Effect of melatonin on production of hydroxyl radical and lactate dehydrogenase during hypoxia in rat cortical slices[1]

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KEY WORDS melatonin; cell hypoxia; lactate dehydrogenase; hydroxyl radical; cerebral cortex

ABSTRACT

AIM: To study the effect of melatonin on the production of hydroxyl radical (·OH) and lactate dehydrogenase (LDH) following hypoxia in cortical slice. **METHODS**: Cortical slice was incubated with artificial cerebrospinal fluid (ACSF) in tube. Hypoxia was achieved by substituting 91.6 % N_2 and 8.4 % O_2. The salicylate trapping method was used to measure hydroxyl radicals generated. The content of LDH in medium after hypoxia was measured by International Federation of Clinic Chemistry (IFCC) method. **RESULTS**: The contents of dihydroxybenzoic acid (DHBA) were increased significantly during hypoxia and reoxygenation in cortical slice. The production of DHBA in reoxygenation was decreased concentration-dependently by melatonin, but not during hypoxia 30 min. The release of LDH during hypoxia was steadily elevated and melatonin decreased the content of LDH after hypoxia. **CONCLUSION**: Melatonin decreased the injury and production of ·OH after hypoxia.

INTRODUCTION

The hormone melatonin, *N*-acetyl-5-methoxytryptamine synthesized by the pineal gland participates in many important physiological functions including the control of seasonal reproduction as well as influences on the immune system and circadian rhythms[1,2]. It was a potent scavenger of free radicals and antioxidant[3,4]. Melatonin protected cells, tissues, and organs against oxidative damage induced by a variety of free radical generating agents and processes[5,6,7].

In neural damage, free radicals also play an important role, among which hydroxyl radical is very critical due to its high toxicity and reactivity. In the present study, we investigated the effect of melatonin on the production of hydroxyl radical during hypoxia and reoxygenation in rat cortical slice. Because lactate dehydrogenase (LDH) was released after hypoxia and was considered as an indicator of injury, we then measured the change of LDH content during hypoxia after treatment with or without melatonin.

MATERIALS AND METHODS

Chemicals Sodium salicylate, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, (1s)-*d*-10 camphorsulfonic acid of AR grade were purchased from Sigma. Melatonin, AR grade, was kindly gifted by Prof XIA Qi-Geng (Shanghai Chemical Reagent Factory). HPLC-grade methanol was purchased from BDH Laboratory (UK). Cytotoxicity Detection Kit was purchased from Diasys Co (Germany).

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Preparation of cortical slice Sprague-Dawley male rats (Grade Ⅱ, Certificate № 02-22-2), weighting 180 – 220 g, were purchased from Experimental Animal Center of Shanghai Medical University. Rats were decapitated. The brains were placed in cold oxygen-saturated artificial cerebrospinal fluid (ACSF) consisting of NaCl 118, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25, glucose 10, and sodium edetic acid 0.03 $mmol \cdot L^{-1}$. The cortex was dissected into 2 mm × 2 mm pieces using a razor blade. Cortical slices were transferred to ACSF at 37 ℃ with 95 % O_2 + 5 % CO_2.

Detection of ·OH by salicylate trapping method Cortical slices of similar weights were incubated with ACSF 3 mL containing sodium salicylate 4 $mmol \cdot L^{-1}$ under normoxic conditions (95 % O_2 + 5 % CO_2) at 37 ℃. After 30-min incubation, hypoxia was achieved by substituting 91.6 % N_2 and 8.4 % O_2. At the moment of hypoxia, vehicle or melatonin was added. At 30 min after hypoxia, it returned to normoxic condition with 95 % O_2 and 5 % CO_2 for 15 min. The cortical slices were divided into five groups: treated with vehicle, melatonin 0.01, 0.1, 0.5, and 1 $mmol \cdot L^{-1}$. Samples, 100 μL of supernatant, were collected at normoxic 30 min (control), hypoxia 30 min, and reoxygenation 15 min. After adding HCl 1 $\mu mol \cdot L^{-1}$, 20 μL, samples were centrifuged at 17 530 × g for 10 min, stored at 4 ℃, and measured within 24 h. DHBA contents of supernatant were detected by the salicylate trapping method[8]. Each sample was injected with 20 μL into the HPLC analytical column. The mobile phase consisted of edetic acid 0.4 $mmol \cdot L^{-1}$, NaH_2PO_4 0.2 $mol \cdot L^{-1}$, C_{210}-7 (1 s)-d-10 camphorsulfonic acid 0.7 $mmol \cdot L^{-1}$, and 5 % methanol. The pH was adjusted to 3.2. The flow rate was 0.6 $mL \cdot min^{-1}$. The compounds were detected with the electrode set at +0.7 V against an Ag/AgCl reference electrode. The data were analyzed by Chromatograph Program. Finally, the protein content of each tube was measured by Comassie blue method.

Measurement of LDH during hypoxia In a second set of experiment, the release of LDH was measured without sodium salicylate because it had a protective effect. Slices were incubated with ACSF at 37 ℃ under 95 % O_2 + 5 % CO_2. ACSF was added according to the weight (100 mg : 4 mL ACSF). Melatonin (3 $mmol \cdot L^{-1}$) or vehicle was added at the start. After 5-min interval, hypoxia was induced by switching to 91.6 % N_2 + 8.4 % O_2 for 1 h. Samples were collected at normoxic 5 min, hypoxia 10, 20, 30, and 60 min. After centrifuged at 805 × g for 2 min, supernatant 100 μL was collected. The content of LDH in the supernatant was measured by Cytotoxicity Detection Kit (recommended by International Federation of Clinic Chemistry) at 30 min after hypoxia.

Statistical analysis Differences between groups were analyzed by t-test and ANOVA.

RESULTS

Hydroxyl radical level was elevated during hypoxia and reoxygenation in cortical slice. The concentrations of 2,3-DHBA and 2,5-DHBA were higher in the group of 30 min after hypoxia and 15 min after reoxyenation than that in the group of normoxic 30 min (Fig 1).

The production of DHBA in the group of reoxygenation 15 min was decreased concentration-dependently by melatonin, but not in the group of hypoxia 30 min. To the elevation of 2,3-DHBA after reoxygenation (in comparison with normoxic control), the inhibitory rates of melatonin 0.01, 0.1, 0.5, and 1 $mmol \cdot L^{-1}$ were 9.78 %, 27.02 %, 42.54 %, and 50.30 %, respectively. Melatonin also concentration-dependently inhibited the formation of 2,5-DHBA from 0.01 to 1 $mmol \cdot L^{-1}$ in final concentration (Tab 1).

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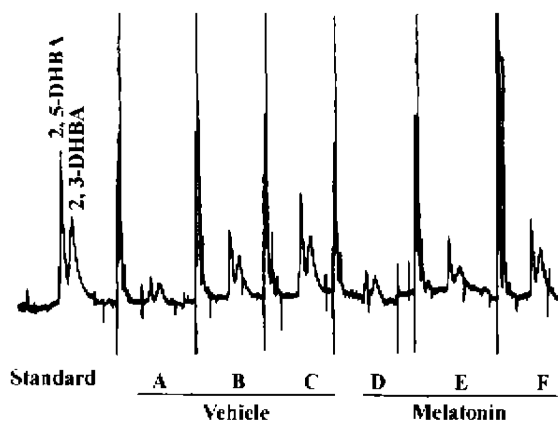

Fig 1. HPLC of 2,5-DHBA and 2,3-DHBA contents after hypoxia 30 min and reoxygenation 15 min in rat cortical slice with or without melatonin 1 $mmol \cdot L^{-1}$. A, D: Control; B, E: Hypoxia; C, F: Reoxygenation.

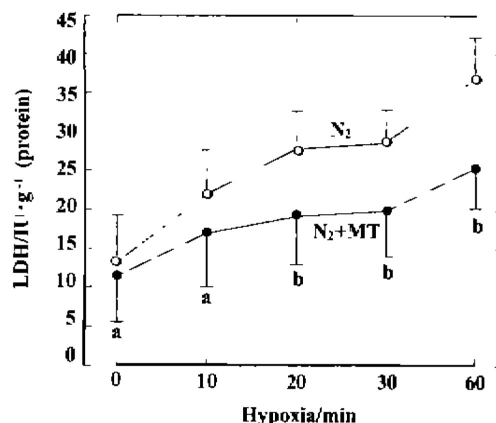

Fig 2. Time-dependent inhibition of melatonin (MT) 3 $mmol \cdot L^{-1}$ on the release of LDH during hypoxia in rat cortical slices. $n = 9$. $\bar{x} \pm s$. $^aP > 0.05$ *vs* vehicle. $^bP < 0.05$ *vs* vehicle.

Tab 1. Effects of melatonin on the production of 2,5-DHBA and 2,3-DHBA [$\mu g \cdot g^{-1}$ (protein)] during hypoxia-reoxygenation in rat cortical slices. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control. $^dP > 0.05$, $^eP < 0.05$ *vs* hypoxia. $^gP > 0.05$, $^hP < 0.05$ *vs* vehicle.

Melatonin/ $mmol \cdot L^{-1}$	n	Control	Hypoxia	Reoxygenation
2,5-DHBA				
0	7	12 ± 3	40 ± 11^b	66 ± 16^{be}
0.01	6	9.9 ± 1.6^g	43 ± 15^{bg}	64 ± 15^{beg}
0.1	7	12 ± 5^g	35 ± 7^{bg}	56 ± 16^{beg}
0.5	6	11 ± 5^g	38 ± 12^{bg}	43 ± 10^{bdh}
1	6	11 ± 5^g	32 ± 7^{bg}	40 ± 8^{bdh}
2,3-DHBA				
0	7	14.1 ± 2.7	32 ± 4^b	56 ± 9^{be}
0.01	6	13.4 ± 2.9^g	35 ± 9^{bg}	51 ± 3^{beg}
0.1	7	15 ± 5^g	31 ± 8^{bg}	46 ± 8^{beg}
0.5	6	15 ± 4^g	32 ± 12^{bg}	40 ± 12^{bdh}
1	6	13.6 ± 2.6^g	26 ± 6^{bg}	34 ± 7^{bdh}

The release of LDH during hypoxia was steadily elevated after hypoxia within 1 h. Treatment of melatonin decreased the content of LDH at 20, 30, and 60 min after hypoxia (Fig 2).

Melatonin decreased the release of LDH at 30 min after hypoxia in a concentration-dependent manner (Tab 2).

Tab 2. Inhibition of melatonin on LDH release following hypoxia in rat cortical slice. $n = 9$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* vehicle.

Melatonin/ $mmol \cdot L^{-1}$	LDH/ $IU \cdot g^{-1}$ (protein)
0	21 ± 5
0.5	18 ± 4^a
1.5	16 ± 4^b
3	14 ± 5^b
4.5	13 ± 5^b

DISCUSSION

Hydroxyl radical is the most toxical radical and plays a critical role in many pathological processes[9,10]. As we mentioned before[3,4], melatonin has been found to be a potent free radical scavenger including ·OH. In the present experiment, we found that melatonin concentration-dependently decreased the production of hydroxyl radical after hypoxia-reoxygenation in rat cortical slice. It suggests that melatonin has a direct effect of scavenging ·OH, which further confirmed our previous *in vivo* study[11].

Furthermore, we would investigate whether

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melatonin has a neuroprotective effect on this model. LDH can be used as an indicator of injury. In the experiment, we observed the changes of LDH with or without treatment of melatonin. As shown in the results, melatonin significantly decreased the content of LDH after hypoxia, showing the evidence that melatonin can decrease the injury induced by hypoxia. *In vivo* study, we also found that melatonin concentration-dependently decreased the neuronal damage in rat brain following ischemia-reperfusion[12].

Melatonin has antioxidative ability especially with hydroxyl radical scavenge activity and cell protection in lung, liver, and brain[5,13,14]. In the present study, melatonin decreased both of the production of ·OH and the release of LDH after hypoxia in the cortical slice. Putting together, it suggests that one of the neuroprotective mechanisms of melatonin may be related to its ability of quenching ·OH. Besides, we have also found that melatonin can inhibit neuronal apoptosis induced by cerebral ischemia through upregulation of *bcl*-2, an apoptotic inhibitory gene[12]. Furthermore, whether melatonin's protective effect is related to other mechanisms needs further investigations.

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褪黑激素对大鼠大脑皮层脑片在缺氧后羟自由基及乳酸脱氢酶生成的影响[1]

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关键词 褪黑激素; 细胞低氧; 乳酸脱氢酶; 羟自由基; 大脑皮质

目的: 研究褪黑激素对大鼠大脑皮层脑片在缺氧后羟自由基及乳酸脱氢酶生成的影响. **方法**: 通过水杨酸捕获法来观察缺氧再给氧中羟自由基含量的变化. 脑片通以 91.6 % N_2 + 8.4 % O_2 造成缺氧. LDH 用细胞毒检测盒测定. **结果**: DHBA 水平在缺氧及再给氧后显著升高; 缺氧时给予褪黑激素可以浓度依赖性地降低再给氧 15 min 时 DHBA 的含量, 但对缺氧 30 min 时 DHBA 的含量没有显著作用. LDH 的含量在缺氧后 1 h内持续升高; 褪黑激素可以显著降低缺氧后 LDH 的释放. **结论**: 褪黑激素降低大鼠缺氧后的损伤以及羟自由基的产生.

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