Role of Cysteine Proteases in the Mechanism of Action of the Anticonvulsants Levetiracetam and Carbamazepine and the Calpain Inhibitor Calpastatin in Pentylentetrazole-kindled Rats

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Abstract: Atypical antiepileptic drugs such as Levetiracetam (LEV) show antiepileptic activity in animals and humans; however their actions do not seem to be similar to the traditional antiepileptic drugs. The present study investigated the possible role of cysteine proteases in the mechanism of action of levetiracetam and the traditional antiepileptic Carbamazepine (CBZ) and calpain inhibitor, Calpastatin (CS) in kindled rats. The effect of increasing doses of CBZ (50, 100 and 200 mg kg⁻¹, p.o.), LEV (13, 27 and 54 mg kg⁻¹, p.o.) and the calpain inhibitor, CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) on calpain, caspase 3 and cathepsin B were studied in normal and in kindled rats. Seizures induced by pentylentetrazole increased the activity level of calpain, caspase 3 and cathepsin B (8.44±1.4 vs. 40.42±0.47 μmol min⁻¹ mg⁻¹ protein, p<0.05), (87.50±0.36 vs. 495.91±3.51 μmol min⁻¹ mg⁻¹ protein, p<0.05) and (88.89±0.38 vs. 500.66±2.51 μmol min⁻¹ mg⁻¹ protein, p<0.05), respectively in brain tissues homogenates. Treatment of fully kindled rats with different doses of CBZ and LEV caused a significant inhibition (p<0.05) of cysteine proteases activity in a significant dose dependent manner in rats brain homogenates. Moreover, the calpain inhibitor, CS reversed calpain activity values to the level of the saline treated group (p<0.05). Besides, the effect on caspase 3 and cathepsin B was more pronounced. Inhibition of cysteine proteases induced by treatment protocol was parallel with marked decrease in seizure severity. These results indicate that both antiepileptic drugs carbamazepine, levetiracetam act partially through the inhibition of cysteine proteases and that the calpain inhibitor, calpastatin might has an important role in the treatment of epilepsy.

Key words: Levetiracetam, calpastatin, calpain, caspase 3, cathepsin B

INTRODUCTION

Epilepsy which is one of the most common neurological disorders that may occur due to disturbances in the normal balance of excitatory and inhibitory neurotransmitters within the seizures focus in the brain (Lothman and Bertram, 1993, McNamara, 1994, 1999). Most behavioral signs and symptoms associated with seizures are related to the normal function of the affected region of the brain (Hauser and Hesdorffer, 1990). Accordingly, most seizures result from discharge, originating from cortical, subcortical and hippocampal structures (Avanzini and Franceschetti, 2003).

Levetiracetam is an atypical antiepileptic drugs that considerably differs from other novel antiepileptic drugs in that it does not interfere with any known target for anticonvulsant activity and appears to have alternative modes of action. In fact, the drug does not affect voltage-dependent channels or receptors for major inhibitory or excitatory neurotransmitters (Klitgaard and Pitkänen, 2003). It was proposed that it binds to a specific, as yet unidentified site on the synaptic plasma membrane (Noyer et al., 1995). Several pharmacological properties of LEV have been reported including binding to synaptic vesicle protein 2A (SV2A) (Lynch et al., 2004). In addition, inhibition of Ca⁺ release from the inositol triphosphate (IP₃) sensitive intracellular storage sites (Fatatis et al., 1994; Cataldi et al., 2005). However, its precise mechanism of action is still not fully elucidated. Carbamazepine (CBZ), a traditional antiepileptic drugs; is widely used as a first-line drug in the management of tonic-clonic seizures. It blocks sodium channels during rapid, repetitive, sustained neuronal firing (Roger et al., 2004).
It is known that neuronal death resulting from seizures can be initiated by excessive glutamate release that activates postsynaptic N-methyl-D-aspartate (NMDA) receptors, thereby triggering large calcium influx. This in turn results in the activation of intracellular proteases such as calpain, caspase and cathepsin (Saido et al., 1994; Zhu et al., 2000; Goll et al., 2003; Friedrich and Bozoky, 2005). In addition, excess intracellular calcium results in mitochondrial damage and cytochrome c release which ultimately leads to caspase activation (Zhu et al., 2000). The intracellular Ca2+-dependent cysteine proteases are widely distributed and have been involved in various physiological and pathological events (Saido et al., 1994; Goll et al., 2003; Friedrich and Bozoky, 2005).

Caspases activate calpain by mediating the degradation of calpastatin, an endogenous inhibitor of calpain proteases (Wang, 2000; Sorimachi and Suzuki, 2001) and additionally caspases might be activated by calpain proteases (Yamashima, 2000). Activated calpain causes a limited degradation of a variety of biologically important proteins including: cytoskeletal proteins, membrane integral proteins, certain enzymes and transcription factors, components in cell adhesion and signaling pathways (Molinari and Carafoli, 1997). Calpain has been implicated in various types of acute neuro-degeneration, particularly those induced by trauma, ischemia and neural excitotoxicity (Blomgren et al., 1999; Ray et al., 2002; Rami et al., 2003; Higuchi et al., 2005). Caspases also have a role in neurofibrillary tangle formation (Rohn et al., 2001; Matsui et al., 2006). Calpain antagonist can prevent breakdown of neurofilaments and spectrin and reduce the traumatic brain injury, suggesting that inhibition of calpain activity can preserve cytoskeletal structure of neurons. The calpain activity in vivo is controlled by calpastatin (Kawasaki et al., 1989; Sorimachi et al., 1997). The upregulation of several members of the calpain family is involved in a diverse range of biological processes and diseases, indicating that this family of proteases has important therapeutic potential. However, little information is available on the role of cysteine proteases; calpain, caspase 3 and cathepsin B pathway in convolution. To our knowledge, calpain antagonist (calpastatin) has not been investigated in fully kindled rats as a model of convolution. Moreover, the information on possible involvements of cysteine proteases in the mechanism of action of atypical antiepileptic drugs; LEV and traditional antiepileptic drugs; CBZ is still lacking.

Therefore, the main objectives of the present study were the followings: firstly to investigate the possible role of calpain, caspase 3 and cathepsin B pathway in the convolution, secondly, to examine the possible effect of calpain, caspase 3 and cathepsin B in the mechanism of action of LEV in comparison with CBZ in PTZ-kindled rats and thirdly, to study anticonvulsant potential of calpastatin, a calpain antagonist.

MATERIALS AND METHODS

Animals: Adult Male Sprague-Dawley rats weighing 200–50 g were obtained from the Animal Care Center, Pharmacology Department, College of Medicine, King Saud University (KSU), Riyadh, Kingdom of Saudi Arabia. They were housed in cages under conventional laboratory conditions. Room temperature was maintained at 22–23°C and a relative humidity of 55% and a regular 12 h light/dark cycle. The animals were fed a standard rat pellet diet and water ad libitum. All animal procedures were undertaken according to the international guidelines of proper experimental animal handling.

Drugs and chemicals: All chemicals and reagents used in this study were of higher analytical grade. The following drugs and chemicals were obtained from commercial sources: Pentylentetrazole (Sigma Chemical Company, St. Louis, MO, USA), Calpastatin peptide powder (Calbiochem Company, Pacific Center Court, San Diego, USA), Carbamazepine (Tegretol) tablets (Novartis Pharma, Basle, Switzerland), Levetiracetam (Keppra) tablets (UCB Pharma, Lake Park Drive, USA). Carbamazepine and levetiracetam were dissolved in distilled, deionized water and the calculated doses of the drugs were based on the rat's average daily intake of water. Calpain, caspase-3 and cathepsin-B activity assay kits (Biovision, Linda Vista Avenue, USA). Total protein kit (Randox, Mississauga, Ontario, Canada).

Induction of kindling: Seizures were induced by intraperitoneal (i.p.) injection of pentylentetrazole (PTZ; 49 mg kg–1 i.p. dissolved in normal saline) every other weekday (Saturday, Monday and Wednesday) for 5 consecutive weeks as described before (Sayyah et al., 2005; Pavlova et al., 2006; Mehla et al., 2010). The selected concentration of PTZ in the present study was based on data from our preliminary experiments. The animals were then placed in isolated cages and observed for 30 min for the onset of convulsions. The intensity of the seizure was scored as follows: 0, immobility; 1, mouth and facial jerks; 2, nodding or myoclonic body jerks; 3, forelimb clonus; 4, rearing, falling down, hindlimb clonus and forelimb tonus and 5, tonic extension of hindlimb,
status epilepticus and/or death. The maximum response was recorded for each animal (De Sarro et al., 1999). Rats that convulsed in response to the kindling treatment on day 1 were excluded from the study. When a rat exhibited stage 4 seizures for three times, it was considered fully kindled, treatment with PTZ was discontinued and the animal was included in the study (De Sarro et al., 2004).

Effects of treatment with different doses of CBZ or LEV or CS on brain proteases activity, seizure severity and motor function in normal and PTZ-treated rats

**Effect of treatment with different doses of the selected drugs on normal rats:** Rats were randomly allocated into 10 groups of 8 animals each. One group of rats served as control (saline treated group) while the other 9 groups were treated with different doses of CBZ (50, 100 and 200 mg kg\(^{-1}\) p.o.) (De Sarro et al., 1999) or LEV (13, 27 and 54 mg kg\(^{-1}\) p.o.) (Sluzewski and Chodera, 1992) or CS (1.59, 3.18 and 6.36 mg kg\(^{-1}\) i.p.) (According to the manufactures instruction, Calbiochem).

**Effects of treatment with different doses of the selected drugs on PTZ-treated rats:** Seizures were induced by PTZ (49 mg kg\(^{-1}\), i.p.) every other weekday (Saturday, Monday and Wednesday) for 5 consecutive weeks as previously detailed. The rats that showed convolution at the end of the treatment period were included in the study. Fully kindled rats were randomly divided into 10 groups of 8 animals each. After a three days PTZ free period, one group was kept as fully kindled rats, served as kindled control and received only vehicle while the other nine groups kindled rats were treated with the different doses of CBZ (50, 100 and 200 mg kg\(^{-1}\), p.o.) 60 min prior to administration of PTZ (Loscher et al., 1998) or LEV (13, 27 and 54 mg kg\(^{-1}\), p.o.) or CS (1.59, 3.18 and 6.36 mg kg\(^{-1}\), i.p.) 30 min prior to administration of PTZ (Bastlund et al., 2005) in a 6 day experiment according to the experimental design illustrated in Table 1. A vehicle control injection was given every second day to ensure that the rats continued to respond with kindled seizures in the absence of anticonvulsant drugs. The animals were then observed for 30 min and the seizures severity test was recorded after treatment with the same above mentioned different doses of CBZ (200, 100 and 50 mg kg\(^{-1}\), p.o.) or LEV (54, 27 and 13 mg kg\(^{-1}\), p.o.) or CS (6.36, 3.18 and 1.59 mg kg\(^{-1}\), i.p.). Moreover, rats were also evaluated for their impaired motor function. Positive effects (+) indicate ability of the rats to maintain equilibrium on Rota rod for at least 3 times while the negative effect (-) indicate inability of the rats to maintain equilibrium on Rota rod for at least 3 times.

<table>
<thead>
<tr>
<th>Experimental days</th>
<th>Day 6</th>
<th>Day 5</th>
<th>Day 4</th>
<th>Day 3</th>
<th>Day 2</th>
<th>Day 1</th>
<th>Day No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Drug</td>
<td>Vehicle</td>
<td>Drug</td>
<td>Vehicle</td>
<td>Drug</td>
<td>Test-dru</td>
<td></td>
</tr>
</tbody>
</table>

**Effects of two weeks pretreatment with different doses of CBZ, LEV and CS on brain proteases activity in PTZ-treated rats:** In another 10 groups of 8 animals each, one group served as control and received only vehicle. The other nine groups were pretreated for two weeks with different doses of CBZ (50, 100 and 200 mg kg\(^{-1}\), p.o.) or LEV (13, 27 and 54 mg kg\(^{-1}\), p.o.) or CS (1.59, 3.18 and 6.36 mg kg\(^{-1}\), i.p.) prior to and during induction of seizures. During kindling acquisition, all the 10 groups received PTZ (49 mg kg\(^{-1}\) i.p.) every other weekday (Saturday, Monday and Wednesday) for 5 consecutive weeks as described previously.

**Preparation of brain tissue homogenates:** At the end of the treatment protocol, the animals were deeply anesthetized using light ether anesthesia and sacrificed by decapitation. Brains were removed, placed on ice and snap-freeze. Both hippocampi were dissected on ice, washed with saline at 4°C, blotted dry on filter paper and weighed (150-160 mg). A 10% (w/v) homogenate was then prepared in ice-cold saline using a Branson sonifier (250, VWR Scientific, Danbury, CT, USA). Extreme care was taken in consideration to keep samples as cold as possible during dissection of hippocampus and preparation of homogenates and to work rapidly to reduce formation of post-mortem artefacts. The brain lysate was then centrifuged at 8000 g for 5 min at 4°C, to remove insoluble debris, snap-frozen and stored at -80°C until further use.

**Measurement of proteases activity (Calpain, Caspase 3 and Cathepsin B):** The level of calpain, caspase and cathepsin B activity were determined fluorometrically using fluorescent assay commercial kits according to the manufactures instruction (Biovision, Linda Vista Avenue, USA).

**Histopathology:** At the end of the treatment protocol, 5 rats from each group were anesthetized with sodium pentobarbital (100 mg kg\(^{-1}\)). Rats were then transcardially perfused with cold saline followed by 10% formalin in phosphate-buffered saline (0.1 M; pH 7.4). The brains were removed from the skull and fixed in the same fixative for 24 h. Thereafter, the brains were embedded in paraffin and then 5 μm thick sections were coronally cut at the level of the dorsal hippocampus by a rotatory microtome.
Statistical analysis: Data are expressed as Mean±SEM. The one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test was used to estimate the difference between various groups. The level of statistical significance was taken at p = 0.05.

RESULTS

Seizure severity response in PTZ treated rats: Table 2 shows that induction of seizures using PTZ resulted in freezing response during the evoked ictal discharge. With repeated activation, the seizures response became generalized to the point of driving bilateral clonic seizures (typically referred to as stage 5 seizures). The early stages 1 and 2 were primarily associated with facial and oral activity, including eye closure and blinking followed by head bobbing and drooling. As focal seizures activity increased during the early kindling trials, mild stage 3 forelimb clonus appeared. With the time, the seizures become more fully generalized and presented with stronger clonus and rearing (stage 4) and rearing and falling (full stage-5).

Effect of treatment with different doses of CBZ or LEV or CS on seizures severity in PTZ-treated rats: Seizures were induced by PTZ (49 mg kg⁻¹, i.p.) every other weekend (Sat, Mon and Wed) for 5 consecutive weeks. The animals were then placed in isolated cages and observed for 30 min for the incidence and onset of convulsions.

Table 2: Seizure severity evaluation each week in PTZ treated rats (n = 8)

<table>
<thead>
<tr>
<th>Week no.</th>
<th>Drugs</th>
<th>Doses (mg kg⁻¹)</th>
<th>Score no. (response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PTZ</td>
<td>49</td>
<td>0.95±0.01</td>
</tr>
<tr>
<td>2</td>
<td>PTZ</td>
<td>49</td>
<td>1.05±0.03</td>
</tr>
<tr>
<td>3</td>
<td>PTZ</td>
<td>49</td>
<td>2.05±0.02</td>
</tr>
<tr>
<td>4</td>
<td>PTZ</td>
<td>49</td>
<td>3.05±0.07</td>
</tr>
<tr>
<td>5</td>
<td>PTZ</td>
<td>49</td>
<td>4.05±0.03</td>
</tr>
</tbody>
</table>

Seizures were induced by PTZ (49 mg kg⁻¹, i.p.) every other weekend (Sat, Mon and Wed) for 5 consecutive weeks. The animals were then placed in isolated cages and observed for 30 min for the incidence and onset of convulsions. The intensity of the seizure response was scored on the following scale: 0, immobility; 1, mouth and facial jerks; 2, nodding or myoclonic-body jerks; 3, forelimb clonus; 4, rearing, falling down, hindlimb clonus and forelimb tonic; and 5, tonic extension of hindlimb, status epilepticus and/or death. Results are expressed as Mean±SEM.

Table 3: Effects of treatment with different doses of carbamazepine, levetiracetam or calpastatin on seizures severity in PTZ-treated rats (n = 8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drug</th>
<th>Dose (mg kg⁻¹)</th>
<th>Score no. (response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle+Drugs</td>
<td>NS</td>
<td>9.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CBZ</td>
<td>20.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CEZ</td>
<td>100.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CEZ</td>
<td>50.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+LEV</td>
<td>54.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+LEV</td>
<td>27.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+LEV</td>
<td>13.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CS</td>
<td>6.36</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CS</td>
<td>3.18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>NS+CS</td>
<td>1.59</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>PTZ+Drugs</td>
<td>PTZ</td>
<td>49.00</td>
<td>4.90±0.03</td>
</tr>
<tr>
<td></td>
<td>PTZ+CBZ</td>
<td>20.00</td>
<td>1.95±0.08</td>
</tr>
<tr>
<td></td>
<td>PTZ+CEZ</td>
<td>100.00</td>
<td>1.90±0.07</td>
</tr>
<tr>
<td></td>
<td>PTZ+CEZ</td>
<td>50.00</td>
<td>1.90±0.08</td>
</tr>
<tr>
<td></td>
<td>PTZ+LEV</td>
<td>54.00</td>
<td>0.95±0.01</td>
</tr>
<tr>
<td></td>
<td>PTZ+LEV</td>
<td>27.00</td>
<td>0.90±0.02</td>
</tr>
<tr>
<td></td>
<td>PTZ+LEV</td>
<td>13.00</td>
<td>0.90±0.08</td>
</tr>
<tr>
<td></td>
<td>PTZ+CS</td>
<td>6.36</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>PTZ+CS</td>
<td>3.18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>PTZ+CS</td>
<td>1.59</td>
<td>0.00±0.00</td>
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</table>

Seizures were induced by PTZ (49 mg kg⁻¹, i.p.) every other weekend (Sat, Mon and Wed) for 5 consecutive weeks. The animals were then placed in isolated cages and observed for 30 min for the incidence and onset of convulsions. The seizure severity test was repeated after administration of different doses of CBZ (200, 100 and 50 mg kg⁻¹, p.o.) or LEV (54, 27 and 13 mg kg⁻¹, p.o.) or CS (6.36, 3.18 and 1.59 mg kg⁻¹, i.p.) to PTZ-administered rats. Results are expressed as Mean±SEM.

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Effect of treatment with different doses of CBZ or LEV or CS on seizures severity in PTZ-treated rats: Seizures were induced by PTZ (49 mg kg⁻¹, i.p.) every other weekend (Sat, Mon and Wed) for 5 consecutive weeks. The animals were then placed in isolated cages and observed for 30 min for the incidence and onset of convulsions. The seizure severity test was repeated after administration of different doses of CBZ (200, 100 and 50 mg kg⁻¹, p.o.) or LEV (54, 27 and 13 mg kg⁻¹, p.o.) or CS (6.36, 3.18 and 1.59 mg kg⁻¹, i.p.) to PTZ-administered rats. Results are expressed as Mean±SEM.

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increase in both caspase 3 and cathapsin B activities (Fig. 1a, 2a, 3a). Similarly, in the prophylactic group, the administration of PTZ (49 mg kg⁻¹, i.p.) every other weekday (Sat, Mon and Wed) for 5 consecutive weeks to normal rats induced three fold elevation in calpain activity and 3.8 times elevation in caspase 3 and cathapsin B (Fig. 1b, 2b, 3b).

Effects of treatment and prophylaxis administration with different doses of CBZ (200, 100 and 50 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) or CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) on the relative calpain activity in seizures induced rats: The administration of increasing doses of CBZ (50, 100 and 200 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) or CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) to normal control rats did not induce any significant changes in calpain activity (Table 4). However, calpain activity was markedly increased in the PTZ-treated rats (49 mg kg⁻¹, i.p.) as compared to saline treated control rats (Fig 1a). Treatment of fully kindled rats with aforementioned doses of CBZ and LEV provoked a significant inhibition of calpain level in a dose dependent manner (p<0.05) as compared to their respective PTZ treated group (Fig 1a). As expected, treatment of fully kindled rats with various doses of CS reversed the calpain level to normal similar to that observed in saline treated group. Moreover, two weeks pretreatment of animals with various doses of CBZ or LEV or CS produced a similar pattern of inhibition. Prophylactic administration of CBZ (50, 100 and 200 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) impeded the activation of calpain in dose dependent manner while value of calpain was kept at level similar to the saline treated group with different doses of CS, even with the lowest dose (p<0.05) (Fig. 1b). It is noteworthy that inhibition of calpain activity in the treatment groups induced by selected doses of both drugs was much higher than in the two weeks pretreatment group (Fig 1a, b). Moreover, LEV was much more effective than CBZ in calpain inhibition in both groups (Fig. 1a, b).

Effects of different treatments with CBZ or LEV or CS on caspase 3 activity of fully kindled rats: Treatment of normal rats with increasing doses of CBZ (50, 100 and 200 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) or CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) did not modulate caspase 3 activity, as compared to their control saline treated group (Table 4). Chronic administration of PTZ (49 mg kg⁻¹, i.p.) to the animals resulted in a profound increment in caspase 3 activities that was significantly

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**Fig. 1a:** Effects of treatment with antiepileptic drugs on calpain activity in hippocampus of PTZ-kindled rats. Fully kindled rats were treated with CBZ (50-100-200 mg kg⁻¹, p.o.) or LEV (13-27-54 mg kg⁻¹, p.o.) or CS (6.36-3.18-1.59 mg kg⁻¹, i.p.) as explained in the Method section. Each column represents the mean of eight rats with vertical bar showing SE. *Significant difference from the control group at p = 0.05, # Significant difference from the PTZ-treated group at p = 0.05, @ Significant difference between different doses of the selected drugs at p = 0.05.

**Fig. 1b:** Effects of two weeks pretreatment with antiepileptic drugs on calpain activity in hippocampus of PTZ-kindled rats. Fully kindled rats were pretreated for two weeks with CBZ (50-100-200 mg kg⁻¹, p.o.) or LEV (13-27-54 mg kg⁻¹, p.o.) or CS (6.36-3.18-1.59 mg kg⁻¹, i.p.) as explained in the Method section. Each column represents the mean of eight rats with vertical bar showing SE. *Significant difference from the control group at p = 0.05, # Significant difference from the PTZ-treated group at p = 0.05, @ Significant difference between different doses of the selected drugs at p = 0.05.
Table 4: Effects of treatment with different doses of carbamazepine or levmetacain or calpastatin on relative calpain, caspase and cathepsin B activities in normal rats (n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Calpain</th>
<th>Caspase</th>
<th>Cathepsin-B</th>
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<tr>
<td>NS</td>
<td>8.4±0.14</td>
<td>87.5±0.36</td>
<td>88.8±0.38</td>
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<tr>
<td>NS+CBZ(200 mg kg⁻¹, p.o.)</td>
<td>8.4±0.19</td>
<td>87.5±0.45</td>
<td>88.6±0.42</td>
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<tr>
<td>NS+CBZ(100 mg kg⁻¹, p.o.)</td>
<td>8.4±0.19</td>
<td>87.4±0.22</td>
<td>88.8±0.35</td>
</tr>
<tr>
<td>NS+CBZ(50 mg kg⁻¹, p.o.)</td>
<td>8.4±0.21</td>
<td>87.4±0.40</td>
<td>88.8±0.34</td>
</tr>
<tr>
<td>NS+LEV(54 mg kg⁻¹, p.o.)</td>
<td>8.4±0.21</td>
<td>87.4±0.51</td>
<td>88.7±0.29</td>
</tr>
<tr>
<td>NS+LEV(27 mg kg⁻¹, p.o.)</td>
<td>8.35±0.22</td>
<td>87.35±0.43</td>
<td>88.76±0.42</td>
</tr>
<tr>
<td>NS+LEV(13 mg kg⁻¹, p.o.)</td>
<td>8.33±0.20</td>
<td>87.37±0.56</td>
<td>88.73±0.34</td>
</tr>
<tr>
<td>NS+CS(6.36 mg kg⁻¹, i.p.)</td>
<td>8.48±0.16</td>
<td>87.14±0.32</td>
<td>88.68±0.38</td>
</tr>
<tr>
<td>NS+CS(3.18 mg kg⁻¹, i.p.)</td>
<td>8.49±0.13</td>
<td>87.27±0.47</td>
<td>88.73±0.61</td>
</tr>
<tr>
<td>NS+CS(1.59 mg kg⁻¹, i.p.)</td>
<td>8.56±0.15</td>
<td>87.73±0.45</td>
<td>89.12±0.42</td>
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Fig. 2a: Effects of treatment with antiepileptic drugs on caspase 3 activity in hippocampus of PTZ-kindled rats. Treatment of fully kindled rats with CBZ (50-100 mg kg⁻¹, p.o.) or LEV (13-27-54 mg kg⁻¹, p.o.) or CS (6.36-3.18-1.59 mg kg⁻¹, i.p.) as explained in the Method section. Each column represents the mean of eight rats with vertical bar showing SE. *Significant difference from the control group at p = 0.05, # Significant difference from the PTZ-treated group at p = 0.05 @ Significant difference between different doses of the selected drugs at p = 0.05.

Fig. 2b: Effects of two weeks administration with antiepileptic drugs on caspase 3 activity in hippocampus of PTZ-kindled rats. Two weeks pretreatment of fully kindled rats with CBZ (50-100-200 mg kg⁻¹, p.o.) or LEV (13-27-54 mg kg⁻¹, p.o.) or CS (6.36-3.18-1.59 mg kg⁻¹, i.p.) as explained in the Method section. Each column represents the mean of eight rats with vertical bar showing SE. *Significant difference from the control group at p = 0.05, # Significant difference from the PTZ-treated group at p = 0.05, @ Significant difference between different doses of the selected drugs at p = 0.05.

Reduced or even prevented with the treatment of the animals with antiepileptic drugs and calpastatin, calpain inhibitor. Treatment of fully kindled rats with CBZ (50, 100 and 200 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) resulted in a 48, 60, 63 and 62, 64, 72 % inhibition of caspase 3 activity, respectively (p<0.05), as compared to the PTZ treated group (Fig. 2a). Similarly, two weeks pretreatment with antiepileptic drugs produced a profound reduction of caspase 3 activity. There was a statistically significant and dose dependent inhibition of caspase 3 in kindled rats brain in response to prophylactic administration of the selected doses of CBZ or LEV (Fig 2b). LEV was still more effective in the inhibition of caspase 3 than CBZ. Different doses of CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) reversed the caspase 3 activity of kindled rats brains in both types of treatment to that observed in saline treated group, even with the lowest dose of CS (p<0.05) (Fig. 2a, b).

Effects of treatment and prophylaxis administration with CBZ or LEV or CS on cathepsin B activity of seizures induced rats: Treatment of normal rats with increasing doses of CBZ (50, 100 and 200 mg kg⁻¹, p.o.) or LEV (13, 27 and 54 mg kg⁻¹, p.o.) or CS (1.59, 3.18 and 6.36 mg kg⁻¹, i.p.) did not induce any change in cathepsin
Fig. 3a: Effects of treatment with antiepileptic drugs on cathapsin B activity in the hippocampus of PTZ-kindled rats. CBZ (50-100-200 mg kg⁻¹, p.o.) or LEV (13-27-54 mg kg⁻¹, p.o.) or CS (6.36-3.18 -1.59 mg kg⁻¹, i.p.) treatment of fully kindled rats as explained in the Method section. Each column represents the mean of eight rats with vertical bar showing SE. *Significant difference from the control group at p = 0.05, # Significant difference from the PTZ-treated group at p = 0.05, @ Significant difference between different doses of the selected drugs at p = 0.05

B activity as compared to the saline treated group (Table 4). Cathapsin B activity was markedly increased in chronically PTZ-treated rats (49 mg kg⁻¹, i.p.) as compared to saline treated rats. Treatment with antiepileptic drugs CBZ or LEV or and the calpain inhibitor CS caused a significant attenuation of cathapsin B activity. A profound inhibition of cathapsin B activity was observed on treatment of fully kindled rats with selected doses of CBZ or LEV as compared to the PTZ treated group (Fig. 3a). Likewise, the different doses antiepileptic drugs CBZ or LEV provoked a significant reduction of cathapsin B activity during two weeks pretreatment of seizures induced in rats by PTZ (Fig. 3b). LEV was much more effective than CBZ in cathapsin B inhibition in both groups. The values of cathapsin B in both types of treatments were kept at a level similar to the saline treated group, when treated with the different doses of CS (Fig. 3a, b).

Histopathological evaluation: The histopathological findings of the control, PTZ-treated and protective agents (CS, LEV) given animals are displayed in Fig. 4a-d. The findings showed marked improvement in the arrangement and appearances of the hippocampal neurons which was clearly evident following selected drugs administration.

Fig. 4a: (Normal animal): Cross section through the Ammon's horn of the Hippocampus of a normal animal. Note the normal thickness of the neuronal layer and appearances of the neurons (arrow head). Haematoxylin and eosin stain x400
Fig. 4b: (PTZ treated rats): Cross section through the Ammon’s horn of the hippocampus of an animal treated with PTZ alone. Note the decreased thickness of the neurons layer with “disarray” in their arrangement. Some neurons are also showing chromatolysis. Haematoxylin and eosin stain ×400

Fig. 4c: (PTZ and CS 400): Cross section through the Ammon’s horn of the hippocampus of an animal treated with PTZ and CS. Note the improvement in the thickness of the neuronal layer and neuronal morphology. The arrow head does, however, points towards occasional remaining degenerate neurons with chromatolysis. Haematoxylin and eosin stain ×400

Fig. 4d: (PTZ+LEV): Cross section through the Ammon’s horn of the hippocampus of an animal treated with PTZ and LEV. Note the improvement in the thickness of the neuronal layer. There is also relative reduction in the number of neurons showing chromatolysis. Haematoxylin and eosin stain ×400

**DISCUSSION**

Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures. A seizure is the symptomatic, behavioral manifestation of abnormal, disordered, spontaneous and synchronized, high-frequency firing of populations of neurons in the central nervous system (McNamara, 1999). PTZ kindling is used as a model of seizure-induced hippocampal neurodegeneration (Pavlova et al., 2006). It has been reported that PTZ-kindling in rats induced moderate neuronal cell loss in hippocampal fields CA1, CA3, CA4 and dentate gyrus. The majority of damaged cells in hippocampi of PTZ-kindled rats were cyclin B1 positive, with no expression of other cell cycle markers (Pavlova et al., 2006). Since cyclin B1 expression has been identified in hippocampal neurons of patients with temporal lobe epilepsy (Nagy and Esiri, 1998). Therefore, the similarity of neuronal cell death features in PTZ-kindling with those in temporal lobe epilepsy suggested that PTZ kindling may be a suitable model to study role of cysteine proteases; calpain, caspase 3 and cathepsin B pathway in convulsion and a possible role of calpastatin as a treatment of convulsion.

The result of the present study revealed that the development of kindled convulsion was directly proportional and cumulative with repeated exposure to PTZ which agrees with earlier report (De Sarro et al., 1999; Pavlova et al., 2006). Induction of seizure using PTZ resulted in a freezing response during the evoked ictal discharge. With repeated activation, the seizure response becomes generalized to the point of driving bilateral clonic seizures typically referred to as “stage 5 seizures”. The early stages 1 and 2 were primarily associated with facial and oral activity, including eye closure and blinking followed by head bobbing (Pavlova et al., 2006).
Caspases and calpains are among the best-characterized cysteine proteases that are activated in brain disorders (Ono et al., 1998). During the last decade, extensive research revealed that the deregulation of calpains activity is a key cytotoxic event in a variety of neurodegenerative disorders (Saito et al., 1994; Huang and Wang, 2001). Moreover, interest in the role of calpain in neurodegenerative processes is growing (Camins et al., 2006). Calpain activation has been demonstrated in brain specimens of Alzheimer’s Disease (AD) (Taniguchi et al., 2001), calpain 2 was shown in approximately 75% of neurofibrillary tangles (Adamec et al., 2002) and calpains may promote cell cycle activation, a potential source of cell injury in AD, through the activation of cyclin-dependent kinase 5 (Taniguchi et al., 2001).

Induction of seizure using PTZ in our study elicits a highly significant increase in the relative calpain, caspase 3 and cathepsin B activities which are parallel with previous studies (Henshall et al., 2000; Kondratyev and Dale, 2000, Taniguchi et al., 2001; Adamec et al., 2002). Obay et al. (2008) showing that PTZ at a convulsive dose induced an oxidative stress response by depleting the antioxidant defense systems and increasing lipid peroxidation in the brain of rats and thus may increase neuronal damage in the brain during seizures. Likewise, Naseer et al. (2009) reported that PTZ treated rats showed scattered and shrunken neurons with markedly condensed nuclei confirming the apoptotic neurodegeneration. Therefore, the marked elevations in the activity level of cysteine proteases such as calpain, caspase 3 and cathepsin B in the kindled rats’ brain, in the present study may uncovers new indicators of neuronal damage caused by PTZ induced seizures as reported earlier (Pavlova et al., 2006; Obay et al., 2008; Naseer et al., 2009). These results could be explained by previous observations showing that the production of a pathological increase in the concentration of extracellular glutamate in neuronal death is associated with an increase in intracellular calcium induced activation of intracellular proteases (Taniguchi et al., 2001; Adamec et al., 2002, DeLorenzo et al., 2005). Interestingly, treatment of normal rats with different doses of CBZ, or LEV or CS were without any effect on cysteine proteases calpain, caspase 3 and cathepsin B on the normal rat’s brain. However, two weeks pretreatment or treatment with different doses of CBZ or LEV elicit a marked inhibition of calpain, caspase 3 and cathepsin B activity in fully kindled rats in a statistically significant dose dependent manner. LEV was more effective than CBZ. However, CS was most potent than CBZ and LEV in reducing cysteine proteases activities in rats brain homogenates. Inhibition of cysteine proteases activities by selected drugs conform to the effect of these drugs on the kindled behavior as indicating by decrease the seizures severity. The data from our current study show that CS is more effective and potent than LEV since it prevented the occurrence of seizure after the administration of the first dose while the effect of LEV was evident after the administration of third dose to kindled rats. In contrast, CBZ was the least effective in decreasing the kindling behavior in rats. These finding suggest that the inhibition of the activity of these proteases may be utilized for the evaluation of the therapeutic effect of anti-epileptic drugs.

The involvement of calpain in neuronal cell death has been implicated in various neuropathological circumstances (Ray et al., 2003). Takano et al. (2005) suggested that calpain plays a central role in the excitotoxic signal transduction cascade leading to DNA fragmentation. Higuchi et al. (2005) found that the balance between the levels of calpain and calpastatin activities determines the fate of neurons following excitotoxic challenges. Therefore, calpastatin overexpression clearly inhibited both calpain-dependent proteolysis of cytoskeletal proteins and subacute neurodegeneration. These observations led us to speculate that proteolytic disorganization of cytoskeletal structures by calpain may contribute to the morphological degeneration of neurons. Moreover, the significant involvement of caspase-1 in seizures is strongly supported by the drastic reduction in seizures susceptibility observed in mice with deletion of the caspase-1 gene. Dos Santos et al. (2011) clarified recently the role of caspase in epilepsy and declared that alpha-lipoic acid inhibit both caspase-dependent and-independent apoptotic pathways and therefore, induced a neuroprotective effect against hippocampal damage during pilocarpine-induced seizures. Likewise, Seo et al. (2009) showed that caspase pathways contribute to excitotoxic neuronal necrosis in primary neuronal cultures. Recently Lopez-Meraz et al. (2010) investigated the role of caspase-3, -8 and -9 in neuronal injury, using a lithium-pilocarpine model of status epilepticus and showed that caspase-8 upregulation preceded caspase-3 activation in morphologically necrotic neurons. Pretreatment of animals with the pan-caspase inhibitor Q-VD-OPH reduced neuronal injury in CA1-subiculum showing that caspase contribute to status-epilepticus-induced necrosis. Cystatin B (CSTB) acts as an inhibitor of the lysosomal cathepsins. Deficiency of CSTB protein results in increased activity of cathepsins with increased apoptosis in specific neuronal cell types (Rinne et al., 2002). Loss-of-function (mutations) in the gene encoding CSTB underlies an inherited neurodegenerative disorder, progressive
myoclonus epilepsy (Rinne et al., 2002). The effects of decreased CSTB activity may, at least in part, be mediated by cathepsins through increased activity of cathepsins S and L.

Many calpain inhibitors have been tested in vitro and in vivo for their neuroprotective potential (Saez et al., 2006) and the inhibition of calpain activity has a major therapeutic potential. It has been reported that the continuous perfusion of calpain inhibitor I on picrotoxin induced seizures in chronic freely moving rats has no effect on basal Electroencephalograph (EEG) but doubled average seizures duration, increased more than five-fold the total seizures time and three times the seizures offset time compared to picrotoxin alone, in each individual rat (Sierra-Paredes et al., 1999). These revelations suggest strongly that a calpain mediated mechanism may be responsible for seizures offset, probably through AMPA glutamate receptors internalization and further degradation (Dargelos et al., 2008). Ravizza et al. (2006) reported that inhibition of caspase-1 in the brain provides significant protection from acutely induced seizures in rodents. The pharmacologic inhibition of caspase-1 was achieved in rodent brain by using pralnacasan or VX-765 (Ravizza et al., 2006). The powerful anticonvulsant effect achieved in rats after peripheral administration of pralnacasan or VX-765 opens the perspective of a clinical use of selective caspase-1 inhibitors for controlling seizures. Pralnacasan or VX-765 was the first caspase-1 inhibitor to enter clinical development.

The results of the present study together with earlier findings can demonstrated clearly that inhibition of cysteine protease has a powerful anticonvulsant effects. Therefore, inhibition of cysteine proteases by LEV or CBZ was not epiphenomenon but may be a part of their mechanism of actions. Moreover, CS completely prevented the rise in the activity level of these proteases to the saline treated group thereby give strong support to the possible uses of calpain inhibitor; CS in the treatment of epilepsy. It is possible, therefore, that calpain antagonist may have promising antiepileptic effect.

**CONCLUSION**

Results showed that PTZ induced seizures in rats resulted in an increase in the levels of relative calpain, caspase 3 and cathepsin B activity. Treatment of fully kindled rats with both atypical antiepileptic drugs; LEV and traditional antiepileptic drugs; CBZ inhibited the levels of calpain, caspase 3 and cathepsin B in a dose dependent manner. CS produced more potent inhibition on the measured proteases as compared other anti-epileptic drugs used in the study. It is possible that inhibition of cysteine protease by CBZ and LEV may be a part of their mechanism of actions and the calpain antagonist may have promising antiepileptic effect.

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**DISCLOSURE**

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure of conflicts of interest: The authors of this manuscript (Mahmoud A. Mansour, Awatif B. Al-Baker and Abdulqader Alnaimy) hereby declare we have no conflicts of interest and have received no support, financial or otherwise, in conjunction with the generation of this submission.

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