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Lovastatin decreases the synthesis of inflammatory mediators during epileptogenesis in the hippocampus of rats submitted to pilocarpine-induced epilepsy

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1. Introduction

Epilepsy is a brain disorder characterized by recurrent, spontaneous seizures. A particularly common form of epilepsy is temporal lobe epilepsy (TLE). The presence of spontaneous seizures may trigger inflammatory cascade in specific brain regions, raising the possibility that inflammatory mediators could modify the processes by which a normal neuronal network becomes a seizure-generating focus. This abnormal network generation may occur after a brain insult, and this process is called epileptogenesis [1], which results in spontaneous seizure onset [2–4]. According to these authors, this epileptogenic process occurs during a silent period, with the absence of abnormal clinical and electroencephalographic (EEG) signals, being followed by the appearance of spontaneous and recurrent seizures.

Previous reports from our group have described an important role of inflammatory processes in epilepsy, showing an increased expression of several inflammatory markers such as kinin receptors, cytokines, and nitric oxide in the hippocampus of humans and rodents submitted to experimental models of epilepsy [5–10]. Thus, cytokines have been linked to the pathogenesis of epilepsy, since their levels are increased after limbic seizures [3].

Molecules such as IL-1β, TNF-α, and IL-6 are expressed at very low levels in a normal brain. In contrast, their mRNA and protein levels are quickly increased (≤30 min) after the induction of seizures, declining to basal levels within 48–72 h after seizure onset [11]. Thus,
antiinflammatory strategies have been tested, and new evidence has have emerged showing that antiinflammatory molecules may protect the brain during the epileptogenesis. Consequently, the mechanism of action of several drugs has been studied in brain, after a severe insult.

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase inhibitor and are orally administered as cholesterol-lowering drugs. The enzyme HMG–CoA reductase catalyzes the conversion of HMG–CoA to α-mevalonate. Statins prevent biological activities downstream of α-mevalonate because of their inhibitory effect [12]. Furthermore, several lines of evidence have associated lipid metabolism with inflammatory processes linked to the progressive nature of neurodegenerative diseases [13].

Statin intake has been associated with a decrease in the incidence of Alzheimer’s disease (AD) [14], cerebral ischemia [15], and multiple sclerosis [16].

In epilepsy, previous data from our group have shown that lovastatin is able to decrease the neuronal death rate induced by long-lasting status epilepticus, reducing the levels of IL-1β, TNF-α, IL-6, kinin B1, and B2 receptor mRNA production in the hippocampus of rats during SE [7].

Thus, this work was delineated to understand how lovastatin can exert its long-lasting action in the hippocampus of rats during the epileptogenic process triggered by SE. In this context, the present work analyzed the effect of lovastatin in the modulation of IL-1β, TNF-α, IL-6, and IL-10 production during the silent (latent) and chronic periods of pilocarpine-induced temporal lobe epilepsy.

2. Materials and methods

2.1. Animal treatment

The animal experiments were performed under UNIFESP Ethical Committee Institutional and approved (Hospital São Paulo/Universidade Federal de São Paulo, process n. 0653/10), and all efforts were made to minimize animal suffering. Wistar adult male rats, weighing 250 g, were housed in groups of three to four per cage and maintained in controlled room temperature, humidity, and light–dark cycle (12:12 h) with chow pellets and tap water available ad libitum. The rats received a single dose of pilocarpine (350 mg/kg, intraperitoneal [i.p.]). To prevent peripheral cholineric effects, scopolamine methyl nitrate was injected subcutaneously at a dose of 1 mg/kg, 30 min before pilocarpine administration. Only rats that displayed SE were included in this study. The SE was monitored and terminated with an injection of diazepam (10 mg/kg ip; Roche, Brazil) 3 h after SE onset.

2.2. Drug administration

We analyzed the latent period (15 days after pilocarpine injection) and chronic period (30 days after first spontaneous seizure), and each group was composed of the following: saline (CONTROL, n = 5), lovastatin (LOVA, n = 5), pilocarpine (PILO, n = 8), and pilocarpine plus lovastatin (PILO + LOVA, n = 8). After pilocarpine injection (350 mg/kg, i.p.), the rats were treated with 20 mg/kg of lovastatin via an esophageal probe after 2 h of SE onset. All rats were continuously treated during 15 days, twice daily.

2.3. Quantitative real-time PCR

Rats from the above groups (n = 5 for each group) were sacrificed, and their brains were isolated and dissected, and the hippocampi were frozen in liquid nitrogen and stored at −80 °C. Thawed tissue was homogenized in 1–ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was isolated according to the manufacturer’s instructions. Ribonuclease acid was subjected to DNase I digestion followed by reverse transcription to cDNA, and PCR was performed in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBR Green core reaction kit (Applied Biosystems). Primers used for cytokine mRNA quantification were as follows:

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward Primer</th>
<th>Reversed Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5′-CCACCTCTACACAGGACAC-3′</td>
<td>5′-GGTTTCACTGGTAACAC-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-AAATGGGCTCCCTCTATAGTC-3′</td>
<td>5′-TCTCTCTGGTGGTTCCTGAC-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-TCTTACCCCAACTCCAATGCT-3′</td>
<td>5′-TTGAGTGGCTTTGCTTACC-3′</td>
</tr>
<tr>
<td>IL-10</td>
<td>5′-AAAGCAAGCATTGCAGCAG-3′</td>
<td>5′-TCAAACATTCATGATGTC3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGCCAGCCCGAGAATAT-3′</td>
<td>5′-CGGTTCACCTGCTGAGATG-3′</td>
</tr>
</tbody>
</table>

Quantitative values for cytokine (IL-1β), TNF-α, IL-6, and IL-10 mRNA transcription were obtained from the threshold cycle number, where the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of GAPDH expression as endogenous RNA control. ΔCt values of the samples were determined by subtracting the average Ct value of cytokine mRNA from the average Ct value of the internal control GAPDH. To express the relative expression data, we used 2-(ΔΔCt) parameter.

2.4. Statistical analysis

Expression levels of cytokine mRNA were presented as an n-fold difference relative to the levels expressed during the inflammatory process, and the different treatments were compared using the one-way ANOVA followed by the Student–Newman–Keuls r-test. The data were presented as means ± S.D., and p < 0.05 was considered statistically significant. Only data that showed a normal distribution were considered.

2.5. Immunohistochemistry

The rats were anesthetized with 0.1 mL of chloral hydrate (4%) and subjected to transcardiac perfusion with a solution of paraformaldehyde 1% (pH 7.4, 15 mL/rat, infusion rate: 15 mL/min) followed by a solution of paraformaldehyde 4% (pH 7.4, 150 mL/rat, infusion rate: 15 mL/min). After perfusion, the brain was carefully removed from the skull, postfixed in paraformaldehyde (4%) for 48 h, and immersed in a solution of sucrose (30%) for cryoprotection for 48 h. Forty-micrometer-thick coronal slices were obtained using a cryostat (HM 505E Micromeria, Zeiss) and stored in 0.1-M phosphate buffer (pH 7.4). The slices were collected throughout the hippocampus and stored in 0.1-M phosphate buffer. The slices were mounted on gelatin-coated slides for immunohistochemistry with the antibodies anti-IL-1β (IBL, 1:100), IL-6 (Abcam, 1:50), IL-10 (R&D, 1:100), and TNF-α (IBL, 1:100).

The immunohistochemistry was performed in the same way for all primary antibodies in all groups. Briefly, free-floating slices were treated with hydrogen peroxide (1%) for 10 min, washed with phosphate-buffered saline (PBS, pH 7.4) several times, and then treated with Triton X-100 (0.4%) for 30 min. Slices were washed with PBS, preincubated with albumin (10%) for 2 h, and incubated with primary antibody overnight at 4 °C. The next day, the slices were washed and then incubated at room temperature with appropriate secondary antibodies (1:200, biotinylated immunoglobulin G, Calbiochem) for 2 h. Sections were finally washed and incubated in avidin–biotin–peroxidase complex (ABC Kit, Vector) for 90 min, washed again with Tris–HCl (pH 7.6), and finally developed with diaminobenzidine (DAB, 1 tablet/15 mL of Tris–HCl). Slices were finally washed in PBS and mounted on histological slides.
3. Results

3.1. Animal behavior

Pilocarpine administration induced the following behavioral changes: akinesia, facial automatism, and limbic seizures consisting of forelimb clonus with rearing, salivation, and masticatory jaw movements and falling. This type of behavior built up progressively into motor limbic seizures that recurred repeatedly, evolving to long-lasting SE [19].

3.2. Real-time PCR analysis for cytokines

3.2.1. Latent period

Cytokine mRNA levels were analyzed using a quantitative RNA assay, and IL-1β, TNF-α, and IL-6 showed increased expression in the hippocampus of the PILO group when compared with the CONTROL group. In contrast, the PILO + LOVA group showed an important decrease in IL-1β (p < 0.05), TNF-α (p < 0.001), and IL-6 (p < 0.01) when compared with the PILO group. By contrast, IL-10 mRNA presented similar values in all groups (Fig. 1).

3.2.2. Chronic period

Interleukin-1 beta and TNF-α mRNAs showed an increased expression in the PILO group when compared with the CONTROL group. However, the PILO + LOVA group showed an important decrease in IL-1β (p < 0.05) and TNF-α (p < 0.001) levels in the hippocampus when compared with the PILO group. Interleukin 6 mRNA expression showed no difference between studied groups. Interestingly, -10 mRNA expression levels were increased in the PILO + LOVA group when compared with the PILO group (p < 0.05) (Fig. 2).

3.3. Immunohistochemistry

Interleukin-1 beta was localized in all hippocampal formations, mainly in experimental animals. In the PILO group, during silent and chronic periods (Figs. 3A II and 4A III), this staining was increased when compared with the CONTROL (Figs. 3A I and 4A I) and LOVA groups (Figs. 3A II and 4A II), mainly in regions such as CA1, CA3, and stratum lacunosum–moleculare. However, in the PILO + LOVA group, this immunoreactivity was decreased in the same regions of hippocampal formation during the latent (Fig. 3A IV) and chronic (Fig. 4A IV) periods.

Tumor necrosis factor-alpha was visualized in the hippocampal formations as staining of neurons from the dentate gyrus and hilus. However, no difference between the PILO and PILO + LOVA groups was found in either period.

Regarding IL-6 distribution in the hippocampus of PILO group, in both the silent and chronic periods (Figs. 3C III and 4C III), we found cells with the shape of astrocytes stained with more intensity, when compared with CONTROL (Figs. 3C I and 4C I) and LOVA groups (Figs. 3C II and 4C II). In contrast, the PILO + LOVA group (Figs. 3C IV and 4C IV) presented the same staining observed in the CONTROL and LOVA groups in both periods, showing a disappearance of cells with the shape of astrocytes.

Interleukin-10 was also localized throughout all hippocampal formations of all groups. This immunoreactivity was visualized in major intensity in the PILO group during latent (Fig. 3D III) and chronic (Fig. 4D III) periods when compared with the CONTROL (Figs. 3D I and 4D I) and LOVA groups (Figs. 3D II and 4D II). However, the PILO + LOVA group (Figs. 3D IV and 4D IV) presented more cells with the shape of microglia stained by IL-10 antibody when compared with the CONTROL (Figs. 3D I and 4D I) and LOVA groups (Figs. 3D II and 4D II) during the chronic phase.

4. Discussion

Pilocarpine-induced SE ([18,19]) simulates a brain injury and is associated with the development of temporal lobe epilepsy in rodents and is an appropriate model to study epileptogenesis. Thus, events occurring during this period are critically important and may reveal targets for pharmacological intervention. Successfully modifying these targets during epileptogenesis will likely reduce neuronal death and inflammation.

The present study demonstrated that continuous treatment with lovastatin, after induced SE, reduced several important hippocampal cytokines related to brain inflammation during epileptogenesis and during the period of spontaneous seizures. In addition, an increased expression of IL-10, an antiinflammatory cytokine, was increased during the chronic phase, in those rats treated with lovastatin.

Studies with statins have shown that these drugs activate several neuroprotective signaling pathways. Zacco et al. [20] reported that several statins render mouse primary cortical neurons more resistant to excitotoxicity in a cholesterol-dependent manner. This action is related to the efficacy of statins in blocking HMG–CoA reductase [21]. This idea was reinforced by the reversal of this effect that was observed by the addition of mevalonate or cholesterol into cell culture [20].

However, in vivo experiments have demonstrated that statin treatment increases neurogenesis and synaptogenesis without altering serum cholesterol levels after brain damage [22–25].
Hering et al. [26] also observed that treatment with statins would lead to instability of the AMPA receptor in cultured neurons. This effect occurs due to modification of cholesterol and sphingolipid-rich microdomains known as insoluble membrane domains or “lipid rafts.” However, in vivo studies suggested the possible involvement of cholesterol biosynthesis in antiexcitotoxic activity mediated by statins. So far, it is not known how the cholesterol biosynthesis could mediate this activity in vivo. However, the reduction of cholesterol levels in fractions of these “lipid rafts” on the plasma membrane of synaptosomes [27] suggested that statin treatment could affect intracellular and extracellular distribution of cholesterol, which is associated with glutamate receptor function during induced excitotoxicity.

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**Fig. 2.** Quantification of mRNA expression by RT-PCR assay. Expression levels of mRNA of IL-1β, TNF-α, IL-6, and IL-10. All data have been normalized for levels of GAPDH expression within the same sample and are expressed relative to levels detected in each experimental group in the chronic period. (#) Comparison between the PILO group with the CONTROL group (\( p \leq 0.05, **p \leq 0.01, \text{and} ***p \leq 0.001 \)). (\( \star \)) Comparison between the PILO + LOVA group with the PILO group (\( *p \leq 0.05, **p \leq 0.01, \text{and} ***p \leq 0.001 \)). The difference between groups was evaluated using one-way ANOVA followed by the Student Newman Keuls t-test.

**Fig. 3.** Immunohistochemical analysis of cytokines in the hippocampus in the CONTROL, LOVA, PILO, and PILO + LOVA groups in the latent phase. A: Interleukin-1β (IL-1β). B: Tumor necrosis factor-α (TNF-α). C: Interleukin-6 (IL-6), and D: Interleukin-10 (IL-10). Scale bar = 100 μm.
In the present study, we observed a significant increase in the IL-1β transcript as well as in its hippocampal distribution in all phases of the model. Intense staining was visualized in CA1 and CA3 in stratum lacunosum–moleculare and in the dentate gyrus of the PILO group, which was reduced in animals from the PILO + LOVA group, suggesting that lovastatin promoted an antiinflammatory action, interfering in the inflammatory cascade triggered by induced SE.

Interleukin-1 beta may involve the induced synthesis of other cytokines as well as IL-6 and TNF-α by astrocytes and microglia [41,42], and many actions of IL-1β in the CNS could be mediated by IL-6 and TNF-α [28].

The highest concentration of the TNF-α transcript was verified in the PILO group, especially in the acute phase of the model, decreasing in lovastatin-treated group [7].

Dolga et al. [29] observed that lovastatin increased the selective expression of TNFR2 induced by excitotoxicity in culture of cortical neurons. Levels of TNFR2 were increased in ischemic tissues, and this TNF receptor has been related to neuroprotective signaling cascade, which includes PKB/Akt and NF-κB [30,31]. However, the mechanism by which the TNFαR2/NF-κB promotes neuroprotection is not yet fully understood.

Additionally, IL-10 has been associated with neuroprotection since this cytokine presents an antiinflammatory action. This mechanism seems mediated by astrocytes and microglia [32]. Indeed, IL-10 mRNA quantification showed a significant increase in the chronic phase in the PILO + LOVA group. This result was confirmed in hippocampal tissue since increased expression of IL-10 was visualized in cells with the shape of microglia.

Cell survival promoted by IL-10 is associated with its antiinflammatory action, protecting the central nervous system against a variety of proapoptotic factors induced during the immune response activation. Interleukin-10 blocks initiator and effector molecules involved in cell death [33]. In addition, IL-10 prevents cell death induced by TNF-α in astrocytes [34]. This cytokine also reduces the death of cerebellar neurons through the inhibition of excitotoxicity induced by NMDA receptor [35,38]. Experiments with IL-10-deficient mice showed the presence of high levels of TNF-α and IL-6 in the brain [39,40], and it is supposed that IL-10 may reduce the expression of proinflammatory cytokines [34]. Interleukin-10 also inhibits mitochondrial-induced cell death in microglia [36], blocking the apoptosis cascade [37]. Thus, our data suggest that lovastin may reduce excitotoxicity during epileptogenesis induced by pilocarpine by increasing the synthesis of IL-10 in the hippocampus.

5. Conclusion

These data have relevance to the development of innovative strategies to block the activation of cytokine-mediated signaling in associated with the development and maintenance of epilepsy, thus highlighting potential new targets for therapeutic intervention. Based on this, our data showed that lovastatin decreases the inflammatory process in the hippocampus induced by long-lasting SE. This action was initially identified during SE [7] and confirmed now during the silent and chronic periods in this epilepsy model.

Conflict of interest

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the


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