Effects of Incomplete Ischemia and Subsequent Recirculation on Free Palmitate, Stearate, Oleate and Arachidonate Levels in Lumbar and Cervical Spinal Cord of Rabbit

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Abstract. The effect of severe incomplete ischemia, induced by abdominal aorta ligation for 40 minutes, and subsequent recirculation for one and four days on accumulation of free fatty acids was studied in the lumbar and cervical part of rabbit spinal cord. Changes in free fatty acid levels were determined separately in gracile fascicle (Fg), dorsal part (Dp, without Fg) and ventral part (Vp) of both spinal cord regions. In lumbar spinal cord increases in free fatty acid levels, especially that of arachidonate, were observed in Fg, Dp and Vp at the end of the ischemic period. During recirculation all values were similar to nonischemic controls. In cervical spinal cord a slight increase in free fatty acid levels was found in Fg after four days of recirculation, and in Dp arachidonate and stearate levels were most markedly elevated after one day of recirculation. No changes at any interval were found in Vp of cervical spinal cord. The present results indicate that the experimental insult induced typical ischemic injury to spinal cord tissue demonstrated by fatty acid liberation from membrane lipids. This injury may affect neurotransmission and other processes and free fatty acids themselves impair tissue metabolism (inhibition of oxidative phosphorylation, edema precipitation, synthesis of eicosanoids) and thus restrict the possibilities to enhance recovery in the recirculation period.

Key words: Severe incomplete ischemia — Spinal cord — Free fatty acids

Introduction

Degradation of neural membrane structure during experimentally induced ischemia begins with lipolysis of membrane lipids (Gercken and Bräuning 1973; Yatsu 1975; Porcellati et al. 1978; Marion and Wolfe 1979; De Medio et al. 1980) accompanied by the liberation of free fatty acids (FFA). Since first

reported by Bazán (1970) and Bazán et al. (1971) a considerable interest has focussed on FFA production and the fate of FFA during ischemia and recirculation. Polyenoic fatty acids, and notably arachidonate (20 : 4), are preferentially released and they are known to be metabolized to prostaglandins and hydroxy fatty acids (Galli et al. 1978) by the cyclooxygenase pathway via endoperoxides, together with thromboxane and prostacyclin synthesis (Wolfe 1982). Another pathway of 20 : 4 metabolism is leukotriene formation by lipoxygenase activity which has recently been demonstrated by Moskowitz et al. (1984) in brain tissue after ischemia and recirculation. The oxygenated biologically active derivatives of arachidonate, defined as eicosanoids, contribute to the pathogenesis of brain edema, postischemic hypoperfusion and also may trigger peroxidative changes of lipid membrane constituents. A liberation of fatty acids from brain tissue lipids has been comprehensively investigated under conditions of decapitative complete ischemia by Nemoto et al. (1982a, b), Dorman et al. (1983) and Shiu et al. (1983). Results obtained from rat tissues consistently indicate that levels of FFA in brain tissue are increased during ischemia, the elevation being proportional to the duration of the insult. Porcellati et al. (1982) induced transient ischemia in gerbils and found a marked production of FFA, especially arachidonate and docosahexaenoate (22 : 6), together with the production of diacylglycerol (DG), rich in arachidonate, probably derived from phospholipid degradation. Rehncrona et al. (1982) studied complete and severe incomplete ischemia followed by 30 minute recirculation and observed similar increases in FFA in both ischemic models that were essentially reversed during recirculation period.

Despite extensive studies of FFA levels during ischemia, and following recirculation in brain, no data on the effects of ischemia on spinal cord FFA levels are apparently available. The present paper is directed to the analysis of effects of severe incomplete ischemia of lumbar spinal cord and subsequent prolonged recirculation together with responses in the cervical spinal cord remote from the ischemic region, with respect to FFA levels in gracile fascicle (Fg), the dorsal part (Dp, without Fg) and the ventral part (Vp) of both spinal cord regions.

Materials and Methods

Rabbits of either sex, weighing 3.0—3.5 kg, had free access to food and water until commencement of the experiment. The following experimental groups were examined: control and sham control animals, animals with severe incomplete ischemia of lumbar spinal cord and animals with ischemia and subsequent recirculation for one and four days, respectively. Each group consisted of 5 animals. Ischemia was induced by occlusion of the abdominal aorta, tightly below the origin of the renal arteries, for 40 minutes. The surgery and tissue removal were performed under thiopental anesthesia

(30 mg/kg). At the end of each particular experimental period with the respective groups lumbar and cervical spinal cords were quickly removed and fixed in liquid nitrogen. Separation of Fg, Dp and Vp was performed on a cooled glass plate, after stripping the envelopes, with instruments cooled in liquid nitrogen. The spinal cord was cut in short segments kept frozen and Fg was removed using a blade at the temperature just sufficient to allow the excision. Each segment was again dipped in liquid nitrogen, and Dp and Vp were separated by vertical cut made between ventral and dorsal horns via central channel. All three parts so obtained were kept in liquid nitrogen. Frozen tissue was weighed and homogenized in 0.25 mol . l^{-1} ice cold sucrose (5 : 1, v : w) for protein determination (Lowry et al. 1951). This step was required for other simultaneous analyses in these experiments. Tissue lipids were then extracted from the homogenate according to Bligh and Dyer (1959) with the additional two step extraction of pellets with 10 volumes of chloroform-methanol (2 : 1, v : v). The two chloroform phases and that from the initial step were pooled, filtered and evaporated in rotary vacuum evaporator. Lipid extracts were redissolved in chloroform-methanol (2 : 1, v : v) and subsequently they were washed with sodium citrate (0.4 mol . l^{-1}), methanol-sodium citrate (0.4 mol . l^{-1}, 2 : 3, v : v), KCl (0.74 mol . l^{-1}) and pure theoretical upper phase (water-methanol-chloroform, 47 : 48 : 3, v : v : v). The chloroform phase was filtered, evaporated to dryness and redissolved in 5 ml of chloroform-methanol (2 : 1, v . v). Samples were stored at −30°C under N_2. The procedure provided extracts convenient for all lipid assays performed in these experiments.

For FFA levels (palmitate 16 : 0, stearate 18 : 0, oleate 18 : 1 and arachidonate 20 : 4) TLC of neutral lipids was performed on Silicagel CH (LACHEMA, Brno) plates (15 × 15 cm, 250 µm) washed with chloroform — methanol (1 : 1, v : v) and activated at 110°C for 30 minutes. Extracts were applied in spots 1 cm from the bottom and the plates were eluted with petrolether-ether-acetic acid (90 : 10 : 1, v : v : v) in preequilibrated tanks lined with filter paper at 4°C to the height 0.5 cm from the top. Lipids were visualised by brief exposition to iodine vapours, spotted and after evaporation of iodine FFA spots were scrapped in test tubes and extracted three times with chloroform-methanol (2 : 1, v : v). FFA extract was evaporated in vacuo and redissolved in 3 ml of benzene-methanol-5 mol . l^{-1} H_2SO_4 (123 : 61 : 5.1, v : v : v) for transesterification. Methyl esters were prepared by gentle shaking of the mixture in stoppered glass flasks at 60°C for 2 hours (Shiu and Nemoto 1981). The reaction was stopped by the addition of 1 ml of water, the mixture was cooled and methyl esters were extracted twice with 3 ml of heptane. Extracts were evaporated and reconstituted in 50 µl of heptane for GC. To follow the efficiency of transesterification pentadecanoic acid was used as an internal standard.

Chloroform, methanol, ether, petrolether and benzene (LACHEMA, Brno) of analytical grade were redistilled before use. Heptane purchased from Ubichem Ltd. (United Kingdon) was of analytical grade as well as all other chemicals used for analysis.

FFA methyl esters were chromatographed on FRACTOVAP 2200 (Carlo Erba, Milano) using a minigrator (Spectra Physics, USA) integrator. A glass column (1.2 m) packed with Chromosorb P AW DMCS with 10 % DEGS and 1 % H_3PO_4, 100—120 mesh, was used with N_2 as a carrier gas (2.2 kp . cm^{-2}, H_2 flow rate 0.05 l . min^{-1}, temperature gradient 195—235°C, 5°C . min^{-1}). Standards (SIGMA, USA) were run parallel to each sample series analysis.

Results were statistically evaluated by Student's *t*-test and given as means ± S.E.M.

Results

In lumbar spinal cord 40 minutes of severe incomplete ischemia resulted in a significant accumulation of all four fatty acids (16 : 0, 18 : 0, 18 : 1, 20 : 4) assayed. The most marked increase was found in gracile fascicle (Fig. 1), where an

approximately 4-fold increase in levels of 16:0 and 18:1 ($p < 0.001$), a 7-fold increase in 18:0 ($p < 0.001$) and about 9-fold increase in 20:4 ($p < 0.001$) was recorded at the end of ischemic period. The ischemic insult resulted in an accumulation of individual FFA also in Dp (Fig. 2, $p < 0.01$ for 16:0, 18:1, and $p < 0.001$ for 18:0 and 20:4) and in Vp (Fig. 3, $p < 0.01$ for 16:0 and 18:1, $p < 0.001$ for 18:0 and 20:4). Less marked elevations of 16:0 and 18:1 were found and increase in 18:0 and 20:4 levels prevailed as compared to control values.

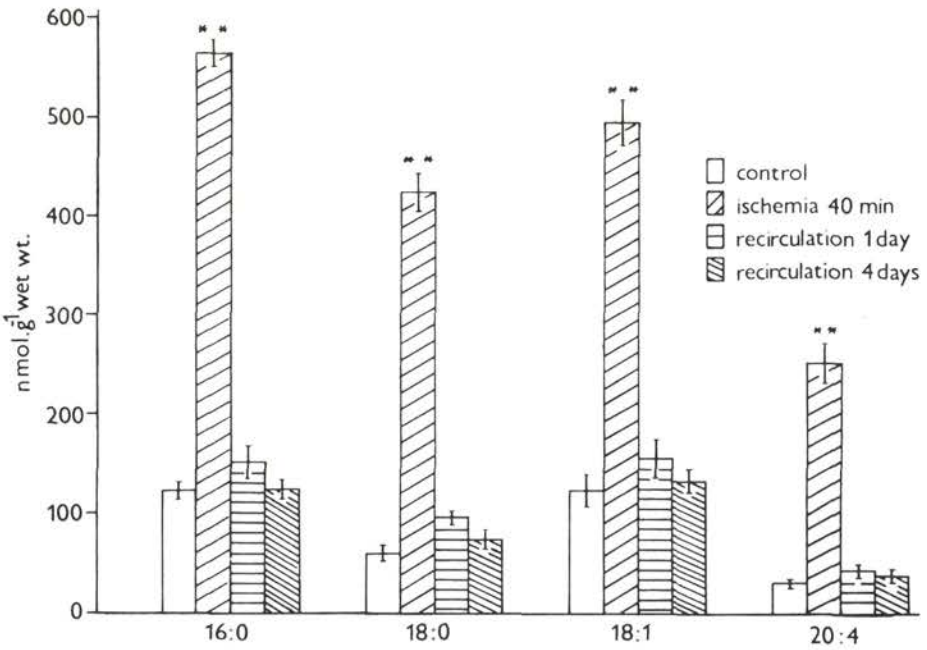

Fig. 1. Free palmitate (16:0), stearate (18:0), oleate (18:1) and arachidonate (20:4) levels in fasciculus gracilis of lumbar spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

The ratio of polyunsaturated 20:4 to saturated FFA was increased in Fg from 0.16 ± 0.02 to 0.25 ± 0.01, in Dp from 0.16 ± 0.03 to 0.43 ± 0.04, in Vp from 0.12 ± 0.02 to 0.39 ± 0.05 (values given as ratio means $\pm$ S.E.M).

After one and four days of recirculation, levels of FFA were similar to those recorded in nonischemic controls in all three parts of lumbar spinal cord.

In cervical spinal cord, levels of FFA were within the range of their control values at the end of 40 minute ischemia in all three parts studied.

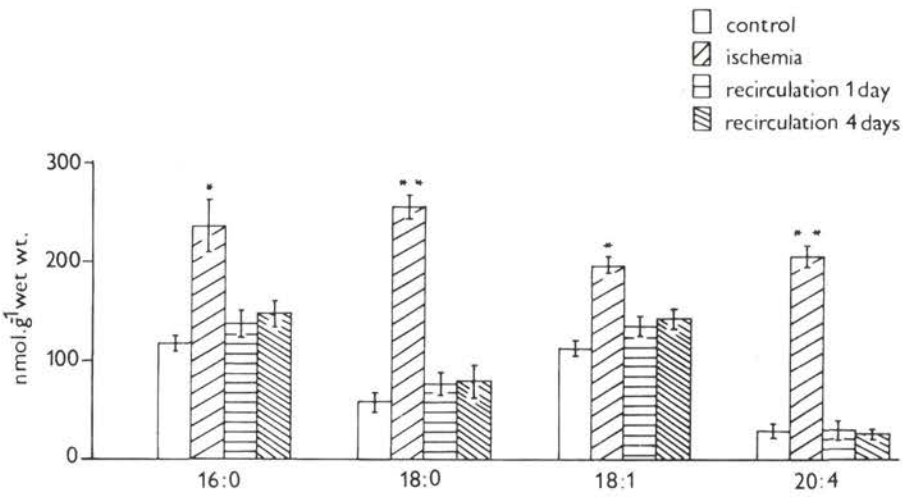

Fig. 2. Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate (20 : 4) levels in dorsal part (without fasciculus gracilis) of lumbar spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

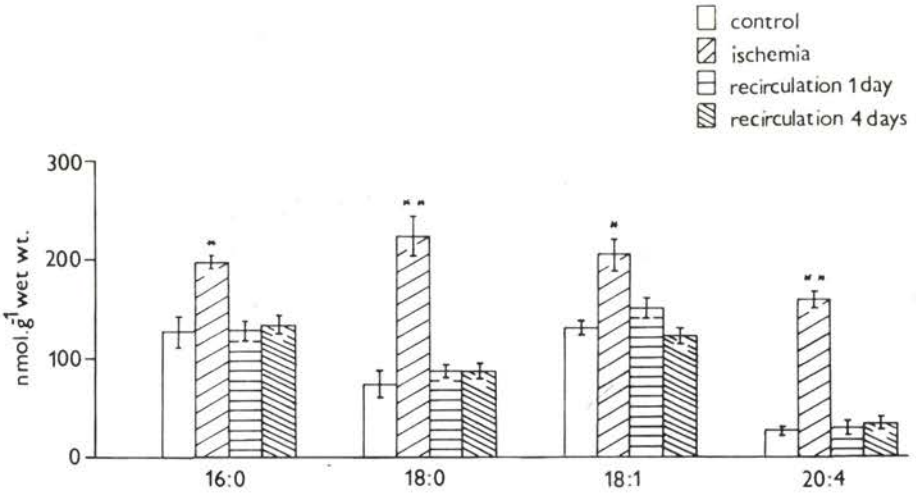

Fig. 3. Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate (20 : 4) levels in ventral part of lumbar spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

In gracile fascicle (Fig. 4) moderately increased levels of FFA, especially 18 : 0 and 20 : 4 ($p < 0.05$), were found after four days of recirculation, with the ratio of polyunsaturated to saturated fatty acids increased from 0.17 ± 0.01 to 0.26 ± 0.02. In Dp (Fig. 5) slight elevations of 16 : 0 and 18 : 1 ($p < 0.05$) were found after 24 hours of recirculation and levels of 18 : 0 and 20 : 4 were about two fold higher ($p < 0.01$) than control values. The ratio of polyunsaturated to saturated fatty acids was increased from 0.17 ± 0.01 to 0.26 ± 0.02. No statistically significant difference was found in Dp after four days of recirculation.

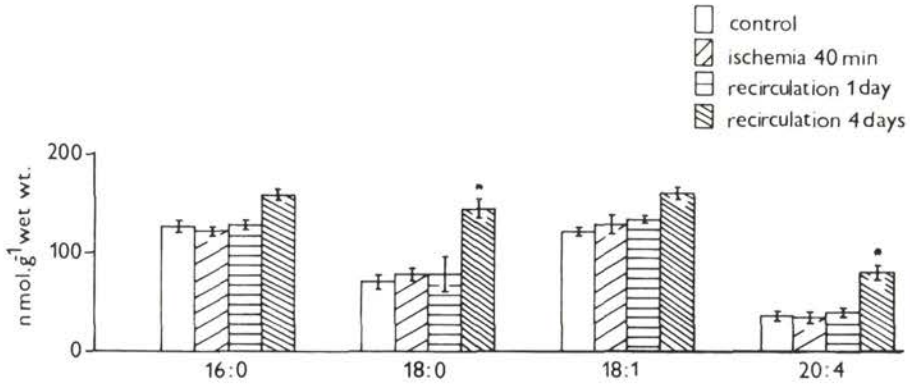

Fig. 4. Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate (20 : 4) levels in fasciculus gracilis of cervical spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

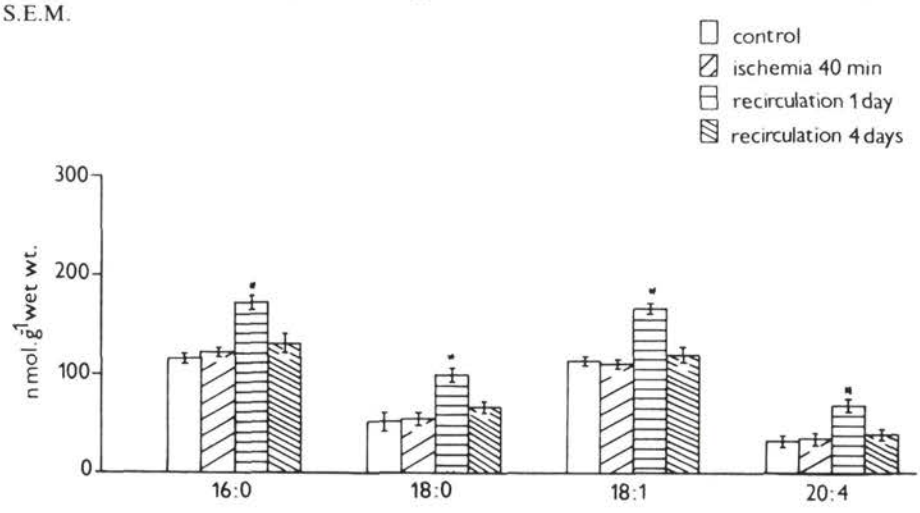

Fig. 5. Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate (20 : 4) levels in dorsal part (without fasciculus gracilis) of cervical spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

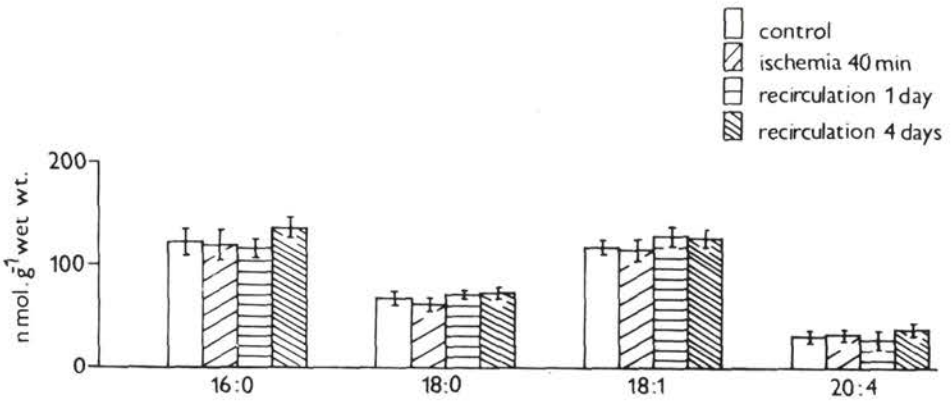

Fig. 6. Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate (20 : 4) levels in ventral part of cervical spinal cord after ischemia, induced by abdominal aorta ligation below the origin of the renal arteries, and subsequent recirculation. Values given as means ($n = 5$) $\pm$ S.E.M.

In Vp (Fig. 6) no accumulation of FFA was recorded.

Values of FFA levels in sham operated controls were within the range of those found in intact controls; hence values obtained from both of these groups are referred to as "controls" and are given in Table 1 and Table 2.

Table 1. Lumbar spinal cord of control animals: Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate concentrations in fasciculus gracilis (Fg), dorsal part (Dp, without Fg) and ventral part (Vp).

	16 : 0	18 : 0	18 : 1	20 : 4
Fg	121 ± 10	59 ± 7	123 ± 14	29 ± 4
Dp	117 ± 8	57 ± 9	113 ± 7	28 ± 9
Vp	125 ± 15	73 ± 5	130 ± 10	24 ± 3

Data are reported as nmol . g^{-1} wet tissue weight as means ± S.E.M.

Table 2. Cervical spinal cord of control animals: Free palmitate (16 : 0), stearate (18 : 0), oleate (18 : 1) and arachidonate concentrations in fasciculus gracilis (Fg), dorsal part (Dp, without Fg) and ventral part (Vp).

	16 : 0	18 : 0	18 : 1	20 : 4
Fg	126 ± 6	70 ± 8	121 ± 4	35 ± 4
Dp	116 ± 5	50 ± 15	112 ± 5	31 ± 5
Vp	122 ± 10	69 ± 3	119 ± 7	30 ± 3

Data are reported as nmol . g^{-1} wet tissue weight as means ± S.E.M.

Discussion

In present experiments severe incomplete ischemia of rabbit spinal cord was used as a model for the study of FFA changes in various parts of the spinal cord. Occlusion of a abdominal aorta below the origin of renal arteries for 40 minutes resulted in the development of clinical symptoms manifested as paraplegia at about 6 hours after surgery.

No data on control values of individual FFA in rabbit spinal cord were in the literature found with which to compare present values. These values are, however, within the range of control values of FFA reported by other authors for brain (Nemoto et al. 1982a, b; Porcellati et al. 1982; Rehncrona et al. 1982; Dorman et al. 1983; Shiu et al. 1983).

Conditions of restricted blood supply to spinal cord are accompanied by a depletion of ATP pool in cells and by an increase in NADH concentration indicating the disturbance of the tissue redox state (Chavko et al. 1978; Chavko and Danielisová 1980). In the brain these events were found to result in disturbed Ca^{2+} homeostasis, i.e. influx of Ca^{2+} into cells (Harris et al. 1981) and impaired mitochondrial sequestration of Ca^{2+} (Siesjő and Wieloch 1983). Disturbed Ca^{2+} homeostasis has been recently documented by electron microscopic studies (Simon et al. 1984). Accumulation of intracellular Ca^{2+} initiates anaerobic enzymatic processes, i.e. activation of phospholipases, especially Ca^{2+}-dependent phospholipase A_2 (Horrocks and Chun Fu 1978; Edgar et al. 1980), resulting in deacylation of phospholipids. This is a turnover cycle producing increased FFA levels. Another such cycle is the action of Ca^{2+}- dependent phospholipase C, inosine phosphoglyceride specific (Hawthorne and Pickard 1979), that involves catabolism of phosphatidyl inositol to diacyl glycerol (DG) and inositol phosphate. DG is deacylated by DG lipase. Reacylation of phospholipids and phosphatidyl inositol resynthesis are ATP-dependent processes blocked due to depletion of ATP pool. Another pathway, proposed by Goracci et al. (1981) and documented by Porcellati et al. (1982) and Dorman et al. (1983), for FFA release is reversal of the choline and ethanolamine phosphotransferase reaction, resulting from a decrease in ATP concentration, accompanied by increased production of DG that is readily hydrolyzed to FFA and glycerol by diacyl glycerol and monoacyl glycerol lipases (Cabot and Gatt 1976).

These processes occur both in complete and incomplete ischemia and are responsible for FFA accumulation during ischemia. The present results are in agreement with the above findings as well as the observations of other authors (Nemoto et al. 1982a, b; Porcellati et al. 1982; Rehncrona et al. 1982; Dorman et al. 1983; Shiu et al. 1983) in various ischemic models in brain, since FFA levels, and notably level of arachidonate, is markedly elevated in lumbar spinal

cord at the end of an ischemic insult. An increased ratio between arachidonate and saturated fatty acids may indicate an activation of phospholipase A_2 during ischemia as proposed by Bazán (1976). The increase in this FFA ratio in Dp and Vp was higher than in Fg in agreement with the reported predominant occurrence of phospholipase A_2 in mitochondria (Woelk and Porcellati 1973). On the other hand, the presence of higher absolute values of FFA concentrations in Fg than in Dp and Vp at the end of an ischemic period may be attributed to markedly greater ATP depletion in white matter occurring prior to gray matter under conditions of incomplete ischemia (Welsh et al. 1978). The extent of FFA liberation exhibited a difference to that observed in brain (e.g. Rehncrona et al. 1982). This difference could be attributed to diverse severity of ischemia, a differential vulnerability of the brain and spinal cord. Furthermore, effect of thiopental anesthesia must also be considered.

In severe incomplete ischemia, with trickling blood supply preserved, and during subsequent recirculation the situation is even more complex as postischemic cell damage appears to be a function of both events. FFA, which are released due to lipolysis during ischemia under conditions whereby some oxygen supply remains, enter, during the insult and also during subsequent recirculation, oxygen dependent metabolic reactions i.e. cyclooxygenase (Galli et al. 1978; Wolfe 1982) and lipoxygenase pathway (Spagnuolo et al. 1979; Moskowitz et al. 1984). In the first pathway polyenoic FFA, notably arachidonate, are metabolized to prostaglandins PGD_2, PGE_2, PGF_2, PGI_2, thromboxanes and prostacyclin. Synthesis of prostaglandins and the imbalance between thromboxane and prostacyclin synthesis appear to promote plasma leakage, blood vessel constriction and platelet aggregation, resulting in delayed hypoperfusion after initial postischemic hyperemia. Polyenoic FFA themselves may induce edema in nervous tissue (Chan and Fishman 1978) and leucotriene synthesis by lipoxygenase activity contributes to its formation; leucotrienes contribute also to changes in postischemic blood flow reduction. Moskowitz et al. (1984) demonstrated increased leucotriene levels even 24 hours following 15 minutes of cerebral ischemia. Endoperoxides produced in both pathways (PGG_2, PGH_2, 12-HPETE), possessing a free radical character, may mediate selfinactivation of prostacyclin synthetase and may also be considered in connection with peroxidative changes of membrane lipids, thus leading to additional cell damage. Increased FFA levels observed in the present experiments provide a substrate for both of the above pathways.

The occurrence of these processes in ischemic lumbar spinal cord and their consequences are manifested also in ultrastructural changes (Badonič 1980; Cirbusová et al. 1982). During recirculation, released FFA are metabolized and their accumulation is reversed as demonstrated after one and four days of

recirculation. In this period additional lipolysis due to the partial secondary ischemic insult (delayed hypoperfusion), as well as cascade character of FFA metabolizing pathways, cannot be excluded.

Changes in FFA levels found in cervical spinal cord in Fg and Dp are difficult to interprete and they will be the subject of further study. Morphometric analysis, however, of synapse at the termination of Fg in dogs showed that incompletete ischemia of spinal ganglion cells in the lumbosacral region was manifested by local edema of pericarya and the corresponding part of the projecting system was affected at the site of its synaptic level with neurons in the gracile nucleus. This remote reaction occurring 2 days after ligation was very marked and led to an almost 100 % increase in the area of synapses of ascending fibres of Fg projecting from the lumbosacral region (Gavelová et al. 1982). In the studied region degenerative changes of a pale type appeared associated with synaptic edema. This may indicate the presence of metabolic events similar to those in the ischemic region. Moreover, the possibility cannot be excluded that Ca^{2+}-dependent lipolysis, partially blocked reacylation with impaired phospholipid synthesis due to decreased ATP concentration, Ca^{2+}-dependent proteolysis and excessive acidosis (Rehncrona et al. 1980) may induce damage to the molecular structures maintaining axoplasmic transport. These events would result in maturation of final postischemic damage along the ascending spinal cord tracts together with terminal degeneration of affected axons. In contrast to lumbar spinal cord increased FFA levels in cervical spinal cord were found after longer time intervals (one and four days). Neurocytes projecting their axons to cervical spinal cord (Fg) are found in the ischemic region (spinal ganglia cells). Due to the considerable distance eventual changes in cervical spinal cord can be expected to occur after longer periods of recirculation. Finally, one must note recent neuroanatomical data supporting the present observations of increased FFA levels in cervical spinal cord (Maršala and Mechírová 1984). These authors, using the ischemic model proposed by Zivin and De Girolami (1980), described the ischemic degeneration of four ascending rabbit spinal cord tracts, the gracile fascicle, the spinoolivary and spinoreticular tracts, as well as propriospinal projections from the lumbosacral ischemic region terminating in the cervical part of the spinal cord as the result of a 40 minute ligation of the abdominal aorta just caudal to the left renal artery followed by 2, 3 and 4 days of survival. All studied tracts achieved a full degree of degeneration at the end of the third postoperative day, a period that is substantially shorter than the time needed for clearly developed degeneration induced by surgical lesion; it was considered that this Nauta-positivity and faster development of the anterograde

degeneration is partly due to increased FFA levels together with a higher rate of axoplasmic flow in the corresponding ascending tracts.

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