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Mechanism of melatonin protection against copper-ascorbate-induced oxidative damage in vitro through isothermal titration calorimetry

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ABSTRACT

Aims: Involvement of oxidative stress in cardiovascular diseases is well established. Melatonin's role as an antioxidant and free radical scavenger via its receptor dependent and receptor independent pathways is well known. The aim of this study is to identify and elaborate upon a third mechanism by which melatonin is able to abrogate oxidative stress.

Main methods: Oxidative stress was induced in vitro, by copper (0.2 mM)-ascorbate (1 mM) in isolated goat heart mitochondria, cytosol and peroxisomes and they were co-incubated with graded doses of melatonin. Similar experiments in a cell-free chemical system involving two pure antioxidant enzymes, Cu-Zn superoxide dismutase and catalase was also carried out. Biochemical changes in activity of these antioxidant enzymes were analysed. Isothermal titration calorimetric studies with pure Cu-Zn superoxide dismutase and catalase were also carried out.

Key findings: Incubation with copper-ascorbate led to alteration in activity of Cu-Zn superoxide dismutase and catalase which were found to be protected upon co-incubation with melatonin (80 μM for catalase and 1 μM for others). Results of isothermal titration calorimetric studies with pure Cu-Zn superoxide dismutase and catalase along with different combinations of copper chloride, ascorbic acid and melatonin suggest that when melatonin is present in the reaction medium along with copper-ascorbate, it restrains the copper-ascorbate molecules by binding with them physically along with scavenging the free radicals generated by them.

Significance: The present study suggests that possibly, binding of melatonin with antioxidant enzymes masks the vulnerable sites of these antioxidant enzymes, thus preventing oxidative damage by copper-ascorbate molecules.

1. Introduction

Melatonin has long been known to regulate circadian rhythms, influence seasonal reproductive behaviour and control retinal function [1]. However, recent reports in the last two decades have unravelled its exceptional ability to act as a broad spectrum antioxidant and free radical scavenger [2]. Melatonin's ability to scavenge free radicals and reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) [3,4] and it's in vivo stimulation of antioxidant enzymes including glutathione peroxidase, superoxide dismutase (SOD) and catalase [5] has generated immense interest and has become a greatly studied property of melatonin.

Numerous in vivo and in vitro studies have reported that melatonin provides protection against lipid peroxidation, DNA and protein damage induced by reactive oxygen species (ROS) [6,7]. Oxidative stress is involved in the pathogenesis of a number of diseases resulting from oxidative modification of macromolecules due to ROS [8,9] mediated damage to vital structures at the cellular level. Abundant data documenting melatonin's ability to overcome oxidative stress has accumulated in recent years [10–13] including its protective action against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl

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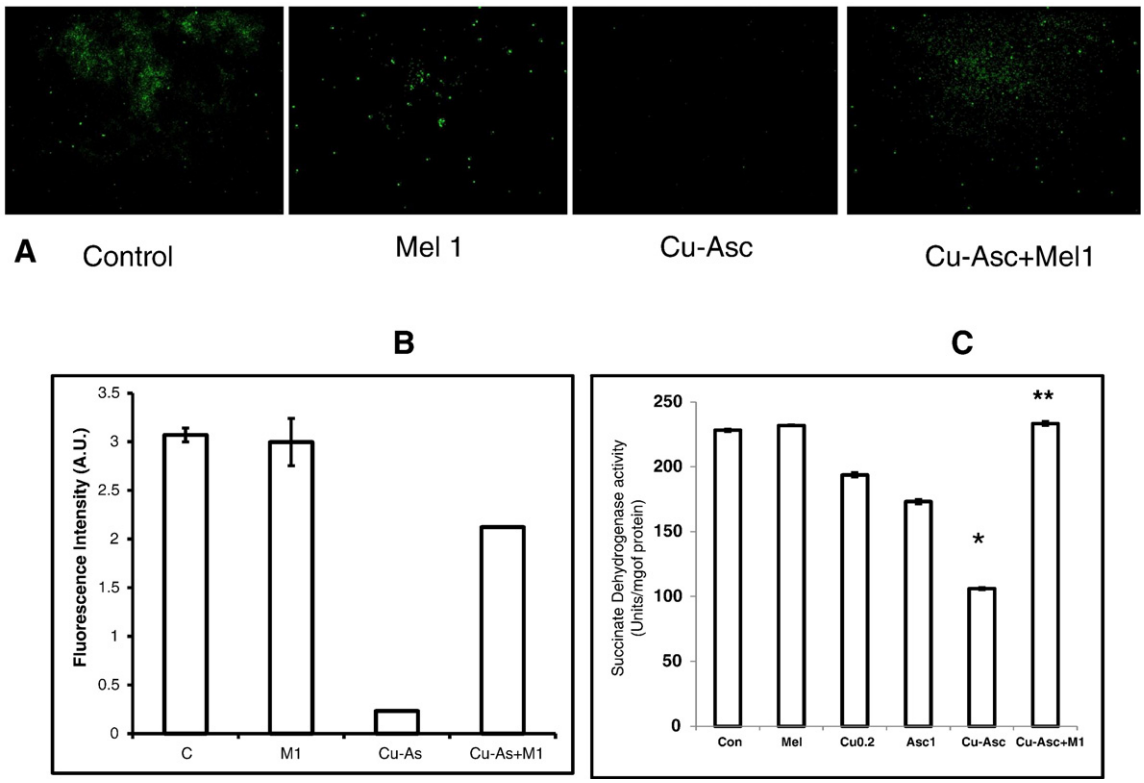

Fig. 1. Protective effect of melatonin against copper ascorbate-induced changes in mitochondrial structure and functional integrity. (A) Janus Green B stained mitochondrial smears of control, only melatonin treated (1 μM), copper-ascorbate treated (Cu-Asc) and copper ascorbate and melatonin treated group (Cu-Asc + Mel1) of isolated goat heart mitochondria taken at 20× magnification. (B) Fluorescent intensity of the various groups of Janus Green B stained mitochondria (C) Succinate dehydrogenase activity (SDH) of goat heart mitochondria in vitro indicating functional integrity of the isolated mitochondria. 0.2 mM $CuCl_2$, 1 mM ascorbic acid, 1 μM melatonin, 0.2 mM $CuCl_2$ + 1 mM ascorbic acid and 0.2 mM $CuCl_2$ + 1 mM ascorbic acid + 1 μM melatonin was incubated with isolated mitochondria for 1 h. Inhibition of SDH activity observed in copper-ascorbate group was found to be restored by 1 μM of melatonin which was found to be the most effective dose from in vitro experiments. Values are expressed as means ± S.E. for six samples for each group: $^{*}p < 0.001$ versus control, $^{**}p < 0.001$ versus copper ascorbate treated cardiac mitochondria.

radicals [14]. It has been reported to efficiently monitor the mitochondrial electron transport chain so as to minimize electron leakage [15]. Studies on its ability to scavenge ROS in cells as well as in cell-free system [16] are also present. The redox properties of melatonin, due to the presence of an electron-rich aromatic ring, are responsible for it to act as an electron donor, which donate electrons to free radicals and neutralize them. Interestingly, melatonin has been shown to be terminally oxidized and hence cannot be recycled for subsequent use [17].

Mitochondria, which houses the electron transport chain (ETC), is the principal site of ROS generation during the partial reduction of oxygen during mitochondrial respiration. We have previously shown that, melatonin ameliorated isoproterenol induced changes in isolated goat heart mitochondria, in vitro, via its antioxidant mechanisms [18].

Presence of excess copper has been reported to cause damage to lipids, proteins and DNA whereas a deficiency results in compromised antioxidant defense status [19]. Free copper ions are known to participate and catalyze the formation of reactive oxygen species via Fenton reaction and Haber Weiss reaction [20] resulting in the generation of an extremely reactive hydroxyl radical (•OH) potent enough to damage any biomolecule present in its vicinity. Elevated levels of copper have been reported to deplete reduced glutathione levels [21]. Melatonin and its metabolites also possess metal chelating ability as well [22]. Parmar et al. [23] suggested that melatonin protects against copper-mediated peroxidation of lipid membranes by binding directly with Cu (I) and Cu (II). Combined treatment with copper and ascorbate is an excellent model for studying oxidative stress in vitro which yields high amounts of reactive oxygen species [24]. The oxidative modification and consequent inhibition/inactivation of antioxidant enzymes by ROS in mitochondria is of fundamental importance as these enzymes provide the basic antioxidant defense against free radical-mediated damage.

Melatonin acts via two identified pathways, i.e., receptor dependent and receptor independent pathways. In mammals, two membrane receptors, classified as MT1 and MT2, are G-proteins coupled high affinity receptors [25,26]. There may also be melatonin binding sites in the nucleus of some cells to synergistically influence the activity of antioxidant enzymes [5]. Studies suggests two receptor independent pathways by which melatonin provides protection to antioxidant enzymes against ROS mediated oxidative damage, namely: (i) through the free radical scavenging ability of melatonin and (ii) through the metal chelating property of melatonin to bind to the free, unbound metal ions itself [27,28]. Here, we identify and elucidate a third mechanism by which melatonin prevents oxidative damage to antioxidant enzymes by the copper-ascorbate system, in vitro, through biochemical analysis in both a cell free/chemical system as well as incubated cellular components (mitochondria, cytosol and peroxisome) and isothermal calorimetric binding studies.

2. Materials and methods

2.1. Reagents and chemicals

Melatonin, pure catalase, pure superoxide dismutase (SOD-1), Janus Green B was purchased from Sigma-Aldrich, St. Louis, MO, USA. Copper chloride, succinate, sodium azide, NAD^{+}, NADPH, pyrogallol were obtained from Sisco Research Laboratories (SRL), Mumbai, India. Thiobarbituric acid (TBA) and ascorbic acid were obtained from Merck Limited, Delhi, India. All other chemicals used were of analytical grade.