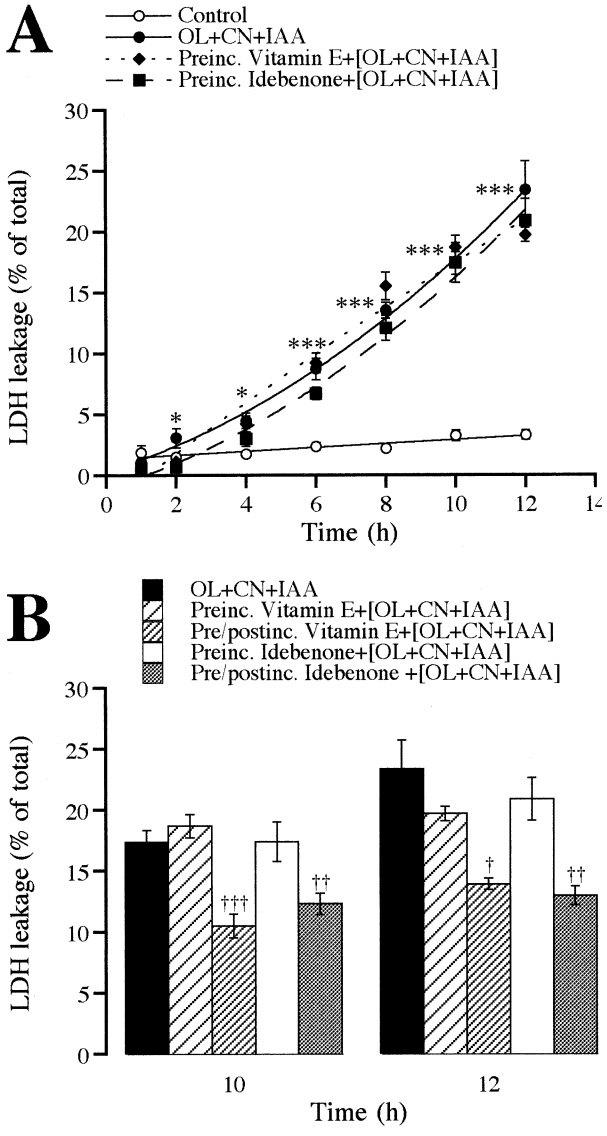

Fig. 4. Ischemia-induced retinal cell injury. Influence of vitamin E or idebenone. Ischemialike conditions were induced in the presence of 5 μg/ml OL, 2 mM CN and 0.5 mM IAA, for 15 min, at 37°C. Control retinal cells were incubated in the absence of the inducers. LDH leakage was measured during the postincubation in BME medium without serum, up to 12 h. Vitamin E (20 μM) or idebenone (10 μM) were pre-incubated (Preinc.) for 20 h as a supplement to the culture medium (A and B). The antioxidants were also incubated prior (20 h), during (15 min) and at 10 or 12 h after the induction of ischemia, in BME medium without serum (Pre/postinc. B). Data are the means ± SEM of triplicates from 6–10 experiments. Statistical significance: *$p < .05$ or ***$p < .001$ as compared to the control; †$p < .05$, ††$p < .01$ or †††$p < .001$ as compared to ischemia (OL + CN + IAA)-like conditions in the absence of the antioxidants.

Na^+ medium without Mg^{2+}, but containing Ca^{2+}, for 15 min plus 12 h postincubation, did not influence significantly the release of LDH (not shown). These results suggested that the activation of NMDA receptors, the entry of Ca^{2+} through the L-type Ca^{2+} channels or the activation of NOS may not be highly involved in delayed retinal cell injury induced by oxidative stress or metabolic inhibition.

Effect of antioxidants, vitamin E and idebenone, on retinal cell viability after chemical ischemia, hypoglycemia or oxidative stress

Because neuronal injury occurring after ischemia/reperfusion has been associated with the formation of reactive oxygen species [30,31], we evaluated the effect of the antioxidants, vitamin E and idebenone, on the release of LDH induced by oxidative stress or metabolic dysfunction situations exacerbated by inducers of hypoglycemia or ischemia, up to 12 h (Figs. 2–4). Vitamin E succinate was previously shown to decrease the extent of lipid peroxidation induced by ascorbate/Fe^{2+} in retinal cells in culture [23,32].

After a preincubation (Fig. 2A), vitamin E and idebenone efficiently reduced oxidative stress-induced cell injury to levels similar to control retinal cells (Fig. 2A). We also tested the hypothesis that postincubation of the antioxidants prevented retinal cell damage. Figure 2B shows vitamin E-mediated decrease ($p < .05$) of retinal cell injury induced by oxidative stress, from 2 h and up to 12 h postincubation. Major differences induced by vitamin E were observed upon 6 h postincubation (about 1.7-fold decrease in LDH leakage). We also analyzed the effect of idebenone postincubation, that decreased cell injury induced by oxidized conditions, but only during the first 2–6 h (about 3 to 1.5-fold decrease in LDH release). Vitamin E and idebenone were less protective upon postincubation (Fig. 2B) than upon preincubation (Fig. 2A). Incubation of vitamin E or idebenone before, during and after exposure to ascorbate/Fe^{2+} showed similar results as compared to those found in Fig. 2A (not shown).

After hypoglycemialike conditions, preincubation of vitamin E was also very effective in reducing retinal cell injury (Fig. 3A) up to 12 h postincubation (2.7-fold decrease in LDH release). Although significant, preincubation of idebenone (10 μM) reduced LDH leakage by only 1.3-fold (Fig. 3A). Postincubation of idebenone was slightly more efficient than vitamin E in reducing the release of LDH after hypoglycemia-like conditions (Fig. 3B). Upon 12 h postincubation, vitamin E decreased IAA-induced retinal cell injury by about 1.2-fold (16.66 ± 0.91% LDH, $p < .05$), whereas idebenone decreased cell injury by about 1.5-fold (13.23 ± 1.17% LDH, $p < .01$). Exposure to vitamin E or idebenone before, during and after the reaction with IAA did not show major effects as compared with the preincubation (data not shown).

Chemical ischemia-induced retinal cell damage was not significantly affected upon preincubation of the antioxidants vitamin E or idebenone (Fig. 4A). Because, in

Table 1. Influence of MK-80, Nitrendipine or Nitro^G-w-Arginine on the Release of LDH Induced by Oxidative Stress, Hypoglycemia or Ischemialike Conditions

	LDH leakage (% of total)			
Postincubation (h)	2 h	4 h	6 h	12 h
Control (C)	1.54 ± 0.34	1.45 ± 0.30	1.42 ± 0.30	2.53 ± 0.42
C + MK-801	0.97 ± 0.24	1.19 ± 0.26	1.57 ± 0.43	1.52 ± 0.25
C + nitrendipine	0.76 ± 0.12	1.69 ± 0.29	2.51 ± 0.46	1.60 ± 0.02
C + nitro^G-w-arginine	1.07 ± 0.14	1.91 ± 0.65	2.21 ± 0.06	1.65 ± 0.05
Oxidative stress (Ox)	4.43 ± 0.47	6.03 ± 0.53	6.77 ± 0.67	7.65 ± 0.76
Ox + MK-801	3.41 ± 0.40	5.08 ± 0.55	5.62 ± 0.64	6.61 ± 0.52
Ox + nitrendipine	2.66 ± 0.58	4.62 ± 0.78	5.57 ± 1.14	5.72 ± 0.15
Ox + nitro^G-w-arginine	3.63 ± 0.96	4.95 ± 1.02	5.04 ± 0.82	8.88 ± 0.74
Hypoglycemia (IAA)	1.07 ± 0.20	5.82 ± 0.51	13.62 ± 0.99	17.88 ± 0.79
IAA + MK-801	0.90 ± 0.28	3.47 ± 0.49*	13.19 ± 1.19	18.14 ± 0.92
IAA + nitrendipine	1.07 ± 0.38	4.29 ± 0.33*	13.46 ± 1.25	16.99 ± 0.53
IAA + nitro^G-w-arginine	0.96 ± 0.29	6.55 ± 1.71	15.73 ± 0.29	17.95 ± 1.05
Ischemia (Isc)	1.10 ± 0.26	4.25 ± 0.58	11.21 ± 1.38	20.74 ± 0.94
Isc + MK-801	0.76 ± 0.20	2.69 ± 0.29*	10.30 ± 0.70	19.11 ± 0.64
Isc + nitrendipine	1.40 ± 0.40	4.12 ± 0.47	10.75 ± 0.48	18.98 ± 0.70
Isc + nitro^G-w-arginine	0.92 ± 0.16	6.22 ± 1.03	17.17 ± 1.21	20.08 ± 2.19

Retinal cells were incubated in the presence of 5 mM ascorbate and 100 μM Fe^{2+}, at pH 6.5 (Oxidative stress-Ox), 0.5 mM IAA (hypoglycemia-IAA) or ischemia (Isc) inducers (5 μg/ml oligomycin, 2 mM cyanide and 0.5 mM IAA), for 15 min. Control (C) retinal cells were incubated in the absence of the chemical inducers. The incubation medium was recovered upon 2, 4, 6 or 12 h of postincubation in BME medium without serum and LDH activity was measured as described under Methods. MK-801 (1 μM), nitrendipine (2 μM) or nitro^G-w-arginine (500 μM) were incubated during and after (up to 12 h) the 15 min reaction. LDH leakage data are the means ± SEM of duplicates or triplicates from 2–3 independent experiments.
* $p < .05$ as compared to hypoglycemia or ischemialike conditions in the absence of MK-801, nitrendipine or nitro^G-w-arginine.

general, postincubation of the antioxidants was less effective than their preincubation, we tested the protective effectiveness of a pre- and postincubation (Fig. 4B). Under such conditions, vitamin E or idebenone decreased retinal cell injury induced by chemical ischemia upon 10 h (reduction of 1.7 or 1.4-fold in LDH release) and upon 12 h postincubation (reduction of 1.8 or 1.7-fold in LDH release).

Formation of reactive oxygen species upon exposure to oxidative stress, chemical ischemia or hypoglycemia

The relative contribution of oxidative stress or chemical hypoxia, hypoglycemia and ischemia to the formation of intracellular ROS, namely peroxides, in retinal cells in culture preloaded with the fluorescent probe $DCFH_2$-DA is shown in Fig. 5 and Table 2. Oxidative stress or hypoglycemia-like conditions significantly increased the production of intracellular peroxides, respectively, by 1.15-fold or 1.09-fold (Fig. 5) upon 6 h, a time period during that retinal cell injury was suggested to be caused by oxidative lesion of membrane components, due to the effective protection afforded by the antioxidants (Figs. 2 and 3). However, no significant differences were observed after exposure to hypoxia or ischemia inducers (Fig. 5). Furthermore, analysis of ROS generation at 2 h postincubation revealed no significant increments in DCF fluorescence (Table 2). Preincubation of vitamin E or idebenone, at conditions that efficiently reduced retinal cell injury (Fig. 2A and 3A), did not affect the formation of intracellular peroxides 6 h upon exposure to inducers of oxidative stress or hypoglycemia-like conditions (Table 2).

Reactive oxygen species formation was also measured

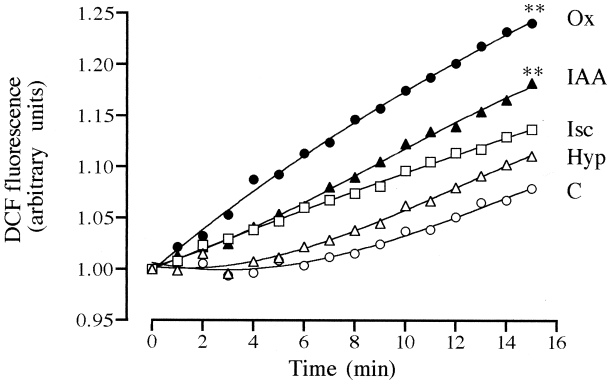

Fig. 5. Comparison of intracellular peroxides formation after oxidative stress, hypoxia, hypoglycemia or ischemialike conditions. Intracellular peroxides or ROS were examined by following the increments in DCF fluorescence in cells postincubated for 6 h after the induction of oxidative stress (Ox), hypoxia (Hyp), hypoglycemia (IAA) or ischemia-like conditions (Isc), for 15 min. Control (C) retinal cells were incubated similarly as described, but in the absence of the chemical inducers. The cells were loaded with $DCFH_2$-DA (20 μM) for 20 min and the peroxides were recorded for 15 min, at 37°C. Data are the means from 5–12 measurements. Statistical significance: ** $p < .01$ as compared to control conditions.

Table 2. Influence of Vitamin E or Idebenone on the Formation of Intracellular Peroxides at 2 or 6 h Postincubation after Oxidative Stress, Hypoglycemia or Ischemialike Conditions

	Increment of DCF fluorescence (AU above the initial values)	
Postincubation (h)	2 h	6 h
Control (C)	1.14 ± 0.02 (4)	1.08 ± 0.03 (8)
C + vitamin E	1.15 ± 0.03 (6)	ND
C + idebenone	1.15 ± 0.02 (7)	ND
Oxidative stress (Ox)	1.18 ± 0.07 (7)	1.24 ± 0.06 (5)*
Ox + vitamin E	1.14 ± 0.02 (8)	1.13 ± 0.01 (5)
Ox + idebenone	1.13 ± 0.01 (6)	1.15 ± 0.02 (7)
Hypoglycemia (IAA)	1.15 ± 0.02 (6)	1.18 ± 0.03 (10)*
IAA + vitamin E	1.06 ± 0.01 (7)	1.13 ± 0.03 (5)
IAA + idebenone	1.12 ± 0.01 (5)	1.13 ± 0.03 (6)
Ischemia (Isc)	1.17 ± 0.05 (11)	1.14 ± 0.02 (12)
Isc + vitamin E	1.10 ± 0.02 (8)	ND
Isc + idebenone	1.17 ± 0.04 (10)	ND

Retinal cells were incubated in the presence of 5 mM ascorbate and 100 μM Fe^{2+}, at pH 6.5 (Oxidative stress-Ox), 0.5 mM IAA (Hypoglycemia-IAA) or Ischemia (Isc) inducers (5 μg/ml OL, 2 mM CN and 0.5 mM IAA), for 15 min. Control (C) cells were incubated in the absence of the chemical inducers. The antioxidants vitamin E (20 μM) or idebenone (10 μM) were pre-incubated in BME culture medium, for 20 h. After 2 or 6 h postincubation in BME medium without serum, the cells were loaded with $DCFH_2$-DA (20 μM), for 20 min. The fluorescence of DCF was monitored for 15 min and the values were expressed as increments of fluorescence, in arbitrary units (AU) above the initial values. Data are the means ± SEM of the number of measurements indicated in brackets. ND = not determined.

* $p < .01$ as compared with the control in the absence of the antioxidants.

with DHR 123 (Fig. 6 and Table 3), a fluorescent probe shown to be oxidized not only by superoxide and hydrogen peroxide [15,18], but also by peroxinitrite [19]. Generation of ROS was detected upon 2 or 6 h (Fig. 6). At 2 h, induction of oxidative stress, hypoglycemia or ischemia enhanced significantly the formation of ROS by about 1.7-fold (Fig. 6A). Upon 6 h postincubation, ROS production increased significantly after oxidative stress (1.6-fold) or chemical ischemia (1.8-fold), although we did not observe significant changes after hypoglycemia-like conditions (Fig. 6B). We also analyzed the effect of preincubation of vitamin E and idebenone upon 2 h, due to the increment in rhodamine fluorescence under all the experimental conditions (Table 3). The antioxidants were without effect in reducing ROS formation, measured with DHR 123, in cultured retinal cells (Table 3).

The formation of TBARS, a common nonspecific indicator of peroxidation byproducts, resulting from free radical attack on double bonds of polyunsaturated fatty acids that comprise membrane phospholipids integrity, was not detected upon 2 or 6 h after chemical ischemia or hypoglycemia (not shown), suggesting that an extensive membrane lipid peroxidation was not involved in retinal cell damage.

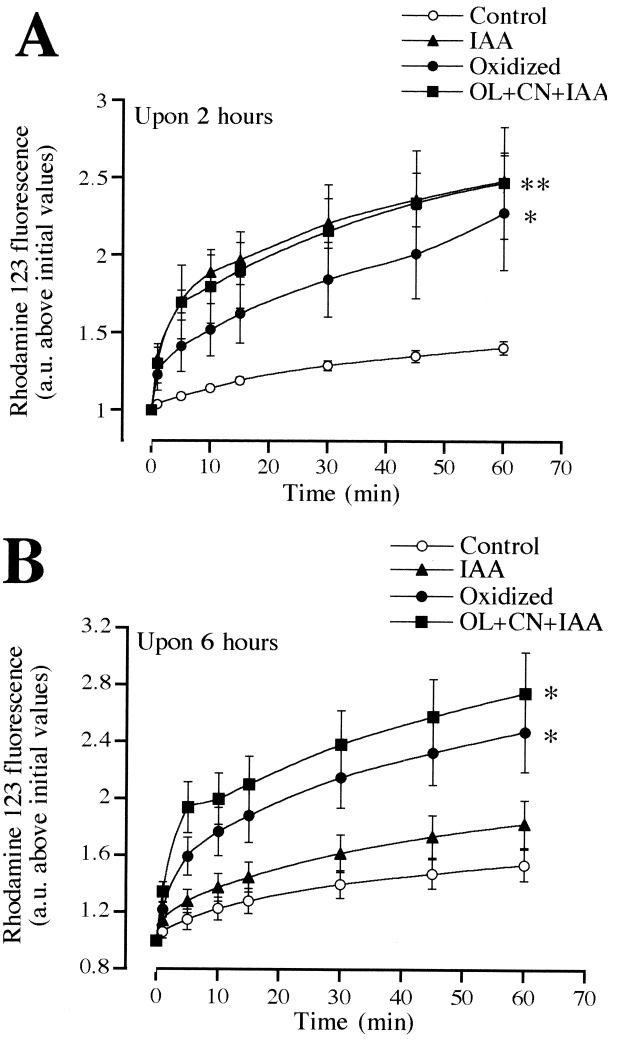

Fig. 6. Comparison of ROS formation after oxidative stress, hypoglycemia or ischemialike conditions. ROS were analyzed for 60 min by determining the increment in rhodamine fluorescence intensity with the probe DHR 123 (5 μM) in retinal cells postincubated for 2 (A) or 6 h (B) after oxidative stress (Oxidized), hypoglycemia (IAA) or ischemia-like conditions (OL + CN + IAA). Data are the means ± SEM from three to nine measurements. Statistical significance: * $p < .05$ or ** $p <$ .01 as compared with control conditions at 2 or 6 h postincubation.

Energy charge of retinal cells after exposure to oxidative stress, chemical ischemia or hypoglycemia

In this study we also evaluated the changes in energy charge upon 2 or 6 h postincubation, in comparison with the alterations occurring after 15 min-reaction (0 h postincubation), as shown previously [5]. Figure 7A shows the energy charge of oxidized retinal cells in comparison with controls and cells submitted to chemical ischemia. As demonstrated previously, oxidative stress did not affect significantly the energy charge even upon 2 h (0.83 ± 0.05) or 6 h (0.91 ± 0.03), as compared to the controls or to the results observed after 0 h postincubation (Fig. 7A). Nevertheless, the energy

Table 3. Influence of Vitamin E or Idebenone on the Formation of ROS at 2 or 6 h Postincubation after Oxidative Stress, Hypoglycemia or Ischemialike Conditions

	Increment in Rhodamine Fluorescence (AU above the initial values)	
Postincubation (h)	2 h	6 h
Control (C)	1.41 ± 0.04 (7)	1.54 ± 0.11 (4)
C + vitamin E	ND	ND
C + idebenone	ND	ND
Oxidative stress (Ox)	2.28 ± 0.37 (5)*	2.48 ± 0.28 (6)*
Ox + vitamin E	2.22 ± 0.31 (6)*	ND
Ox + idebenone	1.99 ± 0.26 (6)*	ND
Hypoglycemia (IAA)	2.48 ± 0.19 (3)†	1.83 ± 0.17 (5)
IAA + vitamin E	2.76 ± 0.31 (3)†	ND
IAA + idebenone	2.31 ± 0.23 (6)*	ND
Ischemia (Isc)	2.47 ± 0.36 (5)†	2.75 ± 0.29 (9)*
Isc + vitamin E	2.76 ± 0.10 (4)†	ND
Isc + idebenone	2.75 ± 0.17 (3)†	ND

Retinal cells were incubated in the presence of ascorbate (5 mM)/Fe^{2+} (100 μM), pH 6.5 (Oxidative stress-Ox), 0.5 mM IAA (Hypoglycemia-IAA) or Ischemia (Isc) inducers (5 μg/ml OL, 2 mM CN and 0.5 mM IAA), for 15 min. Control (C) cells were incubated in the absence of the inducers. The antioxidants vitamin E (20 μM) or idebenone (10 μM) were pre-incubated in BME culture medium, for 20 h. After 2 or 6 h post-incubation in BME medium without serum, the cells were loaded with DHR 123 (5 μM) and the increment in rhodamine fluorescence was monitored for 60 min. Fluorescence data, expressed in arbitrary units (AU) above the initial values, are the means ± SEM of the number of measurements indicated in brackets. ND = not determined.

* $p < .05$ or † $p < .01$ as compared to the controls in the absence of the antioxidants.

charge after chemical ischemia, decreased strongly after 15 min-reaction (0 h postincubation), was maintained upon 2 h postincubation (0.14 ± 0.01) and decreased even further after 6 h postincubation, to 0.01 ± 0.002 (Fig. 7A), that corresponded to a major decrease of the ATP/ADP ratio (not shown). Preincubation of the antioxidants vitamin E or idebenone did not affect the energy charge induced by oxidized conditions (Fig. 7A) or ischemia (not shown). We also analyzed the energy charge in cells submitted to hypoglycemialike conditions (Fig. 7B). Upon 2 h postincubation, in cells submitted to hypoglycemia, the energy charge decreased to 0.53 ± 0.08, whereas upon 6 h postincubation the energy charge decreased to 0.18 ± 0.07. Preincubation with vitamin E or idebenone did not affect the energy charge observed upon the reaction with IAA, for 15 min, or on 2 h postincubation. No significant results were observed in the presence of idebenone upon 6 h postincubation, either (Fig. 7B). However, upon preincubation with vitamin E the decrement in energy charge occurring 6 h after incubation with IAA was reverted to 0.60 ± 0.08 ($p < .001$, as compared to IAA alone).

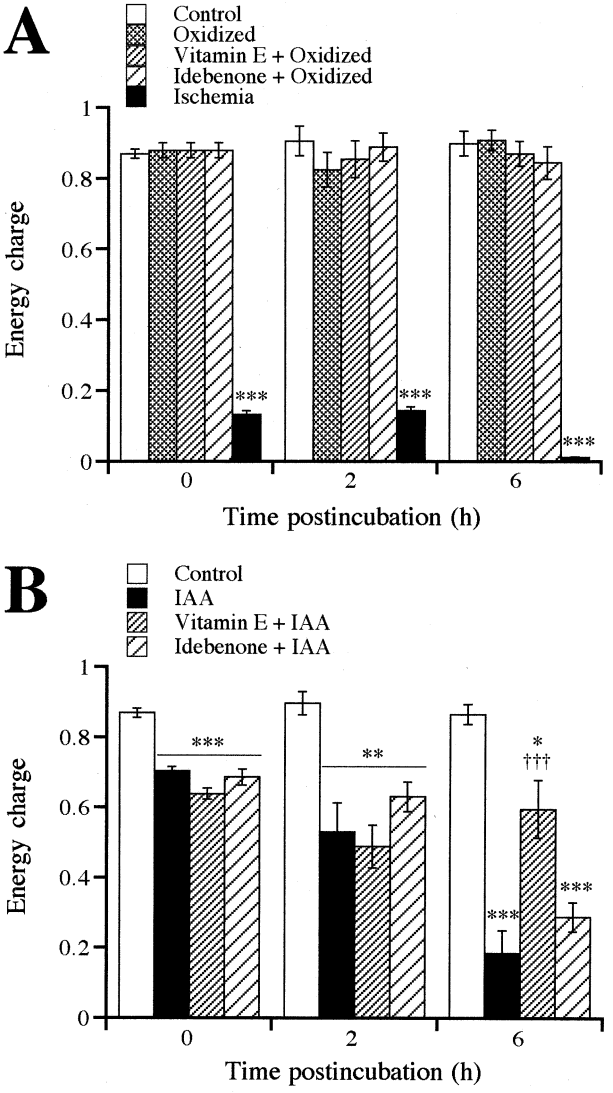

Fig. 7. Energy charge of retinal cells in culture after exposure to oxidative stress, hypoglycemia or ischemia-like conditions. Influence of vitamin E and idebenone. Retinal cells were exposed to inducers of oxidized conditions (ascorbate/Fe^{2+}, pH 6.5), hypoglycemia (IAA) or ischemia-like conditions (OL + CN + IAA), for 15 min (time 0 h). Postincubation in BME medium without serum was performed for 2 or 6 h after the reaction. When present, 20 μM vitamin E or 10 μM idebenone were preincubated for 20 h as a supplement to the culture medium. (A) Data from oxidative stress and ischemialike conditions in comparison with control retinal cells. (B) Data from hypoglycemialike conditions in comparison with the controls. The adenine nucleotides ATP, ADP and AMP were determined by reverse phase HPLC, after acidic cell extraction followed by neutralization. The energy charge (EC) of retinal cells was calculated by using the formula: EC = (ATP + 0.5 ADP)/(ATP + ADP + AMP). Data are the means ± SEM of triplicates from three to five experiments. Statistical significance: *$p < .05$, **$p < .01$ or ***$p' < .001$ as compared to the control; †††$p < .001$ as compared to hypoglycemialike conditions in the absence of antioxidants.

DISCUSSION

Over the last decade many studies have provided evidence that ROS are important contributors to neuronal injury mediated by ischemia [30,31,33]. In this study, by using cultured retinal cells as neuronal models, we have shown that the antioxidants vitamin E and idebenone afford protection against retinal cell injury mediated by

oxidative stress or metabolic dysfunction, mainly associated with hypoglycemia situations. However, the antioxidants were not very effective against retinal cell injury induced by chemical ischemia, probably associated with irreversible damage occurring in the ischemic core.

MK-801, nitrendipine and nitro^G-w-arginine may not be highly effective against delayed retinal cell injury

In early studies, we showed that hypoxic and ischemic conditions, induced in the presence of metabolic inhibitors, increased the release of excitatory amino acids and increased the $[Ca^{2+}]i$ [5]. Under ischemia-like conditions, the release of glutamate was closely associated with a decrease in cellular ATP [6]. Thus, the extracellular accumulation of glutamate can stimulate the glutamate receptors, namely the NMDA receptor, suggested to play an important role in neuronal injury [34]. Significant protection of retinal cells afforded by MK-801, a noncompetitive antagonist of the NMDA receptors, was only observed upon 4 h of posthypoglycemia or postischemia (Table 1). MK-801 failed to reduce retinal cell damage under our experimental conditions, despite used at low concentrations to allow specific interactions with the NMDA receptor, maintained during all the experiment and under conditions that induced the opening of the receptor associated channel, in experiments carried out in the absence of Mg^{2+} (not shown). Nevertheless, Steinberg et al. [35] showed previously that MK-801 could be effective as a neuroprotector against focal cerebral ischemia if the dose applied was maintained. Earley et al. [36] also showed neuroprotective effects of MK-801 against cortical damage induced by cerebral ischemia and, in the retina, Zeevalk and Nicklas [37,38] found protective effects of MK-801 (1 μM) against toxicity induced by 30 min-metabolic inhibition.

Nitrendipine, the L-type Ca^{2+} channel blocker, was not protective against cell injury induced by oxidative stress or metabolic inhibition either (Table 1). This may be in accordance with the reduced involvement of NMDA receptor-mediated injury (Table 1), because the Ca^{2+} channel antagonists were shown to attenuate neurotoxicity induced by activation of NMDA receptors [39]. In retinal cells in culture, Duarte et al. [27] showed that activation of ionotropic glutamate receptors increased $[Ca^{2+}]i$, occurring through the permeation of the receptor-associated channels and through the voltage-gated Ca^{2+} channels, namely of the L-type. Moreover, the L-type Ca^{2+} channels were observed to be highly affected after K^+-depolarization of ascorbate/Fe^{2+}-oxidized retinal cells [28].

Increasing evidences suggest the stimulation of neuronal NOS activity during ischemia. Ischemia-induced damage was substantially reduced in NOS mutants or by using specific NOS inhibitors [25,40,41]. Inducible NOS knockouts also reduced the susceptibility to ischemic brain injury [42]. Moreover, NOS inhibitors protected against excitotoxic neuronal injury [43]. Although we found previously that nitro^G-w-arginine reduced the activity of NOS in retinal cells in culture [29], exposure to nitro^G-w-arginine was not effective against retinal cell injury up to 12 h upon chemical ischemia, hypoglycemia or oxidative stress (Table 1). The failure of nitro^G-w-arginine to increase the viability of retinal cells upon hypoglycemia or ischemia may also be related with a reduced involvement of the NMDA receptors in cell injury (Table 1), because the generation of nitric oxide has been implicated in the excitotoxic neuronal injury mediated by activation of the NMDA receptors [43,44]. Recently, we showed an inhibition of NOS activity after 15 min ascorbate/Fe^{2+}-induced oxidative stress in retinal cells [29], explaining the inefficacy of the inhibitor of NOS after withdrawal of the oxidant inducers.

Extensive delayed retinal cell injury after chemical ischemia

Characterization of time-dependent changes in retinal cells viability showed that depending on how the cells are stressed, their vulnerability to mechanisms associated with cell death may differ. Upon chemical hypoxia there was a maintenance of cell viability (Fig. 1), probably resulting from no significant changes in cellular energy status [5,6] or in the intracellular generation of ROS (Fig. 5), although we observed a significant increase in the extracellular accumulation of excitatory amino acids [5]. Oxidative stress was previously shown to induce the extracellular accumulation of endogenous glutamate and aspartate by a Ca^{2+}-dependent pathway [5]. However, the cellular energy charge was not significantly changed before or after withdrawal of the oxidant pair (Fig. 7A), and reduction of cell viability (Fig. 1) may have occurred as a consequence of ROS generation (Figs. 5 and 6). Surprisingly, LDH leakage was not further enhanced up to 6 h postincubation (Figs. 1 and 2), that suggested a limited source of free radicals generation or the stimulation of endogenous antioxidants. As compared to oxidative stress, a decrement in cell viability occurring after metabolic inhibition, in the presence of hypoglycemia or ischemia inducers (Fig. 1), is imposed due to a decrease in cellular energy charge, that shows a major decrement upon chemical ischemia (Fig. 7). Consequently, delayed cell damage, upon 24 h postincubation, was enhanced after ischemia (Fig. 1). Moreover, the extracellular accumulation of excitatory amino acids occurring by a Ca^{2+}-independent mechanism was larger upon chemical ischemia in comparison with hypoglycemia in cultured retinal cells (unpublished results), and the formation of

ROS, as detected with DHR 123, occurred also in a large extent (Fig. 6).

Formation of ROS and protection by vitamin E and idebenone

In this study, we also evaluated the antioxidant effectiveness of vitamin E and idebenone, to determine the importance of oxidative membrane damage in retinal cell injury occurring upon chemical ischemia or hypoglycemia, and compared with ascorbate/Fe^{2+}-induced oxidative stress. Vitamin E is a potent scavenger of peroxyl radicals within the membranes [45,46]. The polar α-tocopheryl esters, namely vitamin E succinate, have proven valuable tools for supplementing α-tocopherol to isolated cells, increasing the resistance of cells to oxidative damage [47]. Previously, we determined that preincubation of 10 μM vitamin E succinate reduced significantly the release of LDH, the formation of TBARS and conjugated dienes after 5 mM ascorbate/100 μM Fe^{2+} (pH 6.5)-induced lipid peroxidation in retinal cells in culture [23]. Vitamin E was also shown to protect the neurons from deleterious effects induced by cumene hydroperoxide [48]. Idebenone, a benzoquinone derivative, seems to act both as a lipid radical scavenger and as an electron trapping agent. The antioxidant properties of idebenone were determined by the inhibition of lipid peroxidation through its free radical scavenger activity [49]. Furthermore, the protective effects of idebenone are also attributed to an improvement in cerebral energy metabolism [50,51].

In this study we show that the antioxidants vitamin E and idebenone are highly effective in reducing delayed retinal cell injury, especially when present before the induction of oxidative stress or hypoglycemia-like conditions (Figs. 2 and 3). The large effect of the antioxidants implicated the oxidative cell membrane damage in retinal cell injury. After oxidative stress or hypoglycemia, intracellular ROS, detected with the fluorescent probes $DCFH_2$-DA or DHR123, indicated the formation of ROS (Figs. 5 and 6). The inhibition of glycolysis during hypoglycemia may account for a decrease in cellular reducing equivalents and, consequently, an increased formation of intracellular ROS. Preincubation of vitamin E was also shown to significantly increase the energy charge 6 h upon hypoglycemia (Fig. 7B), that may be related with a more effective reduction of retinal cell injury in the presence of vitamin E (Fig. 3A). Besides vitamin E and idebenone, the spin trapping agent α-phenyl-*tert*-butyl nitrone and an inhibitor of lipid peroxidation were shown to protect neurons against hypoglycemic injury [52].

Because in vivo, the antioxidants may be administered after ischemia, we also tested the protection afforded by the antioxidants when postincubated. Under these conditions, idebenone was protective after oxidative stress, up to 6 h (Fig. 2B), but still protected against hypoglycemia-induced cell injury (Fig. 3B). Postincubation of vitamin E was less evident in decreasing cell injury mediated by hypoglycemia (Fig. 3B), but it was still effective in increasing cell viability upon oxidative stress (Fig. 2B). The effectiveness of postincubation of the antioxidants may assume a major importance in demonstrating the "therapeutic window" of these antioxidants required for a clinical application against cell injury associated with hypoglycemia and oxidative stress, probably occurring at the edge of the ischemic core. A significant protective effect of the antioxidants against cell injury, induced by chemical ischemia, was observed only upon their maintenance during all the experimental procedure (Fig. 4), that may have resulted from a strong depletion of energy sources (Fig. 7A) and the formation of ROS at a large extent (Fig. 6). An increase in fluorescence detected with DHR 123 seems to indicate the formation of ROS not only after hypoglycemia, but also after ischemia situations. Nevertheless, because rhodamine redistributes within the cell in response to changes in mitochondrial membrane potential, an increase in fluorescence may also derive from the release of rhodamine from the quenched environment of the mitochondrial matrix to the cytoplasm. Non-detectable formation of ROS upon chemical ischemia, as determined by following the increases in fluorescence of $DCFH_2$, may have resulted from a decrease in intracellular pH.

Vitamin E distributes to cellular membranes and is located in the mitochondrial fractions and in the endoplasmic reticulum [45]. Ray and Fariss [53] proposed the accumulation of vitamin E succinate at the level of mitochondria, where it could exert its antioxidant activity. This could be one of the explanations for vitamin E-induced increase in cellular energy levels upon hypoglycemia-like conditions. Similarly, idebenone incorporates easily in the membranes and was also proposed to interfere with lipid peroxidation in brain mitochondria [49]. Neither vitamin E or idebenone influenced significantly the formation of intracellular ROS (Table 2 or 3), that may be explained by scavenging properties of these lipophilic antioxidants at the membrane level and not in the aqueous medium, where oxidation of $DCFH_2$ or DHR 123 takes place. Nevertheless, the antioxidants vitamin E and idebenone were previously shown to protect against glutamate-induced cytotoxicity and reduced the formation of intracellular peroxides, as determined with the probe 2′,7′-dichlorofluorescin-diacetate [54].

There is compelling evidence that ROS are generated after ischemia [30,31,33], that may constitute the basis to explain the enhancement in brain injury that occurs in the hours after the ischemic onset. Under ischemia, glutamate-mediated generation of mitochondria free radicals was also shown to occur, that decreased after glutamate receptor blockade [55]. The injury induced by reoxygenation after hypoxia was reduced by superoxide dismutase, catalase, trolox and α-tocopherol, indicating the

occurrence of lipid peroxidation [56]. Thus, the antioxidants have proven to be good protectors in experimental stroke models. Synthetic superoxide dismutase/catalase mimetics, catalytically destroying both superoxide and hydrogen peroxide, were also shown to protect brain tissue during a delayed-treatment after the onset of ischemia [57]. Recently, the Mn-superoxide dismutase was demonstrated to play an important role in the mitochondria during ischemia, and the importance of superoxide formation in inducing impairment of mitochondria function was made evident [58,59].

In conclusion, although the antioxidants vitamin E and idebenone did not greatly interfere with retinal neurotoxicity mediated by a severe depletion of energy sources, in cells submitted to chemical ischemia, they efficiently decreased the cellular damage evoked by moderate energy depleting conditions, upon hypoglycemia. This may have important implications for the design of neuroprotective strategies in clinical therapy, aimed at combating oxidative damage during and after acute brain injury, specially associated with the ischemic penumbra area.

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ABBREVIATIONS

BME—basal medium of Eagle
CN—cyanide
$DCFH_2$—2′,7′-dichlorodihydrofluorescein
DHR 123—dihydrorhodamine 123
IAA—iodoacetic acid
LDH—lactate dehydrogenase
MK-801—(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate
NMDA—*N*-methyl-D-aspartate
NOS—nitric oxide synthase
OL—oligomycin
ROS—reactive oxygen species
TBARS—thiobarbituric acid reactive substances