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Melatonin protects mice against stress-induced inflammation through enhancement of M2 macrophage polarization

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ABSTRACT

Stress is known to cause neuropsychiatric diseases, and it has a detrimental impact on the function of the immune system. Melatonin (MLT), a pineal gland hormone, exhibits potent anti-inflammatory properties and it plays a fundamental role in neuroimmunomodulation. In the present study, we investigated the molecular mechanisms of MLT in stress-induced inflammation, focusing on macrophage polarization. MLT (50 and 100 mg/kg) or a vehicle control (5% ethanol in saline) was intraperitoneally administered to mice once a day for 5 days. After the last treatment, mice were subjected to restraint stress (RS) for 2 h. MLT markedly decreased serum levels of corticosterone after RS. RS significantly increased serum interleukin (IL)-1β and IL-6 levels, and it decreased serum IL-10 levels. MLT administration attenuated these changes. After RS, MLT markedly decreased lipid peroxidation and increased hepatic glutathione content. In purified Kupffer cells (KCs) and peritoneal macrophages from mice exposed to RS, the expression levels of M1 marker genes (*NOS2a* and *CD40*) increased, while the expression levels of an M2 marker gene (*Arg1*) decreased. MLT attenuated this increase in expression of M1 marker genes and decrease in the expression levels of the M2 marker gene. Furthermore, in KCs and peritoneal macrophages exposed to RS, MLT decreased the number of $F4/80^+$ $CD86^+$ cells and increased the number of $F4/80^+$ $MRC1^+$ cells. In splenocytes exposed to RS, MLT inhibited the increase in mRNA expression of *NOS2a*. MLT downregulated expression of phosphorylated signal transducer and activator of transcription (STAT) 1 and upregulated STAT3 protein expression. Our findings suggest that MLT reduces stress-induced inflammatory responses by inducing an M1 to M2 phenotype switch in macrophages via activation of STAT3 signaling.

1. Introduction

Stress is an unavoidable part of life in modern society, and it causes alterations in the immune, neuroendocrine, and sympathetic nervous systems. This can lead to disruptions in a host's defenses. Physiological and physical stressors can activate immune and inflammatory responses, and lead to increased cytokine levels and production of reactive oxygen species (ROS) [1]. Acute stress enhances the immune response by stimulating the hypothalamic-pituitary-adrenal axis (HPA) [2], while chronic stress induces immunosuppressive effects by reducing the number of immune cells [3]. A single episode of acute restraint stress (RS) in mice significantly increased interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α levels in both serum and liver tissue [4]. Furthermore, Chen et al. [5] reported that acute RS led to inducible nitric oxide synthase (iNOS) signaling, which coincided with regionally specific increases in immunity and inflammation markers within the rat brain. Despite extensive studies, the effects of stress on the immune system and its underlying mechanisms are still unclear.

Macrophages are crucial for innate immunity, and they are capable of differentiating into cells with a wide range of functions. Diversity and plasticity are the hallmarks of macrophages. In response to microenvironmental cues, macrophages can rapidly switch from one phenotype to another. Macrophages can be activated either by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) to induce the characteristics of the classically activated phenotype (M1) [6], or by IL-4 and IL-13 which lead to an alternative activated phenotype (M2) [7]. The former produces pro-inflammatory mediators, while the latter exhibits anti-inflammatory and immune-regulatory properties. An imbalance between M1 and M2 macrophages occurs in vivo under conditions that are physiological (ontogenesis and pregnancy) or pathophysiological (acute and chronic inflammation, atherosclerosis, and cancer) [8]. In the carotid arteries of high fat-fed Apo E-knockout mice, the M1 markers iNOS and interferon regulatory factor (IRF) 5 were highly expressed in macrophage-rich areas of shear stress lesions [9]. Hepatic ischemia and reperfusion, a complex phenomenon involving an injurious inflammatory response, increased levels of the M1 marker genes, and decreased levels of the M2 marker genes [10].

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Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is a neurohormone synthesized by the pineal gland and many other organs, such as the gut, skin, and bone marrow. MLT acts as a modulator of sleep and sexual behavior, and it possesses anti-inflammatory and antioxidant properties [11]. Likewise, a large number of reports describe MLT as an immunomodulatory compound. Exogenous administration of MLT stimulates monocyte production in both the bone marrow and spleen of mice [12]. Monocytes have both membrane and nuclear melatonin receptors [13]. MLT inhibited the production of NO and IL-6 at the levels of both gene transcription and translation in LPS-stimulated macrophages [14]. MLT ameliorated ischemia/reperfusion-induced liver damage by Toll-like receptor-mediated inflammatory responses [15]. MLT administration in chronic *Trypanosomacruzi*-infected Wistar rats had obvious protective effects, decreasing the percentage of spleen and peritoneal macrophages as well as antigen-presenting cells [16]. However, there is limited information available regarding the effects of MLT on stress-induced inflammation in in vivo animal studies.

In the present study, we aimed to investigate the protective mechanism of MLT in mice subjected to acute RS, particularly with respect to macrophage polarization.

2. Materials and methods

2.1. Animals

All animal care and experimental procedures complied with the guidelines of the National Institutes of Health (NIH publication no, 86–23, revised 1985) and were approved by the Animal Care and Use Committee of Korea University (Approval No. KUIACUC-2016-174), with adherence to the 3Rs (replacement, refinement, and reduction). Male ICR mice weighing 25–27 g were supplied by DaehanBiolink Co. (Eumseong, Korea) and acclimatized under controlled conditions for 1 week before the experiment. The animals were housed in cages (4 mice per cage) located in temperature- and humidity-controlled rooms (25 ± 1°C and 55 ± 5%, respectively) with a 12 h light-dark cycle and received water and food (DaehanBiolink Co.) ad libitum.

2.2. Induction of acute RS

RS was performed with slight modifications in accordance with the method developed by Satoh et al. [17]. Mice were placed vertically with the head down in a 50 ml conical tube with a diameter of 3 cm and length of 10 cm. Gauze was inserted to prevent forward and backward movements and limit side-to-side mobility. A 0.3 cm-diameter hole was made in the center of tube to allow the mice to breathe, and mice were subjected to immobilization stress in the tubes for 2 h.

2.3. Administration of MLT and experimental design

MLT (Sigma-Aldrich, St. Louis, MO, USA) (10 and 50 mg/kg) or vehicle (5% ethanol in saline) was intraperitoneally administered once a day for 5 days. The dose and route of MLT administration were selected according to a previous report [18] and our own preliminary studies. After the last treatment, the mice were placed in conical tubes to induce RS. The animals were randomly assigned to five groups (n = 8 per group): (a) vehicle-treated control (control), (b) 50 mg/kg MLT-treated control (MLT), (c) vehicle-treated RS (RS), (d) 10 mg/kg MLT-treated RS (MLT10 + RS), and (e) 50 mg/kg MLT-treated RS (MLT50 + RS). Mice were anesthetized with ketamine (100 mg/kg, Yuhan Corporation, Seoul, Korea) and xylazine (10 mg/kg, Boehringer-Ingelheim, St. Joseph, MO, USA), and blood samples from the inferior vena cava, spleen and perfused liver were collected. Serum was centrifuged at 10,000 rpm for 10 min at 4°C. The spleen was instantly stored in RPMI 1640 medium (Gibco Life Technologies, Grand Island, NY, USA) at 4°C until used for the assays.

2.4. Isolation of Kupffer cells (KCs)

KCs were isolated by perfusion of the liver using collagenase type IV (Sigma-Aldrich) [19]. After filtration of liver homogenates using a cell strainer, the filtrate was centrifuged at 500 rpm for 3 min, and nonparenchymal sufficient supernatant was centrifuged in 50%/25% Percoll (Sigma-Aldrich). The layer containing KCs was resuspended with RPMI 1640 (Gibco Life Technologies) and 10% FBS (Gibco Life Technologies). The purity of KCs cells exceeded 85%.

2.5. Isolation of splenocytes

Spleens were homogenized with a syringe plunger and filtered through a 70 μm nylon mesh cell strainer (Corning Inc., Durham, NC, USA) to remove debris. Red blood cell (RBC) lysis buffer (eBioscience, San Jose, CA, USA) was used for RBC lysis. Total cell counts were determined by calculating the cell subpopulation.

2.6. Isolation of peritoneal macrophages

Peritoneal macrophages were harvested via peritoneal lavage using phosphate-buffered saline. After lavage, the exudates were centrifuged at 500 × g for 6 min at room temperature. The number and viability of the macrophages were assessed microscopically using a hemocytometer.

2.7. Cell culture and treatment

Murine macrophage RAW 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO_2.The cells were treated either with the vehicle (0.1% dimethylsulfoxide) or various concentrations of MLT (10, 50 and 100 μM). LPS (1 μg/ml) or IL-10 (10 ng/ml) was added 1 h after MLT treatment. After 1 h (IL-10) and 6 h (LPS) incubation, cells were harvested for further analysis. Cell viability was assessed using an MTT assay.

2.8. Serum corticosterone, IL-6, IL-1β, IL-10, and IL-4 levels

Serum corticosterone (Enzo Life Sciences, Ann Harbor MA, USA), IL-6, IL-1β, IL-10, and IL-4(BD Biosciences, San Jose CA, USA) levels were measured using commercially available enzyme-linked immunosorbent assay kits according to the manufacturers' instructions.

2.9. Hepatic lipid peroxidation and glutathione (GSH) content

Hepatic lipid peroxidation was analyzed by assessing malondialdehyde (MDA), which was measured spectrophotometrically at 535 nm by the level of thiobarbituric acid-reactive substances with 1,1,3,3,-tetraethoxypropane as the standard [20]. The total GSH level was measured spectrophotometrically at a wavelength of 412 nm using yeast GSH reductase, 5,5′-dithio-bis (2-nitrobenzoic acid), and NADPH [21].

2.10. Flow cytometry

Isolated KCs, splenocytes and peritoneal macrophages were blocked with anti-mouse CD16/CD32 purified antibody (eBioscience) for 15 min with inversion every 5 min. Flow cytometry buffer (eBioscience) was then added to stop blocking, and the pellet was collected after centrifugation. Isolated KCs, splenocytes and peritoneal macrophages were initially stained with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 antibody (eBioscience) and then stained with a phycoerythrin (PE)-conjugated anti-mouse CD86 antibody (eBioscience) or PE-conjugated anti-mouse MRC1 antibody (AbDSerotec, Raleigh, NC, USA) to confirm M1- and M2-polarized