

# CARNITINE ESTERS PREVENT OXIDATIVE STRESS DAMAGE AND ENERGY DEPLETION FOLLOWING TRANSIENT FOREBRAIN ISCHAEMIA IN THE RAT HIPPOCAMPUS

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## SUMMARY

1. The present study investigated whether propionyl-L-carnitine (PLC) has neuroprotective effects, similar to those reported for acetyl-L-carnitine (AC), against transient forebrain ischaemia-induced neuronal damage and biochemical derangement in the rat hippocampal CA1 region.

2. In total, 105 adult male Wistar albino rats were divided into seven groups of 15 animals each. The first three groups were injected i.p. with normal saline, AC (300 mg/kg) or PLC (300 mg/kg) for 7 successive days. The next three groups were injected i.p. with the same doses of normal saline, AC or PLC immediately after the induction of 10 min forebrain ischaemia and i.p. injections were continued for 7 successive days. Rats in the seventh group were subjected to sham-operated ischaemia and injected with normal saline for 7 successive days.

3. Seven days after treatment, animals were killed and their brains isolated for histopathological examination and biochemical studies.

4. Forebrain ischaemia resulted in a significant decrease in the number of intact neurons (77%), ATP concentration (51%) and glutathione content (32%), whereas there was a significant increase in the production of thiobarbituric acid-reactive substances (TBARS; 71%) and total nitrate/nitrite ( $\text{NO}_x$ ; 260%) in hippocampal tissues.

5. Administration of either AC or PLC attenuated forebrain ischaemia-induced neuronal damage, manifested by a greater number of intact neurons, ATP and glutathione, as well as a decrease in TBARS and  $\text{NO}_x$  in hippocampal tissues.

6. Results from the present study suggest, for the first time, that PLC attenuates forebrain ischaemia-induced neuronal injury, oxidative stress and energy depletion in the hippocampal CA1 region. Propionyl-L-carnitine has neuroprotective effects similar to AC and could have a potential use in the treatment of neurodegenerative diseases.

7. The results of the present study will open up new perspectives for the use of PLC in the treatment of neurodegenerative diseases

associated with, or secondary to, myocardial ischaemia-reperfusion injury and chronic circulatory failure.

**Key words:** adenosine triphosphate, carnitine esters, forebrain ischaemia, hippocampal CA1, oxidative stress.

## INTRODUCTION

Damage to brain tissue resulting from cerebral ischaemia is a major cause of adult disability. It can lead to cognition problems, seizures and death.<sup>1,2</sup> Transient global cerebral ischaemia (forebrain ischaemia), occurring during cardiorespiratory arrest in patients or in experimental animals, induces selective and delayed neuronal cell death. Pyramidal neurons in the CA1 region of the hippocampus are particularly vulnerable and die after global ischaemia.<sup>2–5</sup> Hippocampal CA1 sector injury is observed 3–7 days after untreated forebrain ischaemia in the rat, gerbil and human.<sup>2,6</sup>

In forebrain ischaemia, blood flow is completely interrupted; oxygen in hippocampal neurons is depleted, leading to a decrease in glucose utilization and in the production of ATP and phosphocreatine. Failure of energy production and depleted ATP leads to a major rapid redistribution of ions across the plasma membrane, the membrane potential is changed and neurons and glia depolarize.<sup>7</sup> This triggers the opening of  $\text{Ca}^{2+}$  channels and releases glutamate into the extracellular space, which induces marked expression of several pro-oxidant enzymes or mediators, such as inducible nitric oxide synthase (iNOS).<sup>8</sup> Most of these changes are associated with massive production of reactive oxygen species (ROS), which cause severe oxidative injury to the tissue.<sup>9</sup> Oxidative stress is a primary important factor that exacerbates damage by cerebral ischaemia. The brain is particularly vulnerable to oxidative injury because of its high rate of oxidative metabolic activity, intense production of ROS metabolites and high content of polyunsaturated fatty acids, relatively low anti-oxidant capacity, low repair mechanism activity and the non-replicating nature of its neuronal cells.<sup>10</sup>

Acetyl-L-carnitine (AC) and propionyl-L-carnitine (PLC) are naturally occurring short chain derivatives of L-carnitine, which are synthesised endogenously as well as being obtained from dietary sources.<sup>11</sup> Carnitines play an essential role in transporting fatty acids into the mitochondria, where they are oxidized to produce energy. They are also scavengers of oxygen free radicals in mammalian tissues.<sup>12–15</sup> Although the level of beta-oxidation in the normal adult brain is very low,<sup>16</sup> L-carnitine and its short chain esters are transported through the blood–brain barrier and accumulate in neural cells.<sup>17,18</sup>

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Using an *in vitro* model of the blood–brain barrier, Friedrich *et al.*<sup>19</sup> reported that the organic cation/carnitine transporter OCTN2 is functionally expressed in the brain and may be of relevance for the therapeutic potential of L-carnitine, AC and PLC in the treatment of neurodegenerative diseases. Acetyl-L-carnitine has been shown to have neuroprotective effects in cerebral ischaemia models in rats,<sup>20</sup> gerbils<sup>21</sup> and dogs<sup>22</sup> and anti-apoptotic activity in primary murine cultured neurons.<sup>23</sup> Propionyl-L-carnitine has cardioprotective effects after myocardial ischaemia–reperfusion injury through its anti-free radical effect and has also been shown to have anti-inflammatory effects in some models of vascular inflammation in rodents.<sup>24–26</sup> Under conditions of myocardial ischaemia–reperfusion injury and chronic circulatory failure, PLC exhibited several therapeutic advantages over AC or L-carnitine.<sup>27–29</sup> Propionyl-L-carnitine has higher affinity for carnitine transferase and it increases plasma and cellular carnitine content, thus enhancing substrate oxidation rates.<sup>27,30</sup> During ischaemia, PLC improves the efficiency of the Krebs cycle by providing a readily usable substrate, propionate, which is rapidly transformed into succinate without energy consumption (anaplerotic pathway).<sup>27,29</sup> Finally, owing to the particular structure of the molecule, with a long lateral tail, PLC has a specific pharmacological action independent of its effect on metabolism, resulting in peripheral dilatation and positive inotropism.<sup>31</sup> Although rat brain endothelial cells accumulate PLC similar to AC and L-carnitine, and despite its therapeutic advantages over AC or L-carnitine in many forms of myocardial ischaemia–reperfusion injury, the effects of PLC on brain ageing, cerebral ischaemia and neurodegenerative diseases have not yet been studied. Therefore, the aim of the present study was to investigate whether PLC has neuroprotective effects, similar to those reported for AC, against transient forebrain ischaemia-induced hippocampal CA1 neuronal damage, oxidative stress and energy depletion in rats.

## METHODS

### Animals

In total, 105 adult male Wistar albino rats, weighing 230–250 g, were obtained from the Animal Care Center, College of Pharmacy (King Saud University, Riyadh, Saudi Arabia) and were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless indicated otherwise. The study protocol was approved by the Research Ethics Committee of the College of Pharmacy (King Saud University, Riyadh, Saudi Arabia).

### Drugs and chemicals

Acetyl-L-carnitine and PLC were kindly supplied by Dr Menotti Calvani (Sigma-Tau, Pomezia, Italy). Thiobarbituric acid was purchased from Sigma Chemical (St Louis, MO, USA), whereas Ellman's reagent (5-5'-dithiobis-2-nitrobenzoic acid; DTNB) was purchased from Fluka Chemical (Buchs, Switzerland). All other chemicals were of the highest analytical grade available commercially.

### Experimental protocols

Rats were randomly divided into seven groups of 15 animals each. The first three groups were injected with normal saline (0.5 mL/200 g bodyweight per day, i.p.), AC (300 mg/kg per day, i.p.) or PLC (300 mg/kg per day, i.p.) for 7 successive days. The next three groups were injected with the same doses of normal saline, AC or PLC immediately after induction of 10 min forebrain

ischaemia and i.p. injections were continued for 7 successive days. Rats in the seventh group were subjected to sham-operated ischaemia and injected with normal saline for 7 successive days.

### Transient forebrain ischaemia model

Transient forebrain ischaemia was induced in rats under general anaesthesia (sodium pentobarbital; 30 mg/kg, i.p.) with two-vessel occlusion combined with systemic hypotension, according to the methods of Smith *et al.*<sup>32</sup> and Henrich-Noack *et al.*<sup>33</sup> First, blood was withdrawn gradually from the jugular vein into a heparinized syringe to reduce systemic blood pressure to 45–50 mmHg. With the animal supine, the common carotid arteries were exposed by means of a ventral midline neck incision. Both common carotid arteries were exposed, separated from the vagus nerve and occluded for 10 min using microaneurysmal clips (Dieffenbach Bulldog Clamp; 25 mm straight; CHFIA, Nowy Tomysl, Poland), which consistently resulted in delayed neuronal death in the CA1 region of the hippocampus.<sup>33</sup> At the end of the occlusion period, clamps were released, allowing restoration of carotid blood flow, and the incision was sutured with 2–0 silk sutures. In sham-operated animals, the arteries were freed from connective tissue but were not occluded. Body temperature was kept at 37°C using a controlled heating pad and heating lamps throughout the entire period of ischaemia and postischaemic recovery under anaesthesia. A rectal thermometer was used to monitor body temperature (Apelex Rectal Thermometer; Panlab, Bagneux, France).

### Histological analysis of the hippocampal CA1 region

Seven days after ischaemia, five rats from each group were anaesthetized with sodium pentobarbital (100 mg/kg). Rats were then transcardially perfused with cold saline, followed by 4% formalin in phosphate-buffered saline (0.1 mol/L; pH 7.4). Brains were removed from the skull and fixed in the same fixative for 24 h. Thereafter, the brains were embedded in paraffin and 5 µm sections were cut coronally at the level of the dorsal hippocampus using a rotatory microtome (Leica CM3050S; Leica Microsystems, Bensheim, Germany). Segments of the hippocampal CA1 region per 1000 µm lengths from bregma –3.3, –3.8 and –4.3 were counted for viable cells. Tissue sections were stained with haematoxylin and eosin. Hippocampal damage was determined by counting the number of intact neurons in the stratum pyramidale within the CA1 subfield at a magnification of ×20 and ×40 (Nikon E 600, digital camera DXM1200F; Nikon, Tokyo, Japan). Only neurons with normal visible nuclei were counted. The mean number of CA1 neurons per mm linear length for both hemispheres in sections of dorsal hippocampus was calculated for each group of animals. For identification of neuronal cells within the hippocampal CA1 region, sections from formalin-fixed paraffin-embedded hippocampal tissues were subjected to glial fibrillary acidic protein (GFAP) immunohistochemical staining (an indicator of glial cells), S-100 protein immunohistochemical staining (an indicator of glial cells) and synaptophysin immunohistochemical staining (an indicator of neurons). An observer who was unaware of the condition of each rat assessed all histological sections.

### Tissue sampling

Seven days after ischaemia, 10 rats from each group were decapitated, the brains were removed quickly and the hippocampi were harvested on a cold stage. Hippocampi were washed with saline, blotted dry on filter paper, weighed and then 10% (w/v) homogenates were made in 6% perchloric acid (for assessment of ATP) and in ice-cold saline (for assessment of hippocampal tissue content of reduced glutathione (GSH), thiobarbituric acid-reactive substances (TBARS) and total nitrate/nitrite (NO<sub>x</sub>), using Branson Sonifier (VWR Scientific, Danburg, CN, USA).

### Assay of lipid peroxidation and GSH

The degree of lipid peroxidation in hippocampal neuronal tissue was determined by measuring TBARS in the supernatant from the tissue homogenate.<sup>34</sup>

Homogenates were centrifuged at 1500 g and the supernatant was collected and used for the estimation of TBARS. Absorbance was measured spectrophotometrically at 532 nm and concentrations are expressed as nmol TBARS/g wet tissue. Tissue levels of the acid soluble thiols, GSH, were assayed calorimetrically at 412 nm according to the method of Ellman,<sup>35</sup> using a Shimadzu (Tokyo, Japan) spectrophotometer. The GSH content is expressed as  $\mu\text{mol/g}$  wet tissue.

### Determination of total nitrate/nitrite ( $\text{NO}_x$ ) concentrations in hippocampal tissue

Total nitrate/nitrite ( $\text{NO}_x$ ) concentrations were measured as the stable end-product nitrite according to the method of Miranda *et al.*<sup>36</sup> This assay is based on the reduction of nitrate by vanadium trichloride combined with detection by the acidic Griess reaction. The diazotization of sulphanic acid with nitrite at acidic pH and subsequent coupling with *N*-(10 naphthyl)-ethylenediamine produced an intensely coloured product that is measured spectrophotometrically at 540 nm. Concentrations of  $\text{NO}_x$  are expressed as nmol/g wet tissue.

### Determination of ATP production in hippocampal tissue

Adenosine triphosphate was determined in hippocampal tissue using a HPLC method as reported by Botker *et al.*<sup>37</sup> In brief, hippocampal tissue was homogenized in ice-cold 6% perchloric acid, centrifuged at 110 g for 15 min at 0.5°C and the supernatant fluid was injected into HPLC after neutralization to pH 6–7. Chromatographic separation was performed at a flow rate of 1.2 mL/min, using ODS-Hypersil, 150 × 4.6 mm ID 5  $\mu\text{m}$  column (Supelco SA, Gland, Switzerland) and 75 mmol/L ammonium dihydrogen phosphate as the mobile phase. Peak elution was followed at 254 nm. Concentrations of ATP are expressed as nmol ATP/g wet tissue.

### Statistical analysis

Differences between values obtained (mean  $\pm$  SEM) were evaluated by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test.  $P \leq 0.05$  was taken to indicate statistical significance.

## RESULTS

The effects of 10 min forebrain ischaemia, carnitine esters and their combination on the number of intact neurons in the CA1 region of the rat hippocampus are given in Table 1 and are shown in Fig. 1. Histopathological examination of the hippocampal CA1 region from control (Fig. 1a,c) and sham-operated (Fig. 1d) groups revealed normal intact neurons, counted as 164/1 mm. Forebrain ischaemia, however, resulted in severe neuronal damage, manifested as a highly significant (77%) decrease in the number of intact neurons ( $38.0 \pm 5.4$ ) for ischaemia compared with control ( $164.5 \pm 1.6$ ) (Fig. 1b,e).

**Table 1** Effect of forebrain ischaemia and carnitine esters on the number of intact neurons in the rat hippocampal CA1 region

Treatment group	No. intact neurons/1 mm
Control	$164.5 \pm 1.6$
Sham-operated	$164.0 \pm 2.6^\dagger$
Ischaemia	$38.0 \pm 5.4^*$
Ischaemia + AC	$111.0 \pm 3.0^{*\dagger}$
Ischaemia + PLC	$108.5 \pm 3.1^{*\dagger}$

Data are the mean  $\pm$  SEM of the number of intact neurons per 1 mm ( $n = 5$ ).  $^*P < 0.01$  compared with control;  $^\dagger P < 0.01$  compared with ischaemia.

AC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.

Administration of either AC (Fig. 1f) or PLC (Fig. 1g) immediately after ischaemia and continued for 7 successive days resulted in a significant 2.9-fold increase in the number of intact neurons compared with the ischaemia group, although there was still a significant (23%) decrease in the number of intact neurons compared with the control and sham-operated groups.

Figure 2a–h shows GFAP and S-100 immunohistochemical staining of formalin-fixed paraffin-embedded rat hippocampus. Some glial cells (dark staining) can be seen around the hippocampal CA1 band. Conversely, synaptophysin staining, as shown in Fig. 2i–l, clearly shows many neuronal cells within the hippocampal CA1 band.

Figure 3 shows the effects of 10 min forebrain ischaemia, AC, PLC and their combination on ATP levels in the rat hippocampus. Forebrain ischaemia resulted in a significant (51%) decrease in hippocampal ATP levels compared with the sham-operated and control groups. Administration of AC (300 mg/kg) alone for 7 successive days resulted in a significant (50%) increase in ATP levels, whereas the administration of PLC resulted in a non-significant increase in hippocampal ATP. Interestingly, treatment of ischaemic rats with either AC or PLC immediately after ischaemia and continued for 7 successive days resulted in a complete reversal of the forebrain ischaemia-induced depletion of ATP levels in hippocampal tissues to control values.

The effects of forebrain ischaemia, carnitine esters and their combination on TBARS levels, an index of lipid peroxidation, in hippocampal tissues are shown in Fig. 4. Forebrain ischaemia induced a significant (71%) increase in hippocampal TBARS levels compared with both sham-operated and control groups. Treatment of ischaemic rats with either AC or PLC resulted in reversal of the forebrain ischaemia-induced increase in TBARS levels to control values. However, administration of either AC or PLC alone for 7 successive days resulted in non-significant changes in hippocampal TBARS.

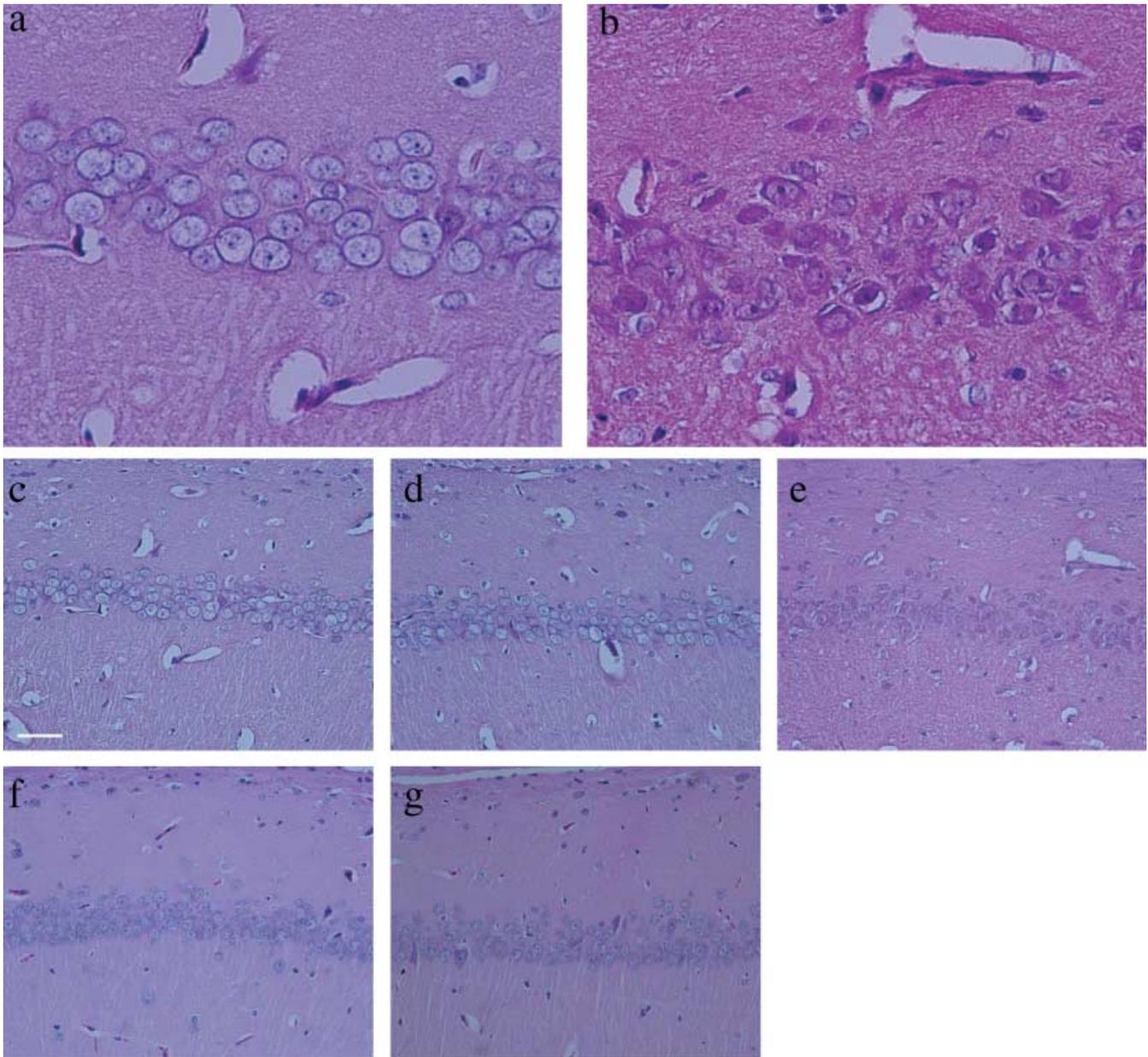
Figure 5 shows the effects of carnitine esters, forebrain ischaemia and their combination on GSH levels in hippocampal tissue. Forebrain ischaemia induced a significant (32%) decrease in hippocampal GSH levels. Administration of either AC or PLC immediately after ischaemia and continued for 7 successive days resulted in reversal of the forebrain ischaemia-induced decrease in GSH levels to control values.

The effects of forebrain ischaemia, carnitine esters and their combination on  $\text{NO}_x$  concentrations in hippocampal tissues are shown in Fig. 6. Forebrain ischaemia induced a significant (260%) increase in  $\text{NO}_x$  concentrations in hippocampal tissue. Treatment of ischaemic rats with either AC or PLC resulted in reversal of the forebrain ischaemia-induced increase in  $\text{NO}_x$  concentrations to control values. Administration of either AC or PLC alone for 7 successive days resulted in non-significant changes in hippocampal  $\text{NO}_x$  concentrations.

## DISCUSSION

It is well documented that transient forebrain ischaemia results in the death of neurons in the CA1 region of the hippocampus. The results of the present study indicate that 10 min forebrain ischaemia induces selective neuronal damage in vulnerable regions, namely the hippocampal CA1 region. Approximately 77% of hippocampal CA1 neurons died. These results agree with those published previously.<sup>3,38</sup>

In the present study, the concentration of ATP in rat hippocampus was measured 7 days after reperfusion and was found to be decreased by 50% of control values. This prolonged decrease in ATP seemingly

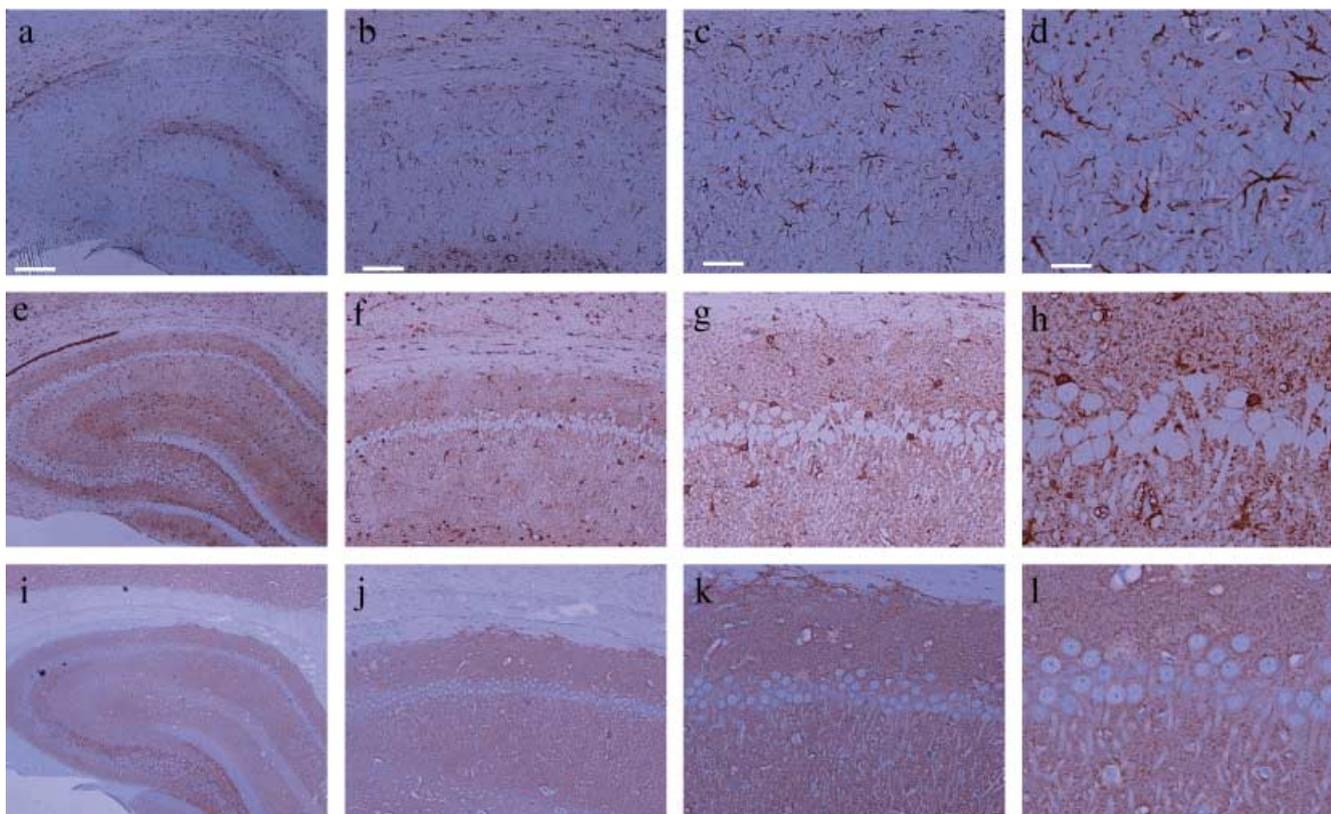


**Fig. 1** (a,b) Photomicrographs showing neurons within the CA1 region of the hippocampus stained with haematoxylin and eosin (HE; original magnification  $\times 40$ ) after transient forebrain ischaemia. (a) Coronal sections showing intact hippocampal neurons of control rats. (b) Neurons in the hippocampal CA1 region after transient forebrain ischaemia. Bar, 25  $\mu\text{m}$ . (c–g) Photomicrographs showing neurons within the CA1 region of the hippocampus stained with HE (original magnification  $\times 20$ ) after transient forebrain ischaemia. (c,d) Coronal sections showing intact neurons in the hippocampal CA1 region of control (c) and (d) sham-operated rats. (e) Most pyramidal cells died in the CA1 subfield 7 days after reperfusion in rats subjected to 10 min forebrain ischaemia. In contrast, administration of either (f) acetyl-L-carnitine or (g) propionyl-L-carnitine immediately after ischaemia and continued for 7 successive days conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield. Bar, 50  $\mu\text{m}$ .

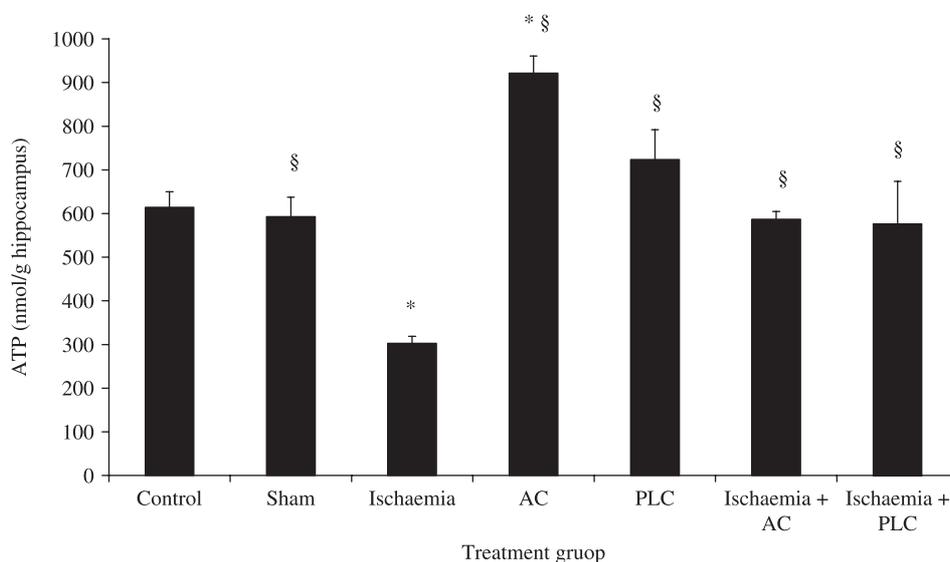
contradicts previous studies that have demonstrated that the loss of phosphocreatine and ATP that occurs during ischaemia recovers to pre-ischaemic values within the first 10–30 min of reperfusion.<sup>39,40</sup> It has been reported that alterations in mitochondrial respiration can induce secondary depletion of both phosphocreatine and ATP,<sup>41</sup> as well as increased levels of brain lactate and decreased activity of pyruvate dehydrogenase (PDH).<sup>42</sup> It has been well documented by several studies that hippocampal injury is not observed until 3–7 days after forebrain ischaemia.<sup>3,40</sup> Such delayed neuronal damage in the hippocampus could explain the prolonged decrease in ATP

observed in the present study 7 days after recirculation. Accordingly, the present results are consistent with those reported previously that, in the CA1 region of the hippocampus, a second decrease in ATP and phosphocreatine occurs beyond 48 h.<sup>40,41</sup>

A cause–effect relationship between forebrain ischaemia and oxidative stress has been confirmed in many experimental models. Forebrain ischaemia is capable of generating ROS, depleting GSH synthesis and increasing malondialdehyde (MDA) levels in experimental animals.<sup>43</sup> The relationship between ischaemia and the induction of oxidative stress in the present study is potent. This is clearly



**Fig. 2** Photomicrographs of (a–d) glial fibrillary acidic protein (GFAP) immunohistochemical staining, (e–h) S100 immunohistochemical staining and (i–l) synaptophysin immunohistochemical staining of the rat hippocampal CA1 region. Bars, 250  $\mu\text{m}$  (a,e,i), 1000  $\mu\text{m}$  (b,f,j), 50  $\mu\text{m}$  (c,g,k), 25  $\mu\text{m}$  (d,h,l).



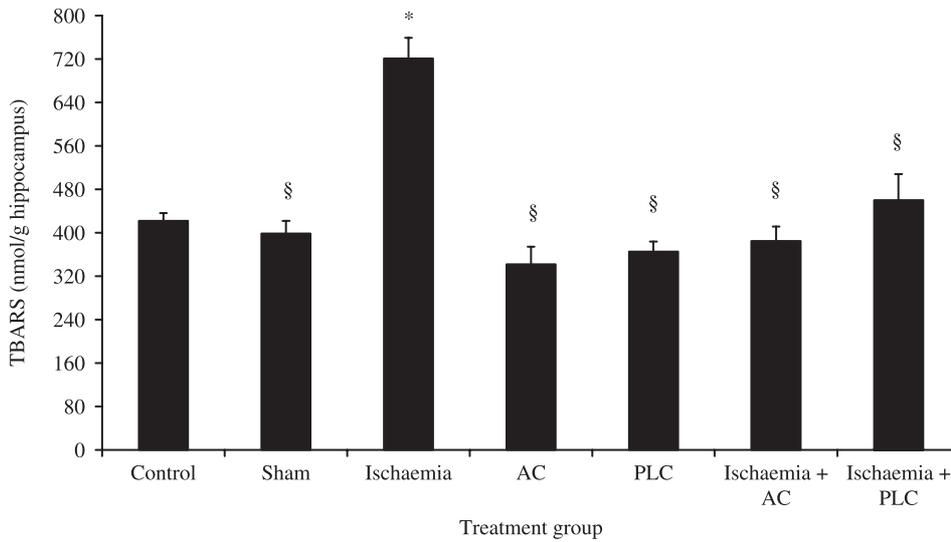
**Fig. 3** Effect of 10 min forebrain ischaemia, carnitine esters and their combination on ATP levels in rat hippocampus. Data are the mean  $\pm$  SEM ( $n = 10$ ). \* $P < 0.01$  compared with control; § $P < 0.01$  compared with ischaemia. AC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.

demonstrated by results indicating that the increase in lipid peroxides and the decrease in GSH levels are positively correlated with hippocampal neuronal damage induced by ischaemia. The present results confirm those reported previously.<sup>42–45</sup>

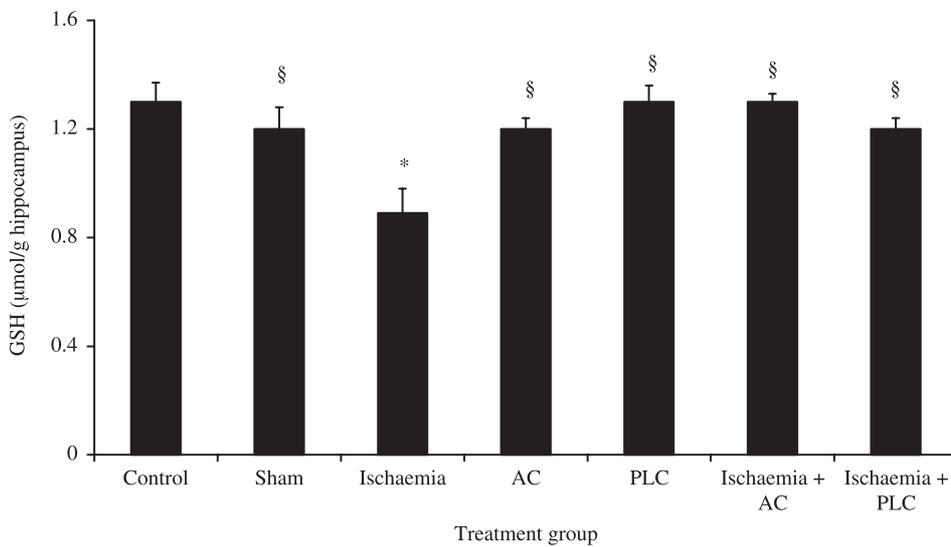
We also demonstrated an increase in  $\text{NO}_x$  concentrations after ischaemia. Nitric oxide (NO) is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage.<sup>46</sup> It has been suggested that NO has the ability to exert multiple cytotoxic effects,

including an increase in arachidonic acid metabolism, as well as the formation of peroxynitrite ( $\text{ONOO}^-$ ). Nitric oxide may also damage DNA through nucleotide base deamination and may trigger programmed cell death.<sup>47</sup>

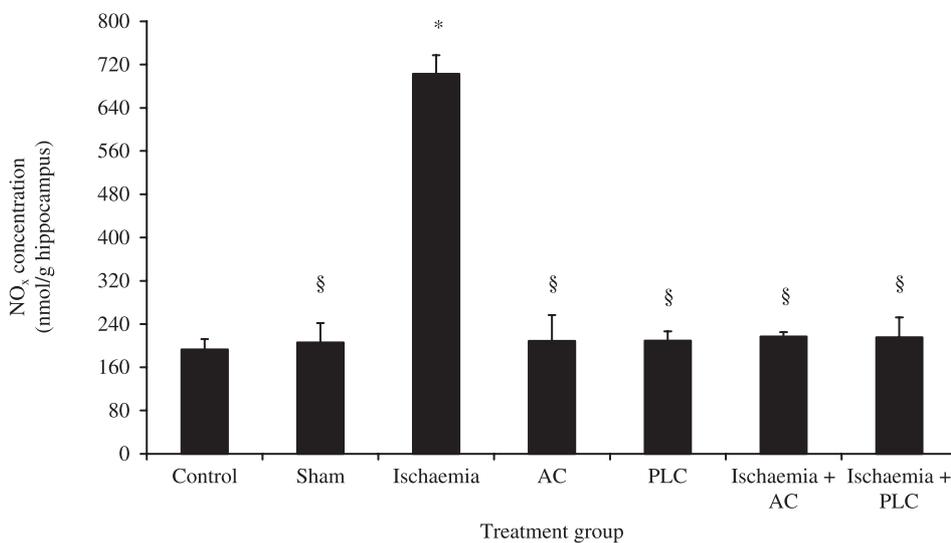
The results of the present study clearly indicate that treatment with both AC and PLC offers protection against hippocampal CA1 neuronal damage induced by 10 min forebrain ischaemia. This was evidenced by the fact that both AC and PLC rescued most of the



**Fig. 4** Effect of 10 min forebrain ischaemia, carnitine esters and their combination on thibarbituric acid-reactive substances (TBARS) in rat hippocampus. Data are the mean $\pm$ SEM ( $n = 10$ ). \* $P < 0.01$  compared with control; § $P < 0.01$  compared with ischaemia. AC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.



**Fig. 5** Effect of 10 min forebrain ischaemia, carnitine esters and their combination on reduced glutathione (GSH) in rat hippocampus. Data are the mean $\pm$ SEM ( $n = 10$ ). \* $P < 0.01$  compared with control; § $P < 0.01$  compared with ischaemia. AC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.



**Fig. 6** Effect of 10 min forebrain ischaemia, carnitine esters and their combination on total nitrate/nitrite (NO<sub>x</sub>) concentrations in rat hippocampus. Data are the mean $\pm$ SEM ( $n = 10$ ). \* $P < 0.01$  compared with control; § $P < 0.01$  compared with ischaemia. AC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.

CA1 pyramidal neurons from ischaemic death. In addition, administration of AC and PLC resulted in complete recovery of the decrease in ATP levels observed after ischaemia compared with control values. The present results are in good agreement with the data presented by Aureli *et al.*,<sup>48</sup> who reported that AC given immediately after ischaemia and at different time intervals during reperfusion restored the decrease in ATP levels and the marked increase in lactic acid induced by ischaemia in rats. Calvani and Arrigoni-Martelli<sup>49</sup> reported that, under conditions of energy crises, the transfer of the acetyl group from AC to coenzyme A (CoA-SH) to form acetyl-CoA as the primary source of energy is a plausible mechanism of action of AC. Reversal of PDH inhibition has been reported in brain tissues from animals treated with AC immediately following ischaemia.<sup>50</sup>

In the present study, administration of PLC to ischaemic rats offered considerable protective effects, manifested by normalization of ATP and an increased number of intact neurons. A possible explanation for this is that, in mitochondria, PLC has a high affinity for CoA-SH/carnitine acetyltransferase and is being converted into free L-carnitine and propionyl-CoA.<sup>27,29</sup> The L-carnitine portion of PLC could increase PDH activity by removing its end-product, acetyl-CoA, which has accumulated during ischaemia in a reaction mediated by carnitine acetyltransferase, thus increasing the CoA-SH/acetyl-CoA ratio, which is essential for PDH activity as well as the activity of many other mitochondrial enzymes.<sup>51</sup> Accordingly, the reversal of forebrain ischaemia-induced depletion of ATP in hippocampal tissues by PLC could be a secondary event following an increase in cerebral glucose utilization with a consequent increase in the production of ATP. In addition, the L-carnitine released from PLC could contribute to the stability of the mitochondrial membrane during ischaemia by stimulating the mitochondrial efflux of long-chain acetyl-CoA accumulated under ischaemic conditions.<sup>30</sup> Conversely, the propionyl-CoA formed in mitochondria from PLC can be converted into succinyl-CoA in a reaction mediated by propionyl-CoA carboxylase, thus increasing the flux through the Krebs cycle and the energy yielding utilization of acetyl-CoA in the absence of oxygen consumption through an anaplerotic reaction.<sup>52,53</sup>

In addition, it is possible that both PLC and AC may increase the concentration of L-carnitine in the brain, which may have been decreased secondary to ischaemia. This hypothesis is consistent with the data presented by Wainwright *et al.*,<sup>54</sup> who reported that treatment with D-carnitine, an agent known to inhibit uptake of L-carnitine in heart,<sup>55</sup> kidney<sup>56</sup> and brain,<sup>57</sup> was associated with a marked increase in mortality during hypoxia-ischaemia in newborn rats. More recently, Tanaka *et al.*<sup>58</sup> have reported that AC supplementation is able to restore decreased hippocampal carnitine levels in aged rats.

In the present study, both AC and PLC produced a significant reduction in ischaemia-induced oxidative stress, as indicated by restoration of the non-protein-SH content and a reduction in TBARS production. In addition, AC and PLC significantly prevented the elevation in NO<sub>x</sub> concentrations. The anti-oxidant activities of AC and PLC have been reported previously.<sup>12,14,15</sup> Using the ischaemic kidney model, Mister *et al.*<sup>59</sup> reported that PLC is of value in preventing the decline of renal function that occurs during ischaemia-reperfusion by decreasing lipid peroxidation and free radical generation. The findings of the present study, that both AC and PLC have the ability to reduce NO<sub>x</sub> concentrations after ischaemia, are consistent with those of Sharman *et al.*,<sup>60</sup> who reported that treatment with AC completely restored the elevated cortical NO, neuronal NOS and nitrotyrosine profile of aged mice.

The mechanism by which AC and PLC prevented any increase in NO concentration is uncertain. However, it is likely that ischaemia-induced oxidative stress may lead to inflammation, resulting in the upregulation of iNOS and overproduction of NO.<sup>61</sup> Thus, AC and PLC may reduce oxidative stress-induced inflammation, leading to the prevention of iNOS upregulation. Alternatively, because PLC possesses anti-inflammatory properties,<sup>26</sup> it can directly reduce inflammation-induced iNOS upregulation and prevent any increase in NO concentration. It is worth mentioning that our results are confirmatory regarding the neuroprotective effects of AC, whereas the potential role of PLC is new and needs to be confirmed. This will open new perspectives for the use of PLC in the treatment of neurodegenerative diseases associated with, or secondary to, myocardial ischaemia-reperfusion injury and chronic circulatory failure. Detailed mechanistic studies are needed to evaluate the role of PLC in many forms of brain damage.

In summary, the results of the present study indicate, for the first time, that PLC attenuates forebrain ischaemia-induced neuronal injury, oxidative stress and energy depletion in the hippocampal CA1 region. In addition, PLC has neuroprotective effects similar to those of AC and is potentially useful in the treatment of neurodegenerative diseases.

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