Ischemia-reperfusion of human skeletal muscle during aortoiliac surgery: effects of acetylcarnitine

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Summary. Our previous study on human skeletal muscle undergoing ischemia and reperfusion has revealed that granulocytes, which infiltrate the muscle tissue in large numbers, play an important role in mediating fibre injuries by producing superoxide anion (O_2^-) which is responsible for membrane lipid peroxidation. In the current study, five patients undergoing aortic reconstructive surgery were given acetyl-carnitine (2 mg/kg i.v. plus 1 mg/kg/min for 30 min) prior to the induction of ischemia. Muscle biopsies and blood samples were examined: a) after anaesthesia; b) at the end of ischemia; and c) 30 min after reperfusion, with the aim of elucidating whether acetyl-carnitine could prevent the infiltration and/or the activation of granulocytes and eventually skeletal muscle injuries. During ischemia and reperfusion complement activation recruited numerous granulocytes into the muscle tissue, but, contrary to the untreated samples, the ability for O_2^--generation of these cells remained at low levels and was comparable to that of ischemia even when molecular O_2 was reintroduced to the tissue. Accordingly, the morphological changes of the postischemic muscle fibers were substantially reduced when compared to the untreated samples; in fact, the mitochondrial swelling was only moderate and the intramitochondrial dense bodies were small and scarce. The current findings support a positive role of acetyl-carnitine in ameliorating the ischemia-reperfusion (I-R)-induced damage of human skeletal muscle.

Key words: Skeletal muscle, Ischemia-reperfusion syndrome, Acetyl-carnitine, Granulocytes

Introduction

There is considerable evidence that skeletal muscle undergoes biochemical and morphological changes

during ischemia-reperfusion (I-R) (Andersson et al., 1979; Kloner et al., 1979; Sjostrom et al., 1982; Harris et al., 1986; Ferrari et al., 1988). Although ischemia by itself may produce irreversible damage, most of the injuries occur after reoxygenation of the tissue, when O_2 is available for generation of the reactive oxygen metabolites, which, in turn, are responsible for membrane lipid peroxidation (Bulkley, 1987). Recently, it has been suggested that granulocytes, which infiltrate, in large numbers, the ischemic reperfused muscle represent an important local source of O_2 free radicals (Engler et al., 1986; Korthuis et al., 1988; Loewe et al., 1988; Romson et al., 1988; Smith et al., 1989).

There are several studies which reveal that L-carnitine and its derivatives -known to be essential cofactors of fatty acid oxidation- are capable of reducing the I-R-induced injuries of the skeletal muscle and myocardium (Goa and Brogden, 1987). Accordingly, it has been shown that L-carnitine reduces superoxide production by circulating granulocytes draining the ischemic and reperfused human lower limbs (Novelli et al., 1990). Based on these observations, a study on human skeletal muscle was carried out with the aim of elucidating whether administration of acetyl-carnitine prevents the infiltration and/or activation of granulocytes and eventually reduces the morphological changes of the muscle tissue undergoing I-R.

Acetyl-carnitine was chosen because recent studies indicated that this derivative of L-carnitine is preferentially taken up by muscle tissue during ischemia (Barlett et al., 1989; Hiatt et al., 1989).

Materials and methods

Fourteen patients, all males, aged 50-72 years, bearing an aortic aneurysm underwent aortic reconstructive surgery. None of them was diabetic or had clinical or angiographic signs of chronic ischemia of legs.

After a premedication with atropine (0.01 mg/kg) and meperidine (1 mg/kg) anaesthesia with thiopental sodium (4 mg/kg) and atracurium besylate (0.6 mg/kg)

was induced and then maintained with nitrous oxide, oxygen and isoflurane. The patients were connected to a volume-controlled ventilator (Draeger, Werck AV1, Lubeck, Germany) and the respiratory parameters were regulated in order to maintain a normal $ETCO_2$ (Capnolog, Draeger, Werck). No steroids were given before or during aortic surgery. No blood was transfused, and fluidotherapy consisted in polysaline solutions.

The aorta was clamped below the renal arteries and its occlusion lasted 45-60 min.

The patients were divided into two grous: a control group of 9 patiens and an acetyl-carnitine-treated group of 5 patients. The patients of the latter group were given an i.v. bolus of acetyl-carnitine (2 mg/kg), followed by an i.v. infusion of the same drug at the dose of 1 mg/kg/min for 30 min before the clamping of the aorta.

From each patient of the two groups, muscle biopsies from the superior third of femoral quadriceps of the right leg, as well as blood samples from the homolateral saphenous vein, were taken: (a) after induction of anaesthesia; (b) 5 min before declamping; and (c) 30 min after reperfusion. For the evaluation of superoxide anion production, blood samples were collected as in a), b), 5 min after reperfusion (b'); in two out of five patients, a further blood sample was taken 30 min after reperfusion (c). In the acetyl-carnitine-treated group the blood samples and the muscle biopsies taken at time a) were collected before acetyl-carnitine administration.

Fully informed consent was obtained from all the patients.

For the morphological analysis small pieces of muscle tissue were immediately fixed by immersion in cold 4% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, at room temperature, and postfixed in 1% OsO_4 in 0.1M phosphate buffer, pH 7.4, at 4 °C. The specimens were dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. The tissue fragments were embedded with the muscle fibres running parallel to the plane of section. Semithin sections 1-2 μm thick were cut and stained with toluidine blue-Na tetraborate and observed under a light microscope. Ultrathin sections were also obtained from the same specimens. They were stained with uranyl acetate and alkaline bismuth subnitrate (Riva, 1974) and examined under a Siemens Elmiskop 102 electron microscope at 80 kV.

Concerning the blood analysis, plasma levels of C3 and C4 complement fractions were determined by immunonephelometric method (Array Protein System, Beckman, Fullerton, CA) and expressed as mg/dl.

The number of neutrophils/mm^3 was evaluated by a standard automatic device (Backer Instr. Corp., Bethlehem, USA).

Superoxide anion (O_2^-) production by the whole blood was measured according to Bellavite et al. (1983) in both the absence and presence of N-formyl-methionyl-leucyl-phenylalanine (FMLP, 10^{-7}M, Serva, Heidelberg, Germany), a formylated polypeptide which stimulates the oxidative metabolism of granulocytes. Blood samples (200 μl) were preincubated for 5 min at 37 °C

with cytochalasin B (5 μg/ml; Aldrich Chimica SRL, Milan, Italy) and then challenged for a further 5 min with FMLP in the presence of cytochrome C (1 mg/ml, Sigma Chemical Co, St. Louis, MO).

Superoxide anion production was assayed by measuring superoxide dismutase inhibitable cyto-chrome C reduction and values are expressed as nmoles cytochrome C reduced/10^6 cells/5 min.

Statistical analysis was carried out by SPSS (Microsoft, USA).

Data obtained at each experimental time were analysed with one-tailed or two-tailed t test. p< 0.05 was considered significant.

Values are expressed as mean ± SD.

Results

Patients not given acetyl-carnitine (controls)

Detailed informations on muscle tissue undergoing I-R have been reported in our previous paper (Formigli et al., 1992). Briefly, after the induction of anaesthesia, the hematological parameters studied were within the normal values, as well as the light and electron microscopic features of the muscle biopsies. Upon ischemia, a substantial accumulation of granulocytes inside the muscle tissue, complement activation and increase in the number of neutrophils in the blood draining the ischemic tissues were found. Moreover, blood neutrophils revealed a reduced ability for O_2^- generation after FMLP stimulation with respect to their basal activity. Muscle fibers showed mitochondria with numerous, large-sized dense bodies within their matrix. At reperfusion, tissue neutrophil infiltration, complement activation and increase in number of blood neutrophils in the blood draining the muscle tissue were still present. However, the latter revealed an enhanced O_2^- generation when compared to ischemia. In addition, focal mitochondrial swelling, diffuse intermyofibrillar oedema and increase in number and size of the intramitochondrial dense bodies were common findings (Fig. 9).
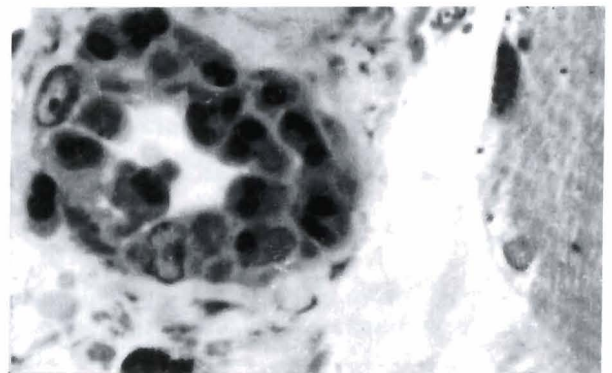
Fig. 1. Acetyl-carnitine-treated patients. Ischemic muscle: numerous granulocytes in the lumen of blood capillary. Semithin section, stained with toluidine Blue-Na tetraborate. x 650
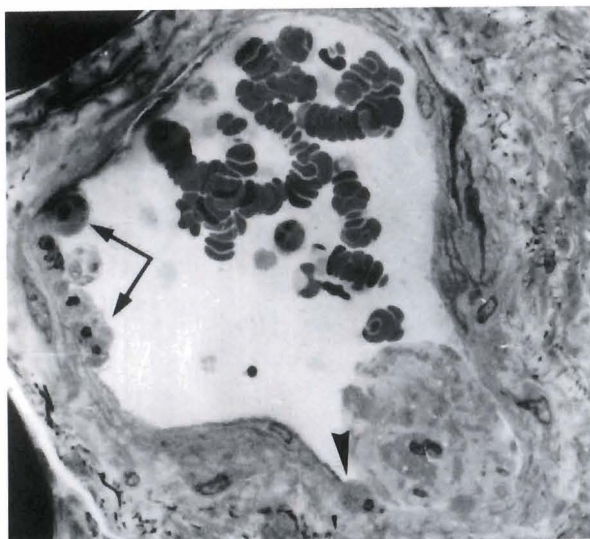
Patients given acetyl-carnitine.

Morphology

After the induction of anaesthesia, the muscle biopsies revealed the same structural and ultrastructural features as those of the control counterparts.

Ischemia: at the end of the ischemic period, light microscopy showed that numerous granulocytes infiltrated the muscle tissue. They formed large clusters in the blood capillaries and sometimes nearly occluded their lumena (Fig. 1). Granulocytes were also found to adhere to the inner and outer blood capillary wall and some of these cells were even caught in transit across the endothelium (Fig. 2). Other granulocytes were also interspersed between the skeletal fibers (Fig. 3). Electron microscopy showed that most of the infiltrating granulocytes were neutrophils which exhibited a normal ultrastructure. They formed many pseudopodia at their cell surface, but none of them was seen to release their granules (Fig. 4).

The connective tissue matrix between the muscle fibres was extremely electron lucent and had few collagen fibres or was completely devoid of them (Figs. 4, 5). The muscle fibres usually showed large-sized mitochondria with zig-zag cristae (Fig. 6) and some of them contained small electron-dense bodies within their matrix.

Reperfusion: 30 min after reperfusion, light microscopy still revealed a conspicuous granulocyte accumulation in the muscle tissue. Electron microscopy showed that intermyofibrillar oedema was only an occasional finding and -when present- it was clearly associated with loss of glycogen particles (Fig. 7). Only

a few mitochondria were moderately swollen (Fig. 7) and others contained scarce and small electron-dense bodies (Fig. 8). The latter retained almost the same size as in the ischemic period, at variance with intra-mitochondrial dense bodies of the reperfused control muscles which instead reached a very large size upon reintroduction of the oxygen to the tissue (Fig. 9).

Blood analysis

After the induction of anaesthesia, hematological parameters investigated were within the normal values.

Ischemia: at the end of the ischemic period there were clear-cut signs of complement activation. In fact, C3 and C4 complement fractions decreased significantly (110 ± 19 mg/dl to 83 ± 10 mg/dl and from 27.3 ± 2.5 mg/dl to 18.2 ± 3.4 mg/dl respectively, $p< 0.001$). This was associated with an increase in the number of neutrophils in the blood draining the ischemic muscle (from $4040\pm 880/mm^3$ to $5753\pm961/mm^3$, $p< 0.05$). Superoxide production from the blood granulocytes after FMLP stimulation resulted to be significantly lower during ischemia (from 18.7 ± 5 nmol/C cyt/10^6 cells/5 min to 10.3 ± 5.7 nmol/C cyt/10^6 cells/5 min, $p< 0.05$). When these data were compared with those of the controls (Figs. 10A,B, 11), no significant differences were
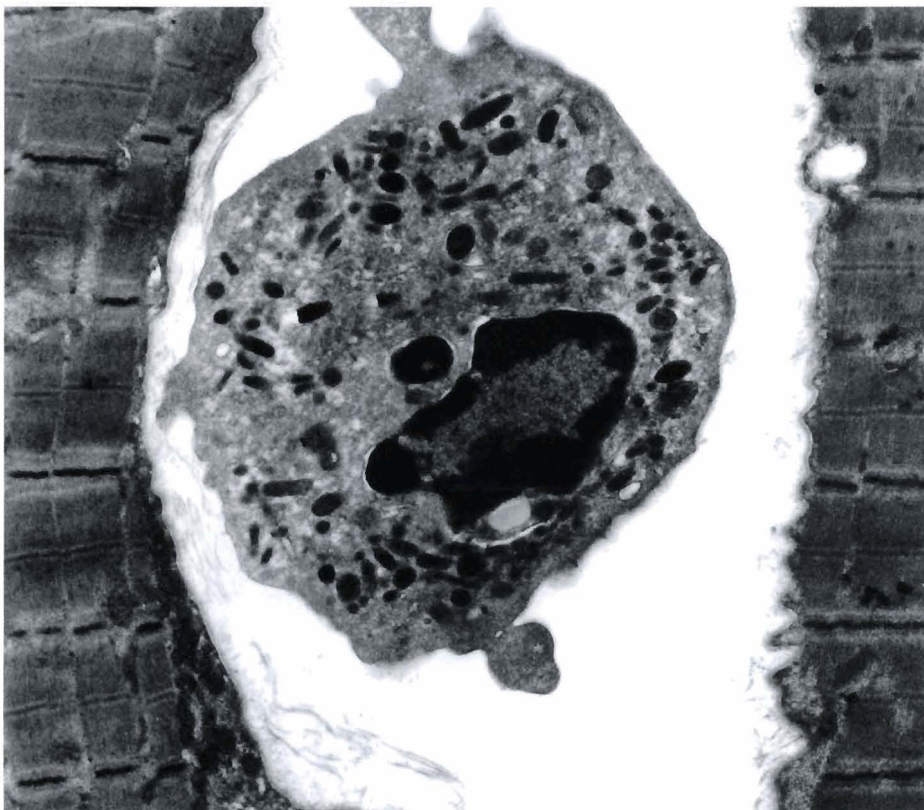

Fig. 2. Acetyl-carnitine-treated patients. Ischemic muscle: granulocytes tightly adhere to the inner face of a blood capillary (arrows). One of them (arrowhead) is seen in transit across the capillary wall. Semithin section, stained with toluidine Blue-Na tetraborate. x 440

Fig. 3. Acetyl-carnitine-treated patients. Ischemic muscle: a large number of granulocytes is found in the connective tissue matrix. Semithin section, stained with toluidine Blue-Na tetraborate. x 1,150
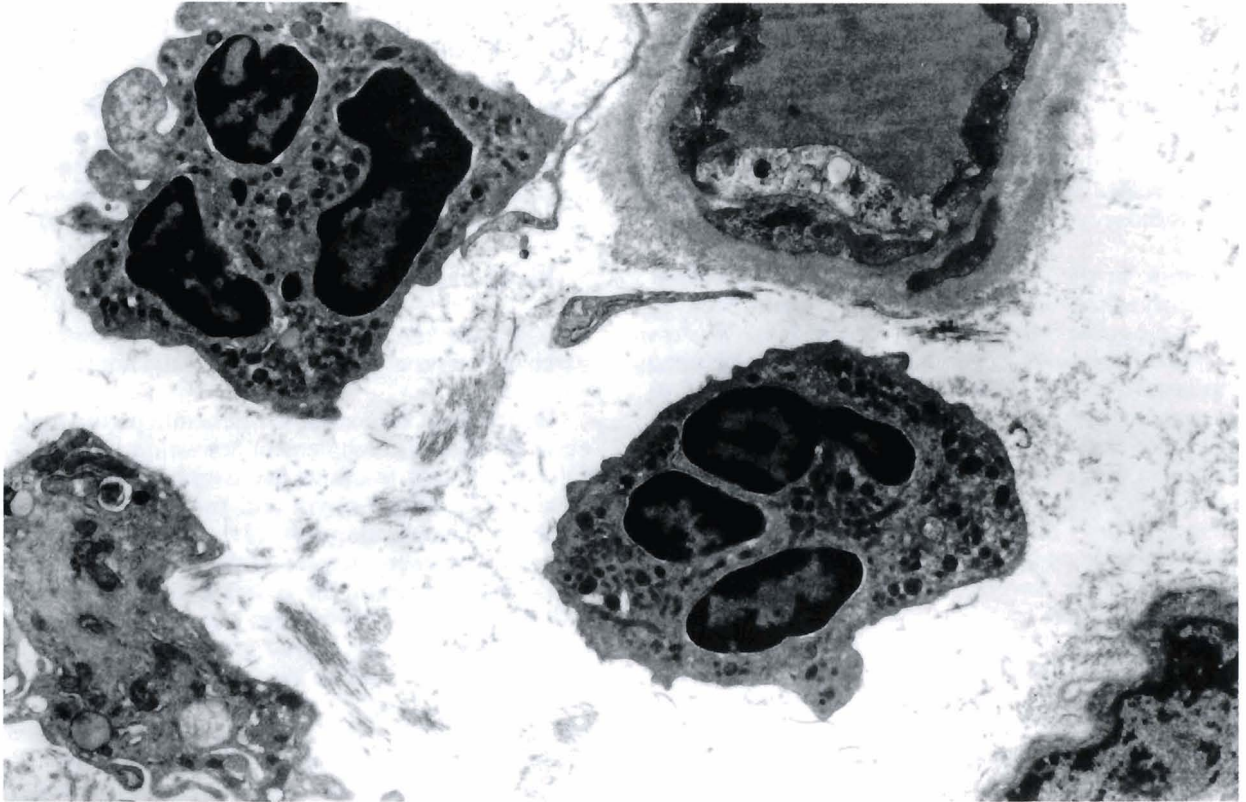
Fig. 4. Acetyl-carnitine-treated patients. Ischemic muscle: neutrophils are in the close vicinity of a capillary vessel. They show many pseudopodia at the cell surface and lie in a very electron-lucent connective tissue matrix. Electron microscopy. x 7,500

Fig. 5. Acetyl-carnitine-treated patients. Ischemic muscle: a neutrophil is found near skeletal muscle fibre. Note the clearing of the connective tissue matrix due to the oedema. Electron microscopy. x 14,000

revealed.

Reperfusion: 30 min after reperfusion, C3 and C4 fractions did not significantly change with respect to ischemia (from 83±10 mg/dl to 69±7 mg/dl and from 18.2±3.2 mg/dl to 15.4±2.6 mg/dl respectively, NS) and neutrophil number in the blood draining the muscle was further increased (from 5753±96/mm^3 to 8549±1812/mm^3, p< 0.05). No significant difference was revealed when compared to the controls (Figs. 10A,B, 11).

Five minutes after reperfusion the O_2^- production from blood granulocytes still remained at a low level without a significant difference when compared to that observed during ischemia (from 10.3±5.7 nmol/C cyt/10^6 cells/5 min to 12.2±5.3 nmol/C cyt/10^6 cells/5 min, NS).

In two out of the 5 patients examined, a further blood sample was taken 30 min after reperfusion. In this sample, the O_2^- production was still at a low level (13.7± 1.3 nmol/C cyt/10^6 cells/5 min) and not significantly different from that of the ischemic period (Fig. 12). In the acetyl-carnitine-treated group O_2^- generation measured five minutes after reperfusion was significantly lower than in the controls (p< 0.05, Fig. 12).

Moreover, in the treated-group the O_2^- generation after 30 min of reperfusion remained at lower levels than in controls (p< 0.05, Fig. 12).

Discussion

Abdominal aortic reconstructive surgery is a suitable model for inducing the I-R syndrome in human skeletal muscles of legs (Novelli et al., 1990; Formigli et al., 1992). In such an experimental condition we have previously revealed that upon reperfusion a large number of granulocytes recruited into the muscle tissue by complement activation was associated with substantial ultrastructural alterations of the muscle fibres (Formigli et al., 1992).

As soon as molecular O_2 was reintroduced to the tissue, the accumulated granulocytes became activated and able to generate superoxide anion. Since there is considerable evidence that O_2^- derived free radicals account for at least part of I-R induced damage (Bulkley, 1987), our previous results strongly suggest that infiltrating granulocytes play an important role in the pathogenesis of I-R syndrome of human skeletal muscle. In the current study we have revealed that in patients

Fig. 6. Acetyl-carnitine-treated patients. Ischemic muscle: a detail of a muscle fibre showing mitochondria with zig-zag cristae (arrow). Electron microscopy. x 50,000
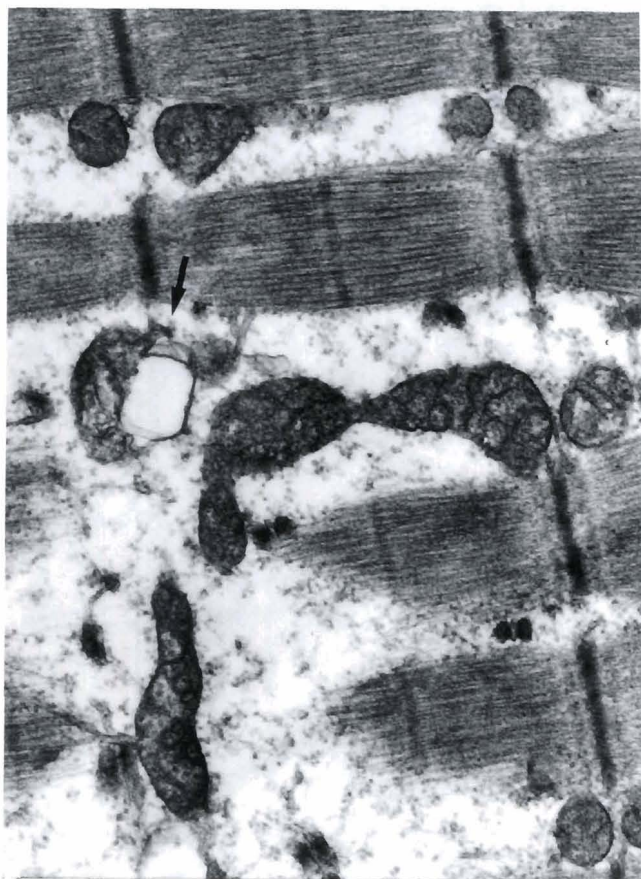

Fig. 7. Acetyl-carnitine-treated patients. Reperfused muscle: a detail of muscle fibre showing intermyofibrillar oedema and one moderately swollen mitochondria (arrows). Electron microscopy. x 28,000

undergoing the same surgery that acetyl-carnitine treatment is capable of preventing -at least in part- the tissue damage induced by I-R in the skeletal muscle of legs. However, complement activation and the consequent neutrophil infiltration into the muscle tissue were still the most distinctive features of the ischemic as well as of the reperfusion period similar to the untreated control muscle. It is well known that complement activated during ischemia (Crawford et al., 1988) produces fragments which play an important role in attracting and activating granulocytes within the injured tissues (Sacks et al., 1978; Jacobs et al., 1980). During ischemia, granulocytes still showed numerous pseudopodia at the cell surface as a sign of intense migratory activity, and the oedema of the connective tissue matrix was the result of an increased vascular permeability

Fig. 8. Acetyl-carnitine treated patients. Reperfused muscle: muscle fibre shows mitochondria containing small-sized electron-dense bodies within their matrix. Electron microscopy. x 28,000

Fig. 9. Control group patients. Reperfused muscle: muscle fibre with mitochondria containing numerous large-sized dense bodies. Compare with those of Fig. 8. Electron microscopy. x 28,000
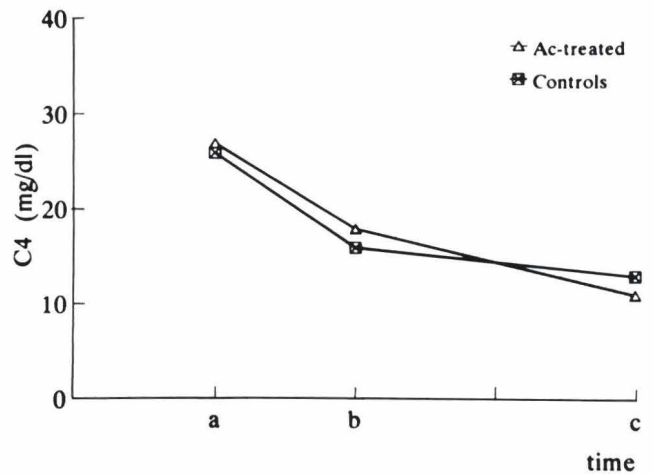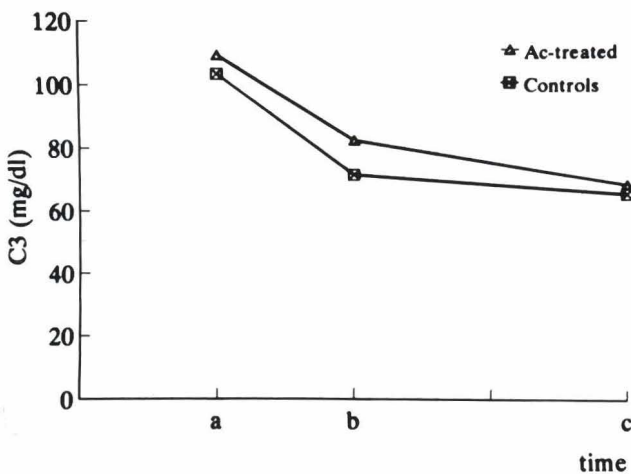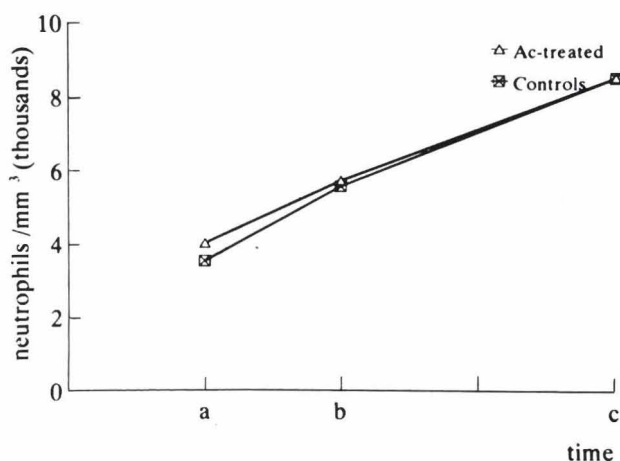

Fig. 10A,B. Changes in C3 and C4 complement fractions in blood draining the muscle after induction of anaesthesia (a), at the end of ischemia (b) and 30 min after reperfusion (c). No statistically significant difference was found between controls and acetyl-carnitine-treated patients (NS).
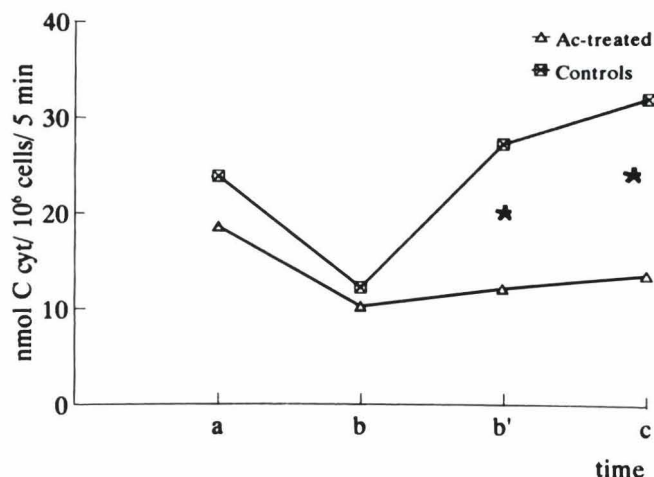
Fig. 11. Changes in neutrophil number in blood draining the muscle after induction of anaesthesia (a), at the end of ischemia (b) and 30 min after reperfusion (c). Values in controls and in acetyl-carnitine-treated group were similar (NS).

Fig. 12. Changes in superoxide anion production by neutrophils in blood draining the muscle after induction of anaesthesia (a), at the end of ischemia (b) at 5 min reperfusion (b') and 30 min after reperfusion (c). At early and later reperfusion (time b', c) superoxide anion production remains at a significantly lower level in acetyl-carnitine-treated patients than in control patients (*, $p < 0.05$).

which accompanied the active diapedesis of these cells as well.

Upon reperfusion, the ultrastructural alterations of the muscle fibres appeared greatly reduced when compared to those of the patients not given acetyl-carnitine. Accordingly, the accumulating granulocytes revealed a reduced ability of O_2 generation both at early and later times of reperfusion when compared to untreated samples. Their reduced function was consistent with previous studies showing that L-carnitine affects superoxide anion production by granulocytes draining the ischemic and reperfused muscle (Novelli et al., 1990). Ultrastructurally, the muscle fibre alterations consisted mainly of a mild mitochondrial swelling with the presence of small and scarce intramitochondrial dense bodies.

These intramitochondrial dense bodies are known to be sites of Ca^{2+} accumulation due to an alteration in the intracellular calcium homeostasis (Khandoudi et al., 1989; Vlessis and Mela-Riker, 1989). Their reduction in size and number, when compared to those of the untreated samples strongly suggests that administration of acetyl-carnitine favours functional integrity of skeletal fibres.

Consistent with these observations are numerous studies indicating that L-carnitine and its derivatives exert a positive effect in ameliorating the I-R injuries (Liedtke et al., 1981; Paulson et al., 1986; Brevetti et al., 1991; Dubelaar et al., 1991) in tissues such as skeletal muscle and myocardium, whose energetic metabolism relies on carnitine-mediated fatty-acid oxidation. Indeed, it has been shown that infusion of acetyl-carnitine (Hulsmann et al., 1988) prevents the leakage of cytosolic enzymes in myocardial fibres of isolated rat hearts. Moreover, the drug administration reduces lactate production and maintains normal ATP levels in patients affected by ischemic heart disease (Ferrari et al., 1984; Bohler et al; 1986). The ability of L-carnitine and its derivatives in preventing I-R injuries may be explained

taking into acount their role in cell energetic metabolism (Goa and Brogden, 1987). In fact, L-canitine and its derivates facilitate the entry of long chain fatty acids into mitochondria where they undergo beta oxidation and eventually produce energy. Hence, carnitines contribute not only to ATP generation, but also limit lactate production and the consequent acidosis. Moreover, it has been recently hypothesized that the protective effect of carnitine may be based, at least in part, on the stabilization of the plasma membrane which, in turn, could prevent the loss of cytosolic enzymes from muscle fibres and thus improve cellular integrity (Hulsmann et al., 1988).

Therefore, it is conceivable that in the presence of acetyl-carnitine the skeletal muscle fibres may improve their energetic metabolism. This hypothesis is supported by the finding of large-sized mitochondria with zig-zag cristae, which are characteristic of metabolically-active tissues (Pappas and Brandt, 1959).

Moreover, recently, it has been demonstrated that several local factors released during ischemia and reperfusion, such as platelet activating factor, phospho-lipase C, calcium, tumor necrosis factor and other cytokines, may converge towards a final activation of membrane-bound NADPH oxidase of granulocytes. Thus, these local factors may be capable of priming neutrophils to enhance their responses to stimuli which would be otherwise uneffective (Braquet et al., 1989). In such a view it is likely that acetyl-carnitine treatment may prevent skeletal muscle from releasing similar local factors so that granulocytes, which still infiltrate the tissue in large numbers upon reperfusion, would not find other stimulants apart from complement fragments to trigger their oxidative metabolism.

In conclusion, the present findings support a favourable effect of acetyl-carnitine on skeletal muscle recovery after a period of I-R.

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