Minireview

REGULATION OF ANTIOXIDANT ENZYMES: A SIGNIFICANT ROLE FOR MELATONIN

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Running title: Melatonin regulation of antioxidant enzymes

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Key words: Melatonin, regulation, antioxidant enzymes, antioxidant enzyme activity, and antioxidant enzyme gene expression.

ABSTRACT

Antioxidant enzymes form the first line of defense against free radicals in organisms. Their regulation depends mainly on the oxidant status of the cell, given that oxidants are their principal modulators. However, other factors have been reported to increase antioxidant enzyme activity and/or gene expression. During the last decade, the antioxidant melatonin has been shown to possess genomic actions, regulating the expression of several genes. Melatonin also influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes. In the present report, we review the studies which document the influence of melatonin on the activity and expression of the antioxidative enzymes glutathione peroxidase, superoxide dismutases and catalase both under physiological and under conditions of elevated oxidative stress. We also analyze the possible mechanisms by which melatonin regulates these enzymes.

INTRODUCTION

Aerobic organisms require ground state oxygen to live. However, the use of oxygen during normal metabolism produces reactive oxygen species (ROS), some of which are highly toxic and deleterious to cells and tissues. The most abundant ROS formed in the course of cellular metabolism is the superoxide radical ($O_2^{\bullet -}$). This radical is mainly produced during electron transport in the mitochondria and in the endoplasmic reticulum, although it is also a byproduct in several enzymatic reactions (oxidases and oxygenases); likewise, it is formed during the hepatic metabolism of some molecules and also as a result of the decomposition of oxyhemoglobin [1].

Dismutation of the $O_2^{\bullet -}$ gives rise to hydrogen peroxide (H_2O_2). This molecule is not a free radical *per se* but, in the presence of transition metals via the Fenton reaction, it is rapidly converted to the hydroxyl radical ($^{\bullet}OH$). The $^{\bullet}OH$ is widely accepted as being the most damaging ROS produced by cells [2]. Free radicals in general and the $^{\bullet}OH$ in particular react with virtually every molecule in living cells (i.e., lipids, sugars, amino acids, nucleotides) with very high rate constants [3]; the resulting damage ultimately may lead to diseases such as cancer, neurodegeneration and autoimmune conditions [4-6].

To protect cells from the damage caused by free radicals and related reactants, organisms have evolved several defense mechanisms to rapidly and efficiently remove ROS from the intracellular environment. When the equilibrium between free radicals (oxidants) and antioxidant defense systems is imbalanced in favor of oxidants, the condition causes what is known as oxidative stress. The oxidants that are not directly scavenged or otherwise not

metabolized attack cellular components producing useless molecular debris and sometimes cell death.

Antioxidant defense systems may be generally classified into indirect enzymatic antioxidant enzymes and into small molecular weight molecules which directly scavenge free radicals and related reactants. The antioxidant enzymes represent a first line of defense against these toxic reactants by metabolizing them to innocuous byproducts.

The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of $O_2^{\bullet-}$ when they are converted to hydrogen peroxide (H_2O_2) and diatomic oxygen. The enzyme at this step is one of two isoforms of superoxide dismutase (SOD); CuZnSOD is present in the cytosol while (MnSOD) is located in the mitochondrial matrix. These enzymes possess transition metals (Cu^{2+} or Mn^{3+}, respectively) at their active sites; this allows for the rapid exchange of electrons between the two superoxides. Although H_2O_2 is not a radical itself, it is reactive and it is rapidly converted into the highly reactive $^{\bullet}OH$ in the presence of ferrous ion (Fe^{++}) via the Fenton reaction unless it is efficiently removed. Two enzymes participate in the removal of H_2O_2 from the cellular environment, peroxidases and catalase. The most abundant peroxidase is the glutathione peroxidase (GSH-Px), which is present in both the cytosol and mitochondria. This enzyme has the transition metal selenium at its active site and uses reduced glutathione (GSH) as a substrate to transfer electrons to H_2O_2 (and other peroxides) thereby converting it into two molecules of water. The second H_2O_2 metabolizing enzyme is catalase (CAT); it is present mainly in the peroxisomes, presents a molecule of ferric ion at its active site and converts two molecules of H_2O_2 into one molecule each of water and diatomic oxygen [7].

Antioxidant enzymes are regulated by multiple factors. Oxidative status of the cell is the primary factor regulating gene expression and activity of these enzymes [8-10]. Both endogenous [11] and exogenous agents [12, 13] act as oxidants and alter cellular oxidative equilibrium and therefore antioxidant enzyme gene expression. There are, however, several other factors which influence antioxidant enzymes. In addition to developmental changes, differentiation and aging influences [14-18], inflammation [19, 20] and hormonal regulation of antioxidative enzymes have been reported [21-23]. Additionally, several antioxidants and cell protectors are believed to regulate gene expression and antioxidant enzyme activity [24-29].

Although, melatonin is known to be an indole secreted by the pineal gland, other organs may produce melatonin where it has functions without being released. Besides its properties as a circadian rhythm transducer [30], several other actions for this interesting molecule have been in uncovered in the last two decades [31, 32]. Its direct free radical scavenging activity [33, 34] and its regulation of gene transcription [35] for antioxidative enzymes are of special interest in the present review. The antioxidant properties of melatonin have been extensively studied and the use of this molecule as a cell protector and as a potential disease-preventing agent have been summarized [36-40]. Melatonin has been proven to be an efficient oxidant scavenger of a variety of radical and non-radical reactants [37, 41]. Control of gene expression by melatonin was initially suggested by Menendez-Pelaez et al. [42, 43]. Thereafter, the regulation of expression of several genes related to antioxidative enzymes was reported [24, 44-58]. Herein, the literature related to the regulation of enzyme activity and gene expression of antioxidant enzymes by melatonin is reviewed.

REGULATION OF ANTIOXIDANT ENZYMES BY MELATONIN

Regulation under basal oxidative stress and conditions

Reports documenting the influence of melatonin on antioxidant enzyme activity were first published in the mid–1990s [59, 60]. These papers described the amplification of GSH-Px activity in the brain of rat and in several tissues of chicks after exogenously administered melatonin (500 μg/kg) [36, 59, 60]. Thereafter, several groups showed that melatonin increases the activity of antioxidant enzymes in other tissues and models. Thus, Ozturk et al. [61] found increased SOD activity in rat liver after administration of 10 mg/kg of melatonin for 7 days, while Liu and Ng [62] reported enhancement of SOD activity in rat kidney, liver and brain after a single melatonin injection (5 mg/kg).

Antioxidant enzyme activities exhibit endogenous rhythms under normal light:dark conditions. This is true both in terms of their activity and gene expression. These changes with time suggested that these cycles might be dependent on the circadian melatonin rhythm [63-65]. Abolition of endogenous the melatonin cycle by exposure of animals to constant light, in fact, also abolished the nighttime rise in antioxidative enzyme activity. This illustrates that changes in physiological levels of melatonin are adequate to alter the antioxidative defense system as reflected in the level of activities of antioxidative enzymes. Continuous exposure to light is known to abolish the nocturnal melatonin rise; this was associated with a reduction in the nighttime increase in GSH-Px and SOD activities in several tissues of chicks [64, 66]. These results were subsequently confirmed by others in rodents [67, 68]. Similarly, Baydas et al [69] reported that melatonin deficiency caused by pinealectomy reduced GSH-Px activity levels in several tissues of rats.

Melatonin administration during pregnancy has also been shown to stimulate antioxidant enzyme activity in the fetuses. Okatani et al. [70, 71] have reported this finding in both rats [70] and humans [71]. They initially showed that relatively high doses of melatonin (10 mg/kg), administered to pregnant rats, caused incremental changes in the concentration of the indole in both maternal serum and fetal brain as early as 1 h after its administration. Concomitantly, GSH-Px and SOD activities were likewise increased in fetal brain. This indicates that melatonin may be potentially beneficial in the treatment of stressful conditions that involve free radical production such as fetal hypoxia and preeclampsia. Subsequently, they administered much lower doses of melatonin (100 μg/kg bw) to pregnant woman before they underwent voluntary interruption of pregnancy and they found an increase in GSH-Px activity in chorionic homogenates with a peak 3 h after indole administration. This again supports the idea that melatonin may have potential usefulness as a fetal protector under conditions of elevated oxidative stress.

Melatonin has also been shown to influence antioxidant enzyme gene expression. As first reported by Antolin et al. [24], melatonin causes incremental changes in mRNA levels for both CuZnSOD and MnSOD in the Harderian gland of female Syrian hamsters after its exogenous administration (500 μg/kg). Increases in antioxidant enzyme gene expression following melatonin injections (50 and 500 μg/kg) were later confirmed by the same group [52] in rat brain cortex. Finally, Mayo et al. [72] showed that mRNA levels for antioxidant enzymes were elevated in non-differentiated PC12 cells and the human neuroblastoma cells SK-N-SH after melatonin was added to the medium in which the cells were grown. These workers reported that the increases in CuZnSOD and gene expression were maximal at 24 and 6 hours, respectively, following melatonin administration. This effect was induced with a

melatonin concentration of 10^{-9}M, the physiological levels of this indole in nighttime serum; conversely, no effect was observed when higher doses of the indole were used. Regulation of antioxidant enzyme gene expression by melatonin is dependent on new protein synthesis, since use of an inhibitor of protein synthesis, i.e., cycloheximide, prevents mRNA increases after melatonin administration. The indole also reduced the half life of CuZnSOD and GSH-Px while it did not affect that of MnSOD indicating that a larger amount of mRNA may be generated for GSH-Px and less mRNA for CuZnSOD. Finally, the presence of melatonin in the culture medium for 1 hour only is sufficient to increase mRNA for antioxidant enzymes 24 h later, indicating a possible role for melatonin receptors in the regulation of antioxidant enzymes by this indole.

Regulation under elevated oxidative stress conditions

When cells are exposed to oxidative stress they increase the activity and expression of antioxidant enzymes as a compensatory mechanism to better protect them from the damage induced by free radicals. In many cases the number of free radicals generated may be so great that even the increased activity of the antioxidative enzymes are insufficient to counteract the potential damage. When antioxidant enzyme activities and/or gene expression were examined under highly elevated oxidative stress conditions, it was found that they are sometimes diminished; thus, it has been proposed that moderate levels of toxic reactants induce rises in antioxidant enzymes while very high levels of reactants reduce enzyme activities due to damage of the molecular machinery that is required to induce these enzymes [18, 73]. Melatonin has a lengthy history of beneficial actions. For example, almost two decades ago it was reported as a protector against glucocorticoid damage [74, 75], against some degenerative neurological conditions [76], as an anticancer agent [31, 77-79], and also

as an enhancer of immune function [32, 79]. Subsequently, the multiple antioxidant properties of melatonin were described [33, 34, 80, 81] and research on its protective effects against oxidative processes have now been identified under a very wide range of conditions in both experimental animals [82-84] and man [85, 86]. Some of the earliest studies documented the antioxidant properties of melatonin in the central nervous system [87], in the prevention of cataract formation [88], and in the reduction in the severity of colitis [89]. At roughly the same time, Pablos et al. [60] described the regulation of antioxidant enzyme activities by melatonin; this was quickly followed by studies confirming the original findings and extending the observations of melatonin's influence on gene expression for antioxidative enzymes.

Antioxidant enzyme regulation by melatonin has been shown to occur concomitant with its protection against elevated oxidative stress in numerous experimental situations. In the first report to document this correlation it was shown that melatonin increased GSH-Px activity and simultaneously reduced free radical damage to the brain and liver of rats treated with lipopolysaccharide (LPS) [90]. In this study, LPS increased total glutathione (tGSH) levels as well as oxidized glutathione (GSSG) concentrations while reducing the activity of GSH-Px. Melatonin (4mg/kg) given to LPS-treated rats enhanced tGSH above basal levels and lowered GSSG concentrations while stimulating the activity of GSH-Px. This indicated that melatonin may act on several points in the antioxidant defense system, not exclusively on GSH-Px. Subsequently, Antolin et al. [24] reported rises in both CuZn and MnSOD gene expression in the Harderian gland after melatonin (500 μg /kg) was administered to female hamsters. The female hamster Harderian gland is in continual jeopardy of experiencing oxidative stress which causes cell damage due to the extremely high content of porphyrins in

this organ. The administration of melatonin lowered porphyrin synthesis and cell damage in this extraorbital tissue and increased gene expression for both isoforms of SOD. In a number of subsequent studies, the activities of both GSH-Px and the SOD were repeatedly shown to be regulated by melatonin with these changes being concurrent with the ability of the indole to reduce oxidative damage.

Multiple reports on neural protection by melatonin via its antioxidant properties have appeared subsequent to the initial reports of this action [81, 90, 91]. In several experiments, antioxidant enzyme activity as well as expression was studied. Mayo et al. [25] found that in an experimental model of Parkinson disease in which dopaminergic PC12 cells were treated with the neurotoxin 6-hydroxydopamine (6-OHDA), low doses of melatonin (10^{-7}M) provided protection against apoptotic death induced by the neurotoxin. In this study, melatonin also prevented the reduction in gene expression for three antioxidant enzymes, GSH-Px, CuZnSOD and MnSOD, which followed 6-OHDA treatments. In vivo experiments have provided results consistent with the in vitro findings. When rodents (rats and mice) were treated with either beta-amyloid peptide 25-35 [92] or with D-galactose [93] both of which cause oxidative damage to the brain, melatonin at doses ranging from 0.1-10 mg/kg restored both SOD and GSH-Px activities. Naidu et al. [94] reported reversal of haloperidol-induced decreases in brain SOD and catalase activities by 1-5 mg/kg melatonin. Melatonin (10 mg/kg or 2 μg/ml in drinking water, respectively) also has been shown to be protective against oxidative stress in both fetal [95] and aging brain of rodents [96], with these beneficial effects being associated with increased GSH-Px activity.

In addition to the brain, antioxidant enzyme activity regulation by melatonin has been shown to be involved in the protection against oxidative damage in other tissues. Restoration

or even augmentation of antioxidant enzyme activity by melatonin has been shown to be associated with prevention of free radical damage induced by several toxins [97-99]. For example, intestinal and gastric damage following ischemia-reperfusion or drug administration [100-103], multiple organ damage resulting from therapeutic and non-therapeutic chemotherapeutic agents [104-110], ultraviolet damage to tissues [111], free radical damage in experimental diabetes [112, 113], as well as chemio- and radiotherapy lesions [114-115] are reduced by melatonin. Finally, it has been recently shown that melatonin may retard aging of the senescence-accelerated mouse with this being associated with augmented antioxidant enzyme activity [96].

INTRACELLULAR PATHWAYS INVOLVED IN ANTIOXIDANT ENZYME REGULATION BY MELATONIN

Mayo et al. [72] provided an insight into the mechanisms by which melatonin regulates antioxidant enzyme gene expression using cultured dopaminergic cells. They found that melatonin induced synthesis of new protein as a condition for regulation of gene expression of all the three antioxidative enzymes, CuZnSOD, MnSOD and GSH-Px. Melatonin also diminished the half-life of mRNAs coding for both CuZnSOD and GSH-Px, without altering that of MnSOD in this study. This indicates that, in the case of the two former enzymes, melatonin in the medium probably induced more abundant levels of mRNAs with shorter half-lives. Finally, nanomolar concentrations of melatonin were adequate to induce antioxidant gene expression with a one-hour exposure to melatonin being adequate to sustain elevated mRNA levels 24 hours later. As noted above, this points to the likelihood of receptors being involved in antioxidant enzyme gene expression.

The mechanisms involved in the regulation of antioxidant enzymes by melatonin in vivo have not precisely determined. It is known, however, that stimulation of antioxidant enzyme gene expression occurs at nanomolar concentrations of melatonin in cultured cells [72]; these melatonin levels are equivalent to the serum concentration of melatonin at its nocturnal peak in vivo. The quantities of melatonin used in most of the in vivo experiments, however, very likely caused circulating levels to exceed physiological concentrations. Thus, melatonin in these studies may have functioned as a direct radical scavenger thereby changing the redox state of cells, which in turn may have altered the specific activity of these enzymes or their level of translation [116]. Only twice, as far as could be determined, has gene expression for antioxidative enzymes under the influence of melatonin been analyzed in in vivo experiments [24, 52] and, surprisingly, changes in enzyme activities after melatonin treatment has not been examined in cell culture experiments.

Kotler et al. [52] found that after chronic administration of melatonin (50 and 500 μg/kg) to rats, the lower dose clearly had a greater stimulatory effect on antioxidant enzyme gene expression than did the 500 μg/kg dose. Antolin et al. [117] reported melatonin protection against in vivo neurotoxicity of MPTP using 500 μg/kg melatonin (the presumed equivalent melatonin used to induce nanomolar concentrations in serum may be roughly 25-50 μg/kg). The work of Barlow-Walden et al. [59] using 500 μg/kg and Kotler et al. [52] using 50 and 500 μg/kg, indicate that antioxidant enzyme activity and expression, respectively, are elevated after the administration of melatonin peripherally.

What intracellular molecular pathways are involved in the regulation of antioxidant enzyme gene expression and/or activity by melatonin is presently unknown. A membrane G-protein-coupled melatonin receptor MT1 was cloned and characterized by Ebisawa et al.

[118]. Subsequently, MT2 and Mel 1c receptors have also been identified, the former mainly differing from MT1 in terms of the tissues in which it is expressed, while Mel 1c is not found in mammals [119]. Melatonin also has been tentatively shown to activate a nuclear orphan receptor belonging to the retinoid Z receptor β and α (RZR β and α), family. Melatonin's action on RORα receptor represses the expression of the 5-lipoxygenase gene [35] and inhibits growth of the breast cancer MCF-7 cells [120]. The results from Mayo et al. [72] suggest that melatonin regulation of antioxidant enzymes is receptor-mediated, thereby most likely implicating the MT1/MT2 receptors via second messengers such as cAMP, phospholipase C or intracellular calcium concentration, being involved. In addition, binding of melatonin to membrane receptors could stimulate MAP kinase cascades thereby activating several transcription factors [121]. The possibility exists that RZR/ROR receptors could also mediate melatonin effects on antioxidative enzymes as suggested by the results of Pablos et al [122]; if so, the pathways involved in their regulation obviously remain unknown. One possibility may relate to MT1/MT2 melatonin binding that, through second messengers and phosphorylation cascades, activates RZR/ROR as reported by Ram et al. [120]. Another possibility by which melatonin may regulate RZR/ROR receptors would be via modulation of the calcium/calmodulin signaling pathway, either by changing intracellular calcium concentrations by binding to MT1/MT2 receptors [123], or by direct binding to calmodulin [124]. The calcium/calmodulin signaling pathway has been reported to regulate transcriptional activity of RZR/ROR receptors via CaM kinases [125].

Antioxidant enzymes are known to be regulated by several factors which induce oxidative stress [12, 13, 19, 126]; these factors presumably activate oxidative stress-sensitive transcription factors. Also, transcriptional activation of antioxidant enzyme genes has been

reported after the treatment of cells with protective agents [29] where non-oxidative stress-dependent transcription factors are involved. Melatonin has been shown to regulate the activation or repression of several transcription factors [55, 127-130], all of them present in the promoter region of the three-antioxidant enzymes reviewed herein. Thus, subsequent experiments should be undertaken in order to shed light on the intracellular pathways and transcription factors involved in the regulation of antioxidant enzyme gene expression and activity by melatonin.

ACKNOWLEDGMENTS

This work was supported by the CICYT grant # SAF00-0010, the FICYT grants FC PB MED 01 12 and FC PC REC 01 11, and ASTURPHARMA SA (CR). JCM and VM were supported by FICYT. FH acknowledges a fellowship of the Institute of Health Carlos III (FIS). RMS was supported by a Fulbright Grant.

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FIGURE LEGENDS

Figure 1.- Hypothetical pathways involved in melatonin regulation of antioxidant enzyme gene expression and activity. 1) Melatonin activation of MT1/2 receptors, vía G inhibitory protein (Gi), inhibits adenylate cyclase and reduces cyclic AMP (cAMP). This results in inhibition of protein kinase A (PKA) and cAMP response element binding protein/activation transcriptor factor (CREB-ATF). This pathway could modulate immediate early gene (IEG) transcription and consequently gene transcription regulation and antioxidant enzyme concentration. 2) MT1/2 binding by melatonin activates the phospholipase C pathway. The consequent increase in Ca^{2+} concentration will phosphorylate protein-kinase C (PKC) which activates CREB/ATF thereby increasing the transcription of IEG. Indeed, PKC activates IEG. PKC activation may also activate NF kappa B (NFκB) and other transcription factors (TF). Melatonin may also, in other systems, induce a Ca^{2+} decrease leading to inhibition of PKC. 3) MT1/2 activation may, through both inhibitory G (Gi) and other G proteins, activate several mitogen activated protein kinases, i.e., extracellular regulated kinase (ERK) and Jun N-terminal kinase (JNK), which regulate IEG activation and thereby gene transcription. 4) Melatonin may inhibit calcium-calmodulin (Ca-CaM) complex by direct binding a lowered Ca^{2+} concentration mediated by MT1/2 receptors has been reported in some models. This would inhibit calmodulin-kinase (CaMK), which in turn may regulate NFκB, the retinoid-related receptor (ROR) and other transcription factor activation, thereby influencing gene transcription. Ca^{2+}-CaM inhibition may also regulate PKC. 5) Melatonin is a free radical scavenger. Although this effect is not receptor-mediated, we should not rule out the possible involvement of receptors the regulation of antioxidant enzymes. Changes in the cellular redox state towards a more reduced environment produces protein reduction which may lead to enzyme activation (a). Also this environment may induce

translational changes which would increase enzyme concentrations (b). Finally, a decrease of free radicals would allow repression of redox-sensitive transcription factors (i.e., NFκB, AP-1) which would regulate gene transcription (c). Continuous lines indicate previously reported melatonin actions. Dashed lines indicate general cellular mechanisms previously known but not probed with melatonin. *These effects of melatonin have not been documented.