Indomethacin treatment prior to pentylenetetrazole-induced seizures downregulates the expression of il1b and cox2 and decreases seizure-like behavior in zebrafish larvae

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Abstract

Background: It has been demonstrated that the zebrafish model of pentylenetetrazole (PTZ)-evoked seizures and the well-established rodent models of epilepsy are similar pertaining to behavior, electrographic features, and c-fos expression. Although this zebrafish model is suitable for studying seizures, to date, inflammatory response after seizures has not been investigated using this model. Because a relationship between epilepsy and inflammation has been established, in the present study we investigated the transcript levels of the proinflammatory cytokines interleukin-1 beta (il1b) and cyclooxygenase-2 (cox2a and cox2b) after PTZ-induced seizures in the brain of zebrafish 7 days post fertilization. Furthermore, we exposed the fish to the nonsteroidal anti-inflammatory drug indomethacin prior to PTZ, and we measured its effect on seizure latency, number of seizure behaviors, and mRNA expression of il1b, cox2b, and c-fos. We used quantitative real-time PCR to assess the mRNA expression of il1b, cox2a, cox2b, and c-fos, and visual inspection was used to monitor seizure latency and the number of seizure-like behaviors.

Results: We found a short-term upregulation of il1b, and we revealed that cox2b, but not cox2a, was induced after seizures. Indomethacin treatment prior to PTZ-induced seizures downregulated the mRNA expression of il1b, cox2b, and c-fos. Moreover, we observed that in larvae exposed to indomethacin, seizure latency increased and the number of seizure-like behaviors decreased.

Conclusions: This is the first study showing that il1b and cox-2 transcripts are upregulated following PTZ-induced seizures in zebrafish. In addition, we demonstrated the anticonvulsant effect of indomethacin based on (1) the inhibition of PTZ-induced c-fos transcription, (2) increase in seizure latency, and (3) decrease in the number of seizure-like behaviors. Furthermore, anti-inflammatory effect of indomethacin is clearly demonstrated by the downregulation of the mRNA expression of il1b and cox2b. Our results are supported by previous evidences suggesting that zebrafish is a suitable alternative for studying inflammation, seizures, and the effect of anti-inflammatory compounds on seizure suppression.

Keywords: Seizure, Zebrafish, Pentylenetetrazol, Interleukin-1 beta, Cyclooxygenase-2, Indomethacin, Inflammation

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Background
Zebrafish experimental models are now widely accepted for investigating human diseases, including epilepsy [1–4]. Importance of this animal model is mainly based on its remarkable features combining exceptionally simple genetic manipulations, which are ideal for forward and reverse genetic investigations, and easy phenotype assessment in a short period of time. Other advantages of this fish species are as follows: low maintenance cost, easy breeding, high fecundity, external fertilization and development, short generation time, and transparency during embryonic and larval stage. Furthermore, their genome shares approximately 70% homology with the human genome comprising large regions of conserved synteny [5].

Zebrafish exposed to chemoconvulsant drugs mimic behavior, electrographic findings, and upregulation of c-fos in brain regions related to neuronal activation [3, 4, 6]. Zebrafish are sensitive to common anticonvulsant drugs; therefore, they are widely used for the high throughput screening of novel antiepileptic drugs (AEDs) [7–13].

Evidence obtained using hippocampal surgical specimens from patients with pharmaco-resistant epilepsy and experimental rodent models demonstrated that proinflammatory cytokines and inflammatory mediators are upregulated after seizures, suggesting that the inflammatory response may play an important role in the pathophysiology of epilepsy [14–18]. Among the proinflammatory cytokines, interleukin-1 beta (IL-1β) is the most widely investigated. IL-1β exerts its action by binding to the IL-1 receptor, which initiates a downstream signaling process that activates the transcription factor nuclear factor-κB (NF-κB). Activation of NF-κB leads to the transcription of multiple inflammation-associated genes, including cyclooxygenase (COX)-2 [19–22]. COX-2 is a key enzyme responsible for the conversion of arachidonic acid into prostaglandins, potent mediators of inflammatory signaling [19–22]. One of these prostaglandins converted by COX-2 is prostaglandin E2 (PGE2), which upregulates the expression of IL-1β [19–24].

Because a relationship between epilepsy and inflammation has been established and inflammatory response in the pentylenetetrazole (PTZ)-seizure model has not been studied, we investigated the expression of the il1b and cox2 transcripts in zebrafish after seizures. In addition, we measured the effects of indomethacin, a nonsteroidal antiinflammatory drug, on seizure latency, number of seizure-like behaviors, and c-fos expression used as a marker of neuronal activity [10].

Results
Temporal expression of il1b in the brain of zebrafish larvae after PTZ-induced seizures
Temporal expression profile of il1b was analyzed 0.05, 1, 6, 12, 24, and 48 h after PTZ-induced seizures by comparing the control (CG) and seizure (SG) groups at each time point. We found a short-term upregulation of il1b mRNA levels 0.05 h (p = 0.02) and 1 h (p = 0.02) after seizures (Fig. 1). However, no significant differences were found between the CG and SG 6, 12, 24, or 48 h after seizures (p > 0.05) (Fig. 1).

The mRNA expression of cox2a and cox2b in the brain of zebrafish larvae after PTZ-induced seizures
Because no significant differences were found in the mRNA expression of il1b pertaining to longer time periods, we chose to evaluate the temporal expression profile of cox2a and cox2b 0.05, 1, and 6 h after PTZ-induced seizures. Our results showed that both cox2a and cox2b were constitutively expressed in the CG (Fig. 2a–e); however, after PTZ exposure, the expression pattern of these genes showed differences. Animals in the CG and SG had similar cox2a mRNA levels after seizures (p > 0.05; Fig. 2a–c). However, cox2b mRNA levels were upregulated 0.05 h (p = 0.004) and 1 h (p = 0.008) after seizures in the SG compared with the corresponding data in the CGs (Fig. 2d, e). No statistical significance was found 6 h after seizures (p = 0.27; Fig. 2f).

Effect of indomethacin administered prior to PTZ on the mRNA expression levels of il1b, cox2b, and c-fos
We used indomethacin at three different concentrations (10, 100, or 307 μM) prior to seizure-induction, and we quantified the mRNA levels of il1b, cox2b, and c-fos. As shown in Fig. 3, expression of il1b was downregulated by indomethacin when we compared the SG with all indomethacin concentration tested (p ≤ 0.001). In addition, il1b mRNA levels in the indomethacin-treated groups were similar to that in the CG. No significant difference was found between indomethacin treatment groups (Fig. 3). The mRNA expression of cox2b was upregulated in the SG (p < 0.05) and 10 μM indomethacin group (p ≤ 0.001) when both were compared to the CG (Fig. 4). Indomethacin treatment at 307 μM significantly downregulated the mRNA expression of il1b, cox2b, and c-fos. As shown in Fig. 3, expression of il1b was downregulated by indomethacin when we compared the SG with all indomethacin concentration tested (p ≤ 0.001). In addition, il1b mRNA levels in the indomethacin-treated groups were similar to that in the CG. No significant difference was found between indomethacin treatment groups (Fig. 3). The mRNA expression of cox2b was upregulated in the SG (p < 0.05) and 10 μM indomethacin group (p ≤ 0.001) when both were compared to the CG (Fig. 4). Indomethacin treatment at 307 μM significantly downregulated the mRNA expression of cox2b compared with the SG (p ≤ 0.001; Fig. 4). Comparisons between indomethacin treatments groups showed that cox2b mRNA levels were downregulated at 100 μM (p ≤ 0.05) and 307 μM (p ≤ 0.001) when compared to 10 μM (Fig. 4). Transcript level of c-fos was upregulated in the SG (p ≤ 0.001) and 10 μM indomethacin pretreated group (p ≤ 0.05) when compared to the CG (Fig. 5). However, 100 μM (p ≤ 0.05) and 307 μM (p ≤ 0.001)
indomethacin treatment downregulated the cfos mRNA level when compared to the SG (Fig. 5).

**Seizure onset latency and the number of seizure-like behaviors**

We evaluated the effect of indomethacin administered prior to PTZ-induced seizures by analyzing seizure onset latency (stage 3, equivalent to the loss of body posture) and the number of seizure-like behaviors. Each larva was observed individually under each experimental condition. In animals pretreated with 10, 100, or 307 μM indomethacin, we found a significant increase of onset latency (give in minutes) compared with animals in the SG (p \(\leq 0.001, p \leq 0.01,\) and p \(\leq 0.01,\) respectively; Fig. 6). Furthermore, all indomethacin concentrations tested reduced the number of seizure-like behaviors compared with the PTZ treatment alone (10 μM, p \(\leq 0.01;\) 100 μM, p \(\leq 0.01;\) and 307 μM, p \(\leq 0.001;\) Fig. 7).

**Discussion**

It has been demonstrated previously that seizures induce the upregulation of IL-1β and COX-2 in clinical specimens and experimental models of epilepsy [14–18]; however, to reveal the main role of inflammatory molecules in epilepsy, further investigations are necessary. Rodent models of epilepsy are widely used in experimental research, but due to its several favorable characteristics, zebrafish seizure models can significantly contribute to understanding the role of inflammation following seizures. In addition, zebrafish models of human diseases are particularly suitable for the pharmacological testing of drugs in a convenient way. Immune and inflammatory responses in zebrafish are comparable to those found in mammals [25–28]; however, to our knowledge, no data is available about the expression of inflammatory biomarkers such as illb and cox2 in zebrafish seizure models.

Our results clearly showed that the expression of illb is upregulated shortly after seizures in the larval brain (Fig. 1). A previous study of Minami et al. [29] showed that the mRNA levels of IL-1β increased more rapidly when the seizure was induced by PTZ compared with kainic acid in rodents. Recently, Järvelä et al. [30] reported that the mRNA level of IL-1β is elevated for up to 24 h after status epilepticus (SE) induced by kainic acid.
in rodents. Our finding suggests that the transcription profile of il1b after PTZ exposure in our zebrafish model is similar to that found in a rodent model of PTZ-induced seizures.

Because of evolutionary gene duplication, the zebrafish genome contains two functional cox2 genes termed as cox2a and cox2b [31, 32]. The mRNAs of cox2a and cox2b are constitutively expressed in numerous tissues, including the zebrafish brain [31]. In this study, we investigated the inducible expression of both cox2 genes after PTZ-induced seizures. Our results revealed a characteristic transcriptional response in both genes. Expression of cox2b, but not cox2a, is upregulated immediately after PTZ-induced seizures (0.05 h) and 1 h after the seizure in the brain of zebrafish (Fig. 2a, b). It is important to note that a study by Ishikawa et al. [31] showed that cox2b is more similar in structure to the mammalian cox2 than cox2a, which may explain our results [31].

Because the mRNA levels of il1b and cox2b were upregulated after seizures, we investigated the effect of an anti-inflammatory drug administered prior to PTZ-induced seizures. Our results showed that indomethacin used at various concentrations downregulated the expression of il1b (Fig. 3). This result is similar to the data obtained using the pilocarpine-induced model of epilepsy, wherein indomethacin administration prior to pilocarpine injection downregulates the expression of IL-1β in rats [33]. In addition, we showed that indomethacin significantly downregulated the mRNA expression of cox2b, but only at a concentration of 307 μM.

After showing that indomethacin was able to reduce the mRNA levels of il1b and cox2b, we investigated if indomethacin reduces the convulsant effect of PTZ (Fig. 4). Therefore, we assessed neuronal activity by measuring the mRNA expression of c-fos, and we investigated seizure behavior during PTZ exposure by analyzing the latency of seizure onset and the number of seizure-like behaviors, which are both well-known characteristics of seizures according to the literature [3]. The c-fos gene is a proto-oncogene, which is expressed rapidly and transiently in neurons following neuronal excitation such as that in seizures; therefore, expression of c-fos has been widely used as a marker for neuronal activity [10]. Baxendale et al. [10] showed that c-fos is a sensitive marker
for investigating anticonvulsant properties of several pharmacological compounds. Our results showed that indomethacin downregulated \( c_{-}fos \) expression at 100 and 307 μM (Fig. 5).

Furthermore, indomethacin administered prior to PTZ reduced the behavioral signs of seizure because it both increased seizure latency (time to reach stage 3, which is characterized by the loss of body posture) (Fig. 6) and decreased the number of seizure-like behaviors for all indomethacin concentrations used in this study compared with the untreated group (Fig. 7).

Although our results clearly suggest that indomethacin has significant effects on several parameters related to seizure activity, we did not demonstrate that the anticonvulsant effect of indomethacin is caused exclusively by its anti-inflammatory action on \( il1b \) and \( cox2b \); therefore, further studies are necessary to address this question.

**Conclusions**

We have shown for the first time that transcriptional levels of two important inflammatory biomarkers related to epilepsy, \( il1b \) and \( cox2 \), are upregulated in the brain of zebrafish after PTZ-induced seizures. Furthermore, we found that indomethacin exposure prior to PTZ-induced seizures had an anti-inflammatory effect by reducing the mRNA expression of \( il1b \), \( cox2b \), and \( c_{-}fos \), and by increasing seizure latency and decreasing the number of seizure-like behaviors.

Taken together, our results demonstrate that the zebrafish seizure model is a valuable alternative model for studying the molecular mechanisms of inflammation and seizures and for the investigation of anti-inflammatory compounds that may have a potential therapeutic effect in seizure suppression.

**Methods**

**Animals**

Wild-type zebrafish (adults, larvae, and embryos) were maintained according to standard procedures [34]. Adult fish were housed in 30–50 L tanks (two animals per liter) filled with non-chlorinated water cleared with mechanical and chemical filtration. Adult fish were maintained at 26 ± 2 °C and in a simulated photoperiod cycle of 10 h dark/14 h light. Adult fish were fed twice a day with commercial flake fish food (Tetramin, Tetra, Blacksburg, VA) and once a day with artemia; larvae were fed with paramecium and artemia twice a day. Fertilized eggs were
collected after natural spawning. Embryos and larvae were housed using Petri dishes filled with water in an incubator system at the same temperature and photoperiods that were used for maintaining the adults. Larvae were staged according to the morphological criteria established by Kimmel et al. [35]. All zebrafish experiments were approved by the Ethical Committee for Animal Research of the University of Campinas (protocol number 3098-1).

Pentylenetetrazole treatment
Larvae at 7 days post fertilization (dpf) were separated in the seizure (SG) and control (CG) groups. Larvae in the SG were placed in a 24-well plate (one larvae per well) containing 15 mM PTZ (Sigma-Aldrich, St. Louis, MO, USA), a γ-aminobutyric acid (GABA) antagonist, for 20 min [3]. After PTZ exposure, animals were washed to eliminate the residual PTZ before being transferred into petri dishes. Fish in the CG were handled identically; however, water was used instead of PTZ. It is important to highlight that each CG or SG presented in this study was composed by a different set of animals.

Monitoring of seizure-like behavior
Seizure-like behavior of the larvae was monitored by visual inspection and was classified based on the criteria established in a previous study [3]. To visualize the swimming behavior of larvae, we used the Stereomaster® microscope (Fisher Scientific, Waltham, MA). Briefly, classification was performed according to the following criteria: stage 1, increased swimming activity; stage 2, rapid circular “whirlpool-like” swimming; and stage 3, seizure-like activity progressing to clonus-like convulsions followed by a brief loss of posture. Animals included in this study exhibited these well-defined behavioral patterns during PTZ exposure. Moreover, we evaluated the latency of seizure onset and the number of seizures during PTZ exposure. It is important to emphasize that latency was determined as the period between the start of PTZ exposure and the appearance of stage 3 seizure-like behavior, and the number of seizure-like behaviors was obtained by counting all stage 3 seizure-like behaviors displayed.

Treatment with indomethacin and its toxicological evaluation
Indomethacin (Sigma-Aldrich, St. Louis, MO, USA) was solubilized in 1% Tris–HCl (pH 8.0) buffer to prepare a
The indomethacin treatment was determined based on a previous AED screening reported by Afrikanova et al. [37]. Zebrafish larvae at six dpf were incubated in 10, 100, and 307 μM indomethacin in petri dishes for 24 h, and then at seven dpf, they were exposed to 15 mM PTZ for 20 min as described above. It is important to highlight that each indomethacin concentration group presented in this study was composed by a different set of animals.

RNA isolation and reverse transcriptase-PCR
Animals were cryoanesthetized and their heads were immediately isolated, quickly frozen in liquid nitrogen, and stored at −80 °C. 0.05, 1, 6, 12, 24, and 48 h after PTZ treatment. We pooled 20 larval heads to obtain sufficient material for RNA extraction. A total of five samples (n = 5 for each time point) were used for each group (CG, SG, and indomethacin treatment). Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and its concentration and quality were determined with the EpochTM spectrophotometer (BioTek, Winooski, VT, USA) and electrophoresis using agarose gels. One microgram of total RNA was reverse transcribed into cDNA using the High Capacity first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reactions without RNA were run as negative controls.

Real-time quantitative PCR
Quantitative PCR was performed using the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA), TaqMan® Universal Master Mix, and TaqMan® Gene Expression Assay (Invitrogen, Carlsbad, CA, USA) for zebrafish (Table 1). All relative quantifications were performed in triplicates and were normalized to the housekeeping gene eukaryotic translation elongation factor alpha 1, like 1 (efla1l1) [38, 39]. The mRNA level of each target gene (il1b, cox2a, cox2b, and c-fos) was normalized to the expression level of the housekeeping gene efla1l1 (Table 1). Efficiency of each quantitative real time PCR assay was assessed using standard curves, which were created by measuring five serially diluted cDNA samples in triplicates. Efficiency was calculated according to the following formula: E = 10[−1/slope]. Relative gene expression quantification (RQ) of the SG samples compared with the CG samples (after normalization to the housekeeping gene) was calculated according to the equation $RQ = 2 − ΔΔCT$ described by Livak and Schmittgen [40]. Each reaction was run without cDNA as negative control. Data were analyzed using the SDS 7500 software (Applied Biosystems) to estimate qPCR efficiency and quantify the relative gene expression.
Table 1 Information about genes and qPCR assays

| Official full name | Gene official symbol | Also known as | Accession | Assay ID |
|--------------------|----------------------|---------------|-----------|----------|
| Eukaryotic translation elongation factor 1 alpha 1a | eef1a1a | eef1a1a | NM_131263.1 | Dr0342748_ml |
| v-fos FBJ murine osteosarcoma viral oncogene homolog Ab | fosab | fos, c-fos | NM_205569.1 | Dr0310089_ml |
| Interleukin 1-beta | il1b | il1-b | NM_212844.2 | Dr03114369_ml |
| Prostaglandin-endoperoxide synthase 2a | ptgs2a | cox2a | NM_153657.1 | Dr03080323_ml |
| Prostaglandin-endoperoxide synthase 2b | ptgs2b | cox2b | NM_001025504.2 | Dr03116323_ml |

Statistical analysis

Data are presented as mean values ± standard error of mean (SEM). Statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software, CA, USA). In all analyses, significance level was set at p ≤ 0.05. Statistical comparisons between two groups were performed using the Mann–Whitney test. Statistical comparisons between three or more groups were performed using one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test.

Abbreviations

CG: control group; cox2a: cyclooxygenase-2a; cox2b: cyclooxygenase-2b; dpf: days post-fertilization; fos: FBJ murine osteosarcoma viral oncogene homolog; il1b: interleukin-1 beta; NSAID: nonsteroidal anti-inflammatory drugs; ptgs2: prostaglandin-endoperoxide synthase 2; PTZ: pentyleneetrazol; qPCR: quantitative PCR; RT-PCR: reverse transcription PCR; SG: seizure group.

Authors’ contributions

PGB performed the experiments, participated in the design of the study, analyzed the data and wrote the paper. ILC participated in the design of the study and revised the manuscript. CVMM conceived the study, participated in the analysis of the data and wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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