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EFFECT OF PROPIONYL-L-CARNITINE ON RAT SPINAL CORD ISCHAEMIA AND POST-ISCHAEMIC REPERFUSION INJURY

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In this study we have examined the effect of propionyl-L-carnitine (PC) on rat spinal cord ischaemia and post-ischaemic reperfusion injury by evaluating two lipid peroxidation indices, thiobarbituric acid reactive substances (TBARS) and diene conjugation, before and after the addition of an ADP-Fe^{+2} complex to spinal cord homogenates. Aerobic, ischaemic, and post ischaemic reperfusion rat spinal cord homogenates from PC treated and untreated animals did not show any statistically significant difference in their TBARS and conjugated diene content. The addition of the ADP-Fe^{+2} complex to these homogenates resulted in an increased production of both the lipid peroxidation indices, though the magnitude of such formation was related to the type of experimental intervention. The post-ischaemic reperfusion samples of untreated rats showed the highest TBARS and conjugated diene content, while ischaemic samples in either treated and untreated rats did not show any statistically significant difference with respect to the aerobic samples. The post-ischaemic reperfusion samples of treated rats showed a statistically significant decrease of TBARS and conjugated diene production in comparison to the untreated samples. In addition, PC was also able to partially inhibit TBARS and conjugated diene formation in linoleic acid micelles exposed to hemoglobin, though it did not protect albumin fragmentation from the irradiation of water with an X-ray source.

KEY WORDS: Ischaemia, reperfusion, spinal cord, propionyl-L-carnitine

INTRODUCTION

In recent years several papers have appeared in the literature concerning the potential role of reactive oxygen species, as a common pathogenetic factor, in the course of ischaemia and post-ischaemic recirculation injury of the central nervous system (CNS), and heart.[1-4] Since reactive oxygen species represent a physiological class of pro-oxidant molecules, cells are equipped with complex antioxidant defence systems able to neutralize the oxidative capacity of reactive oxygen species.[5,6] In some circumstances, however, such as ischaemia-reperfusion injury, the equilibrium between prooxidants and antioxidants seems to be shifted in favor of the former. As a result, reactive oxygen species may express toxicity to various cellular components: biomembranes undergo peroxidative degradation, proteins are denatured, etc.[7] The CNS is

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particularly rich in polyunsaturated lipids which are integral components of the neuronal plasmalemma, and are highly susceptible to peroxidative damage. There does not exist, however, a general agreement in the literature on the involvement of peroxidative processes in the course of CNS ischaemia and post-ischaemic reperfusion injury.[2,8-12] On the other hand, there is a more general consensus on the role of reactive oxygen species during ischaemia-reperfusion injury based on the beneficial effects of enzymatic or non-enzymatic scavengers (in animal studies).[13] In this context, Ferrari *et al.* and Di Lisa *et al.* have demonstrated that propionyl-L-carnitine (PC) has a protective effect against ischaemia and reperfusion injury in the isolated perfused rat heart model.[14,15]

In this work we have evaluated the effect of PC on ischaemia and post-ischaemic recirculation injury in the rat spinal cord both by evaluating two lipid peroxidation indices (the thiobarbituric acid test and conjugated diene content) and by measuring the intrinsic susceptibility to oxidative stress. Our data suggest that PC may decrease the intrinsic oxidative susceptibility in the post-ischaemic recirculated tissue.

MATERIALS AND METHODS

Chemicals

All salts, buffers, and organic solvents were reagent grade quality RPE (ACS) and obtained from Carlo Erba, Milan (Italy). PC (inner sodium salt) was a generous gift of the Sigma Tau Co., Pomezia (Italy). Sodium dodecyl sulfate (SDS), β-mercaptoethanol, bovine serum albumin (Fraction V, essentially fatty acid free), thiobarbituric acid (TBA), linoleic acid (sodium salt), butylated hydroxytoluene (BHT), ADP, bleomycin sulfate, and Chelex 100 were purchased from Sigma Chemicals (USA). Desferrioxamine B methanesulphonate (Desferal) was from Ciba-Geigy, Milan (Italy). All electrophoretic reagents were obtained from Bio-Rad (USA).

Spinal Cord Perfusion

Male Wistar rats (200–250 g) purchased from the Catholic University Breeding Laboratory, Rome, Italy, were anaesthetized with intraperitoneal Diazepam (Valium 2 mg/100 g body weight) and intramuscular Ketamine (Ketalar, 4 mg/100 g body weight). A dorsal laminectomy was performed extending from T8 to L4 to expose the lumbosacral spinal cord. Next, both the abdominal aorta (at the origin of the left renal artery) and the inferior vena cava were microsurgically isolated. Ischaemia of the lumbosacral spinal cord was produced by applying a cerebral aneurysmal clip to the aorta (force of 70 g/mm) just distal to the left renal artery. The inferior vena cava was utilized for injection of either saline solution (0.5 ml) or PC (100 mg/Kg body weight in 0.5 ml of saline). For biochemical investigations, the lumbosacral spinal cord was removed free of the dura mater (within 3 min) and placed in a reservoir containing liquid nitrogen. Finally, the rats were sacrificed by an overdose of thiopental.

Six groups of rats were studied according to the following experimental protocol:
Aerobic: saline injection, 30 min of interval, removal of spinal cord;
Aerobic PC: PC injection, 30 min of interval, removal of spinal cord;
Ischaemic: saline injection, 30 min of interval, clipping of the aorta for 30 min, removal of spinal cord;
Ischaemic PC: PC injection, 30 min of interval, clipping of the aorta for 30 min, removal of spinal cord;

Post-ischaemic reperfusion: saline injection, 30 min of interval, clipping of the aorta for 30 min, removal of the clip and reperfusion for 10 min, saline injection during reperfusion, removal of spinal cord;
Post-ischaemic reperfusion PC: PC injection, 30 min interval, clipping of the aorta for 30 min, removal of the clip and reperfusion for 10 min, PC injection during reperfusion, removal of spinal cord.

Lipid Peroxidation

A portion of the spinal cord tissues was homogenized on ice with a Potter-Elvehjem homogenizer in 6 vol. of ice-cold 5 mM sodium phosphate buffer, pH 7.4, 0.1 mM EDTA. The water used in our experiments was double deionized and Chelex resin-treated to remove any iron salts. To an aliquot of the homogenate (250 μl) was added 100 μl of 7% (w/v) SDS plus 100 μM Desferal. Then, 800 μl of 0.1 M HCl was added, and the samples were allowed to stand for 5 min, followed by 400 μl of 0.5% (w/v) TBA. To this mixture 880 μg of BHT dissolved in ethanol was added. The final concentration of ethanol was $< 0.1\%$. The samples were then placed in a boiling water bath for 45 min using a marble as a condenser, removed, allowed to cool in ice water. To this mixture 2 ml n-butanol was added, mixed, and centrifuged at 1000 $\times$ g for 10 min. The absorbance of the n-butanol layer was measured in a spectrophotometer at 530 nm.

The remaining portion of spinal cord tissue was extracted with chloroform/methanol (2:1, v/v), and the phospholipid fraction were analyzed for conjugated diene content as earlier described.[16]

To evaluate the intrinsic susceptibility to oxidative stress of spinal cord, we preincubated an aliquot of the homogenates at 37°C for 60 min in the presence of ADP (3 mM) and ferrous sulphate (70 μM). We then processed the samples as reported above either for TBA or conjugated diene assays.

Lipid peroxidation of linolenic acid micelles, by measuring either the formation of diene conjugation or TBA reactivity, was performed as ealier reported.[17]

Assay of Non-Protein-Bound Iron Salts

The bleomycin assay was utilized to measure non-protein-bound iron in spinal cord tissues according to the procedure of Gutteridge *et al.*[18]

Evaluation of Hydroxyl Radical Scavenging Activity of PC

The radiolysis of water was used as a source of hydroxyl radical. Irradiation of the samples was carried out employing X-ray (maximum energy of 10 MeV) generated by a Mevatron 74 (Siemens) linear accelerator. The irradiation field was of 10 $\times$ 10cm^2 and homogeneous within $\pm$ 2%. The absorbed doses were 50 and 100 Gy ($\pm$ 3%). The solutions were exposed under 100% oxygen in Pyrex tubes. Exposure of an aqueous solution of bovine serum albumin to hydroxyl radical and superoxide anion, at a protein concentration that does not affect the oxygen radicals yields (0.30 mg/ml), fragments the protein by a direct radical attack.[19]

SDS-PAGE was used to determine the fragmentation of albumin by hydroxyl radical in the presence or absence of PC.[20]

Protein measurements were performed according to Bradford.[21] Statistical significance was determined using Students *t*-test.

RESULTS AND DISCUSSION

Table I shows the TBARS content (mainly MDA) in aerobic, ischaemic, and post-ischaemic reperfused rat spinal cord both in untreated animals and in animals treated with PC. The overall picture emerging from the data presented in this table is that neither the experimental intervention nor PC treatment produced a statistically significant variation of TBARS in any of the samples examined. The analysis of diene conjugation also did not result in any appreciable differences in total phospholipid extracted from the above samples (data not shown). It should be noted that in our TBA assay we have taken two important precautions in order to avoid any iron mediated oxidation and/or to prevent autoxidation of polyunsaturated lipids during heating. Our test mixture contained both desferrioxamine, an iron chelating agent which inhibits iron-dependent lipid peroxidation, and BHT, a well known free radical scavenger. Thus, in our samples, we should detect only those TBARS produced before the assay. The lack of increased lipid peroxidation in the post-ischaemic reperfused rat spinal cord, an experimental model which is believed to be associated with oxygen radical formation, reflects to some extent the difficulty experienced by others in establishing unequivocally the occurrence of a peroxidative phenomena in ischaemia-reperfusion injury of the CNS.[8-10,22] In this respect, Watson *et al.* have demonstrated, by means of a sophisticated technique, that the appearance of diene conjugates in a reversible global ischaemia model in rat brain was highly compartmentalised and not distributed throughout the brain.[11] In other words, it is possible that sampling large amounts of tissue results in dilution of those molecules under investigation. In addition, Bromont *et al.* presented convincing evidence that after transient forebrain ischaemia in rats there was an increase of lipid peroxidation in several brain regions, though the peroxidative event was measurable only after 8 hours of post-ischaemic recirculation.[12]

The incubation of our spinal cord homogenates at 37°C for 60 min in the presence of iron resulted in an extra amount of TBARS production, which may be considered as an index of the intrinsic susceptibility of spinal cord to oxidative stress (see below). The extent of TBARS production was dependent on the type of experimental intervention (Table II). Thus, the post-ischaemic reperfusion of spinal cord of untreated rats resulted in the highest TBARS production. The amount of TBARS generated in aerobic and ischaemic spinal cord of both untreated and PC treated animals did not show any statistically significant changes. The group of post-ischaemic reperfused animals treated with PC showed a statistically significant decrease of TBARS formation with respect to the untreated counterpart, though such production was still significantly elevated when compared to the remaining experimental groups. The reduced amount of TBARS generated in those spinal cord homogenates from PC

TABLE I

TBAR content (nmol/mg of protein) in rat spinal cord homogenates. Values are means of six spinal cord ± SE

	Control	Ischaemic	Ischaemic + Reperfusion
GROUP			
Untreated	2.13 ± 0.26	2.34 ± 0.19	2.71 ± 0.28
Treated	2.42 ± 0.34	2.63 ± 0.44	2.81 ± 0.21

TABLE II

Susceptibility of rat spinal cord homogenates to oxidative stress. The susceptibility was expressed as differential content of TBARS (nmol/mg of protein) after and before the oxidative stress induced with the Fe^{+2}-ADP system. Values are means of six spinal cord ± SE

	Control	Ischaemic	Ischaemic + Reperfusion
GROUP			
Untreated	1.92 ± 0.63	1.82 ± 0.22	5.26 ± 0.25 ($p < 0.001$)* ($p < 0.001$)**
Treated	1.69 ± 0.19	1.93 ± 0.35	3.09 ± 0.45 ($p < 0.010$)* ($p < 0.002$)ª

Comparisons of intragroup values: *P values are comparisons with the control; **P value is comparison with the ischaemic. Comparisons of intergroup values: ªP value is comparison with untreated.

treated rats was not a mere interference effect of PC since, when we carried out the TBA test with pure MDA either in the presence or absence of PC (up to 5 mM) we did not observe any significant difference. In terms of conjugated diene formation, we have observed a similar behaviour to that of TBARS in each of the experimental groups, whereby PC treatment still resulted in a significant decrease in the amount of conjugated diene in the post-ischaemic reperfusion of spinal cord, in comparison to the untreated animals (Figure 1).

The increased susceptibility of the post-ischaemic spinal cord homogenates to the peroxidative system Fe^{+2}-ADP would seem to suggest that these samples contain more lipid hydroperoxide groups than the non-ischaemic controls. In fact, it is well

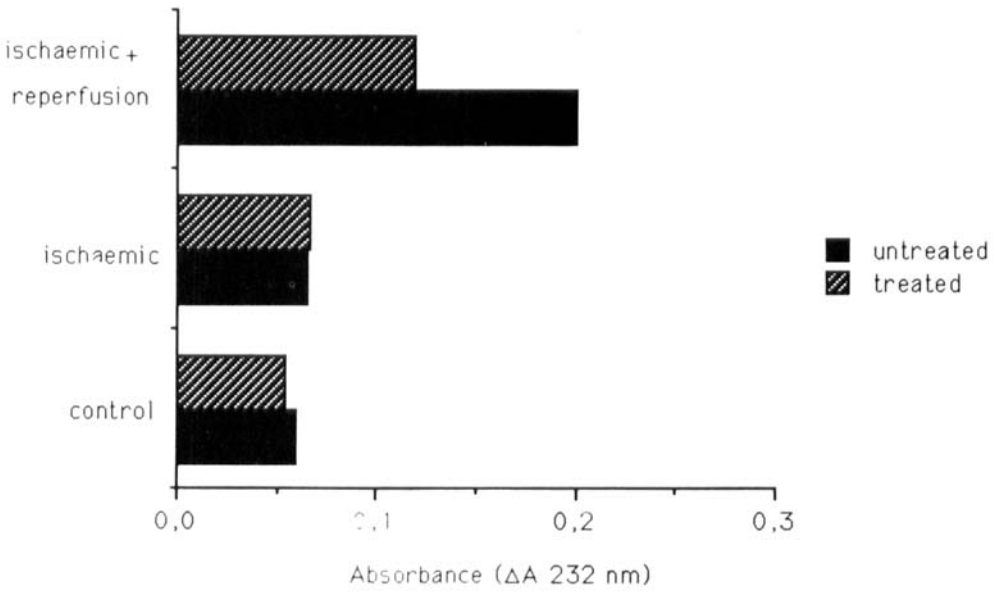

FIGURE 1 Conjugated diene content in spinal cord phospholipids. The values are the average of six experiments. The variation between one experimental value to another was not more than 5%.

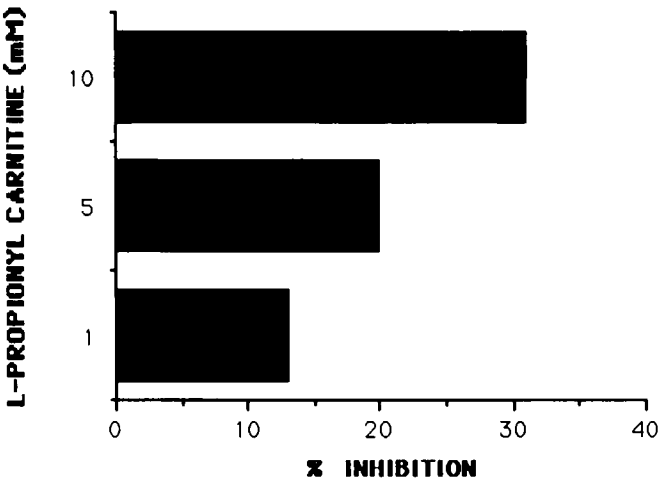

FIGURE 2 The effect of PC on TBARS production from hemoglobin-stimulated lipid peroxidation of linoleic acid micelles. The absolute values are the average of three separate experiments which differed by less than 5%.

known that Fe^{+2}, depending on the type of the chelator, promptly degrades hydroperoxides to generate quite reactive molecules, like hydroxyl radicals or other iron related oxidant species, able to further sustain the perodixation of polyunsaturated lipids.[23,24] Thus, it appears that the simple addition of an Fe^{+2}-ADP complex could be helpful in those cases where it is not possible to demonstrate any modification of lipid peroxidation indices during ischaemia-reperfusion studies. It is interesting to note that we could not detect any free iron in any of the samples examined by means of the bleomycin test, before the addition of the Fe^{+2}-ADP complex.

The reduced susceptibility of the post-ischaemic spinal cord homogenates from PC treated rats may be regarded either as a direct scavenging activity toward some of the reactive species involved in the Fe^{+2}-induced lipid peroxidation or as an ameliorative effect on the antioxidant defence system. In order to obtain more information about the possible mechanism of PC action, we decided to follow-up the scavenging hypothesis. The first test was to evaluate the scavenging activity of PC toward hydroxyl radical and superoxide anion which were generated in the most accurate way: water radiolysis. The irradiation of water with an X-ray source in the presence of albumin, as a target molecule, results in the fragmentation of the protein.[19] This process, then, can be easily followed by means of SDS-PAGE. Any molecule possessing antioxidant capacity toward hydroxyl radical and/or superoxide anion will inhibit the fragmentation process. Albumin samples, after the irradiation, were solubilized in sample buffer (1% sodium dodecyl sulphate, 10% glycerol, 1% β-mercaptophethanol, and 63 mM Tris-HCl pH 6.5), and heated at 95°C for 5 min. Equal amounts of solubilized albumin (100 μg) were loaded onto 10% polyacrylamide gels prepared according to Laemmli. Gels were fixed and stained by either Comassie R250 or Silver procedures. The dye-protein complex intensities were quantified using the Hoefer scanner (GS-300) in the transmittance mode, interfaced with an IBM XT personal computer via a Data Translation A/D card utilizing the GS-360 Data System software (Hoefer).

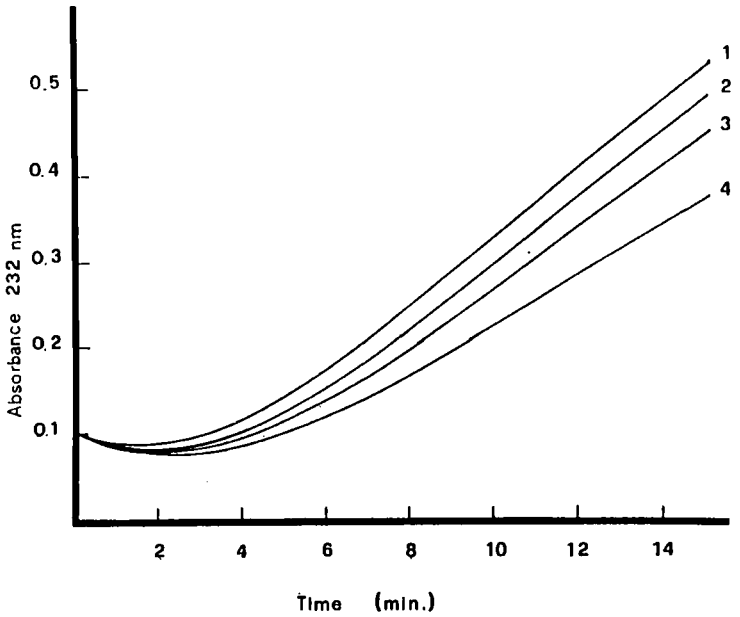

FIGURE 3 The effect of PC on conjugated diene formation from hemoglobin-stimulated lipid peroxidation of linoleic acid micelles. 1, control; 2, control plus 1 mM PC; 3, control plus 5 mM PC; 4, control plus 10 mM PC.

However, PC in all of the concentrations studied did not affect the fragmentation of albumin, thus indicating that PC does not seem to be a good scavenging agent for the hydroxyl radical and/or superoxide anion.

In another set of experiments we analyzed the antioxidant property of PC in a sample peroxidation system consisting of linoleic acid micelles exposed to hemoglobin, which serves as the oxidative agent.[17] The peroxidation of linoleic acid micelles was followed either measuring conjugated diene or TBARS formation. PC was able to inhibit lipid peroxidation partially as evaluated with both of the methods (Figure 2 and 3). The effect was dose dependent, reaching a maximum inhibitory activity of about 30% at 10 mM of PC. Since we have demonstrated that PC is not reacting with hydroxyl radical nor, possibly, with any other related hydroxyl- like radical involved in iron-induced lipid peroxidation, PC may act at some other critical point of the chain-initiating and/or propagation step of the peroxidative pathway. Furthermore, it has been demonstrated that in the course of the iron-induced lipid peroxidation other oxidants such as ferryl and perferryl ion may be responsible for the initiation of lipid peroxidation,[24] though a detailed study on this subject is beyond the scope of the present investigation.

In conclusion, our data suggest that PC may have potential therapeutic implications in those CNS injuries in which it is possible to recognize reactive oxygen species as mediators of the cellular insult.

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