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Trehalose: A biophysics approach to modulate the inflammatory response during endotoxic shock

Letteria Minutoli [a], Domenica Altavilla [a], Alessandra Bitto [a], Francesca Polito [a], Ersilia Bellocco [b], Giuseppina Laganà [b], Tiziana Fiumara [a], Salvatore Magazù [c], Federica Migliardo [c], Francesco Saverio Venuti [d], Francesco Squadrito [a,*]

[a] Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Messina, Italy
[b] Department of Organic and Biologic Chemistry, University of Messina, Messina, Italy
[c] Department of Physics, University of Messina, Messina, Italy
[d] Department of Neuroscience, Psychiatry, and Anaesthesiology, University of Messina, Messina, Italy

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ABSTRACT

We evaluated the effects of trehalose against endotoxic shock, a condition in which the loss of bio-membrane integrity plays a pivotal role. In addition we performed a biophysics experiment by Quasi Elastic Neutron Scattering (QENS) study, to investigate whether the membrane stability effect of trehalose might be correlated with its high capability to switch-off the water diffusive dynamics and, hence, the kinetic mechanisms of interaction. Endotoxic shock was induced in male rats by a single injection of Salmonella enteritidis lipopolysaccharide (LPS; 20 mg/kg/i.p.). Thirty minutes before and 2 h after LPS injection, the animals were randomized to receive vehicle (1 ml/kg/i.p. 0.9%NaCl), sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.). Mean arterial blood pressure, Nuclear Factor-κß (NF-κB) binding activity, Iκ-Bα and Toll-like receptor-4 (TLR-4) activation were evaluated in both liver and lung. Plasmatic tumor necrosis factor-α (TNF-α), Interleukin-1 (IL-1), Interleukin-6 (IL-6) and malondialdehyde (MDA) were also investigated. We studied liver injury by means of blood alanine aminotransferase activity (ALT); inducible nitric oxide synthase (iNOS) expression, myeloperoxidase (MPO) activity and tissue edema evaluation. Lung injury was investigated by means of tissue monocyte chemoattractant protein-1 (MCP-1) levels, MPO activity, iNOS expression and edema formation. Trehalose reduced hypotension, NF-κB binding activity, IκBα protein loss and TLR-4 activation. In addition trehalose reduced TNF-α, IL-1, IL-6 and MDA levels. Trehalose also blunted liver and lung injury. QENS measurements showed also that trehalose possesses a high "switching off" capability. Sucrose did not modify endotoxic shock-induced sequelae. Trehalose blocked the inflammatory cascade triggered by endotoxin shock, stabilizing the bio-membranes and switching off the water diffusive dynamics.

1. Introduction

Severe sepsis and septic shock are common and deadly conditions and their epidemiology, pathogenesis, and management are evolving continuously. Furthermore, those pathological conditions are the leading causes of death in intensive care units in developed countries (Annane et al., 2005). Sepsis and its sequelae represent a continuum of clinical syndrome encompassing systemic inflammation, coagulopathy, and hemodynamic abnormalities followed by tissue injury and multi-organ failure (Sharma and Kumar, 2003). Sepsis is usually initiated by microbial agents or their products, such as lipopolysaccharide (LPS). LPS is the principal component of the outer membrane of Gram-negative bacteria and is recognized by monocytes and macrophages. LPS binds CD14 receptor, a 55-kDa glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein which is present on the surface of monocytes (Schumann, 1992; Goyert et al., 1998; Simmons et al., 1989).

During sepsis, the inflammatory cytokines play a major role, especially tumor necrosis factor-α (TNF-α), a pleiotropic cytokine secreted by different cell type, including macrophages and mastocytes, in response to various stimuli, including LPS (Beutler, 1992). LPS administration in animals has been widely used over many years by numerous researches to induce a state of endotoxic shock that bears many similarities to the clinical condition of human septic shock (Rudkowsky et al. 2004; Demiralay et al. 2006; Madej et al. 2007; Xiao et al. 2007).

Endotoxic shock is also characterized by a marked expression of inducible nitric oxide synthase (iNOS), which catalyzes the production

* Corresponding author. Department of Clinical and Experimental Medicine and Pharmacology, Section of Pharmacology Torre Biologica 5th floor, "AOU" Policlinico G. Martino, Via C. Valeria Gazzi, 98124 Messina, Italy. Tel.:+39 090 2213648; fax: +39 090 2213300.
E-mail addresses: Francesco.Squadrito@unime.it, abitto@unime.it (F. Squadrito).

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of NO (Nussler and Billiar, 1993). NO acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation. Overproduced NO is oxidized to reactive nitrogen species and results in the disruption of cell signalling and uncontrolled systemic inflammation. The iNOS gene is regulated transcriptionally in part by nuclear factor-κB (NF-κB) activation (Xie et al., 1994). NF-κB is sequestered in the cytoplasm in an inactive state because of its association with inhibitory κB (IκB), (Baeuerle and Henkel, 1994). Under stress conditions (Maulik et al., 1999; Armstead et al., 1999) phosphorylation of IκB by IκB kinase (IKK) occurs, which leads to degradation of IκB and disruption of the NF-κB/IκB complex. The dissociated NF-κB subsequently translocates from the cytoplasm to the nucleus, where it binds to the κB promoter region of target genes, including iNOS (Xie et al., 1994; Nathan and Xie, 1994).

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a disaccharide capable of protecting bio-molecules against environmental stress and may inhibit the inflammatory cascade that in turn cause oxidative damage and cytokines production (Benaroudj et al., 2001; Yoshizane et al., 2000; Chen and Haddad, 2004). In fact, previous studies demonstrated that trehalose reduces serum TNF-α and moreover prevents mortality (Minutoli et al., 2006) in LPS injected rats. Several experimental findings obtained by spectroscopic techniques (Branca et al., 2001; Magazù et al., 2001) indicate that the structural properties of water are drastically perturbed by disaccharides, and in particular by trehalose. More specifically, neutron diffraction results (Branca et al., 2002) show for all disaccharides, a strong perturbation of the peaks linked to the hydrogen-bonded network that can be attributed to the destroying of the tetrahedral coordination of pure water.

Aim of the present study was to evaluate the protective effect of trehalose against the pathological sequelae associated with endotoxic shock induced by lipopolysaccharide (LPS). Furthermore we carried out a biophysics experiment by a Quasi Elastic Neutron Scattering (QENS) study, to investigate whether the proposed membrane stability effect of trehalose might be correlated with its high capability to switch-off the water diffusive dynamics and, hence, the kinetic mechanisms of interaction.

2. Materials and methods

2.1. Animals

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD). Male Sprague–Dawley rats (250–300 g) fed on a standard diet and with tap water ad libitum, in a 12 h light–dark cycle were used. The temperature of the animals was maintained at approximately 37 °C using an overhead lamp.

2.2. Endotoxin shock procedure

Rats were anaesthetized with ether and cannulae (PE 50) were inserted into the left common carotid artery for cardiovascular measurements and into the right jugular vein to allow intravenous injection and facilitate blood withdraw. Twenty-four hours after surgery endotoxin shock was induced in animals by a single intravenous injection of Salmonella enteritidis LPS (20 mg/kg; LD_{90}). The dose was chosen in agreement with previous reports (Rudkowski et al. 2004; Demiralay et al. 2006; Madej et al. 2007; Xiao et al. 2007). Sham rats received an equal volume of vehicle (NaCl). Thirty minutes before and 2 h after LPS injection animals were randomized to receive vehicle (1 ml/kg/i.p. of a 0.9% NaCl solution), sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.). Trehalose dose was chosen in agreement with our previously published observations (Minutoli et al. 2006).

A first group of animals was followed for 300 min following endotoxin administration and it was used to monitor blood pressure and to evaluate circulating inflammatory mediators and tissue injury; a second group of animals was sacrificed 1 h after LPS challenge to study NF-κB activation and IκB-α and TLR-4 expression.

2.3. Arterial blood pressure

Briefly, the animals were anesthetized with an intraperitoneal injection of 50 mg/kg of pentobarbital sodium and the cannula inserted into the left common carotid artery was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was monitored for 5 h and displayed on channels of a polygraph (Basile, Varese, Italy). Arterial blood pressure is reported as mean arterial pressure in mmHg.

2.4. Isolation of cytoplasmatic and nuclear proteins

Briefly, pulverized liver and lung samples were homogenized in 1 ml lysis buffer (25 mM Tris/HCL, pH 7.4, 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A (10 ug/ml each) and Na_3VO_4 100 mM,) with a Dounce homogenizer. The homogenate was subjected to centrifugation at 15,000 g for 15 min. The supernatant containing cytoplasmatic protein was collected and stored at −80 °C. The pellets after a single wash with the hypotonic buffer (10 mM Hepes pH 7.9, 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A (10 ug/ml each) and Na_3VO_4 100 mM) were suspended in an ice-cold (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, protease and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently and centrifuged for 15 min at 4 °C. The supernatants were collected as nuclear extracts and stored at −80 °C. The concentration of total proteins was determined using the bio-rad protein assay kit (Bio-Rad, Richmond, CA, USA).

2.5. Determination of NF-κB

NF-κB binding activity was performed in a 15-μl binding reaction mixture containing 1% binding buffer (50 μg/ml of double-stranded poly(dI.dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$ and 10% glycerol), 15 μg of nuclear proteins, and 35 fmol(50,000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT AGG C-3′; Promega, Madison, WI, USA) which was end-labeled with [γ-^{32}P] (3000 Ci/mmol at 10 mCi/ml; Amersham Life Sciences, Arlington Heights, IL) using T4 polynucleotide kinase. The specificity of the binding reaction was assessed using an excess (50-fold over the probe) of unlabeled oligonucleotides added into the reaction mixture. The latter eliminated competitively the induced bands. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis on 5% not denaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at −70 °C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results of each group were expressed as relative integrated intensity compared with the liver and lung protein extracts obtained from sham operated group and measured in the same batch, because the integrated intensity of group samples from different electrophoretic mobility shift assay (EMSA) batches would be affected by the half-life of the isotope, exposure time, and background levels.

2.6. Determination of IκBα, iNOS and TLR-4

Protein samples (50 μg) were denatured in reducing buffer (62 mmol Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol,

0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mmol glycine, 48 mmol Tris pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were stained with Ponceau' S (0.005% in 1% acetic acid) to confirm equal amounts of protein and blocked with 5% non fat dry milk in TBS-0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS-0.1% Tween, and incubated with a primary for the phosphorylated form of IκBα (Cell Signaling, USA) for iNOS (Chemicon, Temecula, CA.) and for TLR-4 (Santa Cruz) in TBS-0.1% Tween overnight at 4 °C, diluted 1:1000. After being washed three times for 10 min each in TBS-0.1% Tween, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, UK) for 1 h at room temperature diluted 1:20,000. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham, UK). The iNOS ,IκBα and TLR-4 protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil, Celbio, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with control measured with the same batch. Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling, USA) diluted 1:500 and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, UK) diluted 1:15,000. All antibodies are purified by protein A and peptide affinity chromatography.

2.7. Nitrite production

Nitrite concentration was measured in a standard Griess reaction. 100 μL of liver and lung lysate were incubated with an equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrocloride in 2.5% phosphoric acid). After 10 min of incubation at room temperature, the absorbance of the cromophore so formed was measured at 540 nm using a microtiter plate reader. Nitrite concentrations were calculated by comparison with a standard calibration curve with sodium nitrite ($NaNO_2$: 1.26 to 100 μmol/l), with control baseline supernatant as the blank.

2.8. Malondialdehyde measurement

Determination of malondialdehyde (MDA) levels was carried out in blood samples. The blood was collected in polyethylene tubes to which has been added 10 μl of heparin solution. Plasma samples were obtained after centrifugation. The assay was carried out by using a colorimetric commercial kit (Calbiochem, USA). Briefly, 0.65 ml of 10.3 mM *N*-methyl-2phenyl-indole in acetonitrile was added to 0.2 ml of samples. After vortexing for 3–4 s and adding 0.15 ml of HCL 37%, samples were mixed well and closed with a tight stopper and incubated at 45 °C for 60 min. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at 586 nm.

2.9. Evaluation of IL-1, TNF-alpha and IL-6

Blood (750 μl) was drawn following endotoxin challenge. Plasma interleukin-1 (IL-1), Tumor Necrosis Factor-α (TNF-α) and interleukin-6 (IL-6) were determined by commercially available ELISA kits (Endogen USA).

2.10. Serum ALT evaluation

Blood (750 μl) was drawn following endotoxin challenge. The collected blood was processed separately to obtained serum to determine alanine aminotransferase (ALT). Serum ALT activity was determined using a commercial available kit (Sigma Chemical CO, St Louis MO).

2.11. MCP1 concentration in the lung

The concentration of monocyte chemoattractant protein-1 (MCP-1) in tissue samples was measured following endotoxin administration using commercially available antibodies and according to procedure supplied by manufacturer (R&D System, Minneapolis).

2.12. Mieloperoxidase activity

Mieloperoxidase activity (MPO) was evaluated as an index of neutrophil accumulation in the injured tissues. Briefly, liver and lung samples were suspended in 1 ml buffer (0.5% hexadeeyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenate was cleared by centrifuging at 12,000 g for 30 min at 4 °C and the supernatants were used to measure MPO activity. An aliquot of the supernatant was incubated with a substrate o-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl 50 mM phosphate buffer, 3 μl substrate solution (containing 20 mg/ml o-dianisidine hydrochloride) and 3 μl H_2O_2 (20 mM). Samples (10 μl) were added to each well to start the reaction. Standard MPO was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 μl sodium azide (30%). The absorbance was measured spectrophotometrically at 450 nm. MPO activity was expressed as U/g tissue.

2.13. Evaluation of pulmonary and hepatic edema

To quantify the magnitude of pulmonary and hepatic edema, we evaluate the wet/dry weight (W/D) ratio of the lung and the liver. Briefly, portions of the harvested wet lungs and livers were weighed and then placed in an oven for 24 h at 80 °C, and weighed again when they were dry. The W/D ratio was then calculated.

2.14. Neutron spectroscopy

Additional experiments dealing with the chemical and physical properties of trehalose, were also performed to elucidate the insights of its protective mechanism. Ultrapure powdered trehalose, maltose, sucrose, D_2O and H_2O purchased by Aldrich–Chemie, were used for both experiments. Measurements were performed in a temperature range of 273–353 K on trehalose, maltose, sucrose ($C_{12}H_{22}O_{11}$) in H_2O and on partially deuterated trehalose, maltose, sucrose ($C_{12}H_{14}\ D_8O_{11}$) in D_2O at weight fraction values corresponding to 19 water (H_2O and D_2O) molecules for each disaccharide molecule. All the tree disaccharides possess hydrogen atoms, belonging to the OH groups, which exchange easily the deuterium atoms of heavy water. To focus our attention on the disaccharide dynamics in D_2O, the exchangeable atoms were substituted with deuterium before preparing the solutions. We estimated that in the deuterated solutions (at the investigated concentration) the coherent contribution to the total scattering cross section is ~5%. In order to obtain partially deuterated samples, the disaccharides were first dissolved in pure D_2O to exchange the eight labile hydrogen atoms of the disaccharides and subsequently the solutions were lyophilised.

The Quasi Elastic Neutron Scattering (QENS) experiment was carried out using the IRIS high resolution spectrometer at ISIS, the worlds leading pulsed neutron and muon source located at the Rutherford Appleton Laboratory (RAL, UK). The high resolution combined with the most powerful pulsed neutron source of the word make IRIS the best instrument to study the diffusive dynamics (The IRIS User Guide (2nd Edition) Adams et al. (2001), Appleton Laboratory, UK). We measured sets of QENS spectra covering a Q, Q,ω-domain extending from $\omega = -0.3$ to 0.6 meV (energy transfer) and $Q = 0.3$ to 1.8 $Å^{-1}$ (momentum transfer) with a mean energy resolution of $\Gamma = 8$ μeV (HWHM). The raw spectra were corrected and normalised

using the standard GENIE procedures and the IRIS data analysis package (MODES — IRIS Data Analysis (1st Edition), Telling and Howells (2003), Appleton Laboratory, UK).

2.15. Statistical analysis

All data are expressed as the mean ± S.D. Data were analyzed by analysis of variance for multiple comparison of results; the Duncan multiple-range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance.

2.16. Drugs

LPS, sucrose and trehalose were obtained from Sigma (Milan, Italy). All substances were prepared fresh daily and dissolved in 0.9% NaCl solution.

3. Results

3.1. Effects of trehalose on endotoxin shock

3.1.1. Trehalose inhibits arterial blood pressure

Rats injected with endotoxin showed a decrease in mean arterial blood pressure (Fig. 1). Trehalose significantly blunted the sustained decrease in arterial blood pressure (Fig. 1), while sucrose did not modify the decrease in arterial blood pressure (Fig. 1).

3.1.2. Trehalose reduces the activation of NF-κB in liver and lung

NF-κB activation in the nuclear extracts of liver and lung was determined by EMSA 1 h after endotoxin challenge. The top of Figs. 2A and 3A shows representative EMSA picture indicating activation of NF-κB. The bottom of Figs. 2A and 3A shows quantitative data. NF-κB binding activity was present at very low levels in liver and lung of sham shocked animals. In contrast, NF-κB was markedly increased in liver and lung of LPS treated animals (Figs. 2A and 3A). The administration of trehalose markedly reduced NF-κB binding activity, while sucrose did not modify the activation of NF-κB in liver and lung (Figs. 2A and 3A).

3.1.3. Trehalose inhibits the loss of IκBα protein in liver and lung

NF-κB activation was also indirectly investigated by studying its inhibitory protein Iκ-Bα in the liver and lung cytoplasm, 1 h following LPS challenge. The top of Figs. 2B and 3B shows representative Western blot analysis indicating the presence of Iκ-Bα protein in the cytoplasm of liver and lung obtained from sham shocked animals. The bottom of Figs. 2B and 3B represents quantitative data. Iκ-Bα levels were significantly reduced in liver and lung cytoplasm of septic rats treated with vehicle. The administration of trehalose blunted the consistent loss of Iκ-Bα protein from the cytoplasm, while sucrose did not modify the loss of this protein in liver and lung (Figs. 2B and 3B).

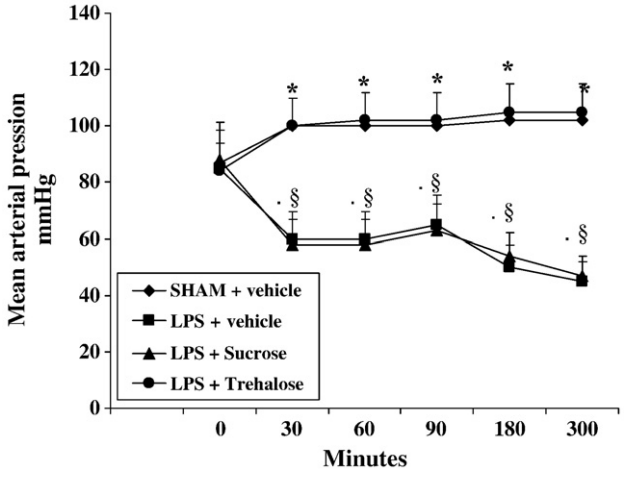

Fig. 1. Effects of vehicle (1 ml/kg/i.p.), sucrose (1 g/kg/i.p.) and trehalose (1 g/kg/i.p.) on mean arterial blood pressure (MAP) in endotoxin-shocked rats. Arterial blood pressure is reported in mmHg. Each value represents the mean ± S.D. of 10 animals. §$P<0.001$ vs SHAM + vehicle; *$P<0.001$ vs LPS + vehicle or sucrose.

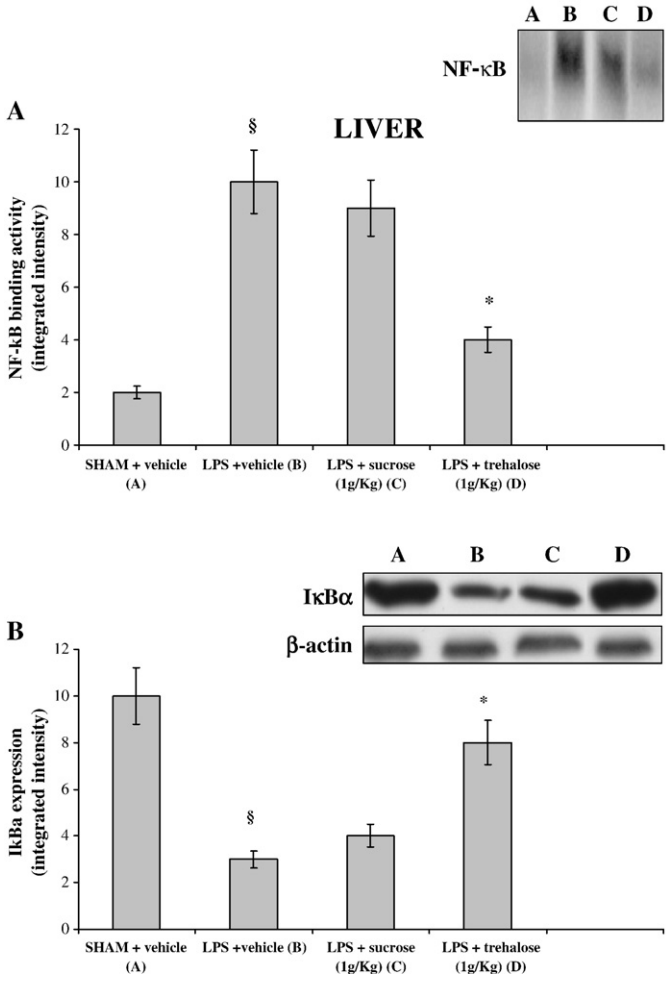

Fig. 2. Electrophoretic mobility shift assay (EMSA) of NF-κB binding activity in the nucleus (panel A) and Western blot analysis of IκBα protein levels in the cytoplasm of rat livers (panel B). Animals received vehicle (1 ml/kg/i.p.) or sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.) 30 min before and 2 h after the LPS challenge (20 mg/kg/iv). Each point represents the mean ± S.D. of 10 animals. §$P<0.001$ vs SHAM + vehicle; *$P<0.001$ vs LPS + vehicle or sucrose.

3.1.4. Trehalose inhibits TLR4 activation in liver and lung

No significant TLR4 expression was observed in liver and lung lysates from sham shocked animals (Fig. 4A and B). The top of Fig. 4A and B shows representative Western blot analysis. The bottom of Fig. 4A and B represents quantitative data. TLR4 activation in liver and lung was significantly increased in rats treated with vehicle, 1 h after LPS injection. In contrast, the treatment with trehalose significantly attenuated the raise in TLR4 expression, while sucrose did not modify LPS-induced TLR4 activation in liver and lung (Fig. 4A and B).

3.1.5. Trehalose inhibits iNOS activation and nitrite production in liver and lung

Neither significant iNOS expression nor measurable nitrite levels in liver and lung were observed in sham shocked animals (Figs. 5A, B, 6A and B). In endotoxin-shocked rats of iNOS expression and nitrite production were significantly increased 5 h after LPS injection in liver

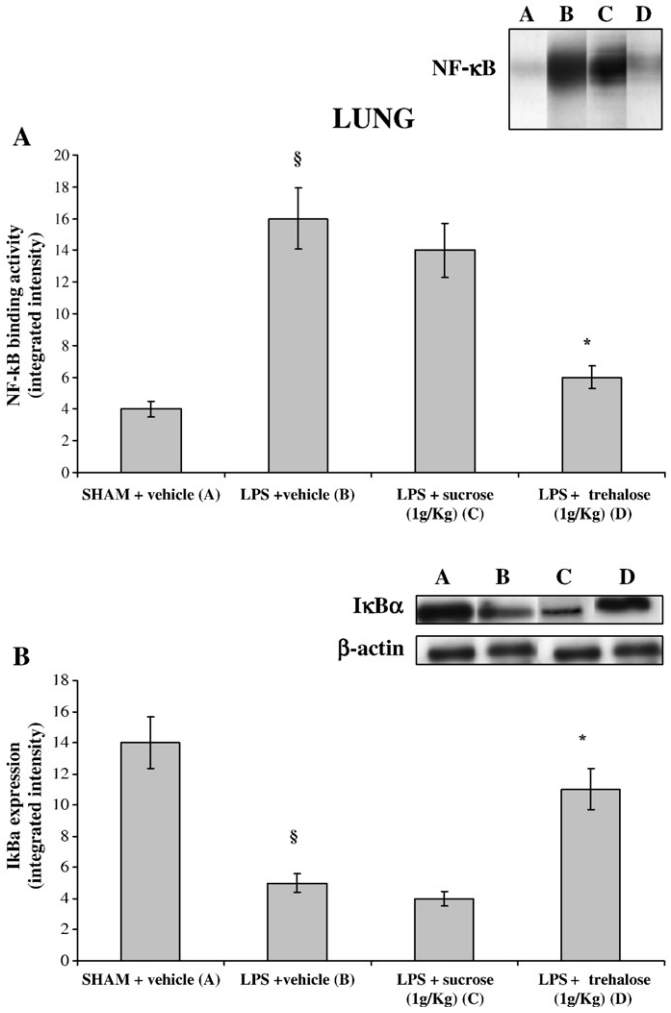

Fig. 3. Electrophoretic mobility shift assay (EMSA) of NF-κB binding activity in the nucleus (panel A) and Western blot analysis of IκBα protein levels in the cytoplasm of rat lungs (panel B). Animals received vehicle (1 ml/kg/i.p.) or sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.) 30 min before and 2 h after the LPS challenge (20 mg/kg/iv). Each point represents the mean ± S.D. of 10 animals. §$P<0.001$ vs SHAM + vehicle; *$P<0.001$ vs LPS + vehicle or sucrose.

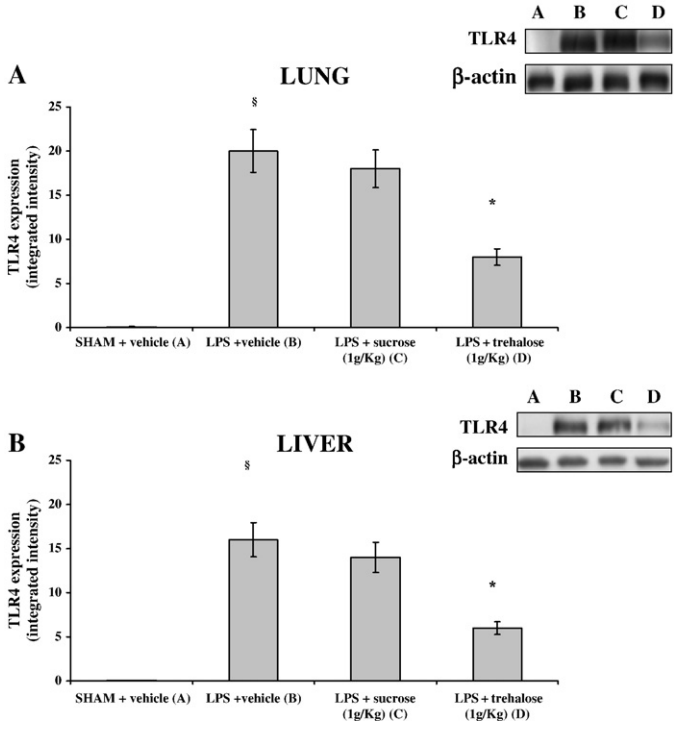

Fig. 4. Western blot analysis of TLR4 protein levels in rat lung (panel A) and liver (panel B). Animals received vehicle (1 ml/kg/i.p.) or sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.) 30 min before and 2 h after the LPS challenge (20 mg/kg/iv). Each point represents the mean ± S.D. of 10 animals. §$P<0.001$ vs SHAM + vehicle; *$P<0.001$ vs LPS + vehicle or sucrose.

and lung (Figs. 5A, B, 6A and B). In contrast, treatment with trehalose significantly attenuated the raise in either iNOS expression and nitrite release in liver and lung (Figs. 5A, B, 6A and B). Sucrose did not modify LPS-induced iNOS expression and did not change nitrite production in rats challenged with LPS (Figs. 5A, B, 6A and B).

3.1.6. Trehalose inhibits shock related pathophysiological parameters

Table 1 shows that a significant increase in the circulating levels of MDA, TNF-α, IL-6 and IL-1 were observed 5 h following endotoxin challenge. Administration of trehalose significantly decreased MDA, TNF-α, IL-6 and IL-1 levels in the bloodstream, while sucrose did not modify those parameters (Table 1).

3.1.7. Trehalose protects against liver injury

Serum ALT was analyzed in order to evaluate liver damage. Very low ALT levels were measured in samples obtained 5 h from sham shocked animals (Table 2). By contrast, LPS caused a marked increase in serum ALT. Administration of trehalose reduced serum ALT, while sucrose did not modify this parameter (Table 2).

Leukocyte infiltration was evaluated by means of the mieloperoxidase activity (MPO) in liver. MPO activity was significantly enhanced in liver 5 h following endotoxin challenge. Treatment with trehalose significantly blunted the increase in MPO activity, while sucrose did not change MPO content (Table 2). Finally, tissue edema was also measured to characterize the severity of endotoxin-induced shock. LPS-induced injury resulted in significant organ edema (Table 2). Administration of trehalose markedly reduced hepatic edema (Table 2), while sucrose did not modify organ injury.

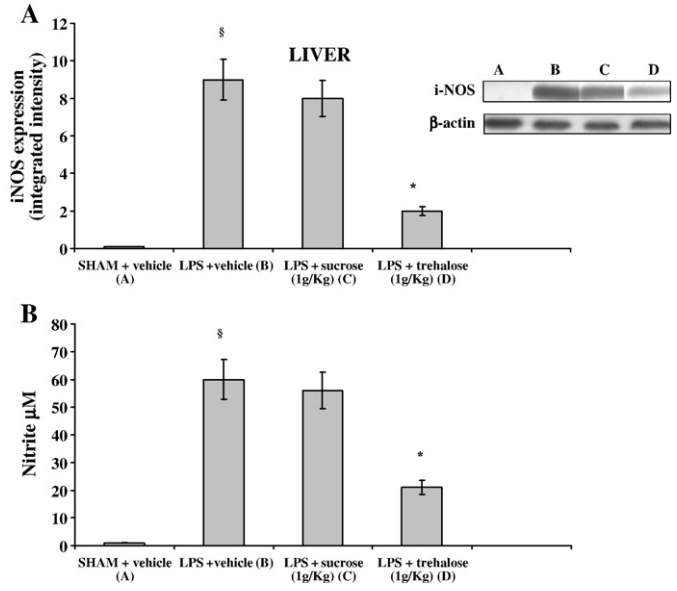

Fig. 5. Effects of vehicle, sucrose and trehalose on iNOS expression (panel A) and nitrite production (panel B) in liver of endotoxin-shocked rats. Animals received vehicle (1 ml/kg/i.p.) or sucrose (1 g/kg/i.p.) or trehalose(1 g/kg/i.p.) 30 min before and 2 h after the LPS challenge (20 mg/kg/iv). Each point represents the mean ± S.D. of 10 animals. §$P<0.001$ vs Sham + Vehicle; *$P<0.001$ vs LPS + vehicle or sucrose.

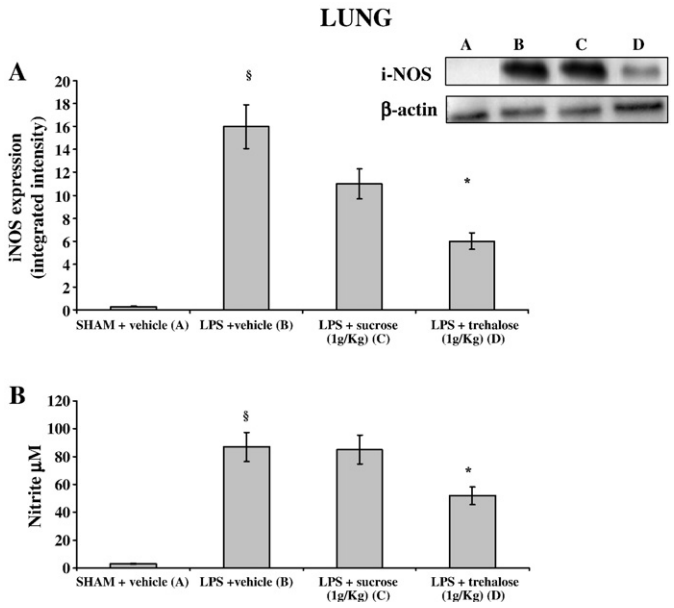

Fig. 6. Effects of vehicle sucrose and trehalose on iNOS expression (panel A) and nitrite production (panel B) in lung of endotoxin-shocked rats. Animals received vehicle (1 ml/kg/i.p.) or sucrose (1 g/kg/i.p.) or trehalose (1 g/kg/i.p.) 30 min before and 2 h after the LPS challenge (20 mg/kg/iv). Each point represents the mean±S.D. of 10 animals. §$P<0.001$ vs Sham+Vehicle; *$P<0.001$ vs LPS+vehicle or sucrose.

3.1.8. Trehalose protects against lung injury

Tissue monocyte chemoattractant protein-1 (MCP-1) was analyzed in order to evaluate lung damage. Very low MCP-1 levels were measured in lung lysates obtained 5 h from sham shocked animals (Table 3). By contrast, LPS caused a marked increase in MCP-1 in the lung (Table 3). Administration of trehalose reduced the chemokine in lung, while sucrose did not modify this mediator (Table 3).

Leukocyte infiltration was evaluated by means of the mieloperoxidase activity (MPO) in the lung. MPO activity was significantly enhanced in the lungs 5 h following endotoxin challenge (Table 3). Treatment with trehalose significantly blunted the increase in MPO activity, while sucrose did not change MPO content (Table 3).

Finally, tissue edema was measured to characterize the severity of LPS-induced injury resulted in significant organ edema (Table 3). Administration of trehalose markedly reduced pulmonary edema (Table 3), while sucrose did not modify organ injury.

3.2. Effects of trehalose on the dynamical properties of water

3.2.1. Quasi Elastic Neutron Scattering (QENS)

QENS measurements, performed both in partially deuterated disaccharides in D_2O and in disaccharides in H_2O, allow to separate the solute dynamics from that of the solvent. On the other hand NSE measurements on the same samples make possible to characterize the dynamics of the entire solute–solvent. The techniques show different aspects of the dynamical properties of the investigated solutions.

Table 1
Effects of trehalose on endotoxin shock (LPS) related pathophysiological parameters

Treatment	MDA (nmol/l)	TNF-α (pg/ml)	IL-1 (pg/ml)	IL-6 (pg/ml)
SHAM+vehicle	3.1±0.6	280±32	150±24	245±31
LPS+vehicle	50±7.0[a]	1820±208[a]	2534±304[a]	1932±149[a]
LPS+sucrose	48±6.5[a]	1765±172[a]	2340±202[a]	1876±178[a]
LPS+trehalose	10±1.2[b]	403±65[b]	524±121[b]	478±176[b]

Animals received vehicle (1 ml/kg/i.p.), trehalose (1 g/kg/i.p.) or sucrose (1 g/kg/i.p.) 30 min before and 2 h after LPS challenge (20 mg/kg/i.v.). Each point represents the mean±S.D. of 10 animals. [a]$P<0.002$ vs SHAM+vehicle; [b]$P<0.001$ vs LPS+vehicle or sucrose.

Table 2
Effects of trehalose on endotoxin shock (LPS) induced liver injury

Treatment	MPO activity (U/g tissue)	Tissue edema (W/D ratio)	Blood ALT (U/l)
SHAM+vehicle	0.45±0.03	2.82±0.8	48±14
LPS+vehicle	12±2.1[a]	5.48±1.5[a]	321±34[a]
LPS+sucrose	11±1.5[a]	5.32±1.2[a]	298±22[a]
LPS+trehalose	2±0.6[b]	2.06±0.9[b]	86±31[b]

Animals received vehzicle (1 ml/kg/i.p.), trehalose (1 g/kg/i.p.) or sucrose (1 g/kg/i.p.) 30 min before and 2 h after LPS challenge (20 mg/kg/i.v.). Each point represents the mean±S.D. of 10 animals. [a]$P<0.002$ vs SHAM+vehicle; [b]$P<0.001$ vs LPS+vehicle or sucrose.

We performed the fitting procedure on deuterated and non deuterated aqueous solutions of disaccharides according to the fitting function

$$S_{\mathrm{inc}}(Q,\omega) = A(Q)\Big\{ f_{\mathrm{Disaccharide}} \times \left[F(Q)\frac{1}{\pi}\frac{\Gamma_1(Q)}{\Gamma_1^2(Q)+\omega^2} + (1-F(Q))\frac{1}{\pi}\frac{\Gamma_2(Q)}{\Gamma_2^2(Q)+\omega^2} \right] + f_{\mathrm{hydr}}\frac{1}{\pi}\frac{\Gamma_3(Q)}{\Gamma_3^2(Q)+\omega^2} \Big\}$$

where the first two terms refer to the translational and rotational contribution of hydrated disaccharide ($f_{\mathrm{Disaccharide}}$ and f_{hydr} represent fraction factors of the total scattering from disaccharide and its strongly bonded water molecules), and the third one refers to hydration water ($f_{\mathrm{Disaccharide}}+f_{\mathrm{hydr}}=1$). Therefore the dynamical information of the diffusive dynamics of disaccharide can be obtained by the analysis of disaccharide+D_2O spectra analysis for which f_{hydr} results are negligible. The dynamical structure factor of disaccharide can be obtained by the spectra of deuterated samples in D_2O. Water dynamics has been analyzed in terms of a third water component. Even if the inclusion of a term describing the rotational diffusion of the mobile water fraction was possible such an analysis does not improve the fit quality.

In Fig. 6 the QENS data for trehalose, maltose and sucrose+$19H_2O$ and for trehalose at different temperature values, together with the fit components, are shown. As it can be seen the model gives excellent fits for both the spectra measured over the entire ω range.

In Fig. 7 the translational linewidth of three disaccharides and of trehalose for different temperature values is reported as a function of Q^2. The data can be fitted using the law:

$$\Gamma_1(Q) = D_s Q^2/(1+D_s Q^2 \pi)$$

where D_s is the self diffusion coefficient of the molecule and τ is the residence time.

This suggests that the HWHM of the trehalose translational dynamics follows the random jump diffusion (RJD) model. The lines represent the best fit according to the model. For trehalose as a function of temperature the RJD model furnishes for the diffusion

Table 3
Effects of trehalose on endotoxin shock (LPS) induced lung injury

Treatment	MPO activity (U/g tissue)	Tissue edema (W/D ratio)	MCP-1 (pg/100 mg of lung)
SHAM+vehicle	0.55±0.08	2.77±0.6	278±20
LPS+vehicle	16±2.4[a]	5.68±1.8[a]	1790±190[a]
LPS+sucrose	15±2.1[a]	5.45±1.5[a]	1830±177[a]
LPS+trehalose	3±0.9[b]	2.58±0.7[b]	568±42[b]

Animals received vehicle (1 ml/kg/i.p.), trehalose (1 g/kg/i.p.) or sucrose (1 g/kg/i.p.) 30 min before and 2 h after LPS challenge (20 mg/kg/i.v.). Each point represents the mean±S.D. of 10 animals. [a]$P<0.002$ vs SHAM+vehicle; [b]$P<0.001$ vs LPS+vehicle or sucrose.

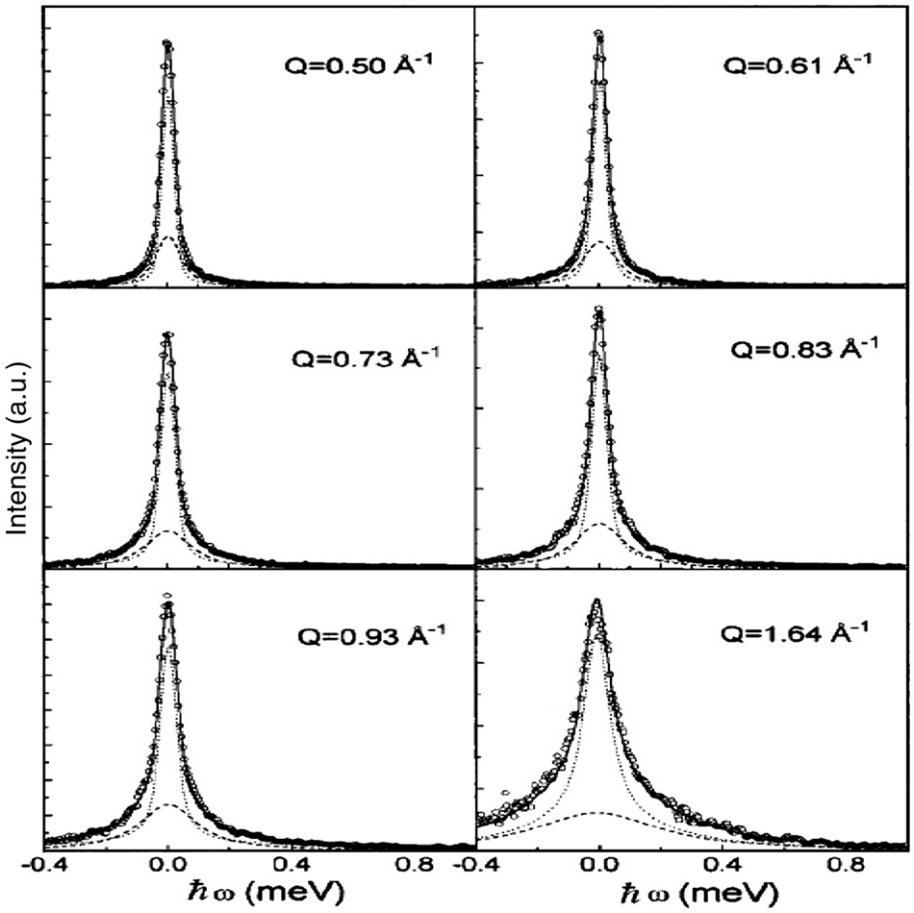

Fig. 7. Best fit of hydrogenated aqueous solutions of (a) trehalose for three temperature values and (b) of trehalose, maltose and sucrose at T=320 K. The total fit (black line) is composed by the disaccharide contribution (grey line), resulting by the fit of disaccharide/D_2O spectra, and the water contribution (light grey line), where only the translational part is present.

coefficient D_s and the residence time τ the values of D_s=2.83×10^{-7} cm^2/s and τ=24.7 ps, D_s=3.82×10^{-7} cm^2/s and τ=20.6 ps, D_s=5.35×10^{-7} cm^2/s and τ=19.1 ps and D_s=8.50×10^{-7} cm^2/s and τ=18.3 ps for T=283, 295, 308, 320 K, respectively. From the relation $<l^2>=6D\tau$ we obtain the values $<l^2>^{1/2}$=0.64 Å, 0.68 Å, 0.78 Å and 1.00 Å T=283, 295, 308, 320 K, respectively.

In Fig. 8 the translational linewidth behavior of water as a function of Q is reported for the three disaccharides and for trehalose for different temperature values. By the behavior of the translational linewidth of water, we conclude that the water dynamics in trehalose solutions for T=283, 295, 308, 320 K resembles that of water at ~256 K, ~261 K, ~263 K and ~268 K, indicating that the water has a diffusive behavior strongly triggered by trehalose molecules and suffers of a noticeable frozen effect, whereas it is similar to that of water at ~271 K in the case of maltose solution and at ~277 K in the case of sucrose solution. Analogously to trehalose aqueous solutions, all disaccharides show a slowing down effect on water dynamics, which is stronger for trehalose than for other disaccharides (Fig. 9).

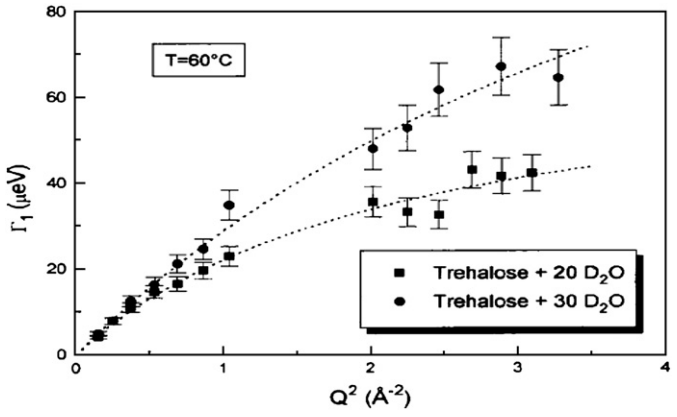

Fig. 8. Translational linewidth of (a) trehalose for different temperature values (b) of the three disaccharides is reported as a function of Q^2.

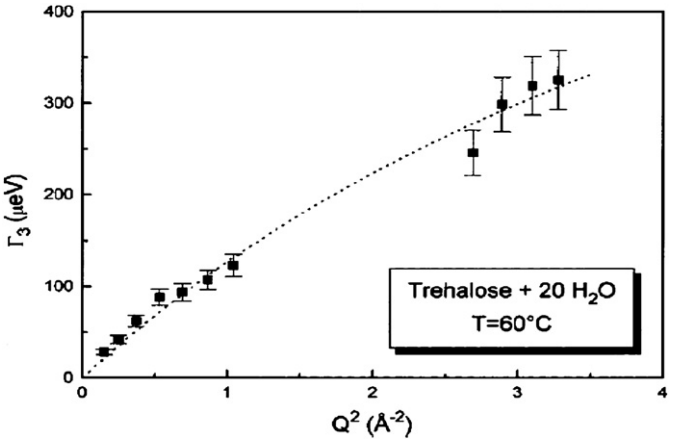

Fig. 9. Translational linewidth behavior of water as a function of Q^2 is reported in the presence of (a) the three disaccharides and of trehalose for different temperature values. In the insert the translational linewidth behavior of pure water as a function of Q^2 at the same temperature values is reported.

4. Discussion

Septic shock is a very serious disorder characterized by a high rate of morbidity and mortality and it frequently demands appropriate antibiotic and supportive therapy system. It is considered a generalized inflammatory response (Galley and Webster, 1996) that it has usually originated from an infectious nidus, which either invades the bloodstream, leading to proliferate locally, and/or releases various microbial products into the bloodstream patient.

The development of septic shock and the consequential multi-organ failure/dysfunction syndrome (MOF/MODS) correlate with poor outcome. The pathways leading to organ failures during sepsis can involve up-regulation of inflammatory responses and over-activation of the neuroendocrine system (Pathan et al., 2004; Schrier and Wang, 2004; Singer et al., 2004).

Several mechanisms are involved in the inflammatory response of septic shock, including the release of cytokines, the discharge of reactive oxygen species (ROS) (Pattanaik and Prasad, 1996) and over-production of NO, a mediator that, by amplifying oxidative stress (Sies and Mehlhorn, 1986; Ji et al., 1999), contributes to the pathophysiology of various disease, including septic shock, autoimmune diseases and chronic inflammation (Moncada et al., 1991; Arteel et al., 1999).

In the recent years trehalose has received increasing attention. It has been shown that trehalose can inhibit the inflammatory cascade and can prevent injury related to reactive oxygen species (Benaroudj et al., 2001; Yoshizane et al., 2000). Recently, this sugar has been accepted as a safe food ingredient by the European regulation system following approval by the US Food and Drug Administration. Experimental studies also suggest opportunities for trehalose as an excipient in the pharmaceutical industry, as a non-toxic cryoprotectant of vaccines and organs for surgical transplant (Colaco et al., 1992). All these data, taken together, would indicate that trehalose may represent a good candidate for halting in vivo the inflammatory potential.

To test this hypothesis we investigated the effects of trehalose on the inflammatory sequelae associated with endotoxin shock. LPS administration decreased mean arterial blood pressure and caused an increase in the circulating levels of several cytokines and MDA, a marker of oxidative stress. Trehalose administration succeeded in improving the drop in mean arterial blood pressure and in halting the inflammatory response caused by the endotoxin shock state.

Indeed the importance of several cytokines such as IL-1, IL-6 and TNF-α in the pathogenesis of endotoxic shock is well recognized (Beutler, 1992) and therefore the beneficial effects of trehalose could be linked to its ability to block the inflammatory response.

However we decided to further analyze trehalose mechanism of action. A common signalling molecule involved in several pathways is the ubiquitous inflammatory transcription factor, nuclear factor (NF)-κB. Our results demonstrate that trehalose inhibits NF-κB translocation to the nucleus in liver and lung and prevents the loss of the inhibitory protein IκBα from the cytoplasm. TLR-4 is a member of the Toll-like receptors (TLRs) family (Medzhitov and Janeway, 2000): its activation by endotoxin represents the up-stream signal that triggers all the inflammatory cascade in LPS-induced shock. Moreover NF-κB activation is regulated and induced by TLR4. Our findings also revealed that LPS enhanced TLR4 expression and trehalose decreased the expression of this receptor. These experimental findings led us to hypothesize that trehalose has protective effects against LPS-induced injury through a down-regulation of TLR4, which in turn attenuates NF-κB activation.

NF-κB can be also activated by elevated levels of reactive oxygen species (ROS), a situation that occurs in sepsis. In fact, administration of LPS augmented the production of MDA, a marker of lipid peroxidation and trehalose blunted the rise in MDA, thus indicating an alternative pathway by which the disaccharide may blunt the transcription factor activation.

Multi-organ failure/dysfunction syndrome (MOF/MODS) correlates with poor outcome during endotoxin shock. Therefore we also investigated end-organ damage in the liver and lung. The disaccharide treatment reduced leukocyte accumulation and edema in liver and lung and reduced the blood levels of ALT and the lung content of monocyte chemoattractant protein-1, a marker of pulmonary damage (Yeh et al. 2007).

Trehalose attenuated also the increase in either inducible nitric oxide synthase (iNOS) expression and NO content, both key components of a molecular pathway involved in tissue inflammation and injury (Kleinert et al., 2003).

However, the exact mechanism by which trehalose causes the molecular events able to block LPS-induced cellular activation is still far from being completely understood. It could be hypothesized that the disaccharide induces a slowing down of the diffusive dynamics of the surrounding matrix giving rise to hydrated complexes able to interact, via water molecules, with the polar head-groups of the lipid that constitute bio-membranes, in such a way to increase the stability of the biological structure and to enhance its resistance to external noxious stimuli. In this regard the QENS findings show that the dynamical properties of water result drastically perturbed by trehalose. More specifically these results clearly indicate that the disaccharide-water molecule interaction strength is much higher in respect to that between the water molecules. The disaccharides investigated in the present work, and trehalose with a greater extent, by the slowing down effect on the dynamics of water, at all the investigated temperatures, show a high "switching off" capability. This circumstance also implies a trehalose higher ability to hold volatile substances, coherently with its bio-protective effectiveness. However our "in vivo" data suggest that trehalose may have peculiar features that are not shared by other disaccharides such as sucrose. In fact in our experiment this other disaccharide had no effect on the pathological sequelae associated with endotoxin shock.

In summary these experimental evidences candidate trehalose as an attractive and safe biophysics approach to modulate inflammatory response in the critical ill patient.

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