Journal of Neuroscience Research 20:451–456 (1988)

Role of Arachidonic Acid and Other Free Fatty Acids in Mitochondrial Dysfunction in Brain Ischemia

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The aim of the present investigation was to evaluate the possible role of arachidonic acid and other free fatty acids in ischemia-induced mitochondrial dysfunction. Respiratory activities were measured in mitochondria isolated from rat brains subjected to 15–30 min of decapitation ischemia. Addition of bovine serum albumin (BSA) to the mitochondria, isolated in BSA-free media, abolished an ischemia-induced increase in substrate-stimulated (state 4) respiration but only partly reversed a marked inhibition of substrate-, phosphate-, and ADP-stimulated (state 3) respiration caused by the ischemia. Individual free fatty acids were measured in aliquots of the same mitochondrial preparations before and after treatment with BSA. There was a significant increase in arachidonic (20:4), stearic (18:0), palmitic (16:0), and docosahexaenoic (22:6) acid during ischemia. BSA treatment removed all 20:4 and reduced the amount of 18:0 and 16:0, but had no significant effect on 22:6. The main conclusions were 1) that 20:4, 18:0, and 16.0 were responsible for the partial uncoupling (increase in state 4 respiration) of mitochondrial respiration during ischemia, 2) that the inhibition of state 3 respiration caused by ischemia could only partly be attributed to an effect of FFAs, and 3) that the ischemia-induced mitochondrial dysfunction was caused by a combination of factors including 20:4.

Key words: mitochondrial function, brain damage, bovine serum albumin, cerebral ischemia

INTRODUCTION

It is well established that global cerebral ischemia is associated with a marked increase in free fatty acids (FFAs) in the tissue [Bazán, 1970; Marion and Wolfe, 1979; Yoshida et al., 1980, 1983, 1985; Rehncrona et al., 1982; Bhakoo et al., 1984; Blomqvist et al., 1985; Yasuda et al., 1985]. According to several other studies global cerebral ischemia in the rat is associated with a reduced ATP-producing capacity of mitochondria isolated following the ischemia [for references see Hillered et al., 1984]. The mechanism of this type of mitochondrial dysfunction remains unclear although a number of hypotheses have been proposed [for reviews see Mela, 1979; Hillered, 1986] including the direct effect of FFAs liberated during the ischemia [Ozawa et al., 1966a; Lazarewicz et al., 1972; Kuwashima et al., 1976]. We have recently reported on profound adverse effects of arachidonic acid (20:4), in concentrations relevant to cerebral ischemia, on brain mitochondrial respiratory function in vitro [Hillered and Chan, 1988]. The reason for focusing on 20:4 in that study was the unique ability of this fatty acid to induce brain edema in vivo [Chan et al., 1983] and neuronal and astrocytic dysfunction in vitro [Yu et al., 1986; Chan et al., 1988]. The aim of the present study was to explore the possible role of 20:4 and other free fatty acids in mitochondrial dysfunction caused by cerebral ischemia.

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). 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-ethylether) N,N,-N′N′-tetraacetic acid (EGTA), 2,4-dinitrophenol (DNP), D-mannitol, L-glutamic and L-malic acid, and oligomycin. Solvents for the lipid analyses were of highest purity as manufactured

Received January 11, 1988; revised May 6, 1988; accepted May 9, 1988.

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This paper was presented in part at the 17th Annual Meeting of the Society for Neuroscience, New Orleans, November 16–21, 1987.

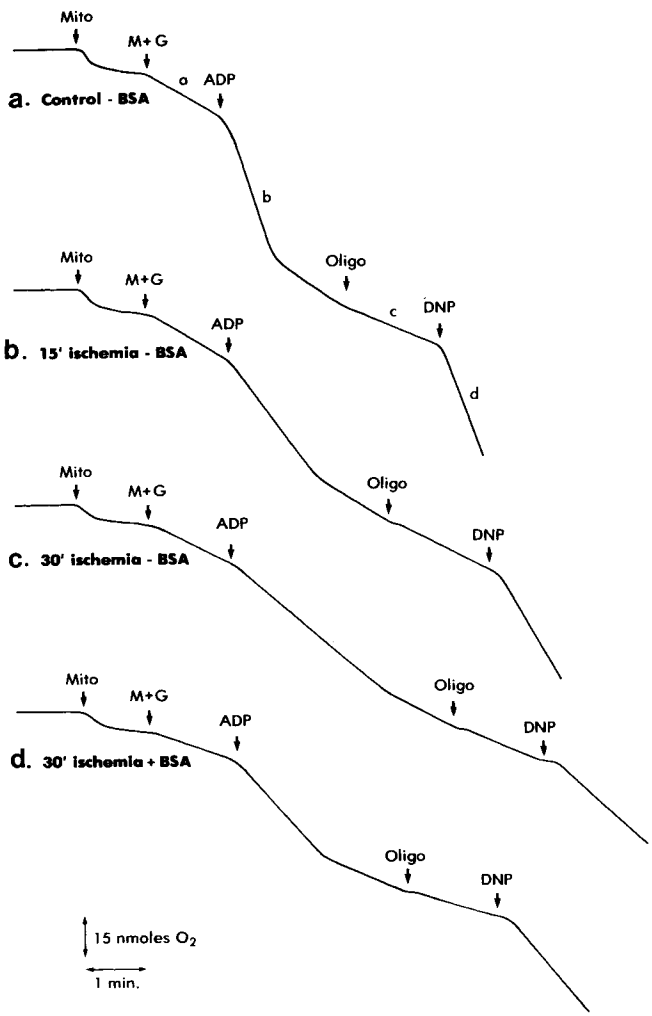

Fig. 1. Examples of recordings from the oxygen electrode illustrating oxygen consumption rates of mitochondria isolated (in the absence of bovine serum albumin, BSA) from rat brains subjected to post-decapitative ischemia. Respiration was measured without (−BSA) or with (+BSA) BSA in the reaction medium at 23°C. Mitochondria (Mito, 1.5 mg of protein/ml) were added to 0.54 ml of the reaction medium. State 3 (**b**) respiration was initiated by the addition of 9 mM malate + 9 mM glutamate (M + G) and by 0.04–0.06 mM ADP, state 4 (**c**) by 2.3 μg/ml oligomycin (Oligo) following consumption of the ADP, and state 3u (**d**) by 37-56 μM 2,4-dinitrophenol (DNP).

by Fisher Scientific Company (Pittsburgh, PA). Other chemicals were commercial products of highest available purity.

Isolation of Mitochondria

Ischemia was induced by decapitation of the rats (without anesthesia) by using a guillotine. The heads were kept in double surgical gloves, preinflated with argon, and put in a 37°C waterbath for 15 or 30 min. 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 (*without* BSA). The crude mitochondrial pellet was purified by using a Ficoll density gradient. This method consistently yields mitochondrial preparations with respiratory control ratios between 7 and 10. The maximal respiratory rates and the purity (0.17–0.23 nmol cytochrome a + a_3 per mg of protein) of the preparations are similar to those reported in other studies using the same technique [Clark and Nicklas, 1970; Rehncrona et al., 1979; Hillered and Ernster, 1983; Hillered et al., 1984].

Mitochondrial Respiratory Activity

Respiratory activity of the mitochondrial suspension was measured as the oxygen consumption rate recorded by a Clarke-type of oxygen electrode operated at 23°C in a closed and magnetically stirred glass chamber (Eschweiler & Co., Kiel, FRG). 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), with or without bovine serum albumin (BSA, 1 mg/ml), to which 20 μl (0.8 mg of 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 (9 mM) malate + 0.5 M (9 mM) glutamate and 2–3 μl 0.1 M (0.4–0.6 mM) ADP (rate (b) in Fig. 1A). State 4 respiration was the rate of oxygen uptake following consumption of the added ADP and the subsequent addition of 1 μl 1.25 mg/ml (2.3 μg/ml) oligomycin (rate (c) in 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 mM (37 μM) 2,4-dinitrophenol (DNP) in the presence of substrate (rate (d) in 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 of mitochondrial protein. Individual values were the means of two separate recordings from the same preparation. The protein concentration of the reaction mixture was measured by the method of Lowry et al. [1951]. The oxygen content of the reaction media was calibrated by the N-methylphenazonium methosulphate method described by Robinson and Cooper [1970]. There was no difference in oxygen content between KCl-Pi and KCl-Pi-BSA media.

Lipid Extraction and Quantitation of Free Fatty Acids

Individual FFAs were measured in mitochondria isolated following ischemia before and after the addition of BSA and rinsing with BSA-free medium. Separation of FFAs (and phospholipids) in lipid extracts from the mito-

TABLE I. Respiratory Activities and Respiratory Control Ratio of Brain Mitochondria Isolated From Rats Subjected to Decapitation Ischemia†

			Ischemia	
	BSA	Control	15 min	30 min
State 3	−	68.5 (2.1)	31.5 (1.2)[a]	25.5 (1.8)[a,b]
	+	70.1 (3.5)	39.6 (2.0)*	30.8 (1.9)*
State 3u	−	63.1 (1.1)	39.3 (2.3)[a]	28.3 (2.3)[a,b]
	+	63.2 (3.4)	46.2 (3.3)*	33.5 (2.4)*
State 4	−	10.2 (0.2)	11.9 (0.4)[a]	11.6 (0.4)[a]
	+	7.8 (0.3)*	7.9 (0.3)*	7.5 (0.2)*
RCR	−	6.7 (0.2)	2.6 (0.0)[a]	2.2 (0.2)[a,b]
	+	9.0 (0.2)*	5.0 (0.1)*	4.1 (0.3)*

†Respiratory rates (nmol $O_2 \times min^{-1} \times mg^{-1}$ protein) and respiratory control ratio (RCR) were measured before (−BSA) and after (+BSA) the addition of bovine serum albumin to the reaction medium. Values were the means of five preparations and SEM: a, indicating statistically significant difference from the corresponding control group; b, from the 15-min ischemia group (one-way analysis of variance, $P<.05$); and *, from the corresponding −BSA group (Student's t-test, $P<.05$). Other conditions were as in Figure 1.

chondrial membrane was based on the method of Horrocks and Sun [1972]; 20-μl samples (0.8–1.0 mg protein) were taken from the mitochondrial preparations and extracted with 4 ml chloroform:methanol (2:1, v/v). The lipid extracts were applied to 20 × 20 cm thin-layer chromatography plates precoated with silica gel H, 250 μl (Uniplate; Analtech, Newark, DE) and developed by two-dimensional chromatography with two solvent systems: 1) chloroform:methanol:15 M-amoniumhydroxide (130:55:10, by vol.), 2) chloroform:methanol:water:acetic acid:acetone (150:30:15:30:60, by vol.). The areas with FFAs were removed from the plates and subjected to methylation by the method of Abood et al. [1978]; 5 ml of 5% HCl in methanol was added to the removed silica gel containing FFAs in Pyrex culture tubes with screw caps and incubated at 100°C for 2 hr. The tubes were allowed to cool to room temperature and were spun for 10 min at 1,200g in a Sorvall tabletop centrifuge (GLC-1) to remove the silica particles before the addition of 10 ml of water to the supernatants. The incubation mixture was extracted with 4 ml of hexane three times. The upper hexane phases were pooled and dried down under N_2.

The fatty acid methyl esters were resuspended in isooctane (2,2,4-trimethylpentane) and 1 μl of the sample was injected into a gas chromatograph (Hewlett Packard 5830A) equipped with a 30-m-long Fused Silica Capillary Column (SP-2330; Supelco, Bellafonte, PA) with an internal diameter of 0.24 mm and a film thickness of 2 μm. The sample was entered by a cool on-column injection with helium as the carrier gas. The temperature program for the column was set at 90–200°C and the Flame Ionization Detector at 250°C. A known concentration of heptadecanoic acid (17:0) was added to the samples routinely (at the time of the first extraction) as an internal standard.

Statistics

Student's t-test was employed to test the statistical significance of differences between nontreated and BSA-treated samples. One-way analysis of variance (Statgraphics, STSC Inc, Rockville, MD) was used for the comparison of multiple means. Normally, differences with a P value $< .05$ were considered statistically significant.

RESULTS

Effect of Ischemia and BSA on Mitochondrial Function

Figure 1 shows examples of recordings from the oxygen electrode illustrating the effects of ischemia on the respiratory rates of mitochondria isolated (in the absence of BSA) from rat brains exposed to 0, 15, or 30 min of postdecapitative ischemia. There was a marked inhibition of state 3 and state 3u respiration during ischemia (trace A–C) as expected from our previous results [Hillered et al., 1984]. The parallel inhibition of state 3 and state 3u respiration suggests that ischemia affected the respiratory chain rather than the ATP synthase or the ATP/ADP translocator.

In Table I (−BSA values), which is a summary of the calculated respiratory rates and RCR values, it can be seen that in addition to the inhibition of state 3 and 3u respiration there was an increase in state 4 respiration during ischemia.

The effect of BSA on mitochondrial respiration following ischemia is shown in Figure 1 (trace C and D) and in Figure 2. Addition of BSA resulted in a significant improvement of state 3 and state 3u and a reversal of the effect on state 4 respiration. RCR, i.e., the ATP producing efficiency of the mitochondria, improved almost twofold

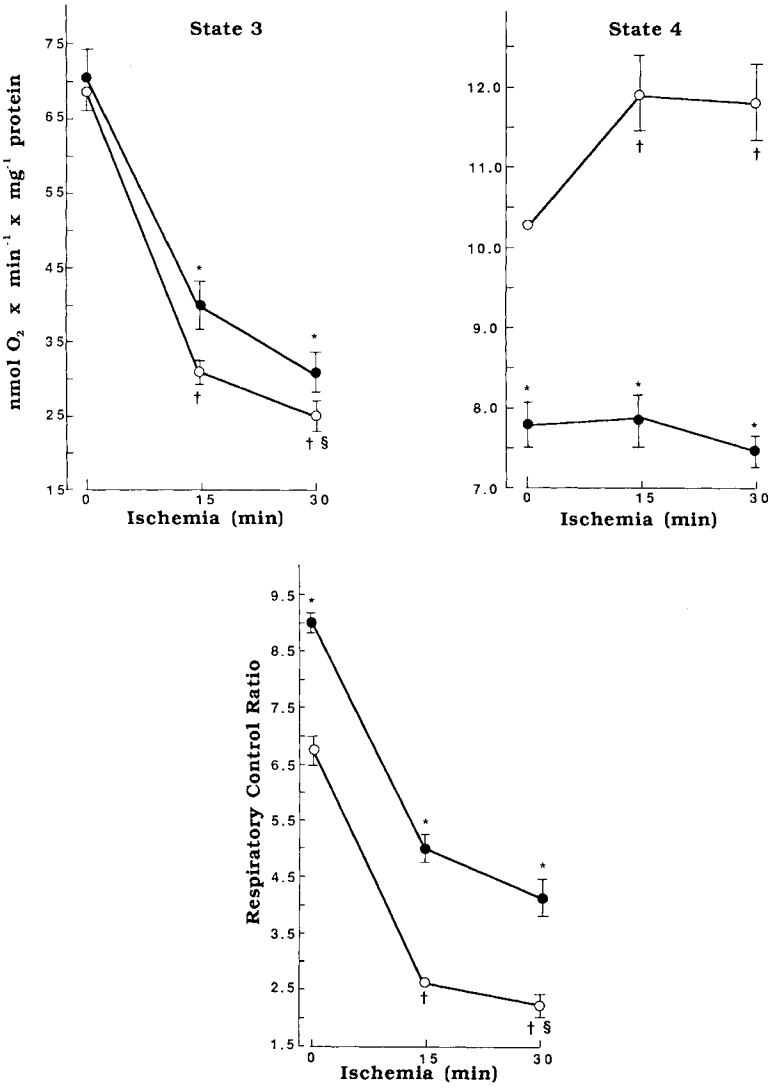

Fig. 2. Effect of bovine serum albumin (BSA) on respiratory rates and respiratory control ratio of rat brain mitochondria isolated (in the absence of BSA) following decapitation ischemia. Open circles, measurements performed before, and closed circles after the addition of BSA to the reaction medium. Statistically significant difference from control (†), from 15 min ischemia (§), and from the corresponding untreated group (*). Values are the means of five preparations and SEM. Conditions were as in Figure 1.

in the presence of BSA. In control preparations BSA had some effect on state 4 (and RCR) but not on state 3 and state 3u respiration.

Effect of Ischemia and BSA Treatment on Mitochondrial FFAs

The changes in the mitochondrial content of individual FFAs during ischemia are summarized in Figure 3 (open circles). In control preparations there were some FFAs present, mainly palmitic (16:0) and stearic (18:0) acid. The control levels of arachidonic (20:4) and docosahexaenoic (22:6) acid were low. During the 30-min ischemia period there was a 30 and 40% increase in 16:0 and 18:0, respectively and a 5.5- to 7.5-fold increase in 20:4. The concentration of 22:6, which was not detectable in control preparations, also increased somewhat during ischemia. The sum of all FFAs measured increased by 30%. Other FFAs measured, including 18:1, 18:2, 18:3, and 20:0, did not increase significantly during ischemia.

Figure 3 (closed circles) illustrates the effect of BSA treatment (addition of BSA, 1 mg/ml, and rinsing with BSA-free medium) on the mitochondrial content of those FFAs that accumulated during ischemia. BSA treatment removed all 20:4 and reduced the amount of 18:0 and 16:0

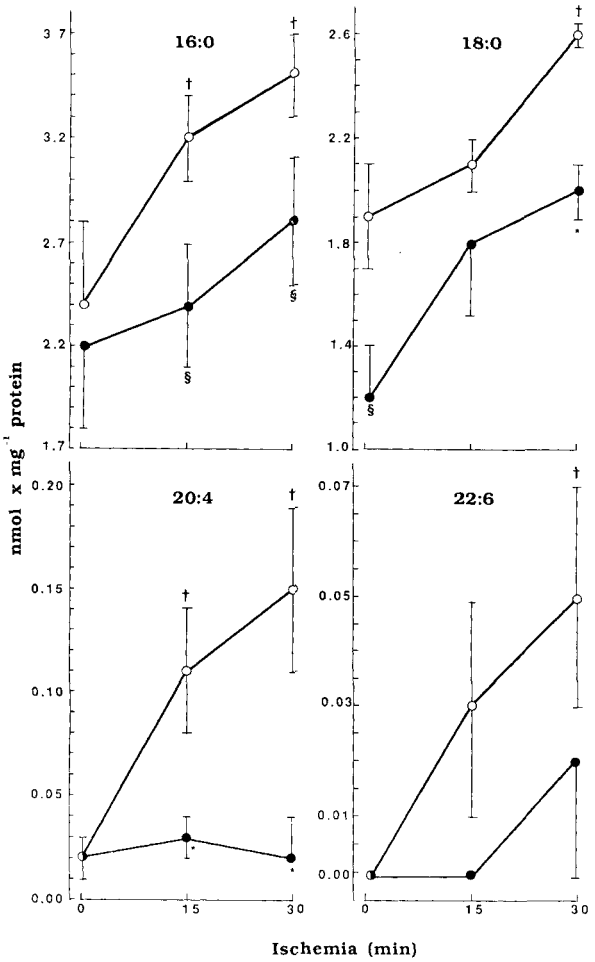

Fig. 3. Free fatty acid (FFA) content of mitochondria isolated from rat brains subjected to postdecapitative ischemia measured before (open circles) and after (filled circles) treatment with BSA. Values were the means of four preparations and SEM. † indicating statistically significant difference from control (one way analysis of variance, $P<.05$), Student's t-test was used for the comparison between untreated and BSA-treated groups (* $P<.05$, § $P<0.10$).

(but not 22:6) significantly. Notably, there was also a reduction of 18:0 in control mitochondria (exposed to 12–20 sec of ischemia).

DISCUSSION

The aim of the present study was to test the hypothesis that 20:4 or other FFAs, liberated from membrane-bound phospholipids, are responsible for the reduction of ATP-producing capacity of mitochondria isolated following cerebral ischemia. We focused on respiratory rates (and RCR) as measures of mitochondrial function since these parameters are more sensitive to ischemia than, for example, P/O ratios [for references see Hillered, 1986]. The time points 15 and 30 min were chosen because previous results have suggested that the point of no return for mitochondrial function following ischemia is within this time range [Hillered et al., 1984]. The special interest in 20:4 was derived from its well-known potency as an edema-inducing agent in vivo and as an inducer of mitochondrial and cellular dysfunction in vitro (for references see Introduction).

Ischemia was associated with a marked inhibition of state 3 (phosphorylating) and state 3u (uncoupler stimulated) respiration and an increase in state 4 respiration (partial uncoupling) of mitochondria isolated in the absence of BSA (Fig. 1). Incubation of the isolated mitochondria with BSA resulted in a reversal of the uncoupling effect and an amelioration of the effect on state 3 and state 3u respiration (Table I, Fig. 2). The combination of these effects was an almost twofold increase in RCR, i.e., the ATP-producing efficiency, of the mitochondria. A similar effect of BSA on state 4 respiration was observed by Lazarewicz et al. [1972] following 5 min of postdecapitative ischemia. No effect was observed on state 3, however, probably owing to the short duration of ischemia.

During ischemia there was an increase in the mitochondrial content of palmitic (16:0), stearic (18:0), arachidonic (20:4), and docosahexaenoic (22:6) acid (Fig. 3). An increase in FFAs in brain mitochondria from guinea pigs has been reported following 5-min postdecapitative ischemia [Strosznajder, 1980]. The occurrence of some FFAs in control preparations (Fig. 3 of the present study) probably reflects the time required to remove the brains following decapitation (i.e., 12–20 sec of ischemia) since the onset of FFA liberation during ischemia is known to be very rapid [Bazán 1970; Majewska et al., 1977; Yoshida et al., 1980]. Furthermore, it has been demonstrated that decapitation and subcellular isolation procedures alone cause a liberation of FFAs, probably from membrane phospholipids [Deshmukh and Radin, 1985]. BSA treatment removed all 20:4 and some of the 16:0 and 18:0 in ischemic mitochondria and reduced the content of 18:0 in control preparations (Fig. 3).

The results suggest, first, that 20:4, 18:0, and 16:0 were responsible for the ischemia-induced increase in state 4 respiration. This is in agreement with previous studies where state 4 respiration was unchanged during ischemia in mitochondria isolated in the presence of BSA [Ginsberg et al., 1977; Rehncrona et al., 1979; Hillered et al., 1984] while Ozawa et al. [1966b] found an increase in state 4 in mitochondria isolated in a BSA-free medium. Taken together, these results indicate that FFAs caused at least a partial uncoupling of mitochondrial respiration in vivo during ischemia. Second, the inhibition of state 3 and state 3u respiration induced by ischemia could only partly be attributed to an effect of FFAs. Although BSA treatment did not remove all of the FFAs accumulated during ischemia it seems more likely that factors other than FFAs were also

involved. This is supported by the finding that an inhibition of state 3 respiration did occur in mitochondria isolated following 15–30 min of ischemia in BSA-containing media [Rehncrona et al., 1979; Hillered et al., 1984]. Third, the increase in state 4 respiration of untreated compared to BSA-treated control mitochondria (Fig. 2) was mediated mainly by stearic acid (18:0). Although our recent results [Hillered and Chan, 1988] suggest that 20:4 may inhibit mitochondrial respiratory function during ischemic conditions in vivo, the present study suggests that the dysfunction of mitochondria isolated following ischemia is caused by a combination of factors including 20:4.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Susan Longar and Dr. Sylvia F. Chen for excellent technical aid and to Dr. Robert A. Fishman for constructive criticism. This study was supported by N.I.H. grants NS-14543, NS-25372, the Swedish Medical Research Council (Project No. 7888), the Swedish Society of Medicine, the Trygg-Hansa Foundation, Pharmacia AB, and Volvo of America Corporation.

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