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Melatonin or ramelteon therapy differentially affects hepatic gene expression profiles after haemorrhagic shock in rat — A microarray analysis

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ABSTRACT

Background & aims: Melatonin has been demonstrated to reduce liver damage in different models of stress. However, there is only limited information on the impact of this hormone on hepatic gene expression. The aim of this study was, to investigate the influence of melatonin or the melatonergic agonist ramelteon on hepatic gene expression profiles after haemorrhagic shock using a whole genome microarray analysis.
Methods: Male Sprague-Dawley rats (200–300 g, n = 4/group) underwent haemorrhagic shock (mean arterial pressure 35 ± 5 mmHg). After 90 min of shock, animals were resuscitated with shed blood and Ringer's and treated with vehicle (5% dimethyl sulfoxide), melatonin or ramelteon (each 1.0 mg/kg intravenously). Sham-operated animals were treated likewise but did not undergo haemorrhage. After 2 h of reperfusion, the liver was harvested, and a whole genome microarray analysis was performed. Functional gene expression profiles were determined using the Panther® classification system; promising candidate genes were evaluated by quantitative polymerase chain reaction (PCR).
Results: Microarray and PCR data showed a good correlation ($r^2 = 0.84$). A strong influence of melatonin on receptor mediated signal transduction was revealed using the functional gene expression profile analysis, whereas ramelteon mainly influenced transcription factors. Shock-induced upregulation of three candidate genes with relevant functions for hepatocytes (*ppp1r15a*, *dusp5*, *rhoB*) was significantly reduced by melatonin ($p < 0.05$ vs. shock/vehicle), but not by ramelteon. Two genes previously known as haemorrhage-induced (*il1b*, *s100a8*) were transcriptionally repressed by both drugs.
Conclusions: Melatonin and ramelteon appear to induce specific hepatic gene expression profiles after haemorrhagic shock in rats. The observed differences between both substances are likely to be attributable to a distinct mechanism of action in these agents.

1. Introduction

The pineal hormone melatonin is known for its physiological function in mediating the photoperiod (Arendt, 1998) but influences on vasomotor tone and thermoregulation have also been described (Doolen et al., 1998). Melatonin synthesis was further discovered in other peripheral organs (Kvetnoy, 1999), although the role in these remains to be elucidated.

Hepatoprotective effects of melatonin have been shown in over one hundred experimental models of stress (Mathes, 2010). In previous studies we established the efficacy of melatonin administration for the improvement of liver function and microcirculation accompanied by reduced tissue damage and neutrophil infiltration in a rat model of haemorrhagic shock with reperfusion (Mathes et al., 2008a,c). Melatonin is thought to act mainly as a direct antioxidant substance and via the activation of antioxidant enzymes (Hardeland, 2005; Tomas-Zapico and Coto-Montes, 2005). Nevertheless, the contribution of receptor-dependent effects to hepatoprotection seems very likely according to

Abbreviations: HS, haemorrhagic shock; DMSO, dimethyl sulfoxide; MAP, mean arterial pressure; SBV, shed blood volume; qRT-PCR, quantitative real-time polymerase chain reaction; DIG-dUTP, digoxigenin-11-2′-deoxy-uridine-5′-triphosphate.

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our previous studies: A) the melatonin receptor agonist ramelteon was shown to possess hepatoprotective potential that appears to be similar to that of melatonin (Mathes et al., 2008b) and B) some of the observed effects were abolished by coincident administration of the melatonin receptor antagonist luzindole (Mathes et al., 2008a,b,c).

Yet, our knowledge on melatonin's influence on hepatic gene expression is sparse. In a model of aflatoxin toxicity, melatonin reduced increased heat shock protein (HSP)-70 expression in rat liver (Meki et al., 2004). After parasite induced liver injury, melatonin counteracted expressional changes of interleukin (IL)-1β and nuclear factor-kappa B (NF-κB) (Laothong et al., 2010). A more extensive evaluation was performed in adriamycin-treated rats, showing the effect of melatonin on the expression of 60 candidate genes in rat liver (Catala et al., 2007). However, all of these studies give only limited insight into the global changes of melatonin-induced gene expression.

The present study aimed to investigate the influence of melatonin on hepatic gene expression profiles after haemorrhagic shock in rat by using a whole genome microarray analysis. In order to differentiate between receptor-mediated and other effects, transcriptional changes by melatonin were compared to those induced by the pharmacological melatonergic agonist ramelteon. The results of this study are likely to provide a basis for further investigations analysing the molecular changes in the liver induced by melatonin or ramelteon administration to septic individuals, in order to understand the impressive effects of the adjunctive therapy with these drugs.

2. Material and methods

2.1. Drugs and chemicals

Melatonin and ramelteon were dissolved in 5% dimethyl sulfoxide (DMSO)/normal saline (0.9%) to a final concentration of 0.02 mg/ml. All chemicals were obtained from Sigma (Sigma-Aldrich, Munich, Germany) unless indicated otherwise.

2.2. Animals

All experiments were carried out after approval of the animal use committee (Landesamt für Gesundheit und Verbraucherschutz, Saarbrücken, Germany; permission no. 31/2006, 16/2007) in accordance with the German Animal Welfare Act. Male Sprague-Dawley rats (body weight 200–300 g) from Charles River (Sulzfeld, Germany) were kept in the institutional animal facility under controlled conditions. Animals had free access to water, but pellet food was withheld for 12 h before surgery. Animals became accustomed to a light–dark cycle of 12:12 h; experiments were started at Zeitgeber time 02.

2.3. Surgical procedures

Animals were anaesthetised (50 mg · 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 ml · kg^{-1} · h^{-1} Ringer-acetate solution (Sterofundin ISO; Braun, Melsungen, Germany). Another catheter was inserted into the left carotid artery for continuous measurement of mean arterial pressure (MAP) and heart rate (Monitor Modul 66S, Hewlett Packard, Wilmington, DE, USA). Haemorrhagic shock was induced by rapid blood withdrawal via the carotid artery (MAP 35 ± 5 mmHg, 90 min). Animals were resuscitated with 60% of shed blood volume (SBV), infused within 5 min, followed by 2 h of reperfusion (fluid management: 200% of SBV as Ringer's acetate solution in the first, 100% of SBV in the second hour). Blood gas analysis was performed before and after haemorrhage and after 2 h of resuscitation using 0.2 ml of heparinised arterial blood (pHOx Plus L, Nova Biomedical, Germany). After 2 h, a midline laparotomy was performed, the liver removed, and the animal sacrificed by an overdose of pentobarbital. Liver tissue was immediately transferred to RNA-later solution (Qiagen, Hilden, Germany) and stored for further processing.

2.4. Experimental protocol

Animals that underwent haemorrhagic shock (HS) were treated with vehicle (HS-V, 5% DMSO) or 1.0 mg·kg^{-1} melatonin (HS-M) or ramelteon (HS-R) at a concentration of 0.02 mg/ml (each n = 4). Sham-operated animals were treated likewise but did not undergo haemorrhage (Sh-V, Sh-M, Sh-R; each n = 4) and were infused with 10 ml·kg^{-1} · h^{-1} Ringer-acetate. The dissolvent DMSO has previously been demonstrated to not affect hepatic microcirculation or liver function (Mathes et al., 2008a).

2.5. Sample preparation for gene expression analysis

For gene expression analysis, 70–80 mg of hepatic tissues from every animal stored in RNA-later solution (Qiagen, Hilden, Germany) was homogenised (Homogenizer, Omni, Warrenton, USA) and digested using proteinase K (20 mg/ml; Invitrogen, Karlsruhe, Germany). Total RNA was isolated (RNeasy Mini kit, Qiagen, Hilden, Germany) and remaining DNA was digested (RNase free DNase Set, Qiagen, Hilden, Germany). An approximate amount of 140 μg total RNA was isolated from each tissue sample. RNA was stored at −80 °C until used for microarray and qRT-PCR analysis. To control quality and purity of isolated total RNA, spectrophotometry, agarose gel electrophoresis, PCR (using β-Actin primers for detecting DNA contamination), and microfluidics-based quality checks (Agilent Bioanalyzer 2100, Agilent, Waldbronn, Germany) were performed. Only total RNA with a ratio A260/A280 >1.9 (spectrophotometry) with 28S ribosomal bands which were present at approximately twice the amounts of the 18S RNA (agarose gel electrophoresis) without detectable contamination of DNA (PCR) and a RNA Integrity Number >7.1 were employed for microarray and qRT-PCR.

2.6. Microarray protocol

RNA from four individuals (10 μg RNA/biopsy) was pooled separately for each group, resulting in 37–43 μg RNA templates for each of six microarrays. These templates were converted into cDNA using a Chemoluminescence RT Labeling Kit™. In short, mRNA was reverse transcribed using oligo (dT) primer and labeled with DIG-dUTP. After RNA degradation, the DIG-labeled cDNA was purified and hybridised on the Rat Genome Survey Microarray. The following day, microarrays were washed and the chemoluminescence reaction performed utilizing the Chemoluminescence Detection Kit™. Chemoluminescence was detected with the AB1700 microarray reader. All materials and instruments were ordered from Applied Biosystems (Weiterstadt, Germany). Quantile normalised gene expression for the interrogation of 26,857 genes were log2-transformed and plotted as a scatter plot after deletion of signals with a signal/noise ratio 2.5 (fold change of the probe's signal relative to the standard deviation of measurements within the spot), giving a 99.4% probability that the measurements performed are correct.

2.7. Microarray analysis

Microarray data was analysed using the PANTHER classification system (http://www.pantherdb.org) (Thomas et al., 2003). All genes were categorised according to their biological processes and molecular functions. Over- or underrepresentation of PANTHER classification categories was statistically determined by use of binomial statistics to compare classifications of multiple clusters to a reference list. In short, based on the reference list derived from Sh-V, an expected value for the number of genes in a certain cluster is determined. Under the null hypothesis it is assumed that genes in the tested list are sampled from