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Research report

The effects of melatonin and curcumin on the expression of SIRT2, Bcl-2 and Bax in the hippocampus of adult rats

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ABSTRACT

Objective: Though the mechanisms are not clearly understood, melatonin and curcumin have been reported to have neuroprotective effects. However, the mechanisms of neuroprotective effects of melatonin and curcumin in the brain are not clearly understood. In the current study, we investigated the effects of melatonin and curcumin treatments on oxidative stress parameters, the expression of SIRT2, Bcl-2 and Bax in the hippocampus.

Methods: A total of thirty adult (13 months-old) male Wistar rats were divided into five groups: Control (1% ethanol:PBS), s.c. for 30 days), dimethyl sulfoxide (10%, s.c. for 30 days), Melatonin (10 mg/kg/day, s.c. for 30 days), Curcumin (30 mg/kg/day, i.p. for 30 days) and Salermide (100 μM, i.p. for 30 days). The levels of malondialdehyde (MDA) glutathione (GSH) were measured as oxidative stress parameters in the hippocampus. The expression levels of SIRT2, Bcl-2 and Bax proteins were tested by western blotting and the SIRT2 protein levels of the hippocampal region was measured by a sandwich ELISA method.

Results: Melatonin and curcumin significantly decreased MDA and SIRT2 expression in the hippocampus ($p < 0.05$). Accordingly, a significant increase in the GSH levels of curcumin-treated group and melatonin-treated group was observed. Melatonin, but not curcumin, significantly increased the Bcl-2 expression of the hippocampal region. There was a significant correlation between SIRT2 and MDA levels ($p < 0.05$).

Discussion: In conclusion, our results suggest that melatonin may increase cell survival in the hippocampus via decreasing oxidative stress and SIRT2 expression and increasing Bcl-2 expression.

1. Introduction

Loss of balance between reactive oxygen species (ROS) produced endogenously in most physiological processes and antioxidant defense systems leads to oxidative stress. Because of its high metabolic activity, the brain tissue is expected to have high ROS production which renders it a major target for free radicals (Bokov et al., 2004). ROS/reactive nitrogen species (RNS) damage in the brain increases apoptosis (Sastre and Pallard, 2000; Pugazhenthi et al., 2003) and conversely, anti-oxidant treatments attenuate apoptosis (Götz et al., 1999; Baydas et al., 2005). Since, the brain has region specific activities, its sensitivity to oxidant and antioxidant markers may vary accordingly (Hill and Switzer, 1984). The hippocampus is one of the most vulnerable brain regions to oxidative damage. This vulnerability explains why neuro-degenerative diseases such as Alzheimer's Disease (AD) primarily influence this brain area (Venkateshappa et al., 2012). Pro-apoptotic and anti-apoptotic members of Bcl-2 family play a significant role in the intrinsic apoptosis pathway, and the ratio of pro-/anti-apoptotic proteins (e.g. Bcl-2/Bax ratio) is regarded as a marker of whether the cells undergo apoptosis or survive.

The pineal gland hormone melatonin has been shown to have neuroprotective effects on spinal cord injury (Esposito et al., 2009), Parkinson's disease (PD) (Tapias et al., 2009), Alzheimer's Disease (AD) (Rosales-Corral et al., 2012), ischemic injury (Wang et al., 2009) and to decrease oxidative stress in the brain (Akbulut et al., 2008). Melatonin binding receptors have been found in different regions of brain including hippocampus (Musshoff et al., 2002). Melatonin has been shown to decrease apoptosis in various tissues including brain (Kireev et al., 2013; Luchetti et al., 2010; Paredes et al., 2015; Akbulut et al., 2012). Likewise, anti-oxidant and neuroprotective effects of curcumin, a naturally occurring phenolic compound of turmeric (Curcuma longa) have been shown in various brain regions, including hippocampus (Bala

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et al., 2006; Jayasena et al., 2013; Motaghinejad et al., 2015). Curcumin is also reported to decrease apoptosis in brain tissue via up-regulating Bcl-2 in pathological conditions (Pan et al., 2008).

Sirtuins (SIRT1-7) are the members of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases III (HDACs III) family. They were shown to play significant roles in several physiological functions including lipid homeostasis, metabolism, oxidative stress resistance, cell cycle, aging, apoptosis, and cancer (Yamamoto et al., 2007; Sidorova-Darmos et al., 2014). The neuroprotective effect of curcumin is reported to be related to the SIRT1 activation in the brain (Miao et al., 2016). SIRT2 is predominantly localized in the cytoplasm and expressed in many tissues such as brain, heart, liver, spleen, kidney, and intestine. SIRT2 is the most abundantly expressed sirtuin in the adult rat brain (Sidorova-Darmos et al., 2014). SIRT2 deacetylates various substrates including p53, FOXO (Forkhead box O) transcription factors, α-tubulin, and histones. SIRT2 has also been reported to play important role in the regulation of oxidative stress responses (Nie et al., 2014), neurodegeneration (Maxwell et al., 2011; Luthi-Carter et al., 2010), apoptosis (Wang et al., 2007), and neurogenesis (Liu et al., 2015). The effects of melatonin and curcumin in the brain with regard to SIRT2 expression are not known.

In the present study, we aimed to study the effects of melatonin and curcumin treatments on the expression of SIRT2, Bcl-2 and Bax and oxidative stress parameters in the hippocampus of adult rats.

2. Methods

2.1. Animals and treatments

A total of thirty adult (13 months-old) male Wistar rats were used in the following groups: Control (CTL, n = 6), Dimethyl sulfoxide (DMSO, n = 6), Melatonin (MEL, n = 6), Curcumin (CUR, n = 6), Salermide (SLM, n = 6). DMSO was used as a solvent control for CUR and SLM groups. Salermide was used as a SIRT inhibitor (Peck et al., 2010). The animals were purchased from Gazi University Laboratory Animals and Experimental Research Center and housed under standard laboratory conditions with a 12 h alternating light/dark cycle (08.00–20.00 h with light period) at 23 °C and were fed commercial rat chow and water ad libitum. Melatonin (Sigma, M5250–1G) was given subcutaneously (s.c.) at the dose of 10 mg/kg/day in 1% ethanol:phosphate-buffered saline (PBS) and curcumin (Sigma, C1385–5G) was given intraperitoneally (i.p.) at the dose of 30 mg/kg/day in DMSO. Salermide (Cayman Chemical, cat. 13178), dissolved in DMSO was given (i.p.) at the dose of 100 μM. Finally, control rats were given equal amounts of 1% ethanol:PBS or 10% DMSO (s.c.). The animals in all five groups were injected at 17:00 h for 30 days. The dose and prolonged duration of the treatments were based on previous studies (Akbulut et al., 2008). On the 31st day of the experiment the rats were sacrificed at 10:00 a.m. and whole brain tissues removed. The division into six regions: thalamus, hippocampus, frontal cortex, parietal cortex, occipital cortex, and amygdala was carried out according to the atlas of Paxinos and Watson and tissues were sampled from the hippocampus, immediately frozen in liquid nitrogen and stored at −80 °C until analyses were performed. The rats were treated according to the guidelines of the European Convention ETS 123 and all the methods used in the current study were approved by the Animal Experiments Ethics Committee of Gazi University (# G.U.ET-11.55).

2.2. Measurements of the tissue lipid peroxidation and GSH levels

Tissue samples were homogenized in ice-cold trichloroacetic acid (40 mg tissue plus 0.72 ml TCA (10% w/v)) in a tissue homogenizer and tissue homogenates were centrifuged at 4000 rpm for 15 min. The formation of thiobarbituric acid reactive substances (TBARS) was used to measure the lipid peroxidation as described by Casini et al. (Casini et al., 1986). After centrifugation, butylated hydroxytoluene (BHT) (1% w/v) and thiobarbituric acid (TBA) (0.67% w/v) were added to the supernatants, and the mixture was heated at 100 °C for 15 min. After cooling, the absorbance at 532 nm was measured immediately and lipid peroxidation levels were expressed as malondialdehyde (MDA) equivalents (nmol/g tissue) using an extinction coefficient of $1.56 \times 10^5\ mol^{-1}\ cm^{-1}$. The glutathione (GSH) level was determined by using a modified Ellman method (Aykaç et al., 1985). Briefly, the supernatants of tissue homogenates were added to 0.3 M Na_2HPO_4 and dithiobisnitrobenzoate solution (0.4 mg/ml 1% sodium citrate). Absorbance was read spectrophotometrically at 412 nm and the GSH levels (μmol/g tissue) were calculated using an extinction coefficient of $13{,}000\ mol^{-1}\ cm^{-1}$.

2.3. Detection of SIRT2, Bcl-2 and Bax expression by western blotting

For western blotting, the total protein concentration of each hippocampal tissue lysates was determined by the Bradford method and equal amounts of protein extract (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (12%) and then transferred to nitrocellulose membrane (Bio-Rad, Germany). The membrane was blocked with 3.5% (w/v) non-fat dry milk (Santa Cruz Biotechnology, TX) and 1.5% (w/v) bovine serum albumin (Bioshop, Canada) dissolved in Tris Buffered Saline (TBS) containing 0.1% Tween 20 (TBST) for overnight at + 4 °C. The blocking was followed by incubation with primary antibodies and then horseradish peroxidase (HRP) conjugated secondary antibodies for 1.5 h at room temperature. The antibodies were diluted in TBST with 5% (w/v) non-fat dry milk at the suggested dilution. All primary and secondary antibodies of interest were purchased from Santa Cruz Biotechnology (TX). Washings were done in TBST at each step, 3 times for 10 min/wash. Following last washing, the membranes were incubated with enhanced chemiluminescence (ECL) detection reagents (Invitrogen Life Technologies, UK) and protein bands visualized on X-ray films. The bands were quantified by using ImageJ software (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/) and normalized to β-actin expression levels. The protein expression levels of the treatment groups (DMSO, MEL, CUR and SLM) were calculated as fold change with regard to the expression level of CTL group.

2.4. Measurement of SIRT2 protein levels by ELISA

The levels of SIRT2 protein were assayed by using commercial ELISA kit (Enzyme-linked Immunosorbent Assay Kit for Sirtuin 2, designed by Cloud-Clone Corp, assembled by USCN Life Science Inc., Germany). The supernatants of hippocampal tissue homogenates were prepared according to the manufacturer's protocol. Then, the samples were applied to a microtiter plate pre-coated with an antibody specific to SIRT2 and incubated for 2 h at 37 °C. Following the addition of detection reagent A (incubated for 1 h at 37 °C) and then detection reagent B (incubated for 30 min at 37 °C), substrate solution was added (incubated for 15–25 min at 37 °C). The blue color development by the addition of substrate solution was stopped with stop solution. Then, the intensity of the color was measured at 450 nm immediately. The concentration of SIRT2 (ng/ml) in the sample was calculated by using the standards provided in the kit, and then normalized to the total protein content of the tissue and expressed as ng/mg protein.

2.5. Statistical analysis

All quantitative results are presented as mean ± standard deviation (SD). The non-parametric Mann Whitney *U* test was used to determine significant differences between the groups. Spearman's rho correlation coefficient was also calculated. $P < 0.05$ was set as the level of statistical significance.