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Effects of Arachidonic Acid on Respiratory Activities in Isolated Brain Mitochondria

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The present investigation was designed to examine the effects of free arachidonic acid (20:4), in concentrations relevant to cerebral ischemia, on brain mitochondrial respiratory activities and the reversibility of these effects. Incubation of brain mitochondria with 20:4 caused a dose-dependent increase in substrate-supported (state 4) respiration (i.e., uncoupling) and a concomitant inhibition of substrate-, phosphate-, and ADP-supported (state 3) or dinitrophenol-supported state (3u) respiration. The temperature dependence of the 20:4 effects on mitochondrial respiration was also studied. It was found that the uncoupling and the respiratory inhibition were at least as pronounced at physiological temperatures as at room temperature. Arrhenius plots of the state 3 respiratory rates suggested that 20:4 did not cause a significant change in membrane fluidity. Addition of bovine serum albumin to the reaction medium following preincubation with 20:4 reversed the uncoupling effect but only partly reversed the inhibition of state 3 respiration. The results suggest 1) that 20:4 may inhibit mitochondrial ATP production during conditions of incomplete cerebral ischemia and 2) that 20:4 may limit the postischemic recovery of mitochondrial function.

Key words: mitochondrial function, recovery, bovine serum albumin, cerebral ischemia

INTRODUCTION

During cerebral ischemia, there is a marked increase in free fatty acids (FFA) in the tissue [Bazán, 1970; Marion and Wolfe, 1979; Yoshida et al, 1980, 1983, 1985; Rehncrona et al, 1982; Bhakoo et al, 1984a; Blomqvist et al, 1985; Yasuda et al, 1985]. Arachidonic acid (20:4), one of the FFA showing the largest increase [Bazán, 1970; Yoshida et al, 1980; Rehncrona et al, 1982; Shiu et al, 1983; Bhakoo et al, 1984a; Ikeda et al, 1986], is receiving increasing attention as a potential mediator of ischemic cell injury [for recent reviews, see Siesjö, 1981; Wolfe, 1982; Chan and Fishman, 1985]. It has been shown that polyunsaturated fatty acids (PUFA), particularly arachidonic acid (20:4), are potent inducers of brain edema when incubated with cortical slices [Chan and Fishman, 1978] and when injected into the brain [Chan et al, 1983; Black and Hoff, 1985]. This unique feature of 20:4 among the FFA released during ischemia may be related to the fact that the brain has the ability to produce both cyclooxygenase products [Gaudet and Levine, 1980; Bhakoo et al, 1984b; Dembin'ska-Kiec' et al, 1984] and lipoxygenase products [Lindgren et al, 1984; Moskowitz et al, 1984] with 20:4 as substrate. This suggestion is supported by the observation that leukotrienes (i.e., LTB_4, LTC_4, LTE_4) have the ability to induce vasogenic edema when injected into the brain [Black, 1984; Black and Hoff, 1985]. Furthermore, oxidation of 20:4 and the 20:4 metabolites is considered to involve highly reactive radical species [Chan and Fishman, 1980; Wolfe, 1982]. Such oxygen radicals have been shown to cause pial arteriolar dilatation [Kontos and Hess, 1983; Rosenblum, 1983] and brain mitochondrial dysfunction [Hillered and Ernster, 1983; Braughler et al, 1985].

The inability of mitochondria to restore normal function during recirculation has been implicated as an important factor in the pathogenesis of irreversible ischemic cell injury [Mergner et al, 1977; Mela, 1979; Rehncrona et al, 1979; Farber et al, 1982; Fiskum, 1983; Hillered et al, 1984b]. FFA, especially the medium- and long-chain saturated ones, are known to have adverse effects on mitochondrial functions [reviewed by Wojtczak, 1976]. However, there is little information on the

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pathophysiological effects of long-chain PUFA on brain mitochondrial function. The aim of the present investigation was to examine the effects of 20:4, in concentrations relevant to ischemic conditions, on brain mitochondrial respiratory activities. The reversibility and the possible mechanisms of the observed effects were also studied.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200–300 g) with unlimited access to rat pellets and tap water were used. Ficoll 400 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and the bacterial proteinase Nagase from Enzyme Development Corp. (New York, NY). The following substances were obtained from Sigma (St. Louis, MO): adenosine 5′-diphosphate (ADP), arachidonic acid (sodium salt), bovine serum albumin (BSA; fatty acid-free), ethyleneglycol-bis-(β-amino-ethyl-ether)-N,N′-tetraacetic acid (EGTA), 2,4-dinitrophenol (DNP), D-mannitol, L-glutamic and L-malic acid, and oligomycin.

Isolation of Mitochondria

Brain mitochondria were isolated by a modification of the method of Clark and Nicklas [1970] as described previously [Hillered and Ernster, 1983]. In summary, the tissue was disintegrated manually and enzymatically in a mannitol-sucrose-EGTA medium containing BSA. The crude mitochondrial pellet was purified using a Ficoll density gradient. This method consistently yields mitochondrial preparations with respiratory control ratios between 7 and 10. The purity of the preparations (0.17–0.23 nmol cytochrome $a+a_3$/mg protein) is similar to that reported in other studies employing the same technique [Clark and Nicklas, 1970; Rehncrona et al, 1979; Hillered and Ernster, 1983; Hillered et al, 1984b; see also Hillered, 1986].

Mitochondrial Respiratory Activity

Respiratory activity of the mitochondrial suspension was measured as the oxygen consumption rate recorded by a Clarke-type oxygen electrode (usually operated at 23°C) in a closed and magnetically stirred glass chamber (Eschweiler & Co., Kiel, Federal Republic of Germany). The reaction mixture consisted of 0.54 ml 150 mM KCl and 10 mM K-phosphate buffer (pH 7.4; referred to below as KCl-Pi medium) containing 1 mM EGTA (pH 7.4), to which 20 μl (0.8 mg protein) of the mitochondrial suspension was added. State 3 respiration, as defined by Chance and Williams [1955], was initiated by the addition of 10 μl 0.5 M (final concentration 9 mM) malate + 0.5 M (9 mM) glutamate as NAD-linked substrates, and 2–3 μl 0.1 M (0.4–0.6 mM) ADP (see Fig. 1A). Alternatively, 18 mM succinate (in the presence of 3.7 μM rotenone) was used as FAD-linked substrate (Fig. 1D). State 4 respiration was measured as the rate of oxygen uptake following the consumption of the added ADP and the subsequent addition of 1 μl 1.25 mg/ml (2.3 μg/ml) oligomycin (see Fig. 1A). Respiratory control ratio (RCR) was the ratio of the state 3 to the state 4 rates of respiration. Uncoupler-stimulated respiration (state 3u) was induced by the addition of 2 μl 10

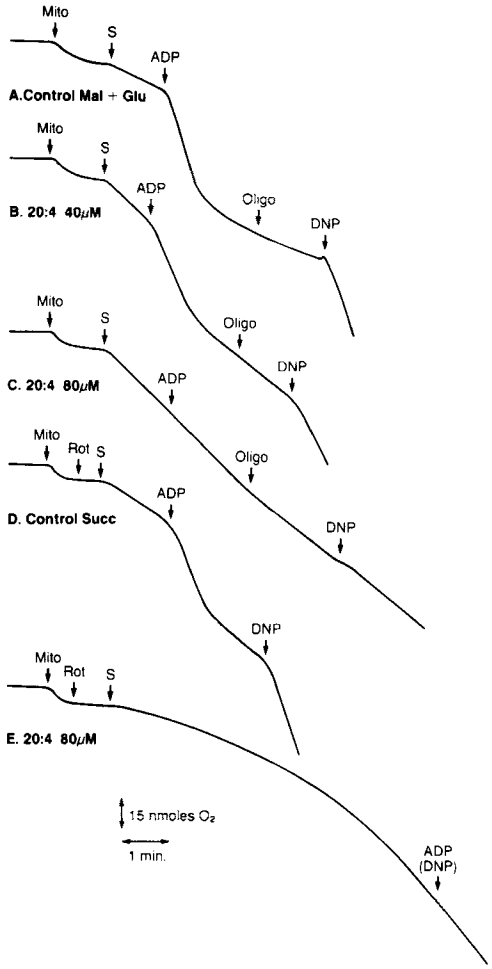

Fig. 1. Examples of recordings from the O_2 electrode illustrating O_2 consumption rates of brain mitochondria incubated with the indicated concentrations of arachidonic acid at 23°C. Mitochondria (Mito; 1.5 mg protein/ml) were added to 0.54 ml of the reaction medium. State 3 respiration was initiated by the addition of 9 mM malate + 9 mM glutamate (traces **A–C**) as substrate (S) and 0.4–0.6 mM ADP (in the presence of phosphate). State 4 respiration was the rate following consumption of the ADP and the subsequent addition of 2.3 μg/ml oligomycin (Oligo) and state 3u the rate after the addition of 37 μM DNP. Traces **D** and **E** illustrate the effect of arachidonic acid on the respiratory rates with 18 mM succinate (in the presence of 3.7 μM rotenone; Rot) as substrate. DNP, no effect was seen after the addition of ADP or DNP.

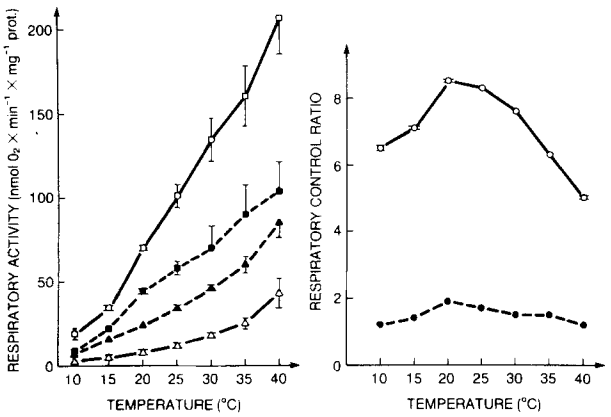

Fig. 2. Effect of temperature on respiratory activities **(left)** and RCR **(right)** of untreated brain mitochondria (open symbols) and mitochondria incubated with 80 μM (53.3 nmol/mg protein) arachidonic acid (filled symbols). Triangles represent substrate supported respiration, and squares represent substrate-, phosphate-, and ADP-supported respiration. Values are the means of two to four experiments ± SEM. Other conditions were as in Figure 1.

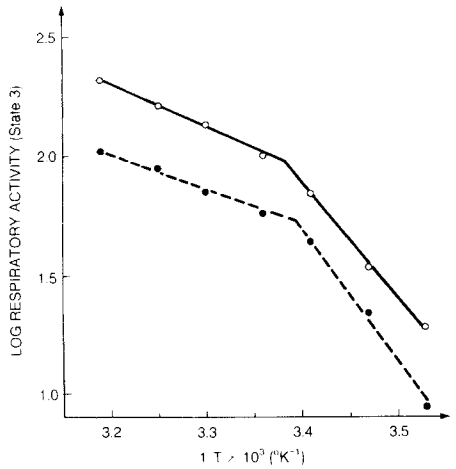

Fig. 3. Arrhenius plots of the mitochondrial respiratory rates in the presence of substrate, phosphate, and ADP (state 3) of untreated mitochondria (open circles) and mitochondria incubated with 80 μM (53.3 nmol/mg protein) arachidonic acid (filled circles). The data from Figure 2 were used for the plots.

mM (37 μM) DNP in the presence of substrate (see Fig. 1A). In experiments with BSA in the reaction medium, 56 μM DNP was required to induce maximal stimulation of respiration. Rates were expressed as nmoles O_2 consumed/min/mg mitochondrial protein. The protein concentration of the reaction mixture was measured by the method of Lowry et al [1951].

Incubation Procedure

Mitochondrial respiration was measured following incubation for 1 min with 10–80 μM 20:4 (sodium arachidonate; final concentration 6.7–53.3 nmol/mg protein). The influence of temperature on the respiratory activities of normal and 20:4-treated mitochondria was studied in separate experiments to ascertain that the effects of 20:4 at room temperature were relevant to physiological temperature conditions. In addition, to reveal a possible effect of 20:4 on membrane fluidity, Arrhenius plots were drawn using the temperature data on state 3 respiration.

Reversibility of the 20:4-induced effect was tested by measuring respiration of mitochondria incubated for 1 min with BSA following preincubation with 20:4 as a function of the concentration of 20:4. The concentration of BSA used in these experiments was 1 mg/ml, yielding a 20:4/BSA molar ratio of about 5 or lower, which has been found effective in preventing 20:4-induced swelling in cortical slices [Chan et al, 1980].

Statistics

Statistical analysis was performed using the Student's t test for the comparison between two means and Duncan's multiple range test (Statistical Analysis System) for the comparison between multiple means. Differences with a P value below 0.05 were considered statistically significant. The lines in Figure 3 were drawn according to best fit using the least-square method (linear regression).

RESULTS

Figure 1 illustrates typical examples of recordings from the O_2 electrode in mitochondrial suspensions incubated with arachidonic acid (20:4). There was a dose-dependent increase in state 4 respiration and a concomitant inhibition of the maximal respiratory capacity (states 3 and 3u respiration) with malate and glutamate as substrate (traces A–C). With succinate, in the presence of rotenone, as substrate there was also an uncoupling effect (increase in state 4 respiration) and an inhibition of the maximal respiratory capacity. In addition, with the FAD-linked substrate, there was a lag time of 5–6 min before a constant rate of respiration was obtained (traces D and E). This is compatible with a 20:4-induced inhibition of substrate transport being the rate-limiting step under these conditions.

Table I is a summary of the calculated respiratory rates and RCR values following incubation with 10–80 μM 20:4 (6.7–53.3 nmol/mg protein) with malate plus glutamate as substrate. The effect of 20:4 was similar with other NAD-linked substrates (e.g., malate-pyruvate) and when EGTA was omitted from the reaction

TABLE I. Effect of Arachidonic Acid on Respiratory Activities and Respiratory Control Ratios of Isolated Brain Mitochondria*

Concentration μM	nmol/mg	State 4 Respiration[a]	(Percent of control)	State 3 respiration[a]	(Percent of control)	RCR	(Percent of control)
0	0	10.6 ± 1.4	(100)	74.8 ± 4.5	(100)	7.1 ± 0.8	(100)
10	6.7	13.8 ± 0.9	(130)	66.0 ± 2.8	(88)	4.8 ± 0.5[b]	(68)
20	13.3	15.5 ± 3.0	(146)	61.7 ± 6.4[b]	(82)	4.0 ± 0.9[b]	(56)
40	26.7	20.3 ± 3.6[b]	(192)	47.7 ± 10.2[b,c]	(64)	2.4 ± 0.5[b,c]	(34)
80	53.3	24.9 ± 6.5[b,c]	(235)	29.9 ± 6.0[b,d]	(40)	1.2 ± 0.1[b,d]	(17)

*Respiratory rates in state 4 (substrate-supported) and state 3 (substrate, phosphate, and ADP-supported) respiration and were measured in isolated brain mitochondria following incubation with (20:4). Values are the means ± SD of three to five experiments.
[a]nmol O_2 min/mg mitochondrial protein with malate and glutamate as substrates.
Statistically significant difference compared to [b] control or [c] 20 μM or [d]or 40 μM experiments. Conditions were as in Figure 1.

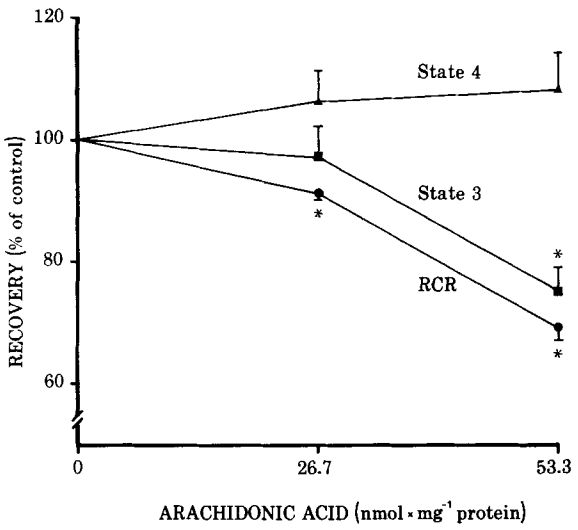

Fig. 4. Recovery of substrate-supported respiration (triangles); substrate-, phosphate-, and ADP-supported respiration (squares); and RCR (circles) of isolated brain mitochondria incubated with BSA following preincubation with various concentrations of 20:4. Values are the means of four experiments ± SD.* Statistically significant differences as compared to control.

medium. Addition of NaCl alone, in concentrations corresponding to those added as sodium arachidonate, did not influence the respiratory rates (data not shown). At concentrations of 20:4 above 80 μM, the uncoupled respiration was progressively inhibited, and, at 1.1 mM (720 nmol/mg), it was almost abolished, the respiratory rate being 7.2 ± 1.2 nmol O_2/min/mg protein (mean ± SD of three experiments).

There was no measurable increase in O_2 consumption upon the addition of 20:4 alone (in the absence of other substrates) to the mitochondrial suspension. This is in agreement with the known inability (or very limited ability) of brain mitochondria to oxidize exogenous FFA [Marquis amd Fritz, 1965; Horrocks and Harder, 1983].

Temperature Studies

The effect of temperature on the respiratory rates and RCR values of normal and 20:4-treated brain mitochondria is illustrated in Figure 2. In untreated mitochondria, as expected, there was an increase in state 3 and state 4 respiration with increasing temperature. The highest RCR values were obtained at 20–25°C (Fig. 2, right). The effect of 20:4 (80 μM, 53.3 nmol/mg protein) on the respiratory rates (Fig. 2, left) was at least as pronounced at 35–40°C as at 20–25°C.

The values of state 3 respiration shown in Figure 2 (left) were used for the Arrhenius plots shown in Figure 3. The plots for treated and untreated mitochondria were similarly shaped, with breakpoints at 22°C and 23°C, respectively.

Reversibility of 20:4-Induced Mitochondrial Dysfunction

Figure 4 shows the effect of incubation with BSA, following preincubation with 20:4, on the respiratory rates and RCR values. The effect of 20:4 on state 4 respiration was reversible at all concentrations tested. However, the 20:4-induced effect on state 3 respiration and RCR was clearly irreversible at 80 μM (53.3 nmol/mg protein). There was a statistically significant, irreversible effect on RCR also at 40 μM (26.7 nmol/mg). Recovery of mitochondrial respiration following preincubation with 20:4 was not improved when the BSA concentration was increased to 2 mg/ml. BSA alone did not influence mitochondrial respiration (data not shown).

DISCUSSION

The present study reports the in vitro effects of 20:4 on individual respiratory rates of isolated brain mitochondria and the reversibility of these effects. The rationale behind the study was the growing interest in the possible role of 20:4 (and its metabolites) in ischemic cell damage and edema in the brain (for references, see Introduction).

At the end of a 30 min period of cerebral ischemia in rodents, the concentration of free 20:4 rose to 0.4–0.6 μmol/g brain tissue homogenate [Yoshida et al, 1980; Rehncrona et al, 1982; Shiu et al, 1983; Ikeda et al, 1986], i.e., an average concentration of 400–600 μM. The distribution of the free 20:4 between the extra- and intracellular compartments is not known. Therefore, it is difficult to determine the true concentration of free 20:4 to which the mitochondria are exposed in vivo. In the present study, we have estimated that the mitochondria may face a concentration of 20:4 of 27–60 nmol/mg mitochondrial protein in vivo during ischemia, based on an average mitochondrial protein content of 10–15 mg/g brain homogenate [Abood, 1969; McIlwain and Bachelard, 1985]. This concentration range corresponds to 40–80 μM in our in vitro system. The results suggest that 20:4 in this concentration range has profound effects on brain mitochondrial function. Already at 13.3 nmol/mg (Table I), there was a significant inhibition of state 3 respiration (and of RCR). At 26.7 nmol/mg, RCR was reduced to 34% of control. This effect was due to a combination of increased proton permeability (increase in state 4 respiration) and an inhibition of phosphorylating (state 3) respiration. At 53.3 nmol/mg 20:4, state 3 was reduced to 40% of control, and respiratory control was virtually abolished. The finding that 20:4 caused a parallel inhibition of states 3 and 3u respiration with NAD-linked substrates (Fig. 1A–C) suggests that an inhibition of the respiratory chain rather than an inhibition of the ADP/ATP translocator or the ATP synthase was rate-limiting for the inhibited state 3 respiratory rate. With FAD-linked substrate, the situation appeared to be more complex. In this case, an inhibition of substrate transport seemed to be rate-limiting (Fig. 1D and E).

The present results are qualitatively in agreement with those of Kuwashima et al [1976], who found that 20:4 inhibited brain mitochondrial ATP production (measured indirectly as glucose-6-phosphate formation in a medium containing mitochondria, glucose, and hexokinase) and O_2 consumption (measured manometrically) in the presence of succinate as substrate. However, in that study, no effect on oxygen uptake was recorded below 80 μM 20:4. This discrepancy probably reflects the differences in methodology. Nevertheless, our preliminary data agree with the findings of Kuwashima et al [1976] that monounsaturated or saturated fatty acids at equal concentration have little or no effect on O_2 consumption of mitochondria. Since increased tissue levels of 20:4 remain in the brain during the initial recirculation period [Yoshida et al, 1980; Rehncrona et al, 1982; Ikeda et al, 1986], the present results suggest that 20:4 delays the recovery of mitochondrial function following transient cerebral ischemia. Furthermore, under conditions of restricted O_2 supply (i.e., incomplete ischemia) 20:4 may, in conjunction with profound intracellular acidosis [Hillered et al, 1984a; Smith et al, 1986] have a detrimental effect on mitochondrial ATP-producing capacity. On the other hand, BSA reversed the uncoupling effect but only partly reversed the inhibition of maximal respiration, suggesting that 20:4 may have produced mitochondrial damage.

Effect of Temperature

The temperature studies summarized in Figure 2 indicate that the effect of 20:4 on states 3 and 4 respiration (and RCR) was at least as pronounced at physiological temperature as at room temperature. Notably, the highest RCR values were obtained at 20–25°C in both treated and untreated mitochondria. The Arrhenius plots of the state 3 respiratory rates of untreated and 20:4-treated mitochondria were similarly shaped (Fig. 3), with breakpoints between 22°C and 23°C. This is within the temperature range (12–25°C) reported for mitochondria from other organs [Smith, 1973; Rottenberg et al, 1980]. The results suggest that no significant change in membrane fluidity was induced by 20:4.

Reversibility of 20:4-Induced Mitochondrial Dysfunction

Incubation of mitochondria with 80 μM (53.3 nmol/mg protein) 20:4 caused an irreversible reduction of state 3 respiration and RCR (Fig. 4). The recovery of RCR also was incomplete at 40 μM (26.7 nmol/mg protein). The protonofore effect of 20:4 (increase in state 4 respiration) recovered to a level not significantly different from control at all concentrations tested. These results suggest that 20:4 is involved in the irreversible inhibition of state 3 respiration reported to occur following 30 min of cerebral ischemia in the rat [Hillered et al, 1984b]. Further experimental work is required to determine the mechanism of the effect of 20:4 on state 3 respiration and whether 20:4 metabolites (see Introduction) are involved.

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