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Research Report

Effects of herbimycin A in the pilocarpine model of temporal lobe epilepsy

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ABSTRACT

Pilocarpine-induced status epilepticus (SE) causes widespread tyrosine phosphorylation in the brain. It has been postulated that this intracellular signal may mediate potentially epileptogenic changes in the morphology and physiology of particular brain regions, including the hippocampus. The present study evaluated the effects of herbimycin A, a protein tyrosine kinase (PTK) inhibitor, over the acute (during which intense biochemical and electrophysiological activation occurs) and the chronic phase (characterized by spontaneous and recurrent epileptic seizures and the presence of synaptic reorganization, e.g., mossy fiber sprouting) of the pilocarpine model of epilepsy. The administration of a single dose of 1.74 nmol of herbimycin A (i.c.v., 5 μL) 5 min after the onset of SE did not change the acute behavioral manifestation of seizures despite significantly decreasing c-Fos immunoreactivity in different areas of the hippocampus and of the limbic cortex. Herbimycin-treated animals developed spontaneous recurrent seizures, as did control animals, with a similar latency for the appearance of the first seizure and similar seizure frequency. Neo-Timm staining revealed that all animals experiencing SE, regardless of whether or not injected with herbimycin, showed aberrant mossy fiber sprouting in the supragranular region of the dentate gyrus. Herbimycin did not obviously affect neuronal cell death as evaluated in Nissl-stained sections. These results indicate that the PTK blockade achieved with the current dose of herbimycin reduced the acute c-Fos expression but failed to alter the spontaneous seizure frequency or to attenuate the morphological modifications triggered by the SE.

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Abbreviations:
BDNF, brain-derived neurotrophic factor
DAB, 3,3′-diaminobenzidine
DG, dentate gyrus
dHilus, dorsal hilus
Ent, entorhinal cortex
GL, grey level
HERB, herbimycin A
NSE, non-status epilepticus
NT-3, neurotrophin-3
OD, optical density
Pir, piriform cortex
PTK, protein tyrosine kinase
SE, pilocarpine-induced status epilepticus
SGi, supragranular region infrapyramidal blade
SGs, supragranular region suprapyramidal blade
vHilus, ventral hilus

1. Introduction

Pilocarpine-induced status epilepticus (SE) produces permanent changes in the morphology and physiology of different brain areas, including the hippocampus thus generating recurrent spontaneous limbic seizures (Turski et al., 1989). Among such changes, it has been described that SE acutely triggers protein tyrosine phosphorylation (Funke et al., 1998). This phosphorylation could occur at least through three different intracellular pathways: (1) the Trk receptors for neurotrophins; (2) the Eph receptors for the ephrins cell-to-cell adhesion molecules and (3) the Src non-receptor tyrosine kinase (Purcell and Carew, 2003). In fact, all of the above-mentioned kinases have been implicated in the epileptogenesis process. McNamara and collaborators have demonstrated the importance of TrkB receptor in the epileptogenesis process (Binder et al., 1999; He et al., 2004). The literature regarding the other two pathways, however, is not so copious. It has been demonstrated, however, the involvement of EphA/ephrin-A family interactions in kindling epileptogenesis and mossy fiber sprouting (Xu et al., 2003). The Src family of protein tyrosine kinase enhances the epileptiform activity generation in the CA_3 region of the hippocampus (Sanna et al., 2000). In addition, it has been previously suggested that the Eph/ephrin effects could be mediated by the Src family of protein kinases (Murai and Pasquale, 2002). Indeed, the Src kinases have recently been described as "a hub for NMDA receptor regulation" (Salter and Kalia, 2004), as it controls synaptic strength and consequently activity-dependent plasticity. Thus, the characterization of protein tyrosine kinase (PTK) inhibitors that could interfere with the process of epileptogenesis might be of clinical interest for human application.

Herbimycin A, a cell permeable ansamycin antibiotic isolated from *Streptomyces sp.*, has specific inhibitory activity on tyrosine residues phosphorylation catalyzed by various protein kinases, in particular by Src kinase (Uehara and Fukazawa, 1991). Herbimycin A is able to attenuate the increase in excitability in different experimental paradigms (e.g., hippocampal LTP (Abe and Saito, 1993) and 5HT-induced potentiation in Aplysia (Purcell and Carew, 2003)).

Here we investigated the effect of herbimycin A on epileptogenesis using the pilocarpine model of epilepsy. We hypothesized that blocking the protein tyrosine phosphorylation with herbimycin A during sustained epileptic activity (i.e., the SE) might prevent epileptogenesis. Animals were evaluated for the development of spontaneous recurrent seizures and associated morphological alterations in the hippocampus (e.g., cell death and mossy fiber sprouting).

2. Results

2.1. *Pilocarpine induced status epilepticus*

The first signs of pilocarpine systemic administration could be observed approximately 10 min after the intraperitoneal injection, as described previously (Longo et al., 2003; Mello et al., 1993). First, the animal freezes and presents some chewing behavior. Piloerection, increased salivation and diarrhea followed by body tremors and stereotyped oral movements are observed after 20 min of the injection. This pattern could progress to partial seizures, characterized by eye blinking, ears and jaw movements and myoclonic twitching of the head muscles. After that, the animals evolved to one of three different states: (1) occasional presence of this behavior for the next hour evolving to rest (normal) behavior (the non-SE group); (2) evolve to a tonic seizure, normally followed by death or (3) evolve to episodic motor generalized seizures (Racine's stage V) with increasing frequency until reaching *status epilepticus* (SE). The SE time course was characterized by robust muscle contractions for 20 min and thereafter with a progressive decline of the intensity of limbs and body jerks. The administration of herbimycin A did not change the behavioral evolution of the SE animals or the frequency of

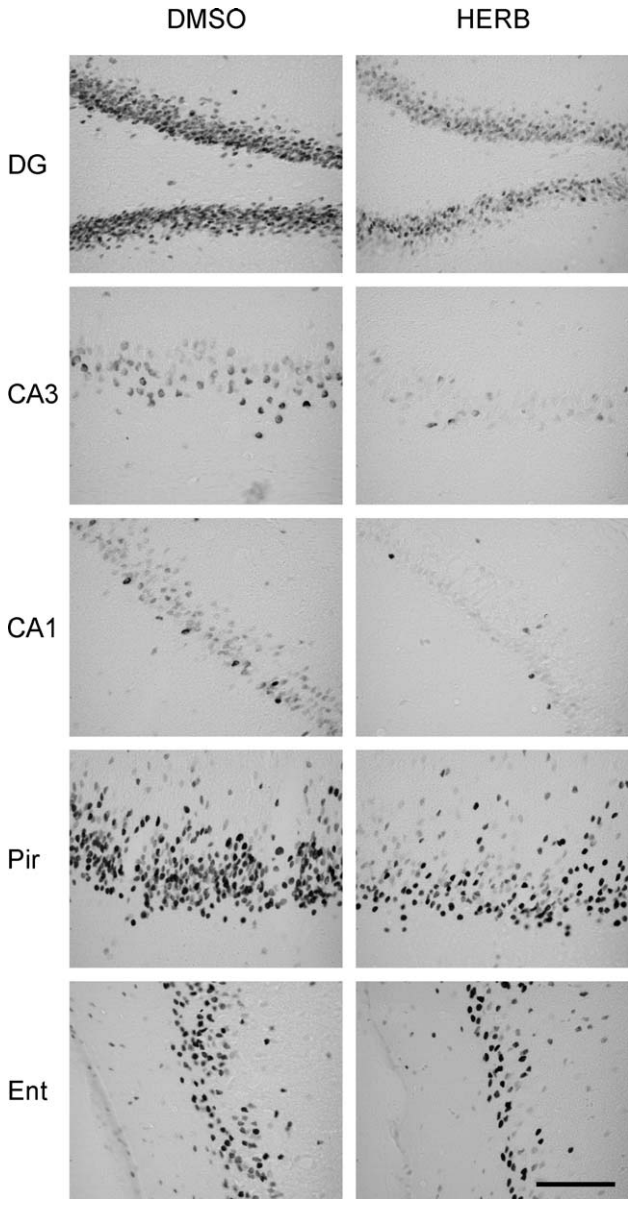

Fig. 1 – Representative microphotographs of coronal sections immunostained for c-Fos. All animals that underwent SE displayed increased c-Fos labeling in the dentate gyrus (DG), CA_3, CA_1, piriform (Pir) and entorhinal (Ent) cortex. The acute i.c.v. treatment with herbimycin A (HERB) clearly reduced not only the intensity of the staining but also the number of positive c-Fos cells in different areas of the limbic system. Scale bar: 100 μm.

NSE (note that herbimycin was injected 5 min after SE onset or alternatively 2 h after pilocarpine injection in NSE animals).

2.2. *c-Fos staining*

Pilocarpine-induced SE increased c-Fos expression in different brain regions, as described previously (Barone et al., 1993; Mello et al., 1996). Five hours of SE resulted in increased c-Fos expression in limbic areas, such as the piriform and entorhinal cortex, amygdala, dentate gyrus, CA_3 and CA_1, as well as neocortex, thalamus and striatum. The immunoreactivity was confined to the soma of the cells and according to its diameters (~10 μm), they are presumably neurons. It is also interesting to note that, in the dentate gyrus, the cells located in the hilar border of the granular cell layer, where the concentration of inhibitory neurons is higher (Ribak and Anderson, 1980), presented a slightly darker staining as compared to the rest of the stained cells in this cellular layer (Fig. 1).

Quantitative analysis of the c-Fos immunoreactivity was performed by means of optical density techniques (Rieux et al., 2002). The i.c.v. treatment with herbimycin A reduced the immunoreactivity of c-Fos in the brain undergoing SE. The total mean OD (the sum of the OD values of each structure in all animals from each group) was reduced by 30% in herbimycin A-treated animals (DMSO1%: 1.20 ± 0.04 and HERB: 0.83 ± 0.02; $P < 0.05$, Student's t test; Saline-injected animals: 0.22 ± 0.01) (Fig. 1). The individual structure analyses revealed a statistically significant decrease in the c-Fos immunoreactivity in the CA_3, CA_1 and the piriform cortex region (Fig. 2) of the herbimycin A-treated animals. c-Fos staining was minimal in the animals injected with saline when compared to pilocarpine-induced SE animals, as described previously (Mello et al., 1996). It was characterized by sparse positive neurons in different areas of the hippocampus and limbic cortex. Optical density analysis of limbic structures showed less staining in the CA_3 region (mean optical density value of 0.015) and maximum staining in the entorhinal cortex (mean optical density value of 0.060).

2.3. *Spontaneous seizure frequency*

All animals undergoing pilocarpine-induced SE later developed spontaneous seizures. Most often, the spontaneous seizures start after a sleeping period and evolves according to the

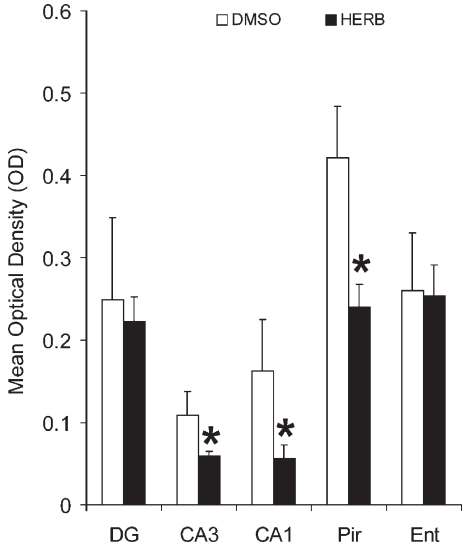

Fig. 2 – Quantitative analyses of c-Fos immunoreactivity in the dentate gyrus (DG), CA_3, CA_1, piriform (Pir) and entorhinal (Ent) following 5 h of SE and treatment with vehicle (DMSO) or herbimycin A (HERB). *$P < 0.05$ compared with the vehicle-treated group (Student's t test). The mean optical density values for saline-injected animals varied from 0.015 (CA_3) to 0.060 (Ent).

Racine's scale (Racine, 1972) with the eyes blinking, ear movements and mouth openings, followed by forelimbs clonus, dorsiflexion of the tail (Straub tail), rearing and falling. Sometimes, at the end of the abovementioned sequence and after a short period of akinesia (circa 10–15 s), bouts of wild-running with vigorous jumps could also be observed. In some animals, from both groups, seizures could also appear in clusters. Thus, one animal could present one seizure each 20 min (one extreme case observed in the present study) for at least 6 h. Table 1 summarizes the mean and standard-error for the frequency (and the amount of seizure clusters) and the latent period for spontaneous seizures in both groups. It should be noted that we observed a huge variation within groups, for the latency for the appearance of the first seizure and for the frequency of spontaneous seizures. Thus, animals from SE-DMSO group presented its first seizure after 9 and 109 days (minimum and maximum, respectively, median: 13), while animals from SE-Herb group presented its first seizure after 17 and 87 days (minimum and maximum, respectively, median: 28). In 4 months, the total number of seizures recorded in the SE-DMSO group varied from 2 to 144 (median: 14) and in SE-Herb group varied from 2 to 61 (median: 10). Treatment with herbimycin A also did not change whether or not clusters were present or its frequency in the animals which presented clusters (Table 1).

2.4. *Limbic cell death and morphological reorganization*

As previously described (Mello et al., 1993), pilocarpine-induced SE produced widespread cell death and pronounced supragranular mossy fiber sprouting. Herbimycin A did not affect cell death in any of the studied brain areas nor the mossy fiber sprouting in the supragranular region. Cell death was more pronounced in the ventral hilus (~80% cell loss), followed by the dorsal hilus and piriform cortex (~60%) and entorhinal cortex (~30%) in both experimental groups (Fig. 3). Mossy fiber sprouting in the inner molecular layer of the dentate gyrus occurred in all SE animals, irrespective of whether or not treated with herbimycin (Fig. 4). The apex and the crests of the inner molecular layer (respectively, positions 1 and 5 in Fig. 4A) presented greater intensity of staining as compared to other parts of this layer, suggesting that the sprouting was not yet at its maximum possible level in all parts of this layer (4 months after SE induction).

2.5. *Regression analysis between spontaneous seizure, cell death and mossy fiber sprouting*

Simple linear regression analysis between different parameters obtained in this study showed a negative correlation between cell counting in the dorsal and ventral hilus and neo-Timm staining in the inner molecular layer of the dentate gyrus ($F(1,18) = 10.47$; $P < 0.01$). A positive correlation was also observed for the cell counting in the piriform cortex and ventral hilus (the most susceptible regions for SE-induced cell death, see Fig. 3) and seizure clusters ($F(1,18) = 7.39$; $P < 0.05$). It should be noted that no correlation was observed between any of the mossy fiber sprouting parameters and aspects related to the spontaneous behavioral seizures (e.g., latency, frequency, clusters).

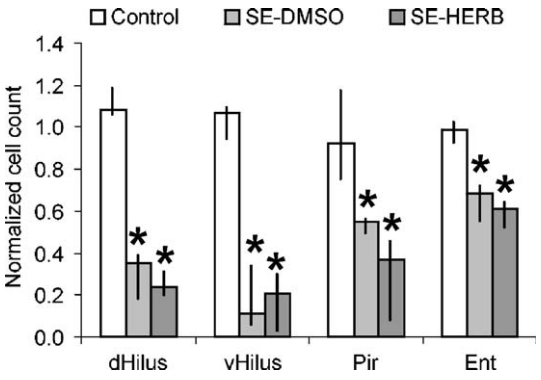

Fig. 3 – Cell counting in the dorsal (dHilus) and ventral hilus (vHilus) and the piriform (Pir) and entorhinal (Ent) cortex of chronic epileptic animals that underwent vehicle (DMSO) or herbimycin A (HERB) treatment. *$P < 0.05$ compared with the control group (Mann–Whitney *U* test).

3. Discussion

The main findings from this study are the following: first, a single dose of herbimycin A significantly reduced c-Fos expression triggered by SE in selected brain areas and second, herbimycin A did not alter the spontaneous recurrent seizure frequency or the supragranular mossy fiber sprouting.

It has been shown that immunostaining for c-Fos could be a good general marker for neuronal activation (Dube et al., 1998; Harvey and Sloviter, 2005; Rieux et al., 2002) and its production may mark specific synapses that undergo plastic changes (Dragunow and Faull, 1989). In the present study, we observed strong c-Fos activation after 5 h of SE in different brain regions, mostly in limbic structures (Barone et al., 1993; Mello et al., 1996). It is assumed that repeated c-Fos induction by periodic afterdischarges may be required for kindling development (Shin et al., 1990; Simonato et al., 1991). In fact, a null mutation for *c-fos* (Watanabe et al., 1996) or the

Table 1 – Latency for the appearance of the first seizure, spontaneous seizure frequency and seizure cluster (total number of days in which animals presented more than one seizure, from the total 58±3 days of video-recording) in SE animals treated with vehicle or herbimycin A

Treatment (i.c.v.)	Latency (days)	Spontaneous seizures (number of seizures/recorded hours)	Seizure clusters (days with >1 seizure)
Vehicle (N = 9)	32 ± 12	0.086 ± 0.039	14 ± 5
Herbimycin A (N = 9)	38 ± 8	0.055 ± 0.020	9 ± 2

Data expressed by means ± standard error. Student's *t* test; non-statistically significant differences between groups.

continued i.c.v. infusion of antisense oligonucleotides (Suzukawa et al., 2003) attenuated kindling development and kainic acid-induced seizures (in ethachrynic acid pre-treated animals), respectively. In this last report, *c-fos* antisense was able to significantly decrease c-Fos immunostaining in limbic structures, the piriform cortex in special (Suzukawa et al., 2003). In the present study, c-Fos activation was significantly attenuated by the i.c.v. treatment with herbimycin A, a protein tyrosine kinase inhibitor, which suggests the involvement of tyrosine phosphorylation in c-Fos activation. The existence of a casual linkage between tyrosine phosphorylation and c-Fos is strengthen by the fact that neurotrophin NT-3 administration (Marsh and Palfrey, 1996) and TrkB receptor activation (through the phosphorylation of the residue Y484) (McCarty and Feinstein, 1999) can increase the c-Fos expression in hippocampal slices. Although not described in neuronal tissue, another protein tyrosine kinase inhibitor, genistein, was able to diminish high pressure-induced c-Fos activation in rat mesenteric arteries (Wesselman et al., 2001).

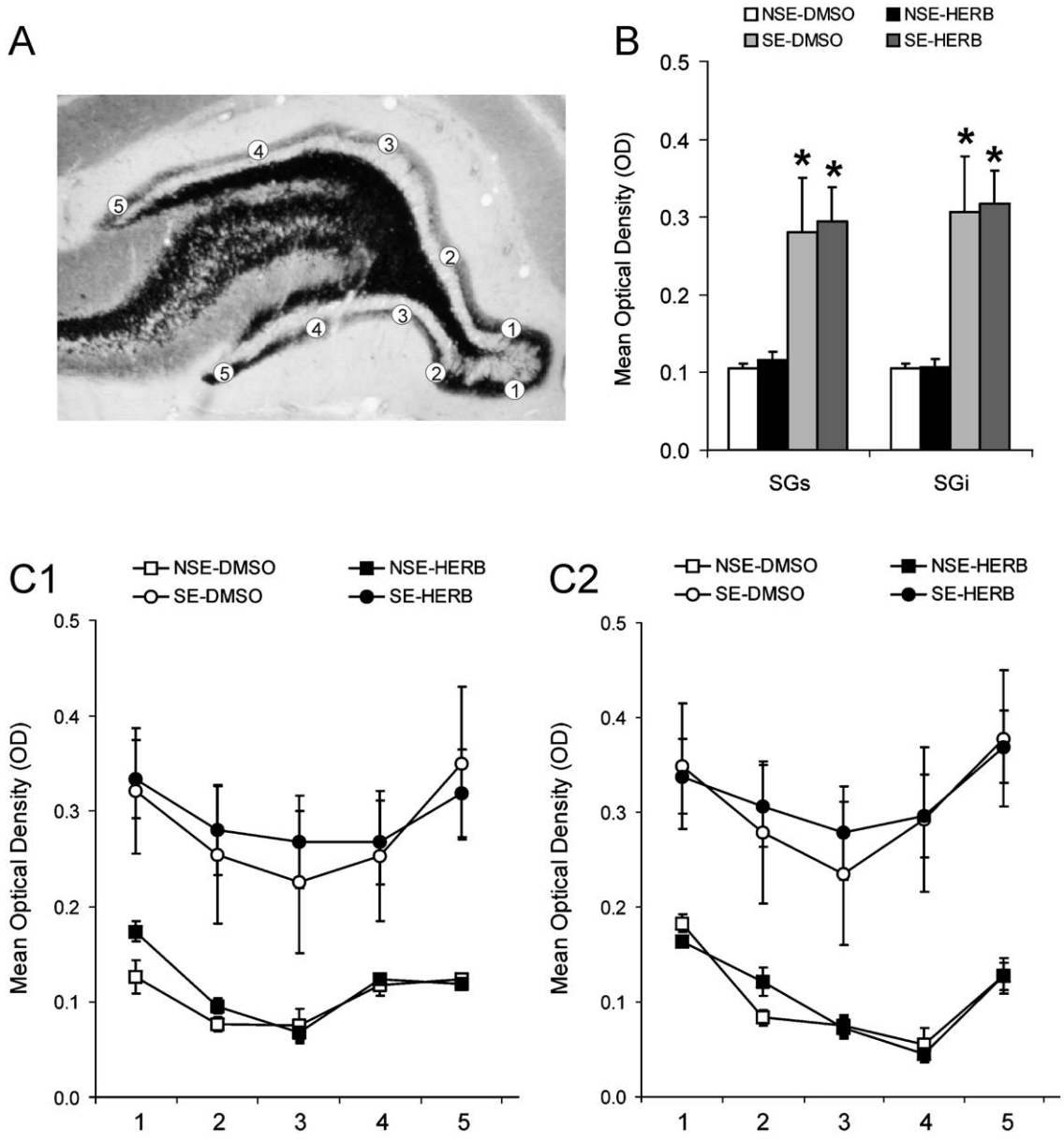

Fig. 4 – Quantification of neo-Timm staining in the supragranular region of the dentate gyrus. In panel A, localization of the sampling areas used to assess the intensity of the neo-Timm staining. In panel B, SGs: supragranular region suprapyramidal blade; SGi: supragranular region infrapyramidal blade. *$P < 0.05$ compared with the NSE-group (ANOVA, followed by Student–Newman–Keuls's post hoc test). In panel C, quantitative analyses of the spatial distribution of the mossy fiber sprouting in the suprapyramidal (C1) and infrapyramidal (C2) blades of the inner molecular layer. In the *x*-axis (shown in panel A), the number 1 represents the medial portion (near the apex), while the number 5 represents the lateral portion (the crest). Induction of SE with pilocarpine was able to trigger mossy fiber sprouting in all animals regardless of any other treatment (herbimycin A or DMSO).

Despite the changes in c-Fos expression, herbimycin A treatment was not able to alter the spontaneous seizure frequency, or the latent period, i.e., the time between SE and the first recorded spontaneous seizure (Table 1). Three possible explanations could account for these results. First, the present experiments used a low dose of herbimycin. This seems unlikely given the ability of 1.74 nmol of herbimycin A administered i.c.v. (thus the same dose and administration route as used here) to attenuate long-term potentiation induced in the dentate gyrus (Abe and Saito, 1993) and in the commissural CA_3 neurons (our results, unpublished observations) in vivo. Also, the dose used here is 300 times higher than the dose normally used for in vitro studies (Kiang, 2003). Furthermore, our results with c-Fos suggest that this pharmacological treatment (single administration, 5 min after the SE onset) was sufficient to affect intracellular biochemical cascades associated to synaptic plasticity. The second possibility is that herbimycin did not affect all limbic structures similarly. This possibility is supported by the observation that only CA_1, CA_3 and piriform cortex showed significant c-Fos reductions after herbimycin treatment (Fig. 2). Thus, pilocarpine treatment might produce tyrosine phosphorylation-mediated epileptogenesis in brain regions not affected by the herbimycin A. The third possibility is that pilocarpine-induced epileptogenesis is not entirely dependent on c-Fos activation during ictogenesis. In fact, this wave of immediate early gene activation could be related to cell stress (Dube et al., 1998) resulted from neuronal activation (Harvey and Sloviter, 2005). Thus, herbimycin A promoted a decrease in c-Fos activation, that in turn might indicate a decreased neuronal recruitment during the SE (Harvey and Sloviter, 2005). Although non-SE animals (pilocarpine-injected animals that did not develop SE) were not included in the present acute c-Fos experiment, Barone et al. (1993) have shown that the subconvulsant dose of 200 $mg{\cdot}kg^{-1}$ of pilocarpine increased c-Fos expression in the piriform cortex but not in the hippocampus itself. This c-Fos activation was only present in animals experiencing focal seizures (Barone et al., 1993).

The fact that the seizure frequency (and latent period) was quantified by sampling video recording could have affected our results. Indeed, although quite robust and less time consuming (Longo and Mello, 1997; Persinger and Belanger-Chellew, 2001), video recordings usually just allow the quantification of behavioral seizures, with a prevalence of generalized stage V seizure. Continuous EEG recordings, associated with behavioral monitoring, could obviously supply a more reliable way for seizure frequency assessment. On the other hand, there is evidence suggesting that in the pilocarpine model most if not all of the spontaneous seizures are stage V seizures (Harvey and Sloviter, 2005; Leite et al., 1990). Thus, the current protocol of herbimycin A treatment did not obviously affect the generalized seizures frequency. Experimental models of partial seizures (i.e., intrahippocampal administration of kainic acid or pilocarpine) should be employed to evaluate the actions of herbimycin A upon mild seizures.

The herbimycin treatment was also not sufficient to attenuate the SE-induced cell death, although it was able to reduce c-Fos expression. It must be mentioned that herbimycin A not only decreased the amount of c-Fos staining (Fig. 2) as it also diminished the number of c-Fos positive cells (Fig. 1). This observation suggests that a smaller number of cells were recruited during SE in herbimycin A-treated animals and consequently, we should have observed some herbimycin A-mediated neuroprotection. This was not the case and argues in favor of a dissociation between c-Fos activation and cell death. On the other hand, herbimycin A has long been known to induce heat shock proteins in different cell types (Kiang, 2003), an effect that could be neuroprotective (Sachidhanandam et al., 2003). Since we were interested in an antiepileptogenic effect of herbimycin A, in our experiments, the treatment occurred concomitantly with the "stressful" event (the SE) making it possible that the amount of heat shock proteins in neurons was not at its maximum. The absence of neuroprotection seen here could also be related to the dose and treatment protocol (see above) or even to the model used since it was recently reported that herbimycin A could protect neuron/glia cultures against Japanese virus encephalitis-induced neurotoxicity (Raung et al., 2005).

Recently, two major families of signalling molecules have been implicated in mossy fiber sprouting (Koyama et al., 2004; Xu et al., 2003). Koyama et al. (2004) have shown that increased excitability of the granule cells leads to mossy fiber sprouting through production and release of BDNF which acts upon TrkB receptors. Xu et al. (2003) have also shown that the mossy fiber sprouting after kindling is regulated by EphA receptors and ephrin-A ligands. Although both of these peptides are tyrosine kinase receptors which activation is inhibited by herbimycin A, the present treatment did not obviously modify the pattern and the intensity of the mossy fiber sprouting triggered by the pilocarpine-induced SE. It could be that administration over a longer time period was required for herbimycin A to alter SE-induced mossy fiber sprouting, given the protracted nature of this phenomenon. In any event, even though controversial (Williams et al., 2002), a single application of cycloheximide is able to significantly affect mossy fiber sprouting (Longo et al., 2003).

The involvement of both hilar cell death and mossy fiber sprouting in the development of the spontaneous seizures is still controversial (Harvey and Sloviter, 2005; Longo et al., 2003; Longo and Mello, 1997; Williams et al., 2002). Using the data produced in the present report, we showed a correlation between the hilar cell death and supragranular mossy fiber sprouting, as well as between hilar and piriform cell death and days with more frequent spontaneous seizures (seizure cluster). Although it is impossible to make a causal linkage between these two processes, it is plausible that the stronger the SE, the greater the cell death the animal will experience and consequently, the more intense all of the consequences resulting from both events including the likelihood of developing frequent spontaneous seizures. In fact, it has already been reported that spontaneous seizure frequency may depend on the duration of the initial status epilepticus (Lemos and Cavalheiro, 1995; van Vliet et al., 2004).

Taken together, these results suggest that herbimycin treatment was able to decrease the acute c-Fos induction seen during and shortly after the SE and yet failed to modify the seizure frequency or mossy fiber sprouting seen in the pilocarpine model. Our data do not support a critical role of tyrosine kinase activation in ictogenesis and epileptogenesis in this model.

4. Experimental procedures

4.1. *Experimental animals*

Male Wistar EPM-1 rats weighting 250–300 g were treated in accordance to ethical guidelines at the laboratory of Neurophysiology of UNIFESP/EPM. The Ethics Research Committee of Universidade Federal de São Paulo (CEP1078/00) previously approved the experimental procedures.

4.2. *Induction of status epilepticus*

Animals were pre-treated with methyl-scopolamine (1 mg kg^{-1}) 15 min before the administration of pilocarpine (320 mg kg^{-1}, i.p.) and after approximately 30 min showed piloerection, salivation, startle and tonic–clonic seizures evolving to status epilepticus (SE group; N = 26). Animals were injected with thiopental 90 min after SE onset to reduce mortality. Some animals that despite receiving pilocarpine did not develop SE (approximately 30% of all animals injected with pilocarpine) were used as additional controls (NSE group; N = 8) in histological analyses. Eighteen animals from the SE group were allowed to survive for 4 months after SE induction while eight animals were sacrificed 5 h after the initiation of the SE for assessment of c-Fos expression, when the tyrosine phosphorylation is at its maximum (Funke et al., 1998). In the acute experiment, animals did not receive thiopental.

4.3. *Herbimycin A administration*

For the i.c.v. administration of herbimycin A or vehicle (DMSO 1%, in saline), a 15 mm guide cannula was previously (5 days prior to SE induction) implanted (stereotaxically from bregma, in mm: AP = −0.8, ML = −1.5, DV = −2.7) (Paxinos and Watson, 1986) under halothane (1%) anesthesia. The cannula was placed 0.2 mm above the right ventricle and fixed to the skull with the aid of 2 screws and dental cement. In the experimental day, 5 min after SE initiation, the ventricle space was reached by gently inserting a 0.3 mm-longer cannula (32G, 18 mm of total length) inside the guide cannula. This procedure was facilitated given that the animal was unconscious due to the SE. The cannula was attached to a 5 μL Hamilton syringe through a polyethylene (PE-20) tube. Animals received herbimycin A 1.74 nmol (dissolved in DMSO 1% followed by dilution in sterile saline solution prior to the injection, N = 13) or vehicle (N = 13) (5 μL, 2 μL/min) and were observed for herbymicn A-induced changes in the behavioral SE. Although no pharmacokinetic studies with herbimycin A in rats are available, it is assumed that this compound displays a long lasting effect since herbimycin-induced heat-shock protein lasts for 3 days in human epidermoid A-431 cells (Kiang, 2003). Also, when administered i.c.v., the present dose is able to attenuate the long-term potentiation in the dentate gyrus of anesthetized rats (Abe and Saito, 1993).

4.4. *Spontaneous and recurrent seizure quantification*

Animal behavior was recorded in VCR for the quantification of spontaneous seizures. The recordings were sampled 6–12 h/day, daytime (8:00 am–8:00 pm), 3 days/week, for 4 months. Video monitoring started 7 days after SE a period normally referred as the latent period in which animals rarely display spontaneous seizures. We evaluated the latency for the first recorded stage V (Racine, 1972) spontaneous seizure, the frequency of spontaneous seizures and the days of recording with more than one seizure event (seizure clusters). In total, each animal was recorded for 345 ± 18 h and during 58 ± 3 days over the 4-month observation period. It should be noted that the seizure frequency was calculated through video recordings for at least 20 h/week during the 4 months period thus allowing an adequate assessment of the seizure pattern and progression for each animal (Longo and Mello, 1997; Persinger and Belanger-Chellew, 2001).

4.5. *Histology: immunohistochemistry, neo-Timm and Nissl*

All histological protocols have been previously and extensively described (Mello et al., 1993). Briefly, in the acute experiment, 8 out of the 26 SE animals were anesthetized (thiopental, 40 mg·kg^{-1}) and subject to transcardiac perfused (50 mL PBS followed by 250 mL PFA 4%) 5 h after SE onset. The experimental groups were equally represented, i.e., four animals received an i.c.v. administration of DMSO 1% and the other four, herbimycin A. Three animals injected with saline were also used as an indication of the basal c-Fos activation. The brains were gently removed from the skull soon after the perfusion and post-fixed in a 30% sucrose solution in PBS. Brains were sectioned on a cryostat at 40 μm-thick coronal slices and one in every five sections was mounted in a glass slide for processing. Immunohistochemistry for c-Fos activation was made with a rabbit monoclonal antibody against c-Fos (Ab5, Oncogene Science) in a dilution of 1:3000, amplified by secondary anti-rabbit antibody (1:200) and avidin–biotin complex and revealed with DAB (0.75 mg·mL^{-1}).

In the chronic experiment, after the behavioral assessment of spontaneous seizure frequency, SE animals (DMSO 1%, N = 9; herbimycin A, N = 9) as well as age-matched non-SE animals (DMSO 1%, N = 4; herbimycin A, N = 4) were anesthetized and transcardiac perfused with 25 mL Millonig buffer 0.12 M followed by 50 mL Na_2S 0.1%, 100 mL glutaraldehyde 3% and 200 mL Na_2S 0.1%. Brain slices were processed as described above for c-Fos. For neo-Timm staining, the slides were immersed in a 360 mL solution (21 °C) containing 240 mL of gum arabic, 10.25 g citric acid, 9.45 g sodium citrate, 3.73 g hydroquinone and 510 mg of silver nitrate for approximately 45 min in two different reaction baths. Visual inspection of slides was used to control the reaction time. Adjacent sections were used to assess cell death using Nissl staining (cresyl-violet 0.4%). To eliminate potential bias associated to different reaction times and a variety of confounding issues, slides from control and experimental were processed in the same batch.

4.6. *Optical density analysis*

Analysis of the grey scale values was performed with a computer-assisted system. Initially, the brain section was magnified by means of a microscope (Olympus BX50) coupled to a video camera (Sony CCD-Iris). The image was then

digitized to the NIH Image 1.62 software. The software analyzed the grey level (GL) of every pixel of the digitized image, which could vary from 1 (white) to 256 (black). For c-Fos quantification, structure contours (granule cell layer of the dentate gyrus, pyramidal layers of the CA_3 and CA_1 and layers II and III of the piriform and entorhinal cortex) were drawn according to a stereotaxic atlas (Paxinos and Watson, 1986) in 3 different sections (200 μm apart) in both hemispheres. The optical density (OD) was then calculated through the relation $OD = \log (GL_{contour}/GL_{background})$ for each section. The grey level from the background was measured in the stratum radiatum (used as background for the evaluation of dentate gyrus, CA_3 and CA_1) and in the adjacent white matter (used as background for the evaluation of piriform and entorhinal cortex). For each animal, a single OD value was obtained from the mean of the 3 different measurements. This technique makes available a fast and accurate measure for c-Fos protein assessment in the central nervous system (Rieux et al., 2002), thus allowing statistical comparison between groups. We choose this method given that direct visual cell counting could be influenced by the increased variability on grey staining levels of individual cells. For the aberrant mossy fiber sprouting assessment, 5 circular frames were positioned in the suprapyramidal (i.e., the area closest to the CA1 region) and infrapyramidal blades of the inner molecular layer of the dentate gyrus (see Fig. 4A) in 3 different sections (200 μm apart) for the evaluation of the mean optical density value, as described above. The grey value for the background was assessed at the *corpus callosum*.

4.7. *Cell counting*

Cell counts were performed in coronal, 40-μm-thick slices stained with cresyl-violet 0.4% (Nissl staining) by an investigator that was unaware of the pharmacological treatment. The identified nucleus of the cell body (Ø ≈ 10 μm, for hilar cells and Ø ≈ 7 μm for cortical cells; with a single apparent nucleolus) was manually quantified in the dorsal hippocampus and piriform cortex (3300–3600 μm posterior to the bregma) and ventral hilus and entorhinal cortex (5600–5800 μm posterior to the bregma) (Paxinos and Watson, 1986) at 400× magnification over a microscope grid (Olympus BX50). Both hemispheres were used in the cell counting. A 4 × 4 grid (10,000 μm^2) in the hilus and a 2 × 4 (5000 μm^2) in the cortex (oriented parallel to the cell layer) were used to perform 4 measurements per section for each given brain structure for each animal. Thus, the total area evaluated for each structure and animal was the same. The hilar region was defined by the granular cell layer borders and by two imaginary lines drawn from the apex of the dentate gyrus and the proximal end of the CA_3 pyramidal cell layer. The cortical counting was performed in layers II (piriform cortex) and II/III (entorhinal cortex) as these layers present some of the most intense neurodegeneration in animal models of temporal lobe epilepsy (Mello et al., 1993; Du et al., 1995). Given that no differences were observed between hemispheres, the number of cells in each structure, in both sides, was averaged for each rat thus yielding a single value per animal for each structure. Thus, cell counting was initially represented as cell numbers divided by the area grid and then normalized to values obtained in control sections (animals injected with pilocarpine that did not develop SE). Although this technique does not allow comparisons with other studies, it is sufficient to uncover differences between treatments and it is less time-consuming.

4.8. *Statistical analysis*

The mean optical density obtained from the c-Fos counts and for seizure frequency and latency was treated by Student's *t* test. Two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls's post hoc test was used to compare the mean optical density for neo-Timm staining. Non-parametric Kruskall–Wallis followed by the Mann–Whitney *U* test was used in the comparison of the normalized cell counts with the Nissl staining. The data were expressed as mean ± standard error unless otherwise stated. A probability of $P < 0.05$ was considered to indicate statistically significant differences.

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