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Carnitine reduces the lipoperoxidative damage of the membrane and apoptosis after induction of cell stress in experimental glaucoma

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The pathological damage caused by glaucoma is associated to a high intraocular pressure. The ocular hypertone is most likely due to a defective efflux of aqueous humor from the anterior chamber of the eye. Ocular hypertension causes apoptotic death of retinal ganglion cells and overexpression of molecular markers typical of cell stress response and apoptosis. In this work, we report on the neuroprotective, antiapoptotic and antioxidant action of a natural substance, L-carnitine. This compound is known for its ability to improve the mitochondrial performance. We analyze a number of cellular and molecular markers, typical of ocular hypertension and, in general, of the cell stress response. In particular, L-carnitine reduces the expression of glial fibrillary acidic protein, inducible nitric oxide synthase, ubiquitin and caspase 3 typical markers of cell stress. In addition, the morphological analysis of the optic nerve evidenced a reduction of the pathological excavation of the optic disk. This experimental hypertone protocol induces a severe lipoperoxidation, which is significantly reduced by L-carnitine. The overall interpretation is that mortality of the retinal cells is due to membrane damage.
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The progression of glaucoma has been described in detail:[1,2] one of its main characteristics consists of a high intraocular pressure (IOP), which raises above statistically normal values. This increase can be ascribed, under a strictly physical point of view, to a defective efflux of the aqueous humor from the anterior chamber of the eye.[3] It has been estimated that glaucoma is the second cause of blindness in humans and that 68 million patients suffer from this pathology, about seven million individuals having bilateral blindness.[4] A common feature of the pathology involves an excavation of the optic disk (cup-to-disk), which is extended to its margins. Several studies indicate that the early lesions occur at the level of the optical nerve, in particular, in the proximity of the head of the optic nerve at the level of the lamina cribrosa; however, it has also been suggested an initial involvement of the retina.[5] Apoptotic death of the retinal ganglion cells (RGCs) has been shown[6] and, in addition, the effect of the intraocular hypertension causes a significant overexpression of caspase 3, which is a commonly accepted marker of programmed cell death.[7,8] Death of a significant number of RGCs most likely reduces the number of the axons forming the optic nerve. The evolution of this phenomenon results in the pathological increase of the normal excavation of the optic papilla, which eventually results in a serious vision deficit.[9] The relevant role of micro- and macroglial cells in the degeneration of RGCs has also been demonstrated.[10,11] Glial cells have a neuroprotective role, however, in a pathological situation; they may contribute to neuron damage. In particular, during progression of glaucoma, astrocytes are involved in the remodelling of the lamina cribrosa and they could act as initiators of the pathology.[12] Studies on experimental models of ocular hypertension and human glaucoma evidenced astrocyte hypertrophy and loss of organization in both retina and optic nerve. The upregulation of the glial fibrillary acidic protein (GFAP) was also observed in cultured astrocytes grown at high hydrostatic pressure.[13,14] The GFAP is considered a very important stress marker in diverse retinal pathologies and in experimental models of ocular hypertension.[8,15,16] Activation of the glial cells may also have noxious consequences on neurons, as they may cause mechanical damages and alterations of the microenvironment.[12,17] This triggers the release and/or production of neurotoxic and proapoptotic compounds such as nitric oxide synthase (NOS).[18,19] The nitric oxide (NO) thus produced is a reactive free radical present in cells as a response to increased intracellular concentrations of Ca^{2+}.[20] It is known that NOS increases in cerebral ischemia[21] and the overexpression of this enzyme causes relevant tissue damage: the overall result is a detrimental action on the cell membrane.[21,22] Recent studies demonstrated that an excess of NO is toxic and this compound increases as a consequence of ocular hypertension.[23] In glaucoma, the involvement of inducible NOS (iNOS) has also been suggested.[24] The oxidative stress and the increase of IOP also causes

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Abbreviations: RGCs, retinal ganglion cells; GFAP, glial fibrillary acidic protein; NOS, nitric oxide synthase; NO, nitric oxide; iNOS, inducible NOS; MTC, methylcellulose; Ub, ubiquitin; MDA, malondialdehyde; IOP, intraocular pressure
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upregulation of ubiquitin (Ub) and stimulation of the Ub-proteasome pathway:[8,25] this possibly derives from the activation of the apoptotic program.[26–28] In this work, we have explored the possibility that L-carnitine may modulate the effects of ocular hypertension. This hypothesis is based on the observation that this substance is able to improve the mitochondrial metabolism[29] and has an antiapoptotic action.[30,31] The action of L-carnitine is related to the improvement of mitochondrial performance as defects of these organelles have a key role in the completion of the apoptotic process.[32,33] Several studies show that L-carnitine has a neuroprotective action in different neurodegenerative pathologies such the syndromes of Parkinson and Alzheimer, wherein mitochondrial malfunction and oxidative stress are also observed.[34,35] The potential therapeutic role of L-carnitine has been reported both for AIDS and ischemia wherein apoptosis is also involved.[36,37] Its protective role has been ascribed to the ability to inhibit the activation of the caspase cascade.[30,31] It has been recently reported that, in addition to L-carnitine, its acetylated derivative is also able to reduce apoptosis in primary neuron cultures and in stable mouse fibroblast lines.[38] The antiapoptotic action was shown to derive from the capacity of L-carnitine to restore and stimulate typical mitochondrial functions such as the activity of pyruvate dehydrogenase.[29,38] Considering all these, we investigated the role of this compound in neuroprotection and in the control of degenerative and apoptotic phenomena. Finally, it has been reported that following ischemia/reperfusion at retinal level, a considerable increase of lipid peroxidation occurs.[39] Our data demonstrate that treatment with L-carnitine significantly reduces the level of membrane peroxidation.

Results and Discussion

Carnitine reduces the expression of stress markers at retinal level. After induction of the hypertensive stress following methylcellulose (MTC) injection in the anterior chamber, the expression of GFAP is increased at the level of RGC and in the Muller cells as evidenced from the morphological alterations of the retinal layers (Figure 1a, 3). After induction of the hypertone and parallel treatment with carnitine, the expression of this marker of glial cells is significantly reduced (Figure 1a, 2). In controls a basal expression of GFAP is indeed observed, but this is topographically limited to the inner retinal layer (Figure 1a, 1) both in controls and in animals not subjected to the hypertensive shock but simply treated with carnitine (not shown). The reduced expression of this commonly accepted marker of neuronal damage[8,16] is a clear indication that carnitine is able to reduce the neurotoxic consequences of the hypertensive stress.

Inducible NOS (iNOS) is known as one of the main enzymes involved in the mitochondrial lipid peroxidation and in neuropathy.[20] As the neuronal damage is reduced, in our experimental model by peroxide scavengers,[8] we examined the possibility that the hypertensive shock could induce upregulation of iNOS. The results reported in Figure 1b show that the hypertension derived by administration of methylcellulose[8] causes a dramatic increase of iNOS, which is involved in the execution of the apoptotic pathway (lane 3). Carnitine administered to the animal along with methylcellulose strongly reduces the expression of this gene product (lane 2). Therefore, carnitine improves the overall homeostatic response to the hypertensive insult and is able to limit apoptotic phenomena.

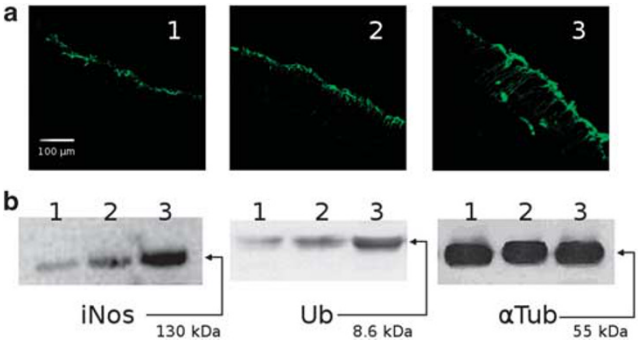

Figure 1 Reduction of the expression of stress markers at retinal level induced by carnitine. (**a**) Immunolocalization of GFAP assessed by immunofluorescence in rat retina. 1: retina from untreated control eye. 2: Contralateral eye treated with methylcellulose in the presence of carnitine. 3: Eye treated only with methylcellulose. Magnification was × 500 in all cases. (**b**) Western blot on total protein from retina extracts to assess the expression of iNOS, ubiquitin and α-tubulin as standard reference. In this and subsequent figures, lanes 1, 2, and 3 report the results obtained in the same experimental conditions as in panel a; that is: control, MTC plus L-carnitine and MTC alone

Inducible nitric synthase is overexpressed in response to an oxidative stress and is also involved in the onset of apoptosis. Literature data exist showing that the Ub-mediated proteasome pathway is activated as a consequence of an oxidative stress in retinal endothelial cells and in cultured cells.[25,26] The result in Figure 1 shows that a basal level of ubiquitin is detected in a total protein extract from control retinas (lane 1). After induction of hypertone, a strong positivity to Ub is detected (lane 3), which is significantly reduced in the retina concomitantly treated with carnitine (lane 2). The reduction of Ub is a signal of improved cell homeostasis, which implies a reduced protein degradation caused by the stress response. As matter of fact, the activation of the Ub/proteasome pathway is directly related to the execution of the apoptotic death.[8,26–28] In conclusion, carnitine is able to mediate a reduction of the stress markers whose upregulation is caused by the hypertensive stress due to the MTC treatment in the experimental glaucoma. As carnitine reduces the intracellular concentration of typical markers of cell sufferance and/or apoptosis, our data strongly suggest that the drug exerts a negative control on the progression of apoptotic death at retinal cell level.

Apoptotic cell death consequent to the ocular hypertone is controlled in the presence of carnitine. To assess weather carnitine can actually reduce the execution of apoptosis after induction of the hypertone, we evaluated the extent of cell death by TUNEL staining and detection of a commonly accepted marker of apoptosis: caspase 3. This double assessment is necessary as the TUNEL fluorescent reaction may generate ambiguous results. As a matter of fact, this method is not sufficiently specific to discriminate

between necrosis and apoptosis in spite of the fact that this method is often utilized to evaluate this type of cell death.[40] Figure 2a shows the results of a typical TUNEL fluorescent reaction in the presence (2) and in the absence (3) of carnitine. Results show that the drug strongly reduces the fluorescent reaction. This can be rationalized as a decrease of DNA fragmentation as this process is associated with cell death at the level of astrocytes and RGCs. Caspase 3 is a protease involved in the triggering and execution of apoptosis. Data shown in Figure 2b evidenced that in hypertensive/MTC-treated retinas, a dramatic expression of caspase 3 occurs (lane 3). The level of this enzyme is strongly reduced if the hypertensive shock is paralleled by treatment with carnitine (lane 2). Therefore we can reasonably conclude that the drug is able to reduce significantly the execution of the cell death program.

It is known that carnitine has a role in membrane stabilization and function. In particular, this compound is involved in the transport of long-chain fatty acid at the level of the mitochondrial membrane.[29–31,35] This results in an improved energy metabolism, which counteracts possible cell death processes activated at mitochondrial level, as also suggested in a previous work on an analogous acetylated molecule. As a matter of fact, the mitochondrial membrane stabilization is supported by the observation that administration of acetylcarnitine to cultured cells decreases the release of cytochrome *c* from the mitochondria.[38] On the basis of the observations, we hypothesized that carnitine, a natural substance normally produced in the organism, could limit apoptosis by a reduction of lipid peroxidation. This reduction would stabilize the mitochondrial membrane thus improving the overall energy metabolism. This hypothesis was probed measuring the production of malondialdehyde (MDA) generated at membrane level after oxidative.

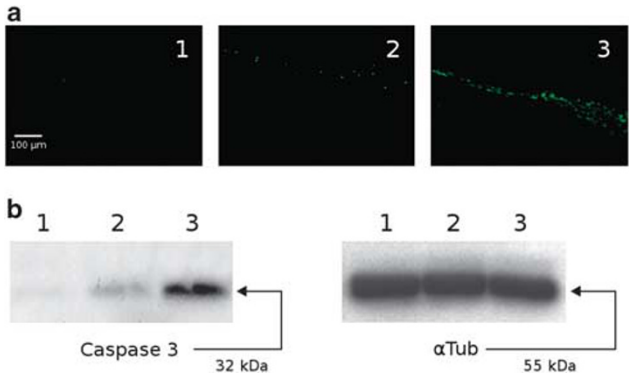

Figure 2 Carnitine reduces apoptotic cell death consequent to the ocular hypertone. (**a**) Evaluation of DNA damage by TUNEL assay. Magnification was × 500 in all cases. (**b**) Western blot on retina total protein extracts to assess the expression of caspase 3. Data shown in 1, 2 and 3 report in the same order the results obtained in the experimental conditions as in Figure 1

Morphological analysis of the glaucomatous cupping of the optic disk. Pathological optic-disk cupping is very often caused by glaucoma. Many different causative agents, not associated with elevated IOP or glaucomatous optic nerve disease, may result in pathological optic nerve excavation. In a previous work a significant pathological cupping of the heads of the optic nerve, as a consequence of loss of astrocytes and nerve fibers, was observed.[8] However, in the case of a hypertensive treatment in the presence of carnitine, a dramatic reduction of the eye to cup phenomenon is observed (Figure 3). Optic-disk cupping is a consequence of myriad disorders, but in this case its reduction is evidently due to a reduced oxidative insult, which also produces a better control of cell homeostasis with consequent rescue of astrocytes and nerve fibers.

The oxidative stress induces an increase of membrane damage. The extent of membrane damage was assessed by measuring the production of MDA, a widely recognized marker of lipoperoxidation whose production occurs in cells directly from a damage to the membrane structure and function. Samples treated with MTC, on the other hand, show extensive lipoperoxidative damage, as shown by the very high cytoplasmic MDA concentration (Figure 4a, bar to the left). However, the data presented here clearly show that carnitine is able to only moderately protect the cells from the apoptotic death due to oxidative stress. As a matter of fact, the MDA levels monitored in retinas treated with this compound in the presence of methylcellulose are only mildly reduced (Figure 4a, central bar), and therefore the apoptotic cell death monitored in our model system is not only due to an oxidative stress. As matter of fact, carnitine reduces the production of MDA but does not restore the cell vitality: thus MTC induces apoptosis by mechanisms that are not solely ascribable to mitochondrial damage.

In summary, the injection of methylcellulose into the anterior chamber of the eye activates diverse cellular and molecular signals of stress at the level of RGCs, such as the upregulation of the GFAP and DNA damage. Methylcellulose essentially hinders the efflux of fluids from the canals of Schlemm. This increases the IOP, which eventually results in the establishment of an oxidative stress. This stress is shown by the overexpression of iNOS, an enzyme primarily involved

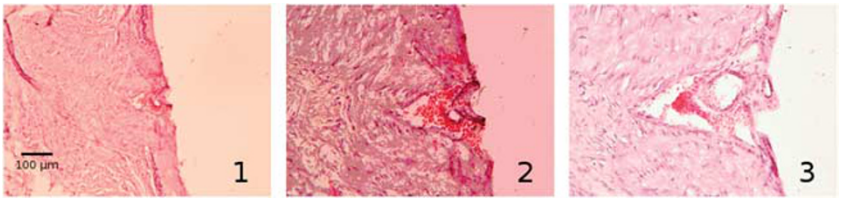

Figure 3 Morphological alterations of cup-to-eye. Control retinal samples (1); methylcellulose treated retinas in the presence (2); or absence of carnitine (3). Magnification was × 500 in all cases

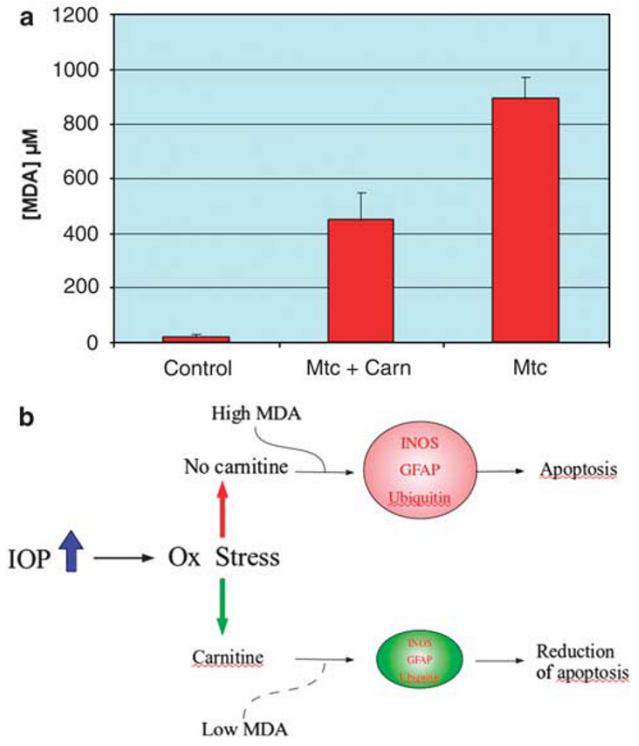

Figure 4 Level of membrane lipoperoxidation in retina samples and role of L-carnitine in the control of apoptosis. (**a**) Bars report the level of malondialdehyde (MDA) evaluated as indicated at the bottom of each bar. Mtc, methylcellulose; Carn, L-carnitine. (**b**) The increase of the intraocular pressure (IOP) results in oxidative stress as shown by the overexpression of inducible nitric oxide synthase (iNOS). Mitochondrial lipid peroxidation causes the accumulation of intracellular MDA: a hallmark of lipoperoxidation. The activation of the ubiquitin (Ub)-mediated proteasome pathway is directly related to the execution of the apoptotic death as also shown by the stimulation of caspase 3 expression. Treatment with methylcellulose in the presence of carnitine reduces the level of all markers of apoptosis, and therefore L-carnitine also improves the overall homeostatic response and limits apoptotic phenomena

in the mitochondrial lipid peroxidation, with consequent damage of the cell membrane, which is validated by the accumulation of intracellular MDA: a hallmark of lipoperoxidation. The Ub-mediated proteasome pathway is also activated and this is directly related to the execution of the apoptotic death as shown by the stimulation of caspase 3 expression. The antiapoptotic role of carnitine is illustrated in (Figure 4b). This compound has a key role in the stabilization and function of the cell membrane. Treatment with methylcellulose in parallel with carnitine reduces the level of all markers of cell sufferance and apoptotis. In conclusion, by enhancing the mitochondrial performance, carnitine also improves the overall homeostatic response to the hypertensive insult and limits apoptotic phenomena.

Materials and Methods

Animal maintenance, induction of hypertone and carnitine treatment. Male adult Wistar rats were used throughout the work. All experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the Institutional Animal Care and Use Committee. General anesthesia was done by intraperitoneal injection of a nonbarbiturate anesthetic in physiological solution administered according to the supplier recommendations (sodium-thiopental solution (Farmotal, Pharmacia & Upjohn, USA)). Local anesthesia was done by administration of eye drops of 0.4% oxibuprocaine (Novesine, Novartis, Basel, Switzerland). Ocular hypertension was induced according to a method developed in our laboratory. Briefly, animals were injected in the anterior chamber of the right eye with MTC (Sigma-Aldrich, St. Louis, MO, USA) in physiological solution. The contralateral eye was used as control and injected only with physiological solution. The IOP was monitored by tonometry (Tonopen XL, Automated Ophthalmics, Ellicott City, MD, USA). For further details refer Calandrella *et al.*[8] Treatments with L-carnitine (Sigma-Tau, Industrie Farmaceutiche Riunite, Pomezia Roma, Italy) were done by dissolving the drug in a methylcellulose suspension at the indicated concentrations. The final concentration of carnitine is referred to the volume, about $300 \pm 20\ \mu$l, as estimated by weighing the content of each control eye.

Morphological, molecular analysis of tissue sections and of retina total protein extracts

Sample preparation. Eyes were removed and the corneas were eliminated at limbus level; vitreous humor and crystalline lens were discarded. The samples thus obtained were fixed in 4% paraformaldehyde for 6 h at 4°C and quickly washed in PBS. Samples were then soaked in a 30% sucrose solution for 6–8 h at 4°C, finally included in freezing resin (OCT, Killik, Bio-Optica, Milan, Italy) and cryostat-cut in longitudinal sections (10-μm thick).

Cell biology assays. Immunolocalization was performed on GFAP. Sections were dehydrated, treated with Triton X 100 (0.1% v/v) for 30 min and blocked in 1% bovine serum albumin for the same time. Endogenous peroxidases were neutralized with hydrogen peroxide (0.3% in PBS for 20 min). GFAP was evidenced by an anti-GFAP (Sigma-Aldrich) and a fluoresceinated fluorescent secondary antibody (Alexa Fluor, Molecular Probes). Fixed sections were stained with 0.1% hematoxylin QS for 1 min (nuclear chromatin); after washing in PBS, tissue sections were incubated for 2 min in 1% eosin to stain the cytoplasm; this step was followed by optical microscope observation.

DNA fragmentation was evidenced by TUNEL reaction (*in situ* cell death detection kit, AP/Roche)

Biomolecular assays. Western blottings and lipoperoxidation assays were performed on control and carnitine-treated retinal cell extracts. Total protein extracts were prepared by standard procedures and analyzed by PAGE using the following antibodies: caspase 3 active form, iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ub (Sigma-Aldrich). α-Tubulin was used as standard reference (Sigma-Aldrich).

To determine the oxidative stress at membrane level, we used a quantitative assay based on the intracellular production of MDA measured by the commercial kit LPO-586 (Oxis Health Research Products Portland, OR, USA). The chromophore, *N*-methyl-2-phenylindole (NMP) reacts with MDA after incubation at 45°C. A single MDA molecule reacts with NMP molecules generating a stable chromophore whose absorbance can be spectrophotometrically measured at a 586 nm. Absorbance values can be directly converted in molar concentration of MDA.

Statistical analysis. All the experiments were repeated at least three times. Statistical analysis of results was made by the Student's *t*-test or by a two-way analysis of variance followed by the Student's *t*-test. *P*-values < 0.05 were considered significant.

Conflict of interest

The authors declare no conflict of interest.

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