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Original Research Communication

Effect of Mitochondrial Electron Transport Chain Inhibitors on Superoxide Radical Generation in Rat Hippocampal and Striatal Slices

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ABSTRACT

In this study, we have compared the generation of superoxide radical in rat hippocampal and striatal slices in the presence of specific mitochondrial electron transport chain (ETC) inhibitors (complexes I and III) under control and depolarization conditions [incubation in artificial cerebrospinal fluid (ACSF) or depolarizing ACSF (dACSF), respectively]. Superoxide radical generation was increased in both ACSF- and dACSF-incubated hippocampal and striatal slices when rotenone and antimycin A were added to the incubation medium. The increase in superoxide radical was dependent on the concentration of ETC inhibitors under control, but not depolarization conditions. Rotenone was found to be more effective than antimycin A in producing superoxide radical from hippocampal and striatal slices. Our results also showed that hippocampal slices were more sensitive to ETC inhibitors compared with striatal slices. Thus, different regions of the brain seem to differ in their capacity to generate free radicals and vulnerability to oxidative stress conditions. This difference should be considered in developing therapeutic modalities against oxidative stress-related disorders and neurodegeneration. Antioxid. Redox. Signal. 3, 1099–1104.

INTRODUCTION

THE BRAIN consumes a disproportionate amount of oxygen in the body as it derives its energy almost exclusively from mitochondrial respiration (1). Mitochondria are involved in intracellular calcium ion storage in addition to their primary role in energy production (32). However, the mitochondrial respiratory chain is also the most important source of superoxide radicals in aerobic cells (15). Mitochondrial respiratory chain shows a functional decline with age, and its dysfunction has been implicated in a variety of degenerative states such as Parkinson's, Huntington's, and Alzheimer's diseases (2, 5, 19, 25, 28).

Huntington's disease is a neurodegenerative disorder that affects striatal spiny neurons. Measurements of respiratory chain activity in Huntington's disease caudate showed deficiency of complexes II, III, and IV (25). In addition, cerebrospinal fluid lactate-to-pyruvate ratios were found to be increased and correlated with impaired energy generation (12).

On the other hand, hippocampal neurons are severely affected in Alzheimer's disease. Although the underlying mechanism remains poorly understood, involvement of reactive

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oxygen species has been suggested (31). Deficiencies of mitochondrial complexes I, III, and IV have been detected in the brain and hippocampus of Alzheimer's disease patients (5, 20, 21).

Several lines of evidence suggest that superoxide radicals play a pivotal role in neurodegeneration and excitotoxic cell death (8, 11, 22, 36). In this study, we have compared superoxide radical generation in rat hippocampal and striatal slices under control and depolarization conditions and in the presence of specific mitochondrial electron transport chain (ETC) inhibitors. Furthermore, we have compared the sensitivity of hippocampal and striatal slices to oxidative stress conditions.

MATERIALS AND METHODS

Three-week-old Sprague–Dawley rats were used in the study, which was approved by the institutional animal care and ethics committee. On each study day, two rats were decapitated and their brains were quickly removed. The hemisphere containing hippocampus and striatum was glued to a vibroslicer stage, and slices of 400 μm were obtained by the vibroslicer (Campden Instruments Ltd., U.K.). Hippocampus and striatum were isolated, and slices were kept in artificial cerebrospinal fluid (ACSF) under carbogen (95% O_2 plus 5% CO_2) aeration for 45 min for functional recovery. ACSF had the following composition: 125 m*M* NaCl, 3.75 m*M* KCl, 1.2 m*M* NaH_2PO_4, 2 m*M* $CaCl_2$, 1.3 m*M* $MgCl_2$, 10 m*M* glucose, 26 m*M* $NaHCO_3$. After equilibration, hippocampal and striatal slices were further incubated for 45 min in either ACSF (control) or depolarizing ACSF (dACSF) containing 50 m*M* K^+. Composition of the dACSF was as follows: 79 m*M* NaCl, 50 m*M* KCl, 1.2 m*M* NaH_2PO_4, 2 m*M* $CaCl_2$, 1.3 m*M* $MgCl_2$, 10 m*M* glucose, 26 m*M* $NaHCO_3$. Mitochondrial ETC inhibitors were added to the incubation medium of ACSF- or dACSF-incubated slices. Rotenone, a complex I inhibitor, and antimycin A, a complex II inhibitor, were used at two different concentrations (1 and 10 μM). Ruthenium red (RuR) a mitochondrial calcium uniporter antagonist, was used at 20 and 50 μM concentrations and only under depolarization condition. Superoxide radical generation was detected by the chemiluminescence (CL) technique using a Mini Lumat LB 6506 luminometer (EG&G Berthold, Germany) as described previously (6, 13, 35). CL intensity was recorded at 15-s intervals 10 min after addition of lucigenin (a CL probe selective for superoxide radical) into tubes containing one slice in Hanks' buffer. Composition of the Hanks' buffer was as follows: 200 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* KH_2PO_4, 1 m*M* $CaCl_2$, 10 m*M* glucose, 15 m*M* $NaHCO_3$, 20 m*M* HEPES, pH 7.2. Lucigenin was added to the tubes at a final concentration of 0.2 m*M*. Results were expressed as relative light units per milligram of tissue. The significance of differences between experimental groups was estimated by one-way analysis of variance with Tukey–Kramer multiple comparison post test.

Lactate dehydrogenase (LDH) activity was determined in the incubation medium of hippocampal and striatal slices. Five slices were incubated in each vial containing 3 ml of ACSF or dACSF under carbogen aeration for 45 min. LDH activities were measured using a commercial diagnostic kit (Biolabo S.A., France).

RESULTS

Figure 1 shows the effects of rotenone and antimycin A on superoxide radical generation in hippocampal and striatal slices incubated in ACSF and dACSF. We have observed a significant increase in superoxide radical generation when rotenone and antimycin A were added to the incubation medium (*i.e.*, ACSF) of hippocampal and striatal slices. The increase in superoxide radical generation was related to the concentration of ETC inhibitors. Rotenone (complex I inhibitor) was more effective than antimycin A (complex III inhibitor). Superoxide radical generation was increased with depolarization (dACSF) in both hippocampal and striatal slices. Addition of lower concentrations (1 μM) of rotenone and antimycin A to dACSF-incubated hippocampal and striatal slices increased superoxide radical generation. However, higher concentrations (10 μM) of rotenone and antimycin A did not enhance superoxide radical generation further and even

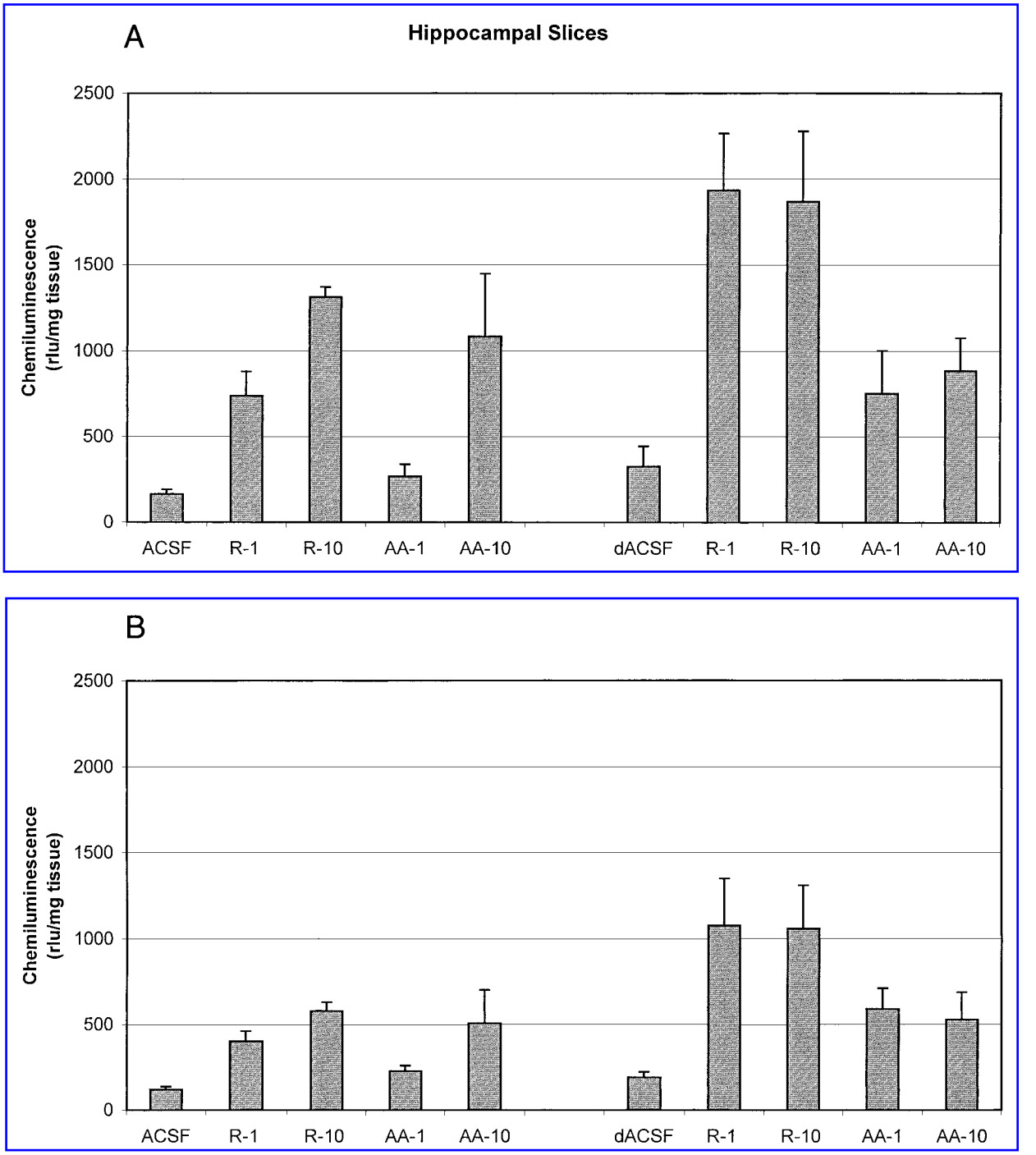

FIG. 1. Effects of rotenone (R) and antimycin A (AA) on superoxide radical generation in (A) hippocampal and (B) striatal slices incubated in ACSF or dACSF. Superoxide radical generation was increased significantly upon application of blockers in a concentration-dependent way in ACSF-incubated slices, whereas it was increased only at lower concentrations upon application of blockers in dACSF-incubated slices. Details of experimental conditions are described in Materials and Methods. rlu/mg of tissue, relative light units per milligram of tissue.

inhibited it. Rotenone was found to generate more superoxide radical under depolarization conditions, and hippocampus was found to be more active in superoxide radical production compared with striatum.

We have used RuR, a mitochondrial calcium uniporter antagonist, under depolarization conditions at two different concentrations (20 and 50 μM). RuR caused almost complete suppression of superoxide radical

Table 1. Effect of RuR on Superoxide Radical Generation in Hippocampal and Striatal Slices under Depolarization Conditions

	*dACSF (rlu/mg)**	*dACSF + 20 μM RuR (rlu/mg)*	*dACSF + 50 μM RuR (rlu/mg)*
Hippocampal slices	276.7 ± 108.3	33.1 ± 12.6	24.6 ± 3.3
Striatal slices	189.5 ± 75.0	20.7 ± 11.8	12.3 ± 3.3

*rlu/mg = relative light units per milligram of tissue.

generation in hippocampal and striatal slices (Table 1).

LDH activity was measured in the incubation medium of slices ($n = 7$) to detect neuronal damage caused by depolarization. LDH activity of hippocampal slices in ACSF was 167 ± 54 IU/L. This was increased to 294 ± 73 IU/L under depolarization conditions (dACSF). LDH activity of striatal slices (ACSF) was 37 ± 5 IU/L and was increased to 157 ± 50 IU/L under depolarization conditions (dACSF).

DISCUSSION

Brain mitochondria play an important role in the etiology of neurodegenerative diseases. In a previous study, it was suggested that mitochondria play a major role as the major buffering compartment against glutamate-induced elevation of intracellular calcium ions in cortical neurons (33). The mitochondrial electrochemical proton gradient controls calcium sequestration, ATP generation, and superoxide radical formation by the respiratory chain. Collapse of the mitochondrial membrane potential was induced by stimulation of NMDA receptors by glutamate and accumulation of calcium ions (17, 26). In addition, intense glutamate stimulation was found to block ETC complexes (I, II/III, and IV) in cultured retinal cells (23). Inhibition of mitochondrial function with specific ETC inhibitors blocked electron transfer and proton extrusion mechanisms, thereby decreasing mitochondrial membrane potential and ATP synthesis (26). Proton translocating complex (complex I) is the largest, containing >40 subunits, and least understood complex of the respiratory chain. Complex I inhibitor rotenone was suggested to interrupt the electron transfer between the iron-sulfur cluster N2 and ubiquinone (27).

In this study, we have shown that addition of rotenone and antimycin A to ACSF-incubated hippocampal and striatal slices increased superoxide radical generation in a concentration-related manner. Hippocampal slices were found to be more active than striatal slices in producing superoxide radical, which might be due to a difference in the mitochondrial number of two structures. Inhibition of complex I by rotenone resulted in more superoxide radical production compared with the inhibition of complex III by antimycin A. In a previous study, heart mitochondria were found to be more sensitive and responsive to antimycin A, whereas brain mitochondria were found to be more sensitive to rotenone (14). Chronic systemic exposure of rats to rotenone was shown to reproduce the anatomical, neurochemical, behavioral, and neuropathological features of Parkinson's disease by causing nigrostriatal dopaminergic degeneration (4).

Novelli *et al.* reported that impaired energy metabolism could result in excitotoxicity (18). Excitatory amino acids were shown to induce profound energy consumption and increased lactate levels in striatal neurons (24). Altered brain energy metabolism and reduction in glucose utilization was detected in Alzheimer's disease patients (16). The mechanism for this was suggested to be reduction of ATP, which is important in the maintenance of neuronal resting membrane potential. Resting membrane potential is dependent on potassium ion concentration; thus, as the extracellular potassium ion concentration increases, membrane becomes depolarized. Upon depolarization and in the presence of glutamate, agonist-operated and voltage-sensitive channels are activated and accumulation of intracellular cal-

cium initiates a series of reactions leading to neuronal death (7, 30).

A relation between oxidative stress, glutamate, and neurodegenerative disorders has been shown previously (8). Excessive excitatory amino acid accumulation may underlie Alzheimer's and Huntington's diseases (11, 36). It was reported that, in bullfrog sympathetic neurons, mitochondrial calcium overload could be generated only with 50 m*M* K^+ stimulation, but not with lower concentrations (10).

Mitochondria are involved in calcium buffering and represent a major source of endogenous reactive oxygen species. It was shown that isolated brain mitochondria can produce free radicals when exposed to elevated concentrations of calcium and sodium ions (9). This was correlated with neurodegeneration. We have previously showed that superoxide radical generation was increased following depolarization of neurons with 50 m*M* K^+ (35). Superoxide radical was also generated during the process of neuronal death in cell culture models (22). Our results show that LDH activity was also increased with depolarization, indicating neuronal injury conditions.

Incubation of depolarized hippocampal and striatal slices with RuR, a mitochondrial calcium uniporter antagonist, effectively blocks superoxide radical generation. RuR was shown to penetrate cells (3). It has several effects on calcium-related functions in biological preparations: it binds to membranal calcium-related sites and interacts with sialic acid residues, thus interfering with calcium-dependent release of neurotransmitters in hippocampal slices (29, 34). The intensive suppressive effect of RuR on superoxide radical generation might be due to a combined effect.

We have observed that, under both control and depolarization conditions, rotenone (complex I inhibitor) was more effective in superoxide production than antimycin A (complex III inhibitor). Depolarization in combination with a low concentration of mitochondrial ETC inhibitors (1 μM) generated a maximum amount of superoxide, because superoxide production did not increase further with a high concentration of ETC inhibitors (10 μM) in both hippocampal and striatal slices. This indicates that complexes I and III are completely blocked under these conditions. Our results also showed that hippocampal slices were more sensitive to ETC inhibitors compared with striatal slices. Thus, different regions of the brain seem to differ in their capacity to generate free radicals and vulnerability to oxidative stress conditions. This difference should be considered in developing therapeutic modalities against oxidative stress-related disorders and neurodegeneration.

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ABBREVIATIONS

ACSF, artificial cerebrospinal fluid; CL, chemiluminescence; dACSF, depolarizing artificial cerebrospinal fluid; ETC, electron transport chain; LDH, lactate dehydrogenase; RuR, ruthenium red.

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