Carnitine-mediated improved response to erythropoietin involves induction of haem oxygenase-1: studies in humans and in an animal model

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Abstract

Background. Carnitine improves erythropoietin (EPO) response and anaemia in haemodialysis patients (HD); however, the mechanism(s) responsible remain unidentified. We have reported that carnitine induces haem oxygenase (HO)-1, which is an antioxidant and antiapoptotic that acts via pathways shared with EPO. Therefore carnitine’s effect on these pathways may account for the improved EPO response. This study evaluates carnitine’s effect on protein expression of HO-1 in unexplained EPO resistant HD. Carnitine’s effect was assessed by HO-1 expression in patients and compared to its antiapoptotic effect via HO-1 induction in a rat model of carnitine-treated heart failure.

Methods. Unexplained EPO resistant HD mononuclear cell HO-1 and rat gastrocnemious muscle HO-1 and Bcl-2 protein expression were evaluated by western blot.

Results. HD’s haemoglobin (Hb) and haematocrit (Ht) were not different before carnitine treatment; 8.8 ± 0.4 mg/dl versus 8.98 ± 0.13 and 30.20% ± 0.84 versus 30.72 ± 1.14, respectively. Carnitine increased HD’s HO-1, Hb and Ht compared with patients not treated with carnitine: 2.40 ± 0.58 versus 1.49 ± 0.41, P = 0.02; 11.22 ± 0.54 versus 8.90 ± 0.15, P < 0.0001; 32.72 ± 1.77 versus 30.66 ± 0.43, P = 0.035, respectively. Carnitine-treated HD’s HO-1 significantly correlated with haemoglobin. HO-1 and Bcl-2 protein levels in untreated heart failure rat’s gastrocnemious muscle were reduced when compared with controls: 3.41 ± 0.49 versus 5.32 ± 0.38 and 0.69 ± 0.11 versus 1.65 ± 0.37, respectively, but were higher in carnitine-treated heart failure rats: 4.8 ± 0.32 versus 3.41 ± 0.49, P < 0.0002 and 1.09 ± 0.08 versus 0.69 ± 0.11, P = 0.0007, respectively.

Conclusions. These results are consistent with an involvement of HO-1 in carnitine’s effect on erythropoiesis. The initial signals or effectors responsible for carnitine’s effect remain to be identified.

Keywords: anaemia; apoptosis; carnitine; dialysis; erythropoietin; haem oxygenase-1

Introduction

More than 90% of chronic kidney disease patients with anaemia respond adequately to erythropoiesis-stimulating agents (ESAs) [1]. However, a number of factors affect the response to ESAs, including the mode of drug administration, type of renal replacement therapy, functional or absolute iron deficiency, systemic inflammatory factors and secondary hyperparathyroidism [2,3]. Erythropoietin (EPO) has been shown to be a major regulator of proliferation and differentiation of erythroid progenitor cells. It acts through the inhibition of proapoptotic caspase activation and attenuation of cell death in response to oxidative stress via effects on JAK2 signalling. In addition to the antiapoptotic activity via JAK2, EPO affects phosphatidylinositol-3-kinase (PI3K) and Akt (protein kinase B) activity [4–6].

Haemodialysis (HD) patients who fail to respond to EPO have an improved response to EPO, resulting in improving anaemia when treated with carnitine [7,8]. L-carnitine is a small water-soluble organic solute and, along with its natural esters acylcarnitines, forms a component of the endogenous carnitine pool in humans and most animal species. L-carnitine, acylcarnitines, various carnitine enzymes located in membranes delimiting subcellular organelles and carnitine transporters devoted to carnitine transfer across the cell membrane constitute the carnitine system playing a pivotal role in cellular energy production [9]. Recently it has been
reported that L-carnitine increased the number of colony-forming unit-erythroid (CFU-E) colonies in mouse bone-marrow cell cultures, suggesting that L-carnitine stimulates erythropoiesis, and thereby improves renal anaemia [10]. However, the nature of the connection between carnitine and response to EPO remains to be clarified [11–13].

The effects of carnitine have been investigated in a variety of systems. There have been reports that have described an antioxidant effect for L-carnitine and its acyl derivative [14] while the specific mechanism is unclear [14,15]. Carnitine has also been recently shown to alter apoptosis, a major determinant of muscle myopathy in heart failure [16], but again the mechanism responsible remains unclear. In addition, carnitine and its derivatives have been shown to be effective in ameliorating the toxic effects of gentamicin, which are thought to be primarily mediated via oxidative stress [17], and carnitine treatment has been shown to have a protective effect on doxorubicin-induced tubular interstitial damage and nephrotic syndrome, attributed to the carnitine-induced reduction of oxidative stress [18]. Finally, L-carnitine, possibly via its free radical scavenging and antioxidant properties, has been shown to ameliorate oxidative organ injury in animal model of chronic renal failure [15].

Recently, we have reported that carnitine and its acyl derivatives appear to have a significant effect on both HO-1 and eNOS, known oxidative stress-related proteins, both at mRNA and protein expression [19]. Interestingly the responses to oxidative stress and apoptosis of HO-1 have been described at the level of transcriptional regulation via pathways similar to those described for EPO [12,20]. This raises the possibility that carnitine’s effect on both EPO and HO-1 are linked and that this effect is linked to carnitine’s ability to improve response to EPO. To examine this possibility, we have explored the effects of carnitine treatment on HD patients who had unexplained EPO resistance, at the level of protein expression of HO-1 and compared it to that of HD patients with unexplained EPO resistance who did not receive carnitine.

In another study, in order to give deeper insight on the recently reported antiapoptotic effect of carnitine in an animal model of heart failure [16], we have evaluated, using the same animal model, the possibility of a link between the carnitine ameliorating effect on apoptosis and the carnitine stimulating effect on HO-1 expression.

The rationale for these two different experimental models (HD patients and rat heart failure) was, therefore, to document the linkage between apoptosis, HO-1 and carnitine-induced HO-1 increase. It is a fact that carnitine may induce HO-1 both in HD patients and in rat with monocrotaline-induced heart failure and this then may reduce apoptosis with a resulting increase of haemoglobin (Hb) in the case of HD patients and decreased muscle loss in the animal model.

**Materials methods**

**Patients**

Five patients under chronic dialysis treatment (210–240 min three times a week, low-flux bicarbonate dialysis using a polysulphone dialyser 1.8 m²) and ultrapure dialysate for at least 1 year, from the patient’ population at the Divisions of Nephrology of the Hospitals of Bolzano, Piove di Sacco, Messina and Padova who had shown unexplained resistance to average EPO treatment and treated with carnitine (1 g i.v. post-dialysis/3 times a week) for at least 8 months and five patients with the same unexplained resistance to epoetin treatment, from the same dialysis units, who had not been treated with carnitine were evaluated in this study.

All patients were under epoetin treatment at a dose ranging between 20 000 and 30 000 U/week; ferritin levels ranged between 200 and 300 µg/L and transferrin saturation was >30%. Vitamin D, PO₄ binders and calcium supplements were also present in the therapeutic regimen for some patients. No patients showed any clinical and biochemical signs of malnutrition. None of the patients was under lipid- lowering treatment; all patients were treated with supplements of folic acid (10 mg) after dialysis session.

Patients’ blood pressure ranged from 135/85 to 150/90 mmHg and antihypertensive treatment included calcium channel blockers, β blockers and α blockers and central anti-adrenergic drugs. One patient from each group was also treated with ACE inhibitors.

The single pool KT/V ratio expressed as mean ± SD of KT/V determinations performed every month for all the patients was 1.46 ± 0.13. Patients with diabetes, malignancies, inflammatory diseases, liver failure, cardio-respiratory insufficiency, severe hyperparathyroidism (PTH > 800 pg/ml) and serum aluminium >30 µg/L were excluded.

For both patients treated with carnitine and those patients not treated with carnitine pretreatment, biochemical and clinical data such as Hb and haematocrit (Ht) levels were derived from clinical records. For the patients treated with carnitine, blood samples were collected after 8 months of treatment with carnitine for determination of Hb, Ht and molecular biology evaluation of the mononuclear cell HO-1 protein level. Blood samples were also collected at this time for patients not treated with carnitine, for the same biochemical and molecular biology evaluations.

To minimize the possibility, although not proven, that a different quantitative protein expression between mononuclear cells subtypes could influence the gene expression of HO-1, i.e. through a fluctuation of the number of the different mononuclear cells subtypes, we checked the patients for the absence of changes of biochemical markers of inflammation such as CRP, α₂-globulins, monocytes and lymphocytes counts as well as for absence of clinical evidence of acute infectious or inflammatory disease.

Informed consent was obtained from all study participants.

**Experimental animal studies**

Three groups of animals were studied for a total of 12 animals. Eight males (80–100 g) Sprague–Dawley rats were injected intraperitoneally with monocrotaline at a dose of 30 mg/kg, which produces severe pulmonary hypertension followed by right ventricular failure [21] without inducing changes in skeletal muscle myosin heavy chain (MHC)
composition and apoptosis. Half of the injected group of rats were treated daily with 50 mg/kg L-carnitine (Sigma Tau, Rome, Italy) given in drinking water. The rats were kept in single cages, and water consumption was measured daily. Ten age- and diet-matched rats were injected with saline and used as controls. After 28 days, when in the monocrotaline-treated animals, overt heart failure developed [16], the rats were killed and gastrocnemious muscles removed and immediately frozen in liquid nitrogen and stored at 80°C until the evaluation of HO-1 and Bcl-2 protein levels.

Molecular biology assays

Mononuclear cells preparation

Dialysis patients’ peripheral blood mononuclear cells (PBM) from 20 ml of EDTA anticoagulated blood were isolated by Ficoll Paque Plus gradient (Amersham Pharmacia Biotech, Sweden).

HO-1 protein expression

HO-1 protein expression from PBM of dialysis patients and rats gastrocnemious muscles was performed using western blot analysis.

Total protein extracts were obtained by cells’ lysis with an ice-cold buffer (Tris– HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na3VO4 1.0 mM, SDS 0.1%) added with proteases inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Protein concentration was evaluated by bichrominic acid assay (BCA Protein Assay, Pierce, Rockford, USA). Proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes (Hybond ECL, Amersham, USA). Proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes (Hybond ECL, Amersham, Uppsala, Sweden) and blocked overnight with no-fat milk (5% in Tween-PBS). Membranes were probed with primary polyclonal antibody anti-HO-1 (1:750) (Stressgen Biotechnologies, Victoria, Canada) and monoclonal antibody anti-GAPDH (Chemicon International, Temecula, CA, USA). After incubation with proper secondary antibodies HRP-conjugated (Amersham Biosciences, Uppsala, Sweden), immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA).

HO-1 protein expression was quantified using a densitometric semiquantitative analysis using NIH image software and were normalized to GAPDH. Ponceau staining was also used as loading control.

Bcl-2 protein expression

Bcl-2 protein expression was evaluated using western blot, which was probed with anti-Bcl-2 (1:1000) (Oncogene, Boston, USA) and monoclonal antibody anti-GAPDH (Chemicon International, Temecula, CA, USA). After incubation, immunoreactive proteins were visualized using HRP-conjugated secondary antibodies (Amersham Biosciences, Uppsala, Sweden) using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA), quantified using a densitometric semiquantitative analysis using NIH image software and were normalized to GAPDH. Ponceau staining was also used as loading control.

Statistical analysis

Data were evaluated on a Macintosh G5 computer (Apple Computer, Cupertino, CA, USA) using the Statview II statistical package (BrainPower Inc., Calabasas, CA, USA). Data were expressed as mean ± SD and were analysed using Student’s t-test for paired and unpaired data and ANOVA for unpaired data. Values at a 5% level or less (P < 0.05) were considered significant.

Results

The high dosage of EPO prior to the start of the study and the relatively small increase upon carnitine treatment did not require any sort of adjustment of EPO dosage through the course of the study. The data used in the study represent determination using an initial sample and then another sample after 8 months of carnitine treatment. No adverse events and intercurrent comorbidities were observed during the study.

Hb and Ht levels in HD patients were not different before carnitine treatment: 8.8 ± 0.4 mg/dl versus 8.98 ± 0.13, P = ns and 30.20% ± 0.84 versus 30.72 ± 1.14, P = ns, respectively.

Hb and Ht from patients treated with carnitine increased from the beginning of the treatment compared with patients not treated with carnitine: 11.22 ± 0.54 (range 10.6–12.0) versus 8.90 ± 0.15 (range 8.6–9.4), P < 0.0001 and 32.72 ± 1.77 (range 29.7–34.0) versus 30.66 ± 0.43 (range 30.0–31.2), P = 0.035.

PBM HO-1 protein level in patients receiving carnitine after 8 months of treatment was increased compared to those patients who did not receive carnitine: 1.49 ± 0.41 densitometric units (d.u.) versus 2.40 ± 0.58, P = 0.02 (Figure 1).

In addition, in patients treated with carnitine after 8 months of treatment, HO-1 protein expression significantly correlated with the Hb level (r = 0.92, P = 0.02) (Figure 2).

ANOVA analysis showed that rat gastrocnemious muscle HO-1 protein level in animals with monocrotaline-induced heart failure was significantly reduced as compared to control rats: 3.41 ± 0.49 d.u. versus 5.32 ± 0.38 while rat gastrocnemious muscle HO-1 protein level was significantly higher in rats with monocrotaline-induced heart failure that received carnitine compared to those who did not receive carnitine: 4.8 ± 0.32 versus 3.41 ± 0.49, P < 0.0002 (Figure 3).

ANOVA analysis also showed that rat gastrocnemious muscle Bcl-2 protein level was significantly reduced in rats with monocrotaline-induced heart failure as compared to control rats: 0.69 ± 0.11 versus 1.65 ± 0.37, while rat gastrocnemious muscle Bcl-2 protein level was higher in rats with monocrotaline-induced heart failure that received
HO-1, carnitine and response to EPO

Fig. 1. Densitometric analysis of HO-1 protein expression in mononuclear cells of dialysis patients not treated with carnitine (white bar) and treated with carnitine (grey bar). The top part of the figure shows representative HO-1 western blot products of two dialysis patients not treated with carnitine and two dialysis patients treated with carnitine. ∗P = 0.02, Student’s t-test.

Fig. 2. Correlation between HO-1 and haemoglobin level in dialysis patients after 8-month treatment with carnitine.

carnitine compared to those who did not receive carnitine: 1.09 ± 0.08 versus 0.69 ± 0.11, P = 0.0007 (Figure 4).

Discussion

Low Ht and Hb levels and poor ESA responsiveness are often linked to inflammation, malnutrition and, most importantly, cardiovascular disease [2]. This triad constitutes the malnutrition–inflammation–atherosclerosis (MIA) syndrome and is strongly associated with a greater risk of death in HD patients [22]. Patients with renal failure and those under renal replacement treatment with HD are exposed to oxidative stress particularly reactive oxygen species (ROS), which arises from activation of endothelial cells [23]. This increase in ROS affects vascular tone via its destruction of vasodilatory NO and increases the risk of cardiovascular disease, which is the major cause of death for renal failure and HD patients [23]. Oxidative stress in these patients also leads to RBC membranes’ lipid peroxidation and RBC destruction, thereby worsening renal failure induced anaemia. Thus, the increased oxidative stress associated with dialysis is of considerable concern in patients undergoing dialysis [23].

Carnitine increased HO-1 expression in both patients and the monocrotaline-induced heart failure animals. This increase in HO-1 is likely mediated by effects on ROS as we have reported that carnitine in cell culture not only increased gene expression of HO-1 but eNOS as well [19]. Given the known effects of HO as an antioxidant and antiapoptotic defence [24], these results point to the ability of carnitine to enhance EPO responses being related to the ability to modulate oxidant status via its effects on HO-1 [12].
Recent reports suggest that cytokine-induced alterations in cellular iron homeostasis are mediated in part by the increased production of ROS [25,26]. This then results in EPO resistance in some HD patients. The ability of carnitine to reduce oxidative stress via its effects on HO-1, which reduces the cytokine-induced alterations in cellular iron homeostasis, may form, at least in part, the basis for the enhancement of EPO responses in those patients. There is robust support from a variety of studies, including the present study, for the inter-related roles of carnitine as both an antioxidant and an antiapoptotic substance [10–19,27,28] and particularly with respect to carnitine’s effect on ROS in EPO signalling in haemopoietic cells in end-stage renal disease and HD patients [10–13,29].

The mechanisms responsible for the effects of carnitine are likely the result of the fact that both EPO and HO-1 antiapoptotic effects occur via activation of the PI3K/Akt pathway and that carnitines induce HO-1 [11–13,19]. In fact HO-1 expression in response to oxidative stress is regulated at transcriptional level by phosphorylated Akt [31,32]. Interestingly, the antiapoptotic action of EPO, which occurs via EPO-mediated inhibition of proapoptotic caspase activation and attenuation of cell death in response to oxidative stress, has been shown to be dependent on JAK2 signalling [4,30] and PI3K-mediated phosphorylation of Akt that, once triggered, activates multiple targets with antiapoptotic effects [4–6,20]. Data from Vescovo et al. [16] demonstrated that carnitine treatment reduced muscle loss, which may be linked to carnitine’s activation of Akt as phosphorylated Akt inhibits FOXO, a transcription factor that activates atrogin-1 (ubiquitin ligase) and atrogenes expression that lead to protein degradation in heart failure [31–33]. That carnitine effects are mediated by antiapoptosis is also strengthened by the findings of the present study obtained in animal experiments, in which carnitine induced an increase in Bcl-2, an antiapoptotic protein, in animals with the monocrotaline-induced heart failure. The findings of the present study obtained in animal experiments, in which carnitine induced an increase in Bcl-2, an antiapoptotic protein, in animals with the monocrotaline-induced heart failure, may be linked to carnitine’s activation of Akt as phosphorylated Akt inhibits FOXO, a transcription factor that activates atrogin-1 (ubiquitin ligase) and atrogenes expression that lead to protein degradation in heart failure [31–33]. That carnitine effects are mediated by antiapoptosis is also strengthened by the findings of the present study obtained in animal experiments, in which carnitine induced an increase in Bcl-2, an antiapoptotic protein, in animals with the monocrotaline-induced heart failure. Thus, the initial signals or effectors responsible for carnitine’s effect remain to be identified. One potential area of investigation may come from carnitine’s well-documented effects on mitochondrial glucose metabolism [34]. These effects may have a profound influence on cell survival as Robey and Hay [35] have suggested that glucose dependence of the antiapoptotic effects of growth factors and Akt plus a strong correlation between Akt-regulated mitochondrial hexokinase association and apoptotic susceptibility point to a major role for mitochondrial glucose metabolism in Akt signalling and cell survival.

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**Conflict of interest statement.** None declared.

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