RESEARCH PAPER

Effect of melatonin co-treatment against kainic acid on thiol redox modulator gene expressions in Rat Hippocampus

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Abstract

Kainic acid (KA)-induced neurotoxicity is mediated by oxidative stress, and melatonin (Mel), an antioxidant pineal hormone, provides neuroprotection against KA. The maintenance of intracellular redox homeostasis in brain is essential for neuronal survival and the regulation of redox homeostasis is provided by thiol containing molecules such as glutathione (GSH), thioredoxin (Trx)/thioredoxin reductase (TrxR) and redox factor-1 (Ref-1). In this study, we aimed to determine the effect of melatonin treatment on gene expressions of Trx, TrxR and Ref-1 against KA in rat hippocampus. Our results show that the levels of TrxR mRNA significantly increased in Mel+KA when compared to controls ($p<0.01$) and Mel only ($p<0.05$). The Mel only treatment significantly decreased the expression of Ref-1 mRNA. Also, there was a significant difference in the expression of Ref-1 mRNA between Mel+KA and Mel only ($p<0.01$). However, there was no significant change in the expressional levels of Trx mRNA in Mel- and Mel+KA-treated rats. Our results may suggest that melatonin plays a role in the regulation of thiol redox modulator genes in the brain in order to maintain cellular redox homeostasis against KA-induced oxidative stress.

Introduction

Oxidative stress occurs when redox homeostasis within the cell is altered. This imbalance may be due to either an overproduction of reactive oxygen species (ROS) or a deficiency in an antioxidant system. ROS are known to damage all cellular biomacromolecules (lipids, carbonhydrates, proteins, DNA) and this damage can lead to secondary products as harmful as the initial ROS. The brain is particularly vulnerable to oxidative insult. It has relatively poor concentrations of antioxidants, the high content of polyunsaturated lipids and redox-active transition metals capable of generation ROS. Thus, oxidative stress is a common discussion point for neurodegener-ative diseases.

Excitotoxicity is widely considered to be a contributing factor in neuronal death associated with a number of central nervous system insults or disorders, including stroke, epileptic seizures, Parkinson's disease etc. Kainate (KA) is a potent excitotoxin isolated from the seaweed Digenea simplex which stimulates subtype of the ionotrophic receptor of the brain neurotransmitter glutamate and results in transmembrane ion imbalance, especially causing calcium influx, which turn generates ROS. Intracellular free Ca^{2+} overload damages the neurons in various ways such as activation of phospholipase A2, phospholipase C, protein kinase C, endonucleases, nitric oxide synthase (Tymianski and Tator, 1996, Cheng and Sun, 1994). Since these enzymes contribute to free radical generation, KA-induced ROS formation can be thought critical in its excitotoxic effects. In addition, administration of kainate to rodents can trigger characteristic limbic seizures and selective neuronal cell death in the hippocampal CA1 and CA3 sub-sectors (Ben-Ari, 1985, Lee et al, 2000).

The regulation of intracellular redox homeostasis is crucial for numerous biological events to occur, such as enzyme activation, DNA synthesis, cell cycle regulation, transcriptional activation of specific genes and apoptosis (Dennery, 2000). Intra-cellular redox homeostasis is regulated by thiol containing molecules such as glutathione (GSH), thioredoxin (Trx)/thioredoxin reductase (TrxR) and redox factor-1 (Ref-1). In many cellular processes, thiol-disulfide exchange is provided by an interaction between Trx and GSH systems (Halliwell, 1999). More importantly, these systems regulate the signal transduction activity of various kinases and phosphatases and modulate the redox control of cell growth, death and the transactivation of redox-sensitive transcription factors (Masutani, 2000). Trx is a small, globular ubiqutious protein of 12 kDa with two redox-active half cysteine residues in its catalytic active center (Holmgren, 1989). It participates in redox reactions by the reversible oxidation of its active center, dithiol, to disulfide and catalyzes dithiol-disulfide exchange reactions, including signal transduction and gene expression. Trx is maintained in its active reduced form by flavoenzyme, TrxR, in the presence of NADPH, which constitutes the thioredoxin system and it enhances the binding of transcription factors to the target DNA more efficiently than GSH. Trx can directly associate in the nucleus with redox Ref-1 which is identical to a DNA repair enzyme, AP endonuclease, and both molecules through their redox-active cysteine residues augment the DNA-binding activity of transcription factors, such as activator protein 1 (AP-1) and p53 (Ueno et al, 1999, Hirota et al, 1997, Sen and Packer, 1996). The components of the Trx system not only scavenge ROS but also play regulatory roles in a

variety of cellular function through protein–protein interaction (Masutani and Yodoi, 2002).

The pineal hormone melatonin has free radical scavenging and anti-oxidant properties. Melatonin as an antioxidant is not only effective in protecting nuclear DNA, membrane lipids and possibly cytosolic proteins from oxidative damage, but is also reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism (Antolin et al, 1996, Barlow-Walden et al, 1995, Reiter et al, 2000, Akcay et al, 2005). This hormone is of particular interest as it can prevent neuronal degeneration induced by neurotoxins such as KA and is potentially useful from a therapeutic point of view. The neuroprotective effect of melatonin has been attributed to its antioxidative properties as shown by its ability to act as a scavenger of the highly cytotoxic hydroxyl radicals (Reiter, 1998). It has also been reported that intraperitoneally (i.p.) administered melatonin reduces DNA damage (Uz et al, 1996), lipid peroxidation (Melchiorri et al, 1995), and apoptotic cell death in KA – induced excitoxicity. In addition, our previous report showed that the pre-treatment with melatonin regulates the expression levels of apoptotic molecules such as Bax and Bcl-2 in hippocampus of rats treated by KA (Yalcin et al, 2004).

Considering melatonin's neuro-protective role and the oxidative stress involved in the excitotoxicity of KA, this study aimed to investigate the regulator effect of melatonin co-treatment against KA on the expression levels of Trx, TrxR and Ref-1 genes in the rat hippocampus.

Materials and Methods

Twelve adult male Sprague–Dawley rats weighing 300–350 g were used for the present study. All animals were kept under the same laboratory conditions of temperature (25±2°C) and lighting (14:10 h light/dark cycle) and were given free access to standard laboratory chow and tap water. The protocol for the experiment was approved by the appropriate Animal Care Committee of Ege University. Melatonin (Mel) was dissolved in 100% ethanol and further diluted in saline, resulting in a final concentration of ethanol of 5%). Animals were divided into 3 groups (4 each): saline, Mel (20 mg/kg), and Mel+KA (20 mg/kg Mel given 15 min after 15 mg/kg KA), administered intraperitoneally (i.p.). In KA+ Mel group, seizure activity occurred approximately 45 min after KA administration. All animals were decapitated at the first hour and the hippocampus was dissected on an ice-cold plate. All samples were stored at −80 °C until use.

Isolation of total RNA from hippo-campus tissues

Total RNA was extracted from the hippocampus using Trizol reagent (Gibco BRL Life Technologies, Grand Island, USA) followed by phenol-chloroform extraction and isopropanol precipitation (Chomczynski, 1993). Prior to the reverse transcription, potentially contaminating residual genomic DNA was eliminated with DNAse I (MBI Fermentas).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (1 µg) was used for first-strand cDNA synthesis by MuMMLV reverse transcriptase (MBI Fermentas). Trx, Ref-1 and GAPDH primers were derived from earlier publications (Yalcin et al, 2004, Yalcin et al, 2005) (Table 1). TrxR primers were newly designed to achieve better sensitivity and assay repeatability. Conditions were optimized in a gradient cycler with regard to Taq DNA polymerase

(Perkin-Elmer), primers (TIBMOL-BIOL, Berlin, Germany), $MgCl_2$ concentrations and various annealing temperatures. The forward and reverse primers for Trx, TrxR, Ref-1 and GAPDH are presented in Table 1. Amplification of 4 µl RT mixture was carried out using 5 µl 10×PCR buffer, 4.0 µl 25 mM $MgCl_2$, 1.0 µl 12.5 mM dNTP mix with dUTP, 1 µl of forward or reverse primers (0.1 µg/µl) and 2.5 units Ampli-Taq DNA polymerase in a total volume of 50 µl. The PCR conditions were: 3 min at 94 °C, 30 s at 94 °C followed by 40 cycles of 30 s at 60 °C and 45 s at 72 °C. Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively.

Table 1 Primer sequences used for reverse-transcription-PCR analysis.

Target gene	Forward (5'-3')	Reverse (5'-3')	Expected PCR product (bp)
Trx	CCGCAACAGCCAAATGGTGAAGC	AGCATGATTAGCCAAACTCCGTAA	339
TrxR	CTCTTTCCGCACACAGCATA	CTGTGGGCTCACTGAACAGA	162
Ref-1	GCCAGAGACCAAGAAGAGTA	TCTGAAGGCTTCATCCCATC	520
GAPDH	AAGGTCATCCCAGAGCTGAA	ATGTAGGCCATGAGGTCCAC	338

Agarose gel electrophoresis

The PCR products were analysed on 1.7% agarose gels in TBE buffer and gels were photographed under UV light after staining with ethidium bromide (EtdBr) (0.5 mg/ml). All reactions were carried out in nucleasefree microcentrifuge tubes. Band intensities were quantified from gel photographs using Biocapt software (Vilber Lourmat, Cedex, France). Quantification for Trx, TrxR and Ref-1 were carried out by comparing with the GAPDH amplification upon densitometric analyses. To control the reproducibility, all amplifications were replicated.

Statistical Analysis

Results are given as means ± SD. A level of $p<0.05$ was considered to be statistically significant. Statistical significance was determined using Fisher's protected least significance difference (PLSD) method following analysis of variance (ANOVA) and by the Pearson's correlation coefficient.

Results

In the present experiments, treatment with melatonin did not completely abolish KA-induced abnormal motor behavior. In KA+ Mel group, KA- induced abnormal motor behaviours of animals were characterized by "staring spells", repetitive head nodding, "wet dog shakes," and subsequent rearing and falling.

Fig. 1. shows the expressions of Trx, TrxR and Ref-1 in rat hippocampus of controls, Mel and Mel+KA groups. Expressional changes in Trx, TrxR and Ref-1 in rat hippocampus following Mel and Mel+KA treatments are presented in Fig. 2. Our results showed that there was no significant change in the expressional levels of Trx mRNA in Mel only group when compared to Mel+KA or controls. The levels of TrxR mRNA significantly decreased ($p<0.05$) in Mel only and increased in Mel+KA ($p<0.01$) when compared to controls. The expression of TrxR mRNA was significantly upregulated in Mel+KA when compared to Mel only ($p<0.05$). The Mel only treatment significantly decreased

the expression of Ref-1 mRNA when compared to controls (p<0.01). Also there was a significant difference in the expression of Ref-1 mRNA between Mel+KA and Mel only (p<0.01).

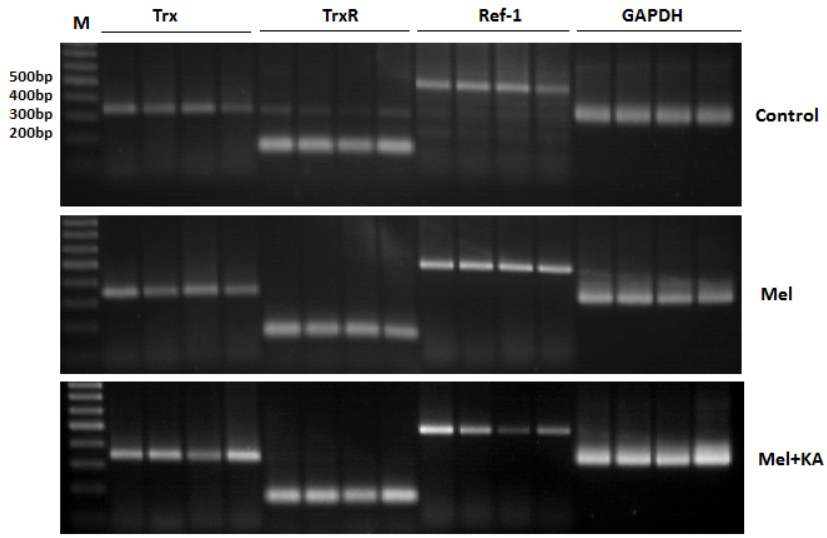

Figure 1 Expressions of Trx, TrxR and Ref-1 in rat hippocampus of control, Mel(melatonin) and Mel+KA. Reverse transcription-PCR products were seperated and visualized by agarose gel electrophoresis representing Trx (330 bp), TrxR (162 bp), Ref-1 (520 bp) and GAPDH (338 bp) in control, Mel and Mel+KA groups. M: 100 bp DNA Ladder molecular size marker. Four individual samples from each experimental group were electrophoresed and visualized for the quantification of target and reference (GAPDH) genes.

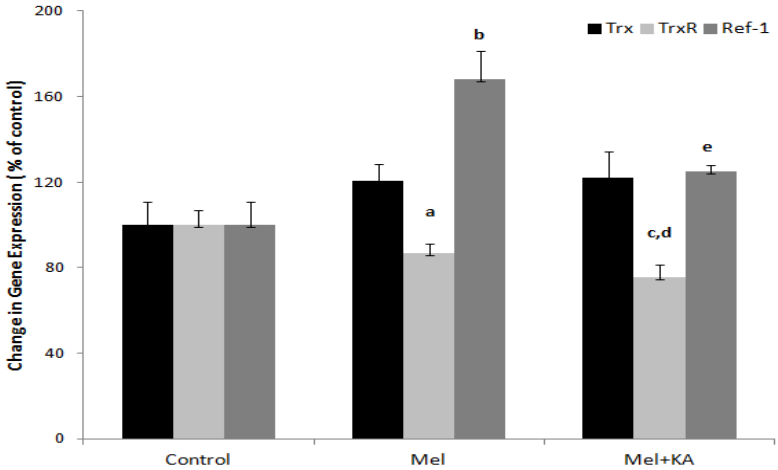

Figure 2 Expressional changes in Trx, TrxR and Ref-1 expressions in the rat hippocampus following Mel and Mel+KA treatments**.** Results are given as mean ±S.D (n=4). [a] p<0.05 Mel *vs* controls; [b] p<0.01 Mel *vs* controls; [c] p<0.01 Mel+KA *vs* controls; [d] p<0.05 Mel+KA *vs* Mel; [e] p<0.01 Mel+KA *vs* Mel.

Additionally, the remarkable correlations were observed between the genes due to their expressional state in Mel and Mel+KA groups. Accordingly, the correlation coefficients between Trx and TrxR genes in Mel and Mel+KA groups were found as r=0.59 and r=0.91, respectively. The negative correlations between the expressions of TrxR and Ref-1 were observed in Mel only and Mel+KA groups as r=-0.12, and r=-0.80, respectively. The correlation coefficients between the gene expressions of TrxR and Ref-1 were determined as r=0.70 and r=-0.53 in Mel only and Mel+KA, respectively.

This study was designed to show the regulator effect of melatonin against KA on thiol redox mdoulators. Our previous studies clearly showed that systemic KA administration produces well-described behavioural changes (Akcay et al, 2005, Yalcin et al, 2004a, Yalcin et al, 2004b, Yalcin et al, 2010, Bojnik et al, 2012, Armagan et al, 2012). Rats exhibit immobility and rigid postures that are replaced by 'staring spells' after approximately 45 min, followed by repetitive head nodding, 'wet dog shakes' and subsequent rearing and falling. In this study we observed that there was no difference in behavioral pattern between Mel only and Mel+KA-treated animals.

Oxygen free radicals and their by-products are capable of causing oxidative damage. ROS are cytotoxic when produced in excess. However moderate concentrations of ROS affect gene expression as well as posttranslational modification of proteins (Sie, 1991). Previous studies have focused on the regulation of gene expression by oxidants, antioxidants, and other determinants of the intracellular reduction-oxidation (redox) state in order to develop better understanding of diseases underlying oxidative stress (Sen and Packer, 1996). Melatonin shows neuro-protective effect dose dependently in KA-induced excitotoxicity (Chen and Chuang, 1999, Chung and Han, 2003). In addition a decrease in melatonin levels in brain increases neuronal damage and degeneration following an excitotoxic process such as stroke and seizure activity (Chung and Han, 2003, Manev et al, 1996). Mainly scavenging of free radicals and protecting from oxidant stress-induced DNA damage and apoptosis mediate the possible protective effects of melatonin.

GSH content and its depletion have been widely reported to regulate a variety of apoptotic signaling pathways. Changes in the intracellular thiol-disulfide (GSH/GSSG) balance are considered major determinants in the redox status/ signaling of the cell (Jones, 2006). Besides direct scavenging activity of melatonin, its protective effect against KA-induced excitotoxicity may also relate to the ability of the indole to maintain glutathione homeostasis in neurons (Floreani et al, 1997). The ability of melatonin to maintain glutathione homeostasis would certainly benefit the neurons considering the important role of GSH in the cell since KA treatment decreases GSH levels in the hippocampus (Yalcin et al, 2010, Turunc et al, 2010). In addition it has been shown that melatonin co-treatment against KA-induced excitotoxicity decreases the levels of lipid peroxidation and restores the levels of CoQ10, an important element of antioxidant defense, both in hippocampal homogenates and mitochondrial fractions (Yalcin et al, 2004b).

Cellular redox is controlled by Trx besides GSH. The exposure of hippocampus to KA results in a concomitant increase in Trx mRNA (Yalcin et al, 2004, Yalcin et al,

2010). It is known that Trx can determine the manifestations of cell death, apoptosis and necrosis. Trx translocates from cytoplasm to nucleus upon stress, and activates the function of transcriptional factors by enhancing their binding capacity to the target DNA. Trx inhibits apoptosis signaling not only by scavenging intracellular ROS in cooperation with GSH system, but also inhibiting the activity of important elements of apoptotic mechanisms in mitochondria such as ASK1 (apoptosis signal-regulating kinase-1) and p38 (Ueda et al, 2002. Pre- (Yalcin et al, 2004a) or co-treatment (Yalcin et al, 2004b) with melatonin against KA do not show a remarkable regulator effect on the expression levels of Trx mRNA in rat hippocampus. Therefore, it can be said that the present results are consistent with our previous findings.

In addition to Trx mRNA, in this study we aimed to determine the expressional changes in the levels of TrxR and Ref-1 mRNA following melatonin or KA treatments in order to evaluate the global status of thiol redox modulators in brain treated with an antioxidant such as Mel, and an oxidative agent, KA. Our results showed that the expressions of TrxR and Ref-1 seem to be more sensitive than Trx to the treatments of Mel or Mel+KA in our experimental conditions. Specifically, we can suggest that TrxR and Ref-1 genes are responsive to oxidative stress-induced by KA since their expression levels significantly upregulated in Mel+KA when compared to Mel only. On the other hand, the low levels of TrxR and Ref-1 gene expression were observed in Mel only group, and this observation may reflect possible activity of melatonin repressing the mechanisms dependent to TrxR and Ref-1 in no stress situation in brain.

Conclusion

In conclusion, our data on the regulation of thiol redox modulator genes by melatonin may support its neuroprotective activity. Therefore melatonin is an important molecule by this point of view since it has been reported that critical steps in the signal transduction are sensitive to antioxidants, and biochemical and clinical studies indicated that antioxidant therapy may be useful in the treatment of diseases (Sen and Packer, 1996).

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Alphabetical order later

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