Effects of doxepin on gene expressions of Bcl-2 family, TNF-α, MAP kinase 14, and Akt1 in the hippocampus of rats exposed to stress

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Abstract

Stress is one of the effective factors in the development of depressive disorders that performs some parts of its effects by affecting hippocampus. Since doxepin has been shown to have neuroprotective effects, in this study, we focused on the effects of doxepin on the expression of involved genes in neuronal survival and plasticity in the rat hippocampus following chronic stress. Male Wistar rats were divided into four groups, the control, the stress, the stress-doxepin 1 mg/kg and the stress-doxepin 5 mg/kg, respectively. To induce stress, the rats were placed within adjustable restraint chambers for 6 h/day, for 21 days. Before daily induction of the stress, rats received an i.p. injection of doxepin. At the end of experiments, expression of Bax, Bad, Bcl-2, tumor necrosis factor alpha (TNF-α), mitogen-activated protein kinase 14 (MAPK14) and serine-threonine protein kinase AKT1 genes were detected by reverse transcription polymerase chain reaction (RT-PCR) in the hippocampus. Results showed significant enhancements in expression of Bax, Bad and Bcl-2 genes in the stressed rats, whereas expression of TNF-α, MAPK14, and AKT1 genes didn’t show significant differences. Doxepin could decrease the expression of Bax and Bad genes in the stress group, but had no significant effects on the expression of other genes. The present findings indicated that doxepin can probably change the pattern of gene expression in the hippocampus to maintain neurons against destructive effects of stress.

Keywords: Doxepin; Stress; Hippocampus; Bcl-2 family, TNF-α, MAP Kinase, AKT

INTRODUCTION

Depression is a physical-mental illness that affects thoughts, moods, feelings, behavior and physical health. Stress is one of the risk factors in depression, but there is little information about its mechanisms (1). Chronic stress changes neural networks and damage brain structure and connectivity (2,3). Studies have shown that stress can directly or indirectly affect genes (4) and consequently cause different effects such as cell death (5). Hippocampus is one of the candidates for the effects of different stresses (6,7). It is an important area for cognitive functions (8,9). It has been demonstrated that stress induces apoptosis and prevents adult neurogenesis in hippocampus (10). Stress damages hippocampal neurons via the adrenal steroids and excitatory neurotransmitters (11). Excitatory neurotransmitters have a role in plasticity in hippocampus; also have an important role in pathological conditions (12). Chronic stress leads to the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) and increased secretion of corticosteroids (13). Also, stress affects the metabolism of the neurotransmitters that are associated with depression such as serotonin, norepinephrine, and dopamine (14,15). Both of these conditions can lead to depression in susceptible individuals (16,17). Recent studies have shown that depression and antidepressants can influence the processes associated with neuronal plasticity and survival through inhibition or stimulation of intracellular pathways (18,19).
Stress caused morphological and functional changes in the structure of different brain regions (20,21), and antidepressants may prevent the effects of stress through influencing these mechanisms or forming appropriate adaptive responses (22).

Doxepin is a tricyclic antidepressant that is also used to treat sleep disorders, acute and chronic pain, chronic urticaria and atopic dermatitis (13). It seems that doxepin performs parts of its antidepressant and protective effects through mechanisms other than inhibition of monoamine reuptake (23). We have previously observed that doxepin can affect expression of genes involved in neuronal survival and plasticity, and prevent stress-induced learning and memory dysfunction, probably via affecting inflammatory cytokines (13,18,24). Therefore, in this study, we focused on the effects of stress on the expression of the genes that the role of their products in inflammation and neuronal apoptosis has been found, and the probable protective effects of doxepin during induction of stress.

MATERIALS AND METHODS

Subjects

The experiments were carried out on male Wistar rats (200-250 g) that were housed under standard conditions of temperature (22 ± 2 °C) and light (12 h light-dark cycle), with free access to food and water. The Ethic Committee for Animal Experiments at Isfahan University of Medical Sciences approved the study and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The animals were randomly divided into four groups of 7 each including the control, the stress, the stress-doxepin 1 mg/kg and the stress-doxepin 5 mg/kg.

Protocols of stress induction and treatment

In the stress groups, the rats were individually restrained for 6 h/day (7:30 AM to 1:30 PM), for 21 days in plastic cylinders (20 × 7 cm) which had holes for air exchange (6). In the doxepin groups, doxepin (1 and 5 mg/kg; dissolved in saline; Ray Chemicals Pvt. Ltd, India) was injected intraperitoneally (i.p.) for 21 days (25). In the stress-doxepin groups, doxepin was injected 5 min before the stress process. Animals in the control groups received the same volume of saline.

At the end of experiment, rats were deeply anaesthetized with chloral hydrates (450 mg/kg, i.p.) (26) and decapitated. Brains were rapidly removed and instantly the hippocampi were dissected in ice-cold artificial cerebrospinal fluid containing 145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 1 mM MgCl₂, 2 mM Na₂Hpo₄ at pH 7.4 (12) and were deep freeze in liquid nitrogen. Then they stored at −80 °C until further studies. Also, the adrenal glands were removed and weighed.

Assessment of gene expression

Real-time polymerase chain reaction (PCR) was used to evaluate the expression of Bax, Bad, Bcl-2, TNF-α, MAPK14, and AKT1 genes. Total RNA was isolated from hippocampus tissues using YTA kit (Yekta Tajhiz Azma, Iran), according to the manufacturer’s instruction protocol; this method first lyse cells by using a chaotropic salt, then binds RNA to silica-based membranes, washes RNA with ethanol-containing wash buffer and then elutes purified RNA by RNase-free ddH₂O. After isolation, the quality of messenger RNA (mRNA) was checked by gel electrophoresis, and RNA quantity was measured using nanodrop (OD 260 nm and 280 nm). At the reverse transcription step, 5 ng of total RNA was used to synthesis the complementary DNA with random hexamers primer using the Reverta-L kit (Amplisens, Moscow, Russia). The real-time PCR was performed using the Step One Plus real-time PCR System (Applied Biosystems, USA). Real Q Plus 2× Master Mix Green with high ROXTM (Ampliqon, Denmark) and specific primers were used (Table 1). Beta-actin (ACTB) was used as an internal control to normalize RNA input. Cycle parameters for real-time PCR included 95 °C for 15 min, 95 °C for 15 s and 60 °C for 60s, the whole process was done 40 cycles and finally melt curve was depicted. All experiments were performed in triplicates for each specimen. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.
Doxepin and the gene expression in stressed rat hippocampus

Fig. 1. Effects of stress and doxepin on adrenal gland weights. Data are expressed as mean ± SEM (n = 10). **P < 0.01 and ***P < 0.001 with respect to the control group, #P < 0.05 with respect to the stress group.

The fold change was calculated using the \(2^{-\Delta\Delta C_T}\) method presented as the fold expression change in treated experiment group relative to their corresponding control group after normalization to the ACTB endogenous control.

**Statistical analysis**

Data were analyzed using both the SPSS 21 (IBM Corporation) for Windows and the REST 2009 (developed by M. Pfaffl (Technical University Munich) and QIAGEN). The results analyzed for gene expression with one sample t-test between the treated and the control groups and with one-way ANOVA between the other groups followed by Tukey’s test. The significant level was set at \(P < 0.05\). Results are expressed as means ± SEM.

**RESULTS**

**Weight of adrenal gland**

The weight of the adrenal glands was increased significantly \((P < 0.001)\) in the stress group compared to the control group. Doxepin significantly \((P < 0.05)\) decreased weight of adrenal glands only in the stress-doxepin 1 mg/kg comparing to the stress group (Fig. 1).

**Levels of gene expression**

As seen in Fig. 2, the mRNA expression of the genes in the stress group was higher than the control group; Bad 727% \((P = 0.024)\), Bax 722% \((P = 0.008)\), Bcl-2 589% \((P = 0.027)\), TNF-α 151% \((P = 0.35)\), MAPK14 497% \((P = 0.27)\), AKT1 328% \((P = 0.3)\).

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**Table 1. Primers used in real-time PCR experiments**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB-F</td>
<td>AGGCCCTCTGAAACCTAAG</td>
</tr>
<tr>
<td>ACTB-R</td>
<td>CCAGGGCATACAGGGACAA</td>
</tr>
<tr>
<td>Bcl2-F</td>
<td>TAAACGGGCTGGGATGC</td>
</tr>
<tr>
<td>Bcl2-R</td>
<td>TGAGCAGGTCTTCAGAGA</td>
</tr>
<tr>
<td>Bax-F</td>
<td>GAGGCGGGCGGTAGTC</td>
</tr>
<tr>
<td>Bax-R</td>
<td>TCCCTGGATGAAACCTGTAGCA</td>
</tr>
<tr>
<td>Bad-F</td>
<td>GACCAGCAGCCACAGTAT</td>
</tr>
<tr>
<td>Bad-R</td>
<td>CGCCTCCATGATGACTGGTTATG</td>
</tr>
<tr>
<td>TNFα-F</td>
<td>ACGTGTTAGCAAACCACCAA</td>
</tr>
<tr>
<td>TNFα-R</td>
<td>CAAGGGCTCTTGATGGCAGA</td>
</tr>
<tr>
<td>MAPK14-F</td>
<td>CCGAGCGATACAGAACCT</td>
</tr>
<tr>
<td>MAPK14-R</td>
<td>CTTCACGTCCACAGATGTC</td>
</tr>
<tr>
<td>AKT1-F</td>
<td>TCGTGTGGCAAGATGTGTAGA</td>
</tr>
<tr>
<td>AKT1-R</td>
<td>CAGGCGGGTGATGGTGAT</td>
</tr>
</tbody>
</table>

CTB was used as a housekeeping gene to compare the samples.
Fig. 2. Effects of doxepin and stress on relative gene expression of Bax, Bad, Bcl-2, TNF-α, MAPK14, and AKT1 in rat hippocampus. The extent of expression was measured by RT-PCR. The mRNA expression data was normalized to the Beta-actin (ACTB) signal. Fold changes relative to the control are presented. Mean ± SEM values of experiments are shown. *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the control group; #P < 0.05 and ##P < 0.01 with respect to the stress group (n = 7).

In the stress-doxepin 1 mg/kg group, doxepin decreased the mRNA expression of Bad 74.14% (P = 0.022), Bax 72.71% (P = 0.017), and TNF-α 52.32% (P = 0.21); and increased the mRNA expression of Bcl-2 22.41% (P = 0.67), MAPK 91.1% (P = 0.43), and AKT 145.12% (P = 0.25) comparing to the stress group.

Doxepin in the stress-doxepin 5 mg/kg group, decreased the mRNA expression of Bad 85.97% (P = 0.006), Bax 75.9% (P = 0.017), Bcl-2 14.26% (P = 0.64), and MAPK 33% (P = 0.83); and increased the mRNA expression of TNF-α 9.93% (P = 0.9), and AKT 1.22% (P = 0.98) comparing to the stress group.

**DISCUSSION**

Results showed that stress caused significant up-regulation in the expression of Bcl-2, Bad, and Bax in the hippocampus. Although, the up-regulation of gene expression of TNF-α, MAPK 14, and AKT1 also observed following the stress, but these changes were not statistically significant.

In accordance with our results, studies have shown that stress increases the expression of Bcl-2 mRNA and its protein in the hippocampus (27,28) and Bad and Bax genes in neurons (29,30). Bcl-2 is anti-apoptotic and Bax and Bad are pro-apoptotic factors (31). These may indicate that stress through the induction of pro-apoptotic factors could damage the neurons, and the up-regulation of Bcl-2 may be a compensatory response (27,28).

In the present study, changes in TNF-α mRNA expression were not significant. But previously we have seen that chronic restraint stress can increase levels of TNF-α protein in the hippocampus (13). Thus, the expression probably increased in the early stages of stress, and then reduced after the production of its protein. However, more studies are needed to prove this assumption.

The MAPK14 gene encodes P38 mitogen-activated protein kinase. This kinase is activated by various environmental stressors and pro-inflammatory cytokines (32). It has been shown that enhancement of the p38 MAPK activity plays a key role in neuronal apoptosis, and its inhibition can block apoptotic pathway in hippocampus (33). Serine/threonine protein kinase is an enzyme encoded by the AKT1. It is an important mediator of growth factor-induced neuronal survival. This enzyme by phosphorylating a large number of proteins inhibits components of the apoptotic machinery (34). The expression of these both genes wasn’t affected by stress after three weeks from initiation of the stress. However, this does not exclude the
possibility of increasing the early and transient expression. Therefore, it is essential to investigate the levels and activity of their proteins.

Secondarily, we evaluated the potential effects of doxepin on the expression of these genes in the hippocampus of the stressed rats. Interestingly, doxepin could decrease the increased levels of expression of the Bad and Bax genes in stress group, but had no significant effects on the Bcl-2 gene. In our previous study, we demonstrated that doxepin could decrease gene expression of Bad in the hippocampus of intact rats (24). This suggests that doxepin mainly decreases the RNA expression of pro-apoptotic genes, however, according to the initial effects of stress on the rise of expression of Bcl-2 gene that inhibits apoptosis, it can be concluded that doxepin could prevent neuronal apoptosis effectively in stress.

Although in our previous study, we observed that low dose of doxepin (1 mg/kg) decreased the increased levels of TNF-α protein in the hippocampus of the stressed rats (13), but present results show that both stress and doxepin have no significant effects on the gene expression of TNF-α, and also MAPK and AKT after a 21-day period of stress and treatments. Therefore, over a long period, the expression of these genes cannot be a candidate for the effects of stress and doxepin, although this suggestion needs to be elucidated.

Another interesting result was the effect of doxepin at 1 mg/kg on suppression of HPA axis and reduction of adrenal glands weight. As both doses of doxepin exhibited similar effects on the gene expression, it can be postulated that its effects on gene expression is independent of HPA axis and glucocorticoids.

CONCLUSION

To determine the potential neurobiological effects of doxepin, we applied a chronic stress protocol and then evaluated the hippocampus for molecular indices that may be involved in this psychopathology. We observed that stress mainly affects the expression of Bcl-2 family genes in the hippocampus and that doxepin changes the pattern of that expression to maintain neuronal survival. Thus, doxepin can be considered as a neuroprotective agent against stress.

ACKNOWLEDGEMENTS

The content of this paper is extracted from the MSc. thesis of student Nastaran Eidelkhani (No. 393045) and a research project (No. 293251) which was financially supported by Applied Physiology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.

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