Melatonin reduces peroxynitrite-induced injury in aortic smooth muscle cells

Jun-lin ZHOU[1], Xiao-guang ZHU[1], Yi-ling LING[2], Qing LI[3]

Department of Hand Suegery, Third Affiliated Hospital, Hebei Medical University, Shijiazhuang 050051;
[2]Department of Pathophysiology, Hebei Medical University, Shijiazhuang 050017;
[3]Department of Physiology, Hebei Medical University, Shijiazhuang 050017,China

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ABSTRACT

AIM: To study the protective role of melatonin (MT) in peroxynitrite-induced injury in cultured aortic smooth muscular cells (ASMC). **METHODS**: Peroxynitrite was synthesized chemically with a quenched flow reaction. Cells were exposed to peroxynitrite 500 μmol/L for 1 h in the absence or presence of various concentrations of MT 100, 300, and 500 μmol/L. Nitrotyrosine (NT), a specific "footprint" of peroxynitrite formation, was detected by immunohistochemical technique. The DNA damage was assayed by TUNEL technique. The levels of MDA in the medium and cell viability were measured. **RESULTS**: Incubation of ASMC with peroxynitrite 500 μmol/L for 1 h elicited the increase in the extent of immunostaining for NT, the rate of the TUNEL-positive cell, the content of MDA in the medium, and the number of dead cell. Pretreatment of ASMC with MT 100-500 μmol/L decreased these peroxynitrite-induced changes in a concentration-dependent manner. **CONCLUSION**: MT attenuated the injury induced by peroxynitrite in ASMC.

INTRODUCTION

The production of large amounts of nitric oxide (NO), a free radical produced by the inducible isoform of NO synthase (iNOS) in vascular smooth muscle cells has been implicated as a cytotoxic factor in a variety of pathophysiological processes, including various forms of inflammation and circulatory shock[1]. The cytotoxic effects of NO are in part, mediated by peroxynitrite, a reactive oxidant species formed from NO and superoxide at an almost diffusion-controlled rate[2]. Peroxynitrite is now generally considered a more toxic oxidant than either NO or superoxide anion alone. The cytotoxic processes triggered by peroxynitrite including initiation of lipid peroxidation, inhibition of mitochondrial respiration, inhibition of membrane pumps, depletion of glutathione, and damage to DNA with subsequent activation of poly (ADP ribose) synthetase and concomitant cellular energy depletion[3,4]. Melatonin (MT, *N*-acetyl-5-methoxytryptamine), a hormone-like substance produced by pineal gland and by other tissues (such as the gastrointestinal tissue)[5], has been considered to be almost exclusively related with the control of circadian physiology and seasonal reproductive events. Nevertheless, during recent years several reports claimed an antioxidant role for melatonin[6]. It is reported that this hormone can scavenge the hydroxyl (·OH)[7], the peroxyl (ROO·)[8], and (after its oxidation to the indolyl cation radical) superoxide anions[9]. Furthermore, MT could inhibit the vasorelaxant action of peroxinitrite in

[1] Correspondence to Dr Jun-lin ZHOU.
Phn 86-311-702-7951, ext 3117. E-mail zhjunlin@yahoo.com
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human umbilical artery[10]. But there is no report about its effect on peroxynitrite-induced injury. The present study was designed to investigate whether MT scavenged peroxynitrite and inhibited its injury to the cultured aortic smooth muscle cells (ASMC).

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle's medium (DMEM) medium and FBS were purchased from Gibco Biochemical Co. Melatonin and TUNEL assay kit were purchased from Sigma Chemical Co. Nitrotyrosine (NT) monoclonal antibody was from Cayman Biochemical Co. ABC kit including normal goat serum, secondary antibody, and the tertiary avidin-biotin-antibody complex was from Zymed Co. Malondialdehyde (MDA) assay kit was the product of Nanjing Jiancheng Biochemical Co. All other chemicals were of analytical grade and commercially available.

Peroxynitrite syntheses Peroxynitrite was synthesized chemically with a quenched flow reaction according to Beckman *et al*[11,12], with minor modification. In brief, 0.6 mol/L HCl+0.7 mol/L H_2O_2 and 0.6 mol/L $NaNO_2$ were mixed by infusing through a Y-type tube and quenched by 1.5 mol/L NaOH. Then 4 g of granular MnO_2 was added and mixed round for 1-4 min to remove the excess H_2O_2. After filtration under the negative pressure, the reacted solution was stored at -20 ºC overnight. The separated yellow liquid in the upper layer was the stock solution of peroxynitrite. The concentration of peroxynitrite was determined by measuring the absorbance at 302 nm in NaOH 1 mol/L.

Cell culture and experimental protocol Rat ASMC were harvested from male Sprague-Dawley rats provided by Hebei Experimental Animal Center of Hebei Province (weighting 200 g±16 g, Grade II, Certificate No 04064) by enzymatic dissociation according to the method of Gunther *et al*[13]. The cells were cultured in DMEM supplemented with 20 % fetal bovine serum (FBS), benzylpenicillin (100 kU/L), and streptomycin (100 mg/L) and passed every 3-5 d. The experiments were performed on cells 4-6 passages from primary culture. At a density of 1×10^9 cells/L, cells were transferred to 24-well tissue culture plates for MDA and cell viability detection and to 6-well tissue culture plates with a glass slide in every well for immunohistochemical and TUNEL assays. When cells grew to confluence, the culture medium was replaced by serum-free medium for 12 h. Then the cells were exposed to peroxynitrite 500 μmol/L for 1 h in the absence or presence of various concentrations of MT 100, 300, and 500 μmol/L. Immediately before use, peroxynitrite was dissolved in the experimental medium and MT was dissolved in ethanol and further dissolved in medium (the final ethanol concentration was 3 %)[14]. Control cells were treated with the same concentration of ethanol vehicle. Preparation of media and incubations were carried out under protection from light.

Immunohistochemical detection of nitrotyrosine Tyrosine nitration, a specific "footprint" of peroxynitrite formation[15], was detected in ASMC by immunohistochemical teqnique. At the end of the experiment, cells grown on the slides in 6-well culture plates were washed twice very gently with PBS at 4 ºC for 5 min and then fixed in cold 95 % v/v ethanol for 10 min. After washed again in PBS for 5 min, slides were immersed in cold 3 % v/v H_2O_2 in PBS for 5 min followed by 5 % v/v normal goat serum in PBS for 20 min at room temperature. Next, slides were covered with rabbit anti-NT antibody (1:50 dilution in PBS) and left for 60 min at room temperature. After incubation with the secondary antibody, slides were washed again with PBS, and the tertiary avidin-biotin-antibody complex was applied for 60 min at room temperature. Slides were washed in PBS and finally incubated with 3,3'-diaminobenzidine (DAB) until suitable staining developed (about 10 min).

TUNEL assay DNA damage was detected *in situ* using the immunohistochemical terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-biotin nick end labeling (TUNEL) technique[12]. After treatment, cells were washed twice with PBS at 4 ºC for 5 min, fixed in cold 95 % v/v ethanol for 10 min and then detected with a TUNEL assay kit according to its instruction. The number of the TUNEL-positive cells and the total cells were counted by the light microscopy at ×200 magnification in each of three representative fields for each well and the TUNEL-positive rate was calculated.

MDA content detection At the end of each incubation, the medium in the 24-well plates were discarded and centrifuged at 3000×*g* for 10 min at 4 ºC. Then the MDA content in the supernatants was measured using MDA assay kit according to its introduction.

Cell viability measurement After the cultured medium was removed, cells in 24-well plates were incubated with 2.5 % trypan blue in phosphate-buffered salinefor 10 min at 37 ºC . The number of dead cells was then determined by light microscopy at ×200 mag-

nification by counting those cells that failed to exclude the dye in each of three representative fields for each well.

Data analysis Values were expressed as mean±SD, and assessed by one-way ANOVA followed the *post-hoc* test . The difference between groups was considered to be significant at $P<0.05$.

RESULTS

Effect of MT on immunohistochemical staining for NT Immunohistochemical analysis revealed negative staining for NT in the vehicle-treated cells. ASMC exposed to peroxynitrite 500 μmol/L for 1 h showed intensive staining for NT. Pretreatment of ASMC with MT even at a concentration of 100 μmol/L before peroxynitrite led to a remarkable decrease in the immunostaining for NT (Fig 1).

Effect of MT on TUNEL staining Sparse TUNEL-positive cells were seen in the control. After incubation with peroxynitrite 500 μmol/L for 1 h, diffusely intense staining was found and the positive-cell rate was increased significantly compared with the control ($P<0.01$). The positive-cell rate of the TUNEL staining was decreased by MT in a concentration-dependent manner ($P<0.01$ *vs* peroxynitrite-treated cells, Fig 2, Tab 1).

Effect of MT on MDA content After incubation with peroxynitrite 500 μmol/L for 1 h, the MDA content in the medium was increased significantly compared with the vehicle-treated control ($P<0.01$). Pretreatment with MT 100-500 μmol/L before peroxynitrite decreased MDA content in a concentration-dependent manner ($P<0.01$, Tab 1).

Tab 1. Effects of melatonin (MT) on the MDA level, cell viability, and the DNA damage in ASMC. ***n*=6 wells. Mean±SD. $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* Peroxynitrite.**

Treatment	MDA (nmol/L)	Cell viability (Number of dead cells/field)	DNA damage (TUNEL-positive rate)(%)
Control	2.32±0.17	10.5±1.6	2.0±0.4
Peroxynitrite	5.16±0.15^c	148±50^c	79±5^c
Peroxynitrite+ MT 100 μmol/L	3. 86±0.14^e	86±4^e	57±3^e
Peroxynitrite+ MT 300 μmol/L	3.20±0.13^f	50.6±2.8^f	41±3^f
Peroxynitrite+ MT 500 μmol/L	3.04±0.11^f	36±4^f	37.0±2.8^f

Effect of MT on cell viability No evidence of cell death was observed in vehicle-treated cells. Confluent cells incubated in the presence of peroxynitrite for 1 h showed a significant loss in cell viability[(144±3) dead cells/field, $P<0.01$ *vs* control]. However, pretreatment with MT 100-500 μmol/L decreased the number of the dead cells elicited by peroxynitrite in a concentration-dependent manner ($P<0.05$, Tab 1).

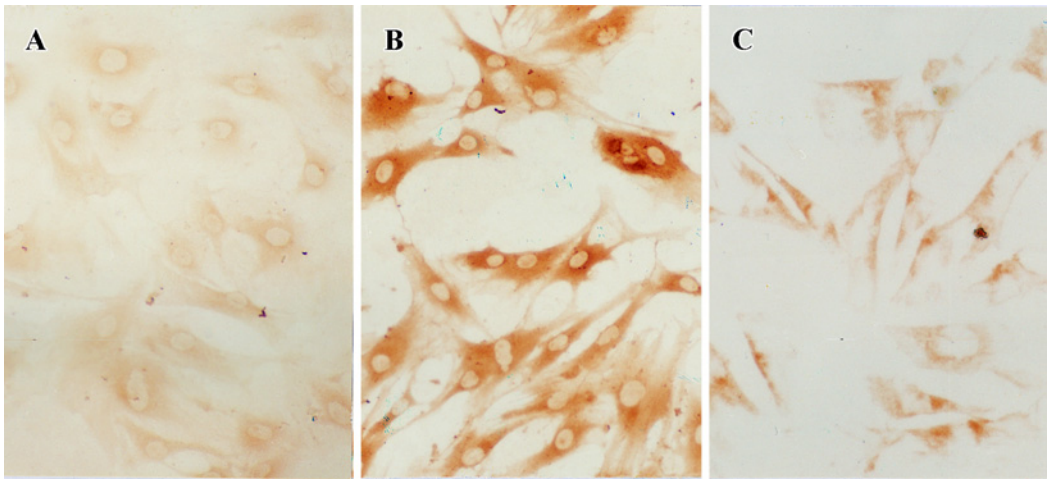

Fig 1. Effect of melatonin on immunohistochemical staining for nitrotyrosine in aortic smooth muscle cells (×400). A) No positive cell was seen in the control. B) Intensively positive cells were seen in the peroxynitrite-treated cells. C) Pretreatment of ASMC with MT before peroxynitrite significantly decreased the extent of positive staining for NT.

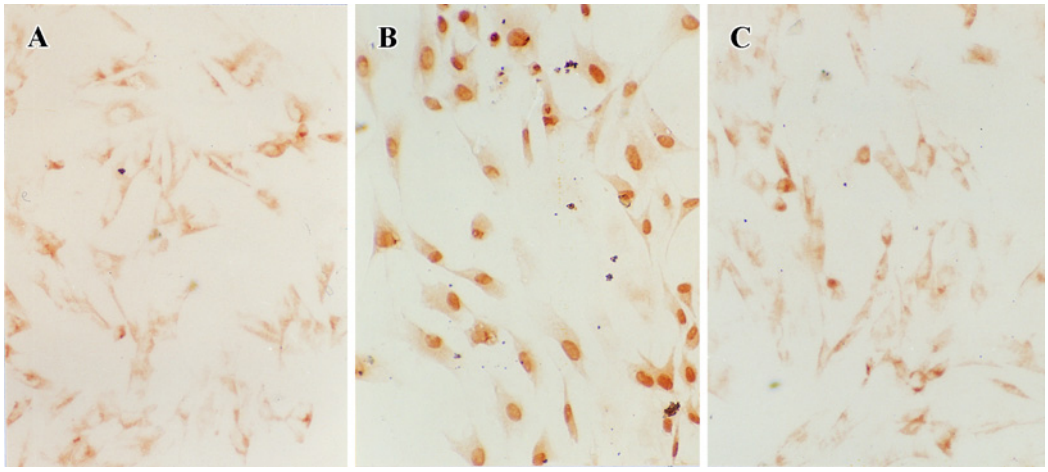

Fig 2. Effect of MT on the DNA damage by TUNEL technique in aortic smooth muscle cells (×200). A) Sparse positive cells were seen in the control. B) Intensively positive staining was seen in peroxynitrite-treated cells. C) The increased-positive staining induced by peroxynitrite was markedly decreased in MT-pretreated cells.

DISCUSSION

MT is a potent direct and indirect antioxidant. *In vitro* experiments carried by Tan *et al*, showed that MT was 5- and 14-fold more potent than glutathione and mannitol, respectively, in scavenging hydroxyl radicals[7]. In addition to its direct free radical scavenging action, MT has been reported to increase tissue levels of some important antioxidative enzymes at molecular level, stimulate their activities, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)[16], and decrease some pro-oxidative enzyme activity, such as nitric oxide synthase (NOS)[17,18] and thereby limit NO production. It is reasonable to speculate that MT was able to inhibit peroxynitrite production and limit its role by scavenging superoxide anions. But the exact and direct effect of MT on peroxynitrite is still to be elucidated. In the present study, we demonstrated that MT markedly inhibited the tyrosine nitration produced from exogenous peroxynitrite in ASMC, indirectly suggesting its scavenging role against peroxynitrite. Further we found that MT prevented the ASMC from oxidant injury induced by peroxynitrite in a concentration-dependent manner. Pretreatment of ASMC with MT before administration of peroxynitrite decreased the number of TUNEL-positive cell and attenuated the ASMC DNA damage induced by peroxynitrite. The cell viability result confirmed the protective effect of MT against peroxynitrite-induced injury in ASMC.

The most devastatingly reactive agents are the hydroxyl radical and the peroxinitrite anion. Besides being capable of directly damaging macromolecules, the peroxinitrite could degrade into the hydroxyl radical[2]. A single molecule of MT scavenges two hydroxyl radicals and the product of this interaction is cyclic 3-hydroxymelatonin[19]. MT was found to directly neutralize hydrogen peroxide, the precursor of the hydroxyl radical[20]. The product of this interaction is *N*1-acetyl-*N*2-formyl-5-methoxytryptamine, which itself is a highly efficient free radical scavenger. So it is possible that MT reacted with peroxynitrous acid or with its highly reactive cleavage products by hemolytic cleavage or heterolytic cleavage.

The biological effects of MT are due to a number of parallel actions. Based on the present data it is possible that scavenging actions of MT against peroxynitrite-induced injury may, in part, contribute to the protective effects of MT in various pathophysiological conditions. It is noteworthy that in many conditions where MT has been shown to have beneficial effects[21,22], the production of peroxynitrite has been demonstrated[4]. Because of its high lipophilicity and diffusibility, MT easily enters cells and gains access to all subcellular compartments[7]. Compared with other traditional antioxidants, such as vitamin E in the membrane and vitamin C in the cytosol[8,9], MT has a distinct advantage to scavenge peroxynitrite and other oxidants. Treatment with MT has been shown to improve the clinical outcome of the septic newborns[23], and therefore it may be a novel pharmacological approach to prevent cell injury in various clinical conditions.

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