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MINI REVIEW

A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes

Abstract: Melatonin, the main secretory product of the pineal gland, is known to collaborate against oxidative stress within cells, but its mechanism of action in terms of stimulating antioxidant enzymes remains unclear. Herein, we propose that melatonin modulates antioxidant enzyme activities via its interaction with calmodulin, which in turn inhibits downstream processes that lead to the inactivation of nuclear RORα melatonin receptor. Eventually, this nuclear transcription factor downregulates NF-κB-induced antioxidant enzyme expression. Therefore, the increment in antioxidant enzyme activities induced by melatonin involves the inhibition of the RORα pathway. Thus, in addition to its direct free radical scavenging activities, melatonin has important actions in oxidative defense by stimulating enzymes which metabolize free radicals and radical products to innocuous metabolites.

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Introduction

Many pathological situations involve free radical generation, which in turn cause oxidative stress. Melatonin is known to protect against oxidative stress in cells, but its mechanisms of action are not totally defined. The indole is known to be a direct free radical scavenger, to increase the efficiency of mitochondrial electron transport, and to promote the activities of antioxidative enzymes. Precisely how melatonin influences the activities of the enzymes which metabolize oxygen-related metabolites to innocuous species is yet to be defined. The basis of this review is to describe a potential mechanism whereby melatonin influences antioxidative enzymes.

Oxidative stress and antioxidant defense

One of the factors that plays a major role in many pathological situations including cancer, ischemia/reperfusion injury, neurodegenerative disorders and even aging, is the excessive generation of free radicals [1–3]. Free radicals are molecules which contain an unpaired number of electrons in their valence orbital; this makes them highly unstable and thus extraordinarily reactive. Among these agents are reactive oxygen species (ROS) which react and damage essential molecules within the cells including lipids, proteins and DNA [4, 5]. In conditions where there is excessive ROS, the resulting molecular destruction is identified as oxidative damage [6].

Reactive oxygen species are formed naturally during many metabolic processes and, thus, cells have developed diverse mechanisms to reduce the deleterious consequences of ROS. To protect themselves against ROS, cells have developed an antioxidant defense which includes enzymatic and nonenzymatic mechanisms. ROS and the antioxidant defense must be in perfect equilibrium to avoid that the relation between ROS formation/depuration being unbalanced. Enzymes involved in the elimination of ROS include superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Besides the enzymatic antioxidant system, there are nonenzymatic antioxidants, or free radical scavengers, which remove ROS directly because of their electron donoring ability thereby neutralizing potential ROS toxicity. The best known nonenzymatic antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbate), glutathione (GSH), β-carotene, and more recently, melatonin.

Melatonin: role on oxidative stress regulation

The main pineal product, melatonin (*N*-acetyl-5-methoxytryptamine), functions as 'time-giver' (Zeitgeber) in the regulation of circadian rhythms [7] and in synchronizing the reproductive cycle with the appropriate season of the year in photoperiodic species [8]. In nonphotoperiodic species such as humans, the melatonin's actions are restricted to other functions of the circadian clock, i.e. consolidation of sleep and regulation of the circadian rhythm of core body temperature [9].

Melatonin's actions, however, are not restricted to its role in the neuroendocrine physiology. Since 1993, melatonin has been known as a radical scavenger with the ability to remove ROS including singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydroperoxide (H_2O_2),

hydroxyl radical ($^{\bullet}OH$) and the lipid peroxide radical ($LOO^{\bullet}$) [10–13]. In some cases, melatonin's efficacy exceeds that of GSH and vitamin E, which are well-known antioxidants [10, 11]. Melatonin's ability to counteract ROS has special relevance as it crosses all morphophysiological barriers and it is widely distributed in tissues, cells and subcellular compartments, because of its distinct physical and chemical properties [14–17]. These allow its localization in cellular organelles and in the cytosol, cellular membranes and nucleus [16, 18–20]. Its widespread subcellular distribution guarantees its ability to interact with toxic molecules throughout the cell, thereby reducing oxidative damage to molecules in both the lipid and aqueous environments of the cells [21–22].

Melatonin also acts as an indirect antioxidant through the activation of the major antioxidant enzymes including SOD, CAT, and GPx [23–27]. This activation is a consequence of antioxidant enzyme mRNA synthesis and eventually enzyme stimulation. These processes probably involve melatonin receptors [24, 28–30], although this remains unknown.

Several specific melatonin receptors have been described in a large variety of mammalian and nonmammalian cell types. These include two members of the group of the membrane G-protein-coupled receptors, designated as mt1 and MT2 [31, 32]. Furthermore, the effects of melatonin on transcriptional regulation clearly depend on the expression of RZR/ROR receptors which are located in the nucleus and support the concept that these receptors are mediators of nuclear melatonin signaling [33]. Likewise, the coexistence of mt1 and RORα_1 receptor has been reported in immune system cells and several other organs [30, 34, 35] and in several cell lines [36, 37].

Proposed mechanism of action of melatonin on antioxidative enzymes

It was first established that a relationship exists between antioxidant enzymes and melatonin, and numerous studies have documented this. The mechanism by which melatonin achieves these actions, however, is still unknown. The aim of this brief review is to integrate current knowledge of melatonin and antioxidative enzymes and to propose a hypothetical mechanism of action for melatonin in these processes. These ideas are summarized in Fig. 1 and discussed in the following sections.

Cellular response to oxidative stress: NF-κB pathway

When cells are exposed to an oxidative challenge, ROS formed act as second messengers and cause the expression of genes involved in inflammatory processes [38]. NF-κB is a transcription factor implicated in the transcriptional upregulation of these inflammatory genes in response to changes in cellular oxidation-reduction status [39]. The target genes of NF-κB in various cell types encode proteins involved in immune, inflammatory and acute-phase responses [40]. The transcription factor NF-κB resides in its inactive form in the cytoplasm due to an inhibitory subunit, I-κB [41, 42]. Among the various conditions that induce NF-κB activation in cells is oxidative stress. Once the cell is stimulated by an oxidative stress inducer, I-κB is phosphorylated and NF-κB is translocated into the

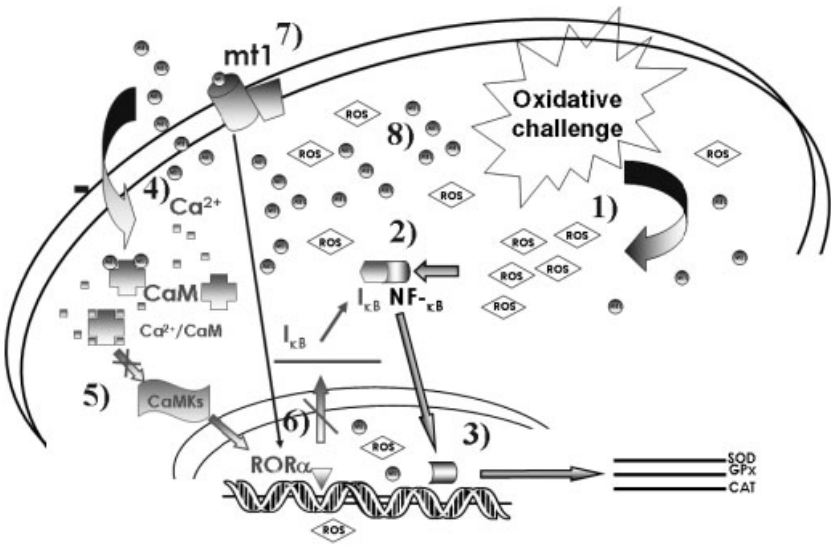

Fig. 1. Proposed melatonin mechanism of action against oxidative stress. When cells are subjected to an oxidative challenge, there is an increment of ROS production which alters the cellular redox state (1). I-κB is phosphorylated and NF-κB translocates into the nucleus (2) and binds to its κB response elements; some of these are located in the promoter regions of the major antioxidant enzymes (3). To maintain this antioxidant pathway, melatonin inhibits the RORα route. The indoleamine blocks the RORα activity through, at least, two different mechanisms. One is the direct melatonin interaction with calmodulin (4), which in turn leads to the inactivation of the calmodulin-dependent kinases (5); this step would repress RORα transcriptional activity on the I-κB gene, allowing the maintenance of the NF-κB pathway (6). Furthermore, melatonin could also restrain RORα constitutive activity through its membrane receptor, mt1 (7). Likewise, melatonin is able to counteract oxidative stress, by means of its direct scavenging activity (8).

nucleus. There, it binds to the κB response element in the enhancer and promoter regions of its target genes. Meanwhile, phosphorylated I-κB is degradated by the proteasome [43, 44].

An early cellular response to oxidative stress is an activation of antioxidant enzymes, including SOD, GPx and CAT [45]. Likewise, the antioxidant enzyme genes are present in the promoter gene region of κB response elements [46]. On the basis of these findings, Zhou et al. [47] characterized antioxidant enzyme activation via the NF-κB pathway within skeletal muscle cells under oxidative stress. From these results, it is known that the transcriptional activation of antioxidant enzymes genes is, at least in part, through the redox-sensitive transcription factor NF-κB. These data seem to indicate a possible role of NF-κB/I-κBα in melatonin's mechanism of action. However, this supposition requires the existence of a connection between NF-κB and melatonin.

Regulation of the inhibitory protein I-κB by the nuclear transcription factor RORα

Delerive et al. [48], by means of primary human aortic and coronary artery cell cultures, showed that RORα negatively interferes with the NF-κB signaling pathway by reducing NF-κB translocation into the nucleus. This action of RORα on NF-κB is through the induction of I-κB, the major inhibitory protein of the NF-κB signaling pathway, whose expression was found to be transcriptionally upregulated by RORα via an ROR response element in the I-κBα promoter. This implies that this nuclear transcription factor is able to negatively regulate antioxidant defense by means of the maintenance of I-κB synthesis.

There are studies which show that the RORα-upregulation of I-κBα synthesis prevents NF-κB activation; meanwhile, the reduction of its production is necessary for the activation of the NF-κB pathway [49–51]. Thus, the nuclear transcription factor RORα, described by some authors as a mediator of nuclear melatonin signaling [33], may be responsible for the increase of I-κB synthesis. These findings along with those discussed in previous paragraphs suggest RORα involvement in antioxidant enzyme gene expression.

We can sum up these data with two important statements: (i) melatonin upregulates antioxidant enzyme activities and gene expression; (ii) RORα is directly implicated in the I-κB rise, which in turn leads to the downregulation of antioxidant enzymes gene expression. Now, however, one question arises: how is it possible that melatonin enhances antioxidant enzymes if its nuclear receptor, RORα, is responsible for the inactivation of the NF-κB pathway? To answer this question, we review the current knowledge concerning RORα regulation.

Melatonin and its relation to the nuclear transcription factor RORα

Becker-Andre et al. [52] reported the identification of melatonin as a ligand of the RZR/ROR, but they subsequently retracted their report [52] and other authors were not able to confirm the existence of the nuclear receptor [36]. However, other data document effects of melatonin on transcriptional regulation which depend on the expression of these receptors [33]. Furthermore, it was shown that the inhibition of the RORα constitutive transcriptional activity was mediated by melatonin and by the melatonin mt1 receptor agonist, AMMTC [36]. This would imply that RORα receptor is not the primary receptor responsible for mediating melatonin's action. Thus, we also include the melatonin membrane receptor (mt1) in our scheme and there are studies that suggest a relationship between mt1 and RORα [36, 53, 54].

Wiesenberg et al. [55] identified a synthetic compound, CGP 52608, which is a RZR-ligand of nuclear receptor RORα. Pablos et al. [56] have demonstrated that this agonist duplicates the stimulatory effect of melatonin on cerebral and cerebellar GPx activity in vivo. As the studies which involve melatonin and oxidative stress implicated a receptor-mediated mechanism, the results obtained by Pablos et al. [56] led to the assumption that RORα, because of the action of melatonin, decreases oxidative stress by stimulating antioxidative enzyme activities. However an explanation of the results obtained by Pablos et al. [56] could be different than proposed. Thus, a feasible mechanism by which CGP 52608 activates GPx activity is through the downregulation of the I-κB gene which would allow for NF-κB activation. Thus, melatonin, as a repressor of RORα transcriptional activity, would achieve its antioxidant action by repressing the transcriptional effect of this nuclear factor [53], in the same way that the indoleamine suppresses the transcriptional activity of other genes [57].

Melatonin is not the only factor that regulates RORα. To identify the actual role of this molecule against oxidative stress, we must take into account other mechanisms which control RORα transcriptional activity, e.g. Ca^{2+}/calmodulin (CaM)-dependent kinases.

RORα is activated by Ca^{2+}/CaM-dependent kinases and inhibited by melatonin

Staggered mice carry a deletion in the RORα gene and have a prolonged humoral response, overproduction of inflammatory cytokines and are immunodeficient [58]. These animals present a phenotype similar to the CaM-dependent kinase IV-deficient mice. For this reason, Kane and Means [59] studied the possible relationship between RORα transcriptional activity and CaM kinases in HaCaT cells. These kinases are Ca^{2+}/CaM dependent.

Elevation of intracellular Ca^{2+} plays an important role in a broad array of cellular functions, including the activation of transcription and regulation of cellular processes [60]. The Ca^{2+} signal is principally mediated by CaM, which serves as the cell's primary Ca^{2+} receptor and is located in both the cytoplasm and nucleus [61]. Ca^{2+}/CaM activates many enzymes including protein kinases and phosphatases, adenylyl cyclases and cyclic nucleotic phosphodiesterases [60]. Within this group of signaling molecules, significant emphasis has been placed upon the CaM-dependent protein kinases (CaMKs), which phosphorylate a variety of substrates in vitro and in vivo

[reviewed in 62]. While CaMK I and CaMK II are widely expressed in many tissues, CaMK IV expression is relatively limited to the brain, T-lymphocytes and postmeiotic male germ cell [63–65].

The studies performed by Kane and Means [59] revealed that RORα is activated by CaM kinase IV and I (CaMK IV, I) and, thus, it is a Ca^{2+}-responsive transcription factor. In support of these findings, Dai et al. [53] studied MCF-7 cells which exhibit a high basal level of RORα transcriptional activity. They have found that the RORα transactivation and DNA-binding activity was repressed by melatonin even though the indoleamine had no effect on RORα protein levels.

Given that Ca^{2+}/CaM-dependent kinases are important for RORα activity, and melatonin has been reported to modulate the Ca^{2+}/CaM signaling pathway in other tissues, it seems reasonable that the connection point between RORα and melatonin, in this pathway, is CaM.

Melatonin can inhibit RORα via CaM

Melatonin has been shown to directly bind to CaM in vitro, via a nonreceptor-mediated process [66–68]. Such binding antagonizes the physiological effects of CaM [69]. Furthermore, Benitez-King et al. [70] demonstrated the in vitro inhibition of CaM-dependent CaMK II activity by physiological melatonin concentrations.

Calmodulin manifests its mechanism of action through a CaM-dependent CaMK series, the same CaMKs that participate in the RORα activation. Thus, melatonin may modulate antioxidant enzyme production via its interaction with CaM, which in turn would inhibit downstream processes, leading to the inactivation of the RORα transcription factor and the consequent downregulation of I-κB transcription.

Significance and conclusions

The actions of melatonin in reducing oxidative stress have been summarized in a number of recent reviews [71–74] and its ability to reduce free radical damage stems from a number of actions. While melatonin's free radical scavenging properties are well known, the mechanisms of antioxidant enzyme activation by the indoleamine have not been elucidated. Many studies have demonstrated that melatonin induces gene expression for and stimulation of the activity of the important enzymes. Little is known, however, about the mechanisms underlying these melatonin-regulated processes. In the current hypothesis, we have proposed a possible melatonin mechanism to reduce oxidative stress which is supported by data summarized in this article. In this proposal, RORα, a nuclear transcription factor, acts as a prooxidant factor which downregulates the majority of the processes implicated in the fight against oxidative stress. Application of melatonin to this pathway leads to the abolishment of the RORα effects, favoring the antioxidant mechanisms displayed by cells. Thus, melatonin could exert its indirect antioxidant actions through several mechanisms, which in turn would lead to the maintenance of the cell integrity and protection against oxidative stress.

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