Experimental and Molecular Pathology 97 (2014) 565–571

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Melatonin modifies cellular stress in the liver of septic mice by reducing reactive oxygen species and increasing the unfolded protein response

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ARTICLE INFO

Article history:
Received 24 October 2014
Accepted 28 October 2014
Available online 29 October 2014

Keywords:
Melatonin
Sepsis
Reactive oxygen species
Unfolded protein response
Acute phase response

ABSTRACT

Background & aims: Melatonin's hepatoprotective actions have numerously been demonstrated in the past but the underlying molecular mechanisms are widely unknown. For a better understanding of melatonin's effects on hepatic stress response this study aimed to elucidate alterations in oxidative stress, unfolded protein response and acute phase response in septic mice.
Methods: Male C3H/HeN mice underwent sham operation or cecal ligation and incision and remained anesthetized for 5 h. Production of reactive oxygen species was determined by electron spin resonance spectroscopy. Protein and mRNA expression levels were determined by western blot analysis and quantitative real-time PCR, respectively.
Results: Production of reactive oxygen species was strongly increased in the aorta and liver after 5 h of polymicrobial sepsis which was entirely inhibited by treatment with melatonin. SOD-1 levels did not differ between the groups. Sepsis also induced the upregulation of VCAM-1 and ICAM-1 independent of melatonin treatment but probably regulated via ERK1/2 signaling. Melatonin triggered the transcriptional upregulation of PERK in septic animals which seems to be independent on ERK1/2 signaling and NR4A1 activation. Melatonin therapy also engendered an increased expression of CHOP, but apoptosis was not initiated. Furthermore, sepsis reduced the expression of the transcription factor CREBH which was entirely suppressed by melatonin.
Conclusions: This study gives new insight into the mechanisms by which melatonin might confer its hepatoprotective actions during polymicrobial sepsis. The results clearly show the melatonin-mediated amelioration of oxidative stress as well as alterations in the cellular stress mechanisms via the unfolded protein response and the acute phase response.

1. Introduction

The pathogenicity of sepsis is expressed in a complex systemic inflammation reaction whose exact mechanisms are still far from being understood. Important vital processes are thrown off balance and alter the redox status of tissues and the immune and coagulation systems. The resulting MODS (multiple organ dysfunction syndrome) frequently leads to death turning the treatment of sepsis to one of the greatest challenges for the intensive care medicine. Apart from an extensive antibiotic therapy there are only few supporting therapies applied in order to reduce organ damage and prevent an aggravation of the patient's condition.

The hormone melatonin has been implicated for the treatment of sepsis (Srinivasan et al., 2012), and its external application seems to influence many fields of sepsis-associated pathology. Melatonin reduces cell- and organ damage as well as oxidative stress after septic challenge in the liver, lung, heart, kidney, and brain (Carrillo-Vico et al., 2005; Crespo et al., 1999; Sener et al., 2005; Shang et al., 2009; Wu et al., 2008). Its positive influence on the liver during sepsis has been demonstrated by the inhibited increase of nitric oxide and lipid peroxidase levels (Carrillo-Vico et al., 2005) as well as reversed decrease of glutathione (Sener et al., 2005), thereby ameliorating oxidative stress in this organ. Additionally, melatonin therapy resulted in reduced infiltration of neutrophils in the liver and lower plasma levels of typical indicators for liver damage (alanine aminotransferase; aspartate aminotransferase) (Sener et al., 2005; Wu et al., 2008). Histological examination of liver damage displayed also a strong hepatoprotection by melatonin therapy after cecal ligation and puncture in rats (Sener et al., 2005). We demonstrated in previous studies that melatonin is able to

Abbreviations: MODS, multiple organ dysfunction syndrome; ER, endoplasmic reticulum; UPR, unfolded protein response; WT, wild-type; KO, knockout; MT, melatonin receptor; CLI, cecal ligation and incision; ROS, reactive oxygen species; ESR, electron spin resonance; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; DETC, diethyldithiocarbamic acid sodium.

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http://dx.doi.org/10.1016/j.yexmp.2014.10.009
0014-4800/

ameliorate liver function, perfusion, damage, and oxidative stress after hemorrhagic shock and reperfusion in rats (Mathes et al., 2008a,b). Additionally, survival of rats and mice after polymicrobial sepsis is significantly improved by a single bolus of melatonin (Fink et al., 2014a). Thus, melatonin's positive influence seems unquestionable, but the molecular and cellular mechanisms are widely unknown.

The endoplasmic reticulum (ER) is an important sensor of cellular stress which might be mediated by altered redox status or calcium concentration and lead to the accumulation of unfolded proteins in the ER lumen. The initiated unfolded protein response (UPR) or ER stress response encompasses several signaling pathways that aim to protect the cell and to resolve the overloading with premature proteins. Notably three ER-membrane bound stress sensors mediate the UPR in order to reduce protein synthesis (PERK), to increase protein folding (ATF6), and to accelerate unfolded protein degradation (IRE1α) (Esposito et al., 2013). In the case of persistent ER stress, the activation of UPR signaling might promote apoptosis and therefore contribute to organ damage (Nishitoh, 2012).

UPR has been shown to be activated in numerous liver diseases such as chronic viral hepatitis (Benali-Furet et al., 2005), insulin resistance (Ozcan et al., 2004), alcoholic liver disease (Ji and Kaplowitz, 2003), ischemia–reperfusion (Duvigneau et al., 2010; Emadali et al., 2005), and acute liver toxin insults (Kim et al., 2010). Considering animal models, lipopolysaccharide administration causes the induction of UPR in different organs of mice including the liver (Hiramatsu et al., 2006). In the past, only some researchers addressed the topic of melatonin's influence on ER stress mediated cell responses. Within those, clear evidence arises that melatonin alters protein and RNA content of the main UPR-players as well as some of their downstream effectors and target genes. Particularly the PERK-mediated signaling pathway seems to be influenced, e.g. the transcriptional downregulation or reduced activation of PERK, EIF2α, ATF4, CHOP and GADD34 was detected by different researchers (Fan et al., 2013; Ji et al., 2012; Tunon et al., 2013; Wang et al., 2011; Zaouali et al., 2013).

This study aims to figure out the influence of melatonin therapy on hepatic ER stress during polymicrobial sepsis in mice.

2. Material and methods

2.1. Drugs and chemicals

Melatonin (Caeco, Munich, Germany) was dissolved in dimethyl sulfoxide (DMSO)/normal saline (NaCl, 0.9%) to a concentration of 4 mg/ml and stored at −20 °C. All other chemicals were obtained from Sigma (Sigma-Aldrich, Munich, Germany), unless indicated otherwise.

2.2. Animals

All experiments were carried out in accordance to the German legislation on protection of animals and the National Institutes of Health guidelines for animal care (approval no. 36/2012 and 02/2013, Landesamt für Verbraucherschutz, Saarbrücken, Germany). Male wild-type C3H/HeN mice (WT, 6 weeks old, >20 g) were obtained from Janvier (Saint-Berthevin Cedex, France). Male knockout-mice (KO, 6 weeks old, >20 g) with deletions of the melatonin receptors MT1 and MT2 (KO ($MT1^{-/-}$/$MT2^{-/-}$), NCBI (http://www.ncbi.nlm.nih.gov/) gene identifiers 17773 and 244701) were bred in the facilities of the Institute for Clinical and Experimental Surgery (Saarland University Medical Center, Homburg-Saar, Germany). Animals had free access to water and food before surgery.

2.3. Surgical procedures

Animals were anesthetized (50 $mg \cdot kg^{-1}$ sodium pentobarbital intraperitoneally) and open tracheotomy was performed to facilitate spontaneous breathing. A polyethylene catheter was placed in the right external jugular vein to allow infusion of 10 $mg \cdot kg^{-1} \cdot h^{-1}$ Ringer-acetate solution (Sterofundin ISO; Braun, Melsungen, Germany). Another catheter was inserted in the left carotid artery and connected to a pressure transducer (PMSET 1DT, Becton Dickinson, Mountain View, CA, USA) for continuous measurement of mean arterial pressure and heart rate (Monitor Modul 66S, Hewlett Packard, Wilmington, DE, USA). Polymicrobial sepsis was induced by cecal ligation and incision (CLI) according to Scheiermann et al. (2009) but modified for mice as described in Fink et al. (2014b). For implementation of CLI, a 1 cm midline laparotomy was performed, the cecum was exteriorized, and both the cecum and paracecal mesenteric blood vessels were ligated below the ileocecal valve. The ligated cecum was incised 0.5 cm on the antimesenteric side, replaced into the abdomen, and the abdominal wall was closed in one layer (WT/KO CLI, n = 10 per group). In Sham-operated animals, laparotomy was performed, the cecum was mobilized but not ligated, replaced into the abdomen, and the abdominal wall was closed (WT/KO Sham: n = 8 per group). All animals remained under general anesthesia, maintained with pentobarbital intravenously (20–30 μl of 8 mg/ml pentobarbital in sodium chloride (NaCl) per bolus) as needed. Five hours after CLI induction, animals were sacrificed by entire blood withdrawal; animals were heparinized 15 min before.

2.4. Determination of reactive oxygen species (ROS) by electron spin resonance (ESR) spectroscopy in the murine liver, aorta and spleen

Superoxide was measured using ESR spectroscopy and the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) as described previously (Speer et al., 2014, 2013). Briefly, murine tissues were incubated in Krebs hepes buffer containing DETC (final concentration 5 μM) and deferoxamine (25 μM) as well as the spin probe CMH (200 μM) for 60 min at 37 °C. Afterwards, ROS production was determined using a Bruker eScan ESR spectroscope. Results were normalized to the dry weight of the tissue.

2.5. Sample preparation for gene expression analysis

For gene expression analysis, frozen liver tissue (approximately 10–15 mg) was homogenized in FastTri buffer (Axon Labortechnik, Kaiserslautern, Germany). Total RNA was isolated according to the manufacturer's instructions, and the remaining DNA was digested with DNase I (Fermentas, St. Leon-Rot, Germany). RNA was stored at −80 °C until it was used for RT-qPCR analysis. To control quality and purity of isolated total RNA spectral photometry and agarose gel electrophoresis were performed.

2.6. Quantitative real-time PCR (RT-qPCR)

cDNA synthesis was performed with 1 μg RNA using 200 U RevertAid™ Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). A cDNA equivalent to 10 ng total RNA was applied to the PCR-Mastermix with the 5× Hot Start Taq Eva Green qPCR Mix (Axon Labortechnik, Kaiserslautern, Germany) and primers in a concentration of 2.5 μM each (unmodified oligos, Eurofins MWG GmbH, Ebersberg, Germany). PCR reaction was performed within 40 cycles using the CFX 96 Real Time PCR Detection System (Biorad, München, Germany). For normalization, the gene expression of succinate dehydrogenase complex, subunit A (SDHA), and ribosomal protein S16 (RPS16) was consulted. Cycle of quantitation (C_q) values were determined by nonlinear regression fitting of Richards function (Guescini et al., 2008). Ratios were calculated by means of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences used in this study and gene identifiers according to NCBI are provided in Table 1.