Abstract

The present study investigated whether the expression of the cAMP response element-binding protein (CREB) in the rat brain is altered following an acute self-limited seizure induced by pentylenetetrazol (PTZ). Male rats were injected intraperitoneally with a single convulsive dose (45 mg/kg) of PTZ, and the matched controls were given saline. For immunohistochemistry, animals were perfused with 4% paraformaldehyde at 24 h following PTZ seizures, and CREB immunoreactivity was examined in rat brain. For real-time RT-PCR, animals were sacrificed at 2 and 24 h and 1 week following PTZ seizures. Tissues from different rat brain regions were micropunched and subjected to real-time RT-PCR using Taqman probe. The CREB immunoreactive profiles were significantly decreased in CA3 and dentate gyrus of hippocampal formation, sensory cerebral cortex and thalamus at 24 h after PTZ seizures. Consistent with changes in CREB immunoreactivity, levels of CREB mRNA were significantly decreased in the hippocampus, cerebral cortex, amygdala and thalamus at 24 h after PTZ seizures. No significant change was found for CREB mRNA expression in these regions at 2 h or 1 week following PTZ seizures. These results show that a brief seizure caused a decline in CREB expression up to 24 h later.

1. Introduction

Epilepsy is one of the most common neurological disorders in man [32], affecting approximately 50 million people worldwide. Various animal models have been developed to study the mechanisms underlying this heterogeneous disease [2,10], including seizures induced by pentylenetetrazol (PTZ) [23]. PTZ can bind to the picrotoxin binding site of the post-synaptic type A gamma-aminobutyric acid (GABA_A) receptor [15] and thus suppress the inhibitory effects of GABA, leading to an easier depolarization of neurons [2,9]. PTZ seizures are mediated by an extensive system involving the reticular formation, diencephalic regions in the vicinity of the anterior medial thalamus and caudal hypothalamus and bulbar regions which give rise to descending motor pathways to the spinal cord [24].

The cAMP response element-binding protein (CREB) is a member of a larger family of structurally related transcription factors, which bind to cAMP-response-element (CRE) promoter sites on target genes. CREB has been implicated in the transcriptional control of several genes, many of which are rapidly expressed in response to an elevation of cytoplasmic cAMP or Ca^2+ levels. CREB is suggested to play an important role in promotion of neuronal survival and differentiation [36]. The phosphorylation of CREB at ser133 can be induced via the protein kinase A pathway [5], or the calcium/calmodulin-dependent kinase (CaMK) pathway, including CaMKI, II, IV and K [4] and/or the mitogen-activated protein kinase pathway [17]. CREB is suggested to be involved in the regulation of immediate early genes [33], neuropeptides [7] and bcl-2 [36] that may either increase biosynthesis of neurotrophic factors or promote cell survival.
Recent studies showed that CREB is activated by acute seizures. In lethargic (lh/lh) mice, a genetic model of absence seizures, gel-shift assays showed that nuclear CRE-DNA-binding activities in the thalamus and cerebral cortex were significantly higher than those of non-epileptic control mice. This higher level of CRE-DNA-binding activity was inhibited by the specific GABAB receptor antagonist CGP 46831, at a dose which suppressed seizure behavior [18,20]. In another study, an acute PTZ seizure caused a significant increase in CRE binding activity in the mouse cerebral cortex and hippocampus [18]. Two hours after a kainic acid-induced seizure in mice, gel retardation electrophoresis revealed potentiated DNA binding for CREB in the hippocampus but not in other regions [1]. CRE-binding activity was significantly enhanced at 2 h in the rat hippocampus following amygdala kindling [21]. The phosphorylation of CREB was significantly increased in the rat neocortex and entorhinal cortex 30 min following acute seizure induced by injection of BAY-K 8644, an L-type voltage-dependent calcium channel agonist [22]. One study has reported that CREB immunoreactivity was significantly decreased in the CA1, CA3 and dentate gyrus of the hippocampus at 90 min following hypoglycemia-induced generalized seizures [28]. However, none of these studies reported whether CREB mRNA is changed at different time points or if CREB protein is changed at longer times (such as 24 h) after an acute seizure. Hence, the present study was done to investigate whether the gene expression of CREB is altered following a brief seizure, using immunohistochemistry and real-time RT-PCR.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (225–250 g) were obtained from Harlan Laboratories (Indianapolis). The animals were kept on a 12-h-light–dark cycle and allowed free access to food and water. All animal procedures were approved by Medical College of Ohio’s Institutional Animal Care and Use Committee.

The animals were randomly divided into two paired groups. The PTZ group was given a single convulsive dose of pentylenetetrazol (PTZ), 45 mg/kg intraperitoneally in saline. Matched controls received saline. At this PTZ dose, rats typically have convulsions, starting in about 1 min, that progress to bilateral front limb clonus (but usually not involving hind limbs). The convulsive episode usually lasts 30 s to 1 min. No rat that progressed to tonic seizures was included in this study.

2.2. Immunohistochemistry

CREB was studied by immunohistochemistry 24 h after PTZ injection. This time point was chosen to correlate with the time of decreased CREB mRNA. At 24 h after injection of saline or PTZ, rats were anesthetized with 65 mg/kg sodium pentobarbital and perfused through the ascending aorta with the descending aorta and vena cava being clamped. The fixative, containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) was infused at 8 ml per min for 35 min. The rat brains were then removed, postfixed in the same fixative for 2 h and soaked for 24 h in 25% sucrose (pH 7.4). Coronal sections of 16 μm were cut in a cryostat at –18 °C and thaw-mounted onto Superfrost/Plus slides (Fisher Scientific). Brain sections were saved at intervals of 96 μm from bregma –0.26 to –4.52 mm. All slides were stored in a cryoprotectant before immunohistochemical procedures [37].

To detect CREB immunoreactivity, tissue sections were rinsed with Tris-buffered saline (TBS; 50 mM Tris–HCl and 150 mM NaCl) containing 0.5% Triton X-100 and incubated for 1.5 h in 10% normal goat serum to block nonspecific background. The endogenous peroxidase activity was quenched with 2% H2O2 for 10 min. Sections were then sequentially incubated with (1) anti-CREB antibody (1:2000, Cell Signaling) overnight at room temperature; (2) 7.5 μg/ml of biotinylated anti-rabbit IgG (Vector) for 12 h at room temperature; (3) avidin-biotinylated horseradish peroxidase complex (1:50, Vector) for 1.5 h at room temperature. The immunoreactivity was visualized by treatment for 5 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) solution in 50 mM Tris–NaCl containing 0.005% H2O2 (Vector). Some tissue sections were counterstained with 0.05% toluidine blue for 5 min before mounting.

Tissue sections from pairs of saline and PTZ-treated rats were processed and analyzed in parallel. The immunoreactivity was viewed with a Spot digital camera system (Diagnostic Instruments) combined with a light microscope. Images were captured into tiff files using a computerized program, “Spot Advanced,” and cell counts were conducted using “Scion Image,” according to a previous method [38]. CREB-positive profiles were identified as round objects containing a dark brown coloration on the computer screen. Parallel sections in the CA1, CA3, dentate gyrus, sensory cerebral cortex and thalamus were selected at bregma –2.80 mm for each PTZ-treated rat and the matched control. For each PTZ-treated rat, a representative section was chosen for analysis, then a corresponding section from the matched control was carefully selected for each brain region. The immunoreactive cells were analyzed with Scion Image software by using the “measure” macro on the images captured at higher magnification objective (×40). For each digitized image, the “threshold” command of the Scion image software was used, and the resulting image was made binary. Then, the total immunoreactive profiles within captured areas (total 0.81 mm²) per section were counted. The person who counted the cells was blinded to the treatment groups.

2.3. Microdissection

For real-time RT-PCR study, rats were euthanized by decapitation at 2 and 24 h or 1 week after seizures. The rat
brains were quickly removed, snap frozen and stored at −70 °C until use. Coronal sections of approximately 500 μm were cut from frozen rat brains in a cryostat at −9 °C and thaw-mounted onto glass slides. Sections were then placed on a precooled microdissection platform. Tissues were micropunched from the hippocampus, cingulate cortex, thalamus and amygdala with blunted stainless-steel needles [26]. Each microdissected brain region from an individual animal (6–10 punches for each brain region) was processed and analyzed as a single datum point.

2.4. RNA extraction and cDNA synthesis

Total cellular RNA was extracted from the various brain regions according to instructions provided with the RNeasy Mini Kit (Qiagen). Briefly, brain tissue was placed in 300 μl of lysis buffer containing 4 M guanidinium salt and β-mercaptoethanol and homogenized using a sonicator. The tissue extract solution was combined with 300 μl of 70% ethanol, and this mixture was loaded onto the RNeasy spin column and centrifuged for 0.5 min at 8000 × g. The column was washed with a buffer containing 70% ethanol and centrifuged two times. Total RNA was finally collected by centrifugation at 14,000 rpm for 1 min. Total RNA was finally collected by centrifugation at 14,000 rpm for 1 min.

About 1 μg of each total RNA sample was reverse-transcribed using Sensiscript RT Kit (Qiagen) in a total reaction volume of 20 μl, containing 1 μM of oligo(dT)12–18, 0.5 mM dNTP, 10 U RNase Out and 200 U of Sensiscript RTase. The reaction was incubated at 37 °C for 1 h and stopped by heating to 93 °C for 5 min. Negative controls including no RNA and no reverse transcriptase were used to confirm that no genomic DNA contamination occurred. We did not find detectable contaminating genomic DNA in any of the controls. The forward and backward primers were also localized in different exons. Hence, routine DNase treatment was not used in this study.

2.5. Taqman probes and primers

The PCR primers and Taqman probes specific for CREB and a housekeeping gene (cyclophilin) were designed according to the manual provided by Qiagen. The specificity of the primers and probes was confirmed by a homology search on Pubmed. The primers for CREB could recognize all subtypes (α, β, δ) of the CREB transcripts. The untagged forward primer for CREB is 5′-CTGAT-TCCCA-AAAAC-GAAGG-3′, which is located in exon 7 and the backward primer 5′-CTGCC-CACTG-CTAGT-TTGTG-3′, which is located in exon 8. The Taqman CREB probe is 5′-FAM-CCATC-ACCAC-TGTAA-CAGTG-CCAAC-C-TAMRA-3′. The expected size of the DNA band for CREB is 188 bp according to published sequences of rat CREB [11,34]. The sequence of upstream primer for cyclophilin mRNA is 5′-ACTGG-GGAGA-AAGGA-TTTTG-G-3′ and downstream primer is 5′-GATGC-CAGGA-CCTGT-ATGCT-3′. The Taqman probe for cyclophilin is 5′-HEX-CACAC-GCCAT-AATGG-CACTG-GTG-TAMRA-3′. The predicted DNA size for cyclophilin mRNA is 171 bp [8].

2.6. Real-time quantitative PCR

PCR was carried out with QuantiTect Probe PCR Kit (Qiagen) in a total volume of 25 μl containing 1 μl of cDNA, 0.4 μM of each PCR primer, 0.2 μM of Taqman probe with passive reference and PCR master mix. The CREB and cyclophilin gene were amplified in separate tubes in duplicate. The amplification parameters were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence emission from individual PCR tubes at each cycle was monitored in an ABI 5700 Sequence Detection System (Perkin Elmer). The cycle of threshold (Ct) values were collected corresponding to the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold, based on baseline data within cycles 3 to 15. The arbitrary threshold was decided to ensure the Ct values were obtained in the exponential phase of PCR where there are no rate-limiting components.

To construct a standard curve, cDNA was synthesized from total RNA extracted from extra tissue of cerebral cortex and then diluted in five steps from 4 to 1024 times. A standard curve of Ct values against the log value of cDNA was generated for both CREB and cyclophilin. The relative concentration of CREB and cyclophilin were obtained from the standard curve, and the ratios of CREB normalized against cyclophilin were calculated.

2.7. Data analysis

All data were expressed as mean ± S.E.M. Correlations were estimated using simple regression analysis. Seizure effects on CREB protein were analyzed by two-way ANOVA using treatment (saline or PTZ) and brain regions as grouping variables, with post hoc analysis using the Tukey test. In the case of the immunohistochemistry results, in which pairs of control and treated brains were processed and analyzed in parallel, the data were treated as matched pairs. Post hoc analysis was done with the Bonferroni test. In all cases, p < 0.05 was considered significant.

3. Results

3.1. Specificity of the immunohistochemistry

Before carrying out immunohistochemistry, the specificity of the antisera against CREB was determined by Western
blot analysis of hippocampal and cortical homogenate (data not shown). The CREB antisera showed a single band at 43 kDa corresponding to the calculated molecular weight of CREB [13,16]. In addition, to determine the specificity of immunostaining, several controls were also used: (1) There was no significant immunostaining in brain tissue sections when primary antibody was omitted from the incubation medium; (2) a dilution series showed a progressive decrease in specific immunostaining with a progressive decrease in concentration of CREB antisera; and (3) an adsorption test was performed with the antibody pre-absorbed with the CREB cell extracts (Cell Signaling), and no significant immunostaining was detected.

3.2. CREB immunoreactivity was decreased at 24 h after PTZ seizures

In the control rats, the CREB immunolabeled cells could be visualized extensively in different brain regions including the sensory and motor cortex, hippocampal formation (Fig. 1), thalamus, hypothalamus, amygdala, striatum and cerebellum. At higher magnification, most neurons showed concentrated staining in the nucleus, whereas only a few had substantial immunostaining in the cytoplasm. In cerebral cortex, CREB immunoreactivity was slightly greater in layer II. Few glial cells were positive for CREB immunostaining. From the counterstained sections, the cell structures in both saline and PTZ treated animals appeared to be very similar, and no obvious neuronal death from PTZ seizure was observed. Representative photomicrographs are shown in Fig. 2, and the number of immunoreactive profiles for CREB is demonstrated in Fig. 3. Two-way ANOVA revealed a significant effect of treatment ($p<0.0001$) and of brain region ($p<0.0001$), but no significant interaction, indicating similar changes in all regions. Post hoc analysis further revealed a significant decrease ($p<0.001$) in the number of immunoreactive cells in CA3, dentate gyrus, cortex and thalamus. At 24 h following PTZ seizures, the number of CREB immunoreactive profiles was significantly decreased in the hippocampal formation, including the CA3 (436 ± 29 vs. 337 ± 18) and dentate gyrus (585 ± 4 vs. 450 ± 28). The possible reduction of CREB immunoreactivity was also compared in other brain regions. The CREB immunoreactivity was significantly decreased in sensory cerebral cortex at 24 h following PTZ treatment (763 ± 29) when compared with the control group (875 ± 5), as well as in the thalamus (449 ± 28 vs. 360 ± 18). In PTZ-treated rats, the shape and size of CREB immunoreactive neurons in different brain regions appeared to be similar to the control rats.

3.3. PCR efficiency and reference gene

To determine the PCR efficiency and calculate the cDNA amount from the Ct value, the standard curves for CREB and cyclophilin were constructed. As exemplified in Fig. 4, a reverse linear relationship was detected for both CREB and cyclophilin primer/probe set when the standard curves for both genes were generated. The numbers of slope ($-3.39$ and $-3.32$) for both genes indicated that our designed primers and probe allowed maximal PCR amplification efficiency. To make sure that the housekeeping gene cyclophilin was not changed by the PTZ treatment, the Ct values for cyclophilin in all the examined brain regions of the control and PTZ groups were collected (data not shown). Student's $t$ test was performed on these Ct values. There was not any significant change detected, indicating the cyclophilin mRNA was not significantly affected by the PTZ treatment in the present study. Thus, we used only one reference gene in our real-time PCR study. To confirm our real-time PCR product, the DNA product from CREB and cyclophilin was subjected to electrophoresis using 2% agarose gel. The gel stained with ethidium bromide showed a single band for each at the expected size (Fig. 4).

3.4. Levels of CREB mRNA were decreased at 24 h after PTZ seizures

At 24 h following PTZ seizures, real-time RT-PCR showed decreased levels of CREB mRNA. Two-way

![Fig. 1. Representative photomicrograph showing CREB immunoreactivity in the hippocampus of male rats in lower magnification (left column) and the dentate gyrus area at higher magnification (right column). (Bar = 500 μm on the left side and 100 μm on right side).](image-url)
ANOVA revealed a significant effect of PTZ treatment \((p < 0.001)\) and no significant difference according to brain region. Post hoc analysis (Tukey test) showed significant treatment effects in cortex \((p < 0.01)\), hippocampus \((p < 0.001)\) and thalamus \((p < 0.05)\). The decrease in amygdala was not quite significant \((p = 0.08)\). The most prominent change occurred in the hippocampus, where there was an average 91% decrease in CREB mRNA levels in PTZ-treated group when compared to control group (Fig. 5, middle). The percent average decrease in CREB mRNA in cerebral cortex, thalamus and amygdala was 70%, 76% and 70%, respectively. Similar changes were found when using the data of CREB mRNA that was not normalized against cyclophilin (data not shown).
3.5. Levels of CREB mRNA were not significantly altered at 2 h or 1 week after PTZ seizures

The CREB mRNA levels at 2 h and 1 week following PTZ seizures were also examined as the reference points for our study. At both time points, no significant change was found in the four brain regions in the PTZ-treated animals. At 2 h following PTZ seizures, CREB mRNA in the hippocampus and sensory cerebral cortex tended to increase but not significantly (Fig. 5 top). It appeared that CREB mRNA was largely returned to normal levels 1 week after PTZ seizures (Fig. 5 bottom).

4. Discussion

The present study was undertaken to investigate the effects of brief, self-limited seizures induced by a single convulsive dose of PTZ on CREB expression in the rat brain, focusing on the hippocampus. The immunohistochemical results showed significant reduction in CREB immunoreactivity in the CA3 and dentate gyrus regions of hippocampal formation, as well as in the sensory cerebral cortex and thalamus, at 24 h following PTZ seizures. Consistent with these results, the real-time quantitative RT-PCR study demonstrated a significant decline in CREB mRNA level in the hippocampus and cerebral cortex at 24 h following PTZ seizures. In addition, real-time RT-PCR also showed significant decrease in CREB mRNA in the amygdala. These findings indicate a widespread decrease in CREB expression 24 h after a single, brief seizure. Levels of CREB mRNA were not significantly changed in the rat brain at 2 h or 1 week following PTZ seizures.

To our knowledge, this is the first report that both CREB immunoreactivity and mRNA levels are significantly decreased in the rat brain at 24 h following a single brief seizure. When comparing real-time PCR data with the immunohistochemical results, it appears that the mRNA levels decreased to a much larger extent than the immunoreactive profiles. This could be due to the sensitivity and limitation of the techniques. The immunohistochemistry detected changes in number of immunoreactive profiles (i.e., cells) and is limited when detecting the strength of immunostaining in individual neurons. Alternatively, it is possible that the decrease in mRNA levels may not be sufficiently prolonged after PTZ seizures to decrease protein to a parallel point. The peak of reduced protein expression may occur somewhat later than the peak decrease in mRNA. Further investigation will be needed to determine the time point after acute PTZ seizures when the CREB immunoreactivity drops to the lowest levels.

The CREB protein is expressed at relatively high levels in normal brain tissues including neuronal and glial cells throughout the neuraxis [14]. In agreement with previous study [12], we observed a slightly higher amount of CREB immunoreactive neurons in the deep layer II in the cerebral cortex. An earlier study could not detect changes in CREB immunoreactivity in the rat brain at 24 h following topical application of KCl to the cortical surface or generalized tonic-clonic seizures evoked by intravenous injection of bicuculline [12], which may have been due to high background when examining total CREB immunoreactivity [6,14]. Before examining the effects of acute PTZ seizures on CREB immunoreactivity, the immunohistochemical protocol for CREB was optimized in the present study by (1) comparing several anti-CREB antibodies; (2) performing Western blotting to ensure that a single protein band corresponding to 43 kDa was shown; and (3) using stringent washing (i.e., 0.5% Triton X-100). Finally, we were able to obtain low levels of background staining and high levels of signal for CREB in the rat brain sections. Unlike the earlier studies [6,14], our immunohistochemistry also showed a low level of glial cell immunoreactive for the CREB, which was consistent with more recent reports [27,28].
To date, data regarding roles of CREB in seizures are very limited. Growing evidence has shown that CRE DNA binding activities were increased by absence-like seizures in brain areas including hippocampus of mouse [1,18–20] and rat [21]. The phosphorylation of CREB was significantly increased in the rat neocortex at 30 min following acute seizure induced by BAY-K 8644, an L-type voltage-dependent calcium channel agonist [22]. Currently, very little is known about the expression of CREB protein after seizures. One previous study reported that the CREB immunoreactivity in the rat hippocampus and cortex was not changed at the time points tested up to 1 h following PTZ seizures [25]. The present study did not examine the CREB immunoreactivity at the time points of 1 or 2 h following PTZ seizures, since our real-time RT-PCR data demonstrated that CREB mRNA was not changed at 2 h following PTZ seizures. In contrast to the earlier report, recent studies showed a significant decrease in CREB immunoreactivity in the hippocampus of both male and female rats at 1.5 h following hypoglycemic seizures [27,28]. It is suggested from these data that the expression of CREB protein may vary depending on the seizure model, the duration following seizures and other unknown factors.

It is not clear what signal pathway might cause the decline in CREB expression after acute PTZ seizures. Acute PTZ seizure has been shown to decrease the expression of many proteins. Between 4 and 24 h following a single convulsive dose of PTZ, mRNA levels for γ-aminobutyric acid type A (GABA_A) receptor α1, β2 and γ2 subunits were significantly decreased in the rat brain [35]. An autoradiographic study reported that a single PTZ administration caused significant decrease in benzodiazepine binding in various rat brain regions [30]. Between 5 min and 24 h following an acute PTZ seizure, the immunoreactivity for vasoactive intestinal polypeptide-related peptides dramatically decreased in the rat brain [31]. Acute PTZ seizures also caused a decline in the concentration of N-acetylaspartate [3] and somatostatin-like immunoreactivity [29]. The present study demonstrated that the gene expression, measured by both protein and mRNA levels of CREB, was significantly decreased in rat brain regions including hippocampus at 24 h following PTZ seizures. No matter what signal pathway caused the decline in CREB expression, our data suggested that CREB is involved in the pathology of epilepsy. It remains for further study to determine whether CREB might play essential roles in the development of epilepsy.

In conclusion, the present study observed that levels of CREB immunoreactivity and mRNA were significantly decreased in the rat brain regions including cerebral cortex and hippocampus at 24 h following injection of a single convulsive dose of PTZ. The levels of CREB mRNA did not show significant changes in the brain regions at 2 h or 1 week after acute PTZ seizures. It is suggested that altering CREB expression may have functional consequences in the development of epilepsy.

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