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The selective COX-2 inhibitor Etoricoxib reduces acute inflammatory markers in a model of neurogenic laryngitis but loses its efficacy with prolonged treatment

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Abstract
Objective A randomised experimental study was used to evaluate the therapeutic effect of a selective cyclooxygenase-2 (COX-2) inhibitor in neurogenic laryngitis.
Materials and methods Male Wistar Han rats were subjected to the nasogastric intubation model (NGI) of laryngitis for 1 and 2 weeks. The NGI animals were divided into three groups: (1) treated with COX-2 inhibitor Etoricoxib, (2) vehicle and (3) non-intubated animals. A fourth group of animals was submitted to NGI only. Laryngeal sections were immunostained for substance P (SP) and calcitonin gene-related peptide (CGRP) fibre-immunoreactivity (IR) and quantification of COX-2 positive cells through stereological analysis. The expression of COX-2, interleukins IL-1β, IL-6, IL-10 and tumour necrosis factor-α (TNF-α) was determined by quantitative real time QRT-PCR.
Treatment Etoricoxib (6 mg/kg/day) was prepared in 0.9% sterile saline with 5% glucose (vehicle) and administered daily during 1 or 2 weeks.
Results Treatment for 1 week with Etoricoxib attenuated the CGRP-IR fibre depletion, the COX-2-IR increased cell number and the TNF-α and COX-2 mRNA increased levels induced by NGI. Two weeks of treatment had no beneficial effect.
Conclusions Etoricoxib is effective in neurogenic laryngitis for limited periods of administration, indicating that selective COX-2 inhibitors should be evaluated in the future.

Keywords Neurogenic laryngitis · Nasogastric intubation (NGI) model · Quantitative real-time QRT-PCR · Selective COX-2 inhibitor · Tumor necrosis factor-α (TNF-α)

Introduction

The aetiology of certain forms of laryngitis is unknown and therapeutics may fail due to the adverse side effects of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) that are usually used [1–3]. Neurogenic inflammation results from interaction between the immune and nervous systems [4]. Neuropeptides like substance P (SP) or calcitonin gene related peptide (CGRP) [4, 5], which are contained in peripheral sensitive nerve fibres, are released by terminals of primary afferents in inflammatory actions induced by several stimuli [5, 6]. Some studies showed a neurogenic component in some pathologies like asthma, rhinitis and rheumatoid arthritis [8]. Recently, we demonstrated a neurogenic factor in a new model of experimental laryngitis [9]. The intraepithelial nerve fibres of the larynx are important for bronchopulmonar defence [10]. These fibres project into the laryngeal lumen and are rich in SP and CGRP [10]. The release of these neuropeptides can induce neurogenic laryngitis as shown by the increased expression of inflammatory cytokines like interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α

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(TNF-α), and the decreased expression of the anti-inflammatory cytokine IL-10 [9].

Nasogastric intubation (NGI) is largely used in clinical practice and can induce important respiratory disorders like aspiration pneumonia or laryngitis [9, 11, 12]. We previously demonstrated that NGI is a good model to study laryngitis in the rat since no direct lesion of the laryngeal epithelium is induced [9]. Additionally, we observed an overexpression of COX-2 enzyme in the laryngeal mucosa [9], which represents the inducible form of the COX enzyme following pathological stimulation in opposition to the constitutive COX-1 isoform [3]. This indicates that selective COX-2 inhibitors may be useful in the treatment of this pathology, similarly to several other pathologies where blocking of COX-2 activity by administration of NSAIDs was shown to have an anti-inflammatory action [13–15]. Classical non-selective NSAIDs and corticosteroids used to treat laryngitis have well known aerodigestive adverse side effects when compared to the selective COX-2 inhibitors [3, 16]. Recent studies have demonstrated the utility and sensitivity of the latter drugs in throat pain [17]. However, to the best of our knowledge, there are no experimental studies evaluating the use of selective COX-2 selective NSAIDs in laryngitis. In order to analyse the potential role of selective COX-2 inhibitors in neurogenic laryngitis, we studied the effect of Etoricoxib upon the inflammatory process in the NGI model of neurogenic laryngitis [9], through immunohistochemical and molecular biology techniques using markers of inflammation, in animals submitted to NGI for 1 and 2 weeks.

Materials and methods

Animals

This study was performed in 66 male rats weighing 350–450 g, obtained from the Wistar Han colony of the Charles River Company (Barcelona, Spain). The experiments were carried out in accordance with the Portuguese regulation for the handling of laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Union Council Directive 86/609/EEC. Animals were lightly anesthetized with inhaled isoflurane in order to allow the swallowing reflex and submitted to the NGI procedure [9]. A 10–12 cm small bore nasogastric aspiration tube used for NGI in premature newborns (cat. No 533.04; Vigon Laboratoires Pharmaceutiques, Ecouen, France) was inserted into the rat oesophagus to the stomach lumen, with the external tip sutured to the nasal lateral cartilages. Animals were given time to recover and then were returned to their cages.

Animals were distributed into five groups, each one formed by 12 animals ($n = 6$ for immunohistochemical processing; $n = 6$ for molecular biology analysis; (6 + 6) ×5 groups, total $n = 60$ rats):

- NGI + $ETORI_{1w}$: submitted to NGI and treated with Etoricoxib from the first day of intubation for 1 week;
- NGI + $ETORI_{2w}$: submitted to NGI and treated with Etoricoxib from the first day of intubation for 2 weeks;
- NGI + $VEHIC_{1w}$: submitted to NGI and administered 0.9% sterile saline with 5% glucose for 1 week;
- NGI + $VEHIC_{2w}$: submitted to NGI and administered 0.9% sterile saline with 5% glucose for 2 weeks;
- CONT: non-intubated rats, whose larynxes were used as naïve controls.

An additional group of six animals were included for additional histopathological comparison of COX-2-IR cells in the laryngeal mucosa:

- NGI_{1w}: submitted to NGI (without treatment; $n = 3$) for 1 week;
- NGI_{2w}: submitted to NGI (without treatment; $n = 3$) for 2 weeks;

After completion of the intubation period, animals for immunohistochemical experiments were perfused under anaesthesia (isoflurane and 35% chloral hydrate, intraperitoneally) through the ascending aorta with 4% paraformaldehyde in PBS 0.01 M. After confirmation that the NGI tube was still inserted to the stomach lumen, the larynx was removed and immersed in the same fixative followed by 30% sucrose in 0.1 M PBS overnight. Animals used for real time QRT-PCR studies were anesthetized with isoflurane and sacrificed with 35% chloral hydrate intraperitoneally. Larynxes were excised immediately, macerated and immersed in 0.8 ml of TRIzol® (Invitrogen®, Carlsbad, CA, USA) and stored at −80°C.

Drug therapy

Etoricoxib (Exxiv® 60 mg, Bial, Bial-Portela & Ca., S.A., Trofa, Portugal) was suspended in 0.9% sterile saline with 5% glucose and administered to each animal in the dose of 6 mg/kg/day, by oral gavage, once daily.

Histology and immunohistochemistry

Laryngeal coronal frozen sections (20 μm) were obtained with a cryostat and processed for immunohistochemistry. Alternate sections were incubated overnight at room temperature with rabbit anti-CGRP (1:6,000; Bachem, San Carlos, CA, USA) or rabbit anti-SP (1:6,000; Bachem, San Carlos, CA, USA) antibodies in a PBS solution 0.1 M at pH 7.2, containing 0.3% triton X-100 (PBST). After

washing with PBST, the sections were then incubated with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA, USA) in PBST for 1 h, washed in PBST and then incubated with avidin–biotin complex (ABC) (1:200; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 h. After rinsing in PBS and 0.1 M Tris–HCl buffer (pH 7.4) the antigen–antibody reaction was visualised with a diaminobenzidine (DAB; Sigma–Aldrich®, St. Louis, MO, USA) solution.

COX-2 immunohistochemistry was carried out according to the streptavidin–biotin-peroxidase complex technique (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, USA), using a primary antibody raised against COX-2 protein (rabbit monoclonal antibody, clone SP21; Neomarkers, Fremont, CA, USA) diluted 1:400. Slides were sequentially washed in PBS 0.02% Tween 20 and incubated with 3% H_2O_2 in methanol for 10 min. This was followed by incubation with blocking solution for 10 min and then the primary antibody solution for 2 h, at room temperature. Sections were then sequentially washed in PBS/0.02% Tween 20 and then incubated with biotinylated goat anti-polyvalent antibody for 10 min and streptavidin peroxidase for 10 min. Immunoreaction was revealed using DAB as chromogen, as above. Slides were counterstained with Mayer haematoxylin (Merck, Darmstadt, Germany). Negative controls were performed by omission of the primary antibody. In this case, no labelled fibers/cells of any type of IR (CGRP, SP and COX-2) were observed in the laryngeal mucosa.

Stereological procedures

Sections were analyzed in an Axioskop 2 plus light microscope and images of laryngeal histological data were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss®, Göttingen, Germany). Cell and fibre numbers were estimated using the optical fractionator method [18]. Briefly, this consists of virtual 3D-boxes (150 μm × 150 μm × 30 μm) with equally spaced grids that were superimposed by the software on every eighth coronal laryngeal section after drawing the limits of the area under study in the laryngeal mucosa. The number of COX-2-IR cells and SP and CGRP immunoreactive fibres that crossed the gridlines in every randomized site was counted, by an experimenter blinded to both the neuropeptide immunoreactivity present and/or the conditions applied (control, vehicle or treatment). The estimated numbers were calculated from the ratio between the total number of counted cells and fibres crossing the grid site and the number of grid sites per area. The coefficients of error were automatically computed by the software according to the formulas of Gundersen for cell numbers [18, 19].

mRNA extraction and QRT-PCR

Total mRNA in the larynx was extracted by adding $CHCl_3$ (Sigma–Aldrich, St. Louis, MO, USA) followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was carefully collected and total mRNA was precipitated using isopropanol (2-propanol, Sigma–Aldrich, St. Louis, MO, USA) followed by centrifugation at 13,000 rpm for 15 min at 4°C. Ethanol 70% was used to wash the mRNA pellet, which was then re-centrifuged at 9,000 rpm for 5 min at 4°C. The supernatant was carefully discarded and the pellet was air dried at 4°C. Finally, the pellet was resuspended with 50 μl of DNAse/RNAse free distilled water (Invitrogen, Carlsbad, CA, USA). Total mRNA was quantified by spectrophotometry using the NanoDrop® equipment (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, samples of total mRNA with the same concentration (2 ng/10 μl) were reverse transcribed in a thermocyclator My Cycler Thermal Cycler® (Bio-Rad, Hercules, CA, USA) using a Superscript Kit II (Invitrogen, Carlsbad, CA, USA), and Oligo dT (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed at 42°C for 60 min followed by RT inactivation at 70°C for 15 min. The cDNA was then subjected to reverse transcription polymerase chain reaction (real time QRT-PCR) procedures for quantification of laryngeal mRNA levels of β-Actin, IL-1β, TNF-α, IL-6, IL-10 and COX-2, using the LightCycler® (Roche, Indianapolis, IN, USA) and a SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Primer sequences used to amplify various cDNAs are shown in Table 1 and have been used previously by our group [9]. A typical QRT-PCR protocol was performed under the following conditions: a 15 min hot start at 95°C, followed by 40 three-temperature cycles (94°C denaturing for 15 s; 58°C annealing for 20 s; and 72°C extension for 15 s), melting at 60°C until 95°C for 90 s and finally cooling to 35°C. Melting point analysis was used to confirm the specificity of the SYBR Green assays. Gene expression of the housekeeping gene β-actin was used for normalization. Target mRNA relative expression was calculated using dCT (change in cycling threshold) [20].

Data analysis

Results are expressed after statistic analysis using SPSS version 17.0 for Windows (SPSS Inc, Chicago, IL, USA). For each neuropeptide, enzyme or cytokine analysed, a double statistical evaluation was performed: (a) data means for each of the five groups (CONT, NGI + $VEHIC_{1w}$, NGI + $ETORI_{1w}$, NGI + $VEHIC_{2w}$ and NGI + $ETORI_{2w}$) were compared using one-way ANOVA statistical

Table 1 Sequences of primers used for real-time PCR

Target	Oligo	Sequence	Gene Bank ACC
β-Actin	Forward primer	5′-GATTTGGCACCACACTTTCTACA-3′	NM_031144
	Reverse primer	5′-ATCTGGGTCATCTTTTCACGGTTGG-3′	
IL-1β	Forward primer	5′-GAAACAGCAATGGTCGGGAC-3′	M98820
	Reverse primer	5′-GAGACCTGACTTGGCAGAGG-3′	
TNF-α	Forward primer	5′-CCAACAAGGAGGAGAAGTTC-3′	NM_012675
	Reverse primer	5′-CCTGGTGGTTTGCTACGAC-3′	
IL-6	Forward primer	5′-CAAGAGACTTCCAGCCAG-3′	NM_012589
	Reverse primer	5′-CTCCGACTTGTGAAGTGGT-3′	
IL-10	Forward primer	5′-GCCAAGCCTTGTCAGAAATGA-3′	NM_012854
	Reverse primer	5′-TTTCTGGGCCATGGTTCTCT-3′	
COX-2	Forward primer	5′-TTTGTTGAGTCATTCACCAGACAGAT-3′	S67722
	Reverse primer	5′-ACGATGTGTAAGGTTTCAGGGAGAAG-3′	

evaluation followed by Tukey HSD post hoc test; (b) a two-way ANOVA was performed to compare the effect of time with the effect of treatment in order to evaluate possible interactions between time and treatment; this second evaluation did not include the control group (CONT). The normal distribution of data was verified using the Kolmogorov–Smirnov test, whereas the equality of variances was verified by the Levene test. When the latter was not observed the Kruskal–Wallis non-parametric test was used. Differences were considered to be significant when $p < 0.05$. Statistical significances are not presented in Figs. 1, 4, 5 and 6 to preserve the clarity of the figure, but are detailed in "Results".

Results

Etoricoxib blocks the decrease of CGRP-IR fibers induced by 1-week NGI

The experimental NGI model of laryngitis and therapeutical protocol using Etoricoxib induced different changes in the expression of neurogenic peptides at the end of both the first and second weeks (ANOVA, CGRP $p < 0.0001$; SP $p < 0.0001$) (Figs. 1, 2, 3).

After the first week, there was a significant decrease in the number of CGRP-IR (Figs. 1a, 2a, b) and SP-IR (Figs. 1b, 2d, e) fibres in the NGI group, when compared with non-intubated animals (NGI + VEHIC_{1w} × CONT, CGRP $p < 0.0001$; SP $p < 0.001$, Tukey test). The 1-week therapy with Etoricoxib reverted the decrease in the number of CGRP-IR fibres to control levels (NGI + ETORI_{1w} × NGI + VEHIC_{1w}, $p < 0.002$; NGI + ETORI_{1w} × CONT, $p > 0.05$, Tukey test) (Figs. 1a, 2c). However, it did not affect the decrease in the levels of SP-IR fibres observed in the NGI plus vehicle group (NGI +

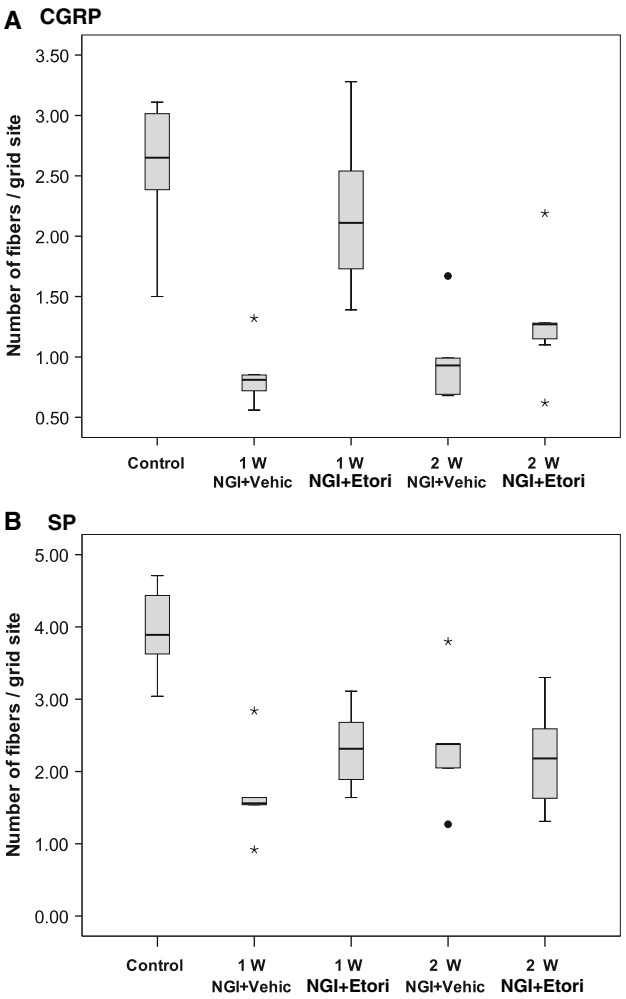

Fig. 1 Effect of Etoricoxib treatment upon CGRP-IR (**a**) and SP-IR (**b**) fibre immunoreactivity after 1 and 2 weeks of NGI. Note that the decrease in CGRP-IR fibres was rescued with Etoricoxib treatment after the first, but not the second week of NGI. The decrease induced in SP-IR fibres was not rescued by the treatment. Values shown as mean ± SD. *Asterisks* and *dots* represent outliers. Statistical significances are not presented for clarity of the figure, but are specified in "Results"

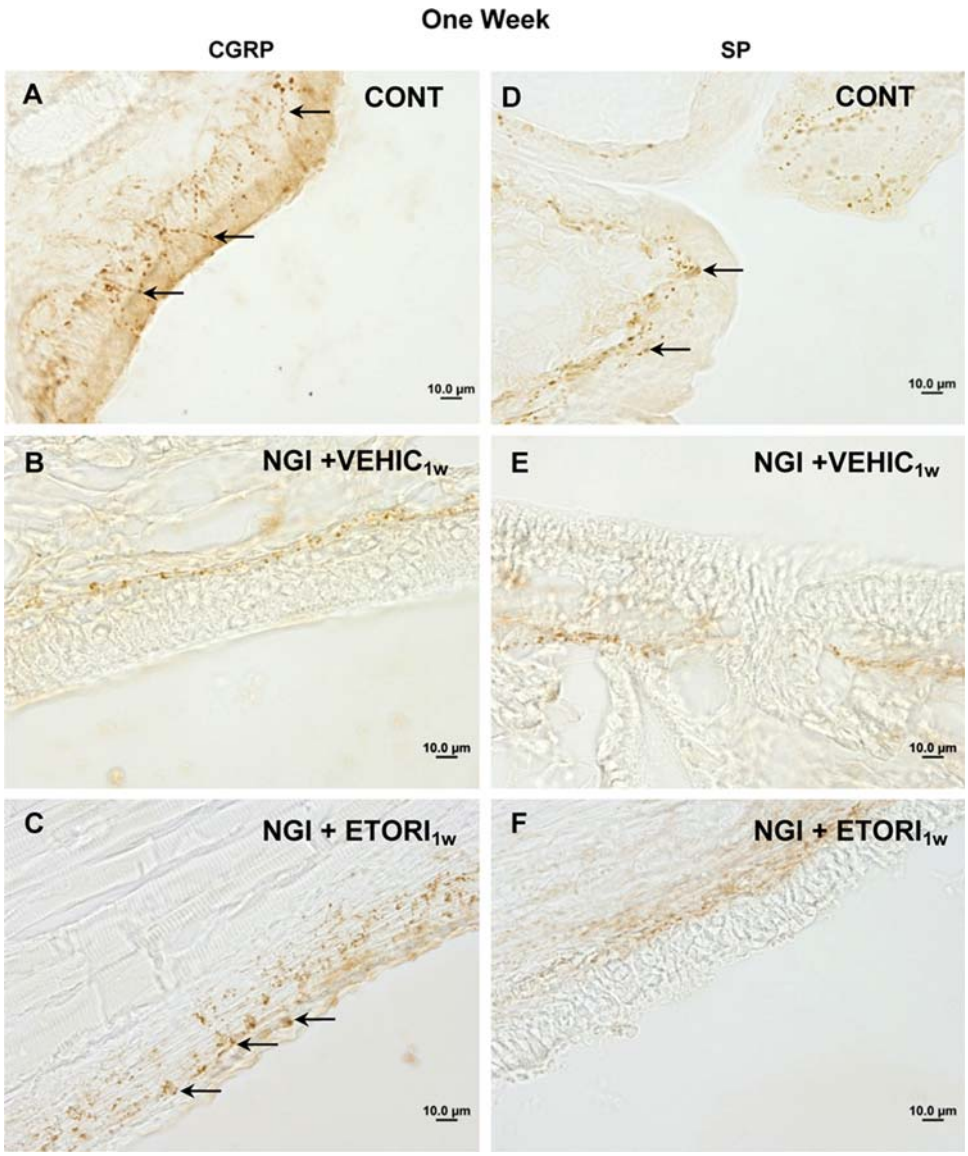

Fig. 2 Photomicrographs of CGRP-IR (**a**–**c**) and SP-IR (**d**–**f**) sensitive fibres in the laryngeal mucosa in naïve (control) animals (**a**, **d**) and following 1 week of NGI in both vehicle-treated (**b**, **e**) and Etoricoxib-treated animals (**c**, **f**). After 1 week, there is a clear reduction in the presence of CGRP and SP immunoreactive fibres in the mucosa induced by NGI (**b**, **e**) and the recovery of CGRP-IR to control levels allowed by the treatment with Etoricoxib (**c**). IR was revealed using DAB as chromogen. *Arrows* fibres

$ETORI_{1w} \times$ NGI + $VEHIC_{1w}, p > 0.05$; NGI + $ETORI_{1w} \times$ CONT, $p < 0.002$, Tukey test) (Figs. 1b, 2f).

At the end of the second week, there was a decrease in the number of CGRP-IR fibres in NGI plus vehicle (NGI + $VEHIC_{2w} \times$ CONT, $p < 0.0001$, Tukey test) (Figs. 1a, 3a, b) and NGI animals treated with Etoricoxib (NGI + $ETORI_{2w} \times$ CONT, $p < 0.001$, Tukey test) (Figs. 1a, 3a, c). Regarding SP-IR fibres, the same results were observed in both groups (NGI + $VEHIC_{2w} \times$ CONT, $p < 0.005$, Tukey test) (Figs. 1b, 3d, e) (NGI + $ETORI_{2w} \times$ CONT, $p < 0.001$, Tukey test) (Figs. 1b, 3d, f).

Finally, there was a significant interaction between time and treatment upon the level of CGRP-IR fibers (time × treatment effect, $p < 0.018$, two-way ANOVA). However, time, treatment or time × treatment had no effect on SP-IR fibers.

Etoricoxib blocks the increase of TNF-α mRNA expression induced by 1-week NGI

The NGI experimental model induced significant changes in inflammatory mediators at the laryngeal mucosa. TNF-α (one-way ANOVA, $p < 0.0001$) mRNA levels increased significantly after 1 and 2 weeks of NGI (NGI + $VEHIC_{1w} \times$ CONT, $p < 0.0001$; NGI + $VEHIC_{2w} \times$ CONT, $p < 0.0001$, Tukey test) (Fig. 4). After a 1-week treatment with Etoricoxib (NGI + $ETORI_{1w}$), the specific COX-2 inhibitor blocked the increase of TNF-α expression induced by NGI, reducing it to values similar to those observed in control animals (NGI + $ETORI_{1w} \times$ NGI + $VEHIC_{1w}$, $p < 0.0001$; NGI + $ETORI_{1w} \times$ CONT, $p > 0.05$, Tukey test). However, no significant reduction of TNF-α mRNA expression was observed after 2 weeks of treatment with

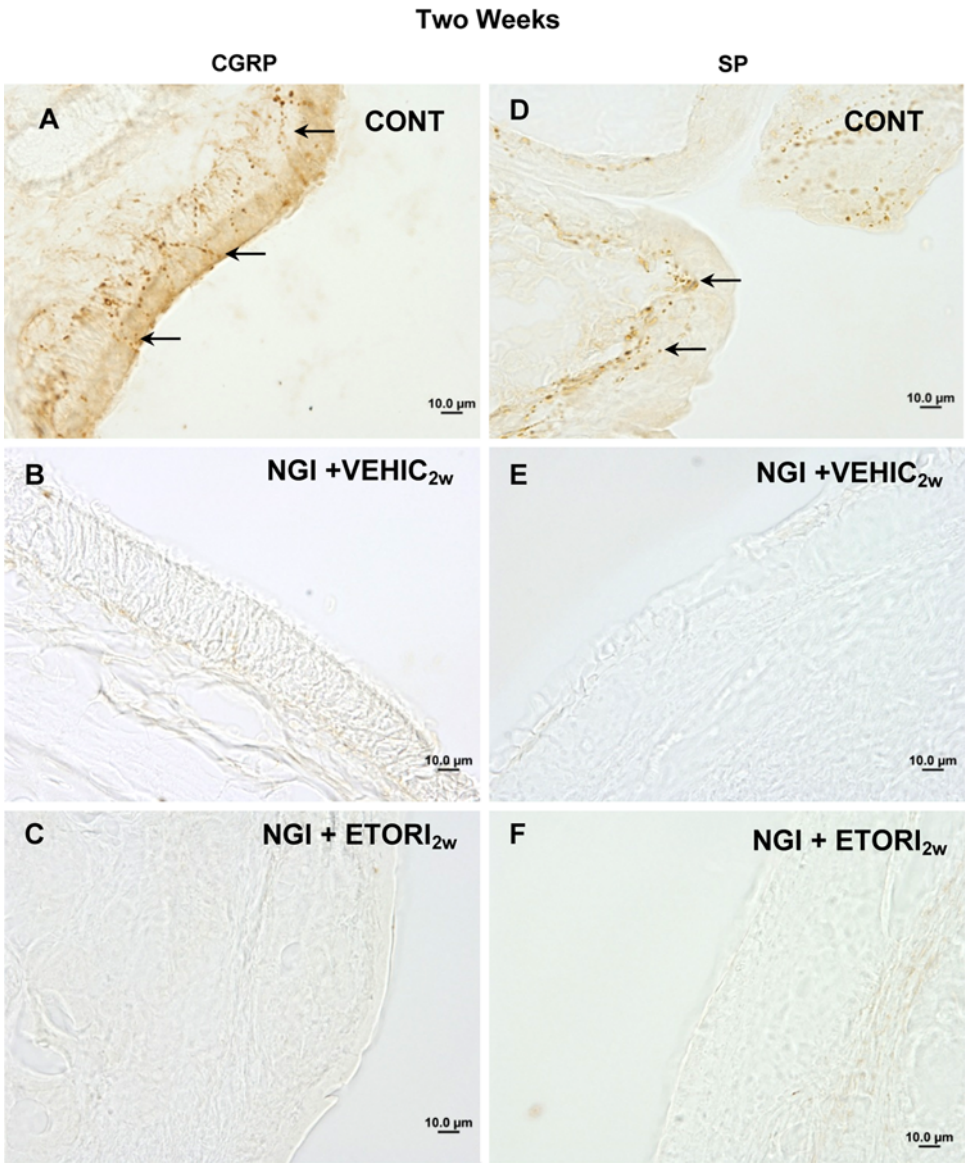

Fig. 3 Photomicrographs of CGRP-IR (**a**–**c**) and SP-IR (**d**–**f**) sensitive fibres in the laryngeal mucosa in naïve (control) animals (**a**, **d**) and in animals submitted to NGI for 2 weeks, treated either with vehicle (**b**, **e**) or with the selective COX-2 inhibitor Etoricoxib (**c**, **f**). Two weeks of treatment with Etoricoxib did not rescue (**c**, **f**) the reduction of laryngeal neuropeptidergic fibres (CGRP-IR and SP-IR) induced by NGI (**b**, **e**). IR was revealed using DAB as chromogen. *Arrows* fibres

Etoricoxib (Fig. 4). Finally, there was a loose interaction between time and treatment reaching the border of significance (time × treatment effect, $p < 0.08$, two-way ANOVA).

Concerning IL-1β expression (one-way ANOVA, $p < 0.0001$), treatment with Etoricoxib did not affect the level of expression after the first week, but increased mRNA values (NGI + $ETORI_{2w}$ × CONT, $p < 0.008$, Tukey test) after 2 weeks (Fig. 5a). No interaction was observed between time and treatment, but there was a significant effect with treatment (treatment effect, $p = 0.03$, two-way ANOVA). IL-6 levels in the larynx of both groups of intubated rats was increased (Vehicle and Etoricoxib, after 1 or 2 weeks) (Kruskal–Wallis, $p < 0.002$), but there was no effect of Etoricoxib treatment upon laryngeal levels of this cytokine, with a trending increase in IL-6 levels after a 2-week treatment (although not statistically significant). A borderline interaction between time and treatment was close to statistical significance (analysis of four groups excluding controls, $p > 0.051$, two-way ANOVA (Fig. 5b). In the case of IL-10 (one-way ANOVA, $p < 0.017$), NGI induced an increase in the expression after 2 weeks (NGI + $VEHIC_{2w}$ × CONT, $p < 0.012$, Tukey test), which was ameliorated after treatment with Etoricoxib (NGI + $ETORI_{2w}$ × NGI + $VEHIC_{2w}$, $p < 0.041$, Tukey test) (Fig. 5c). Even though no interaction between time and treatment was present, there was a significant effect with treatment (treatment effect, $p < 0.046$, two-way ANOVA).

Etoricoxib blocks the increase of COX-2 IR and mRNA levels induced by 1-week NGI

Changes in the expression of COX-2 were detected by increases in both mRNA levels recorded by QRT-PCR

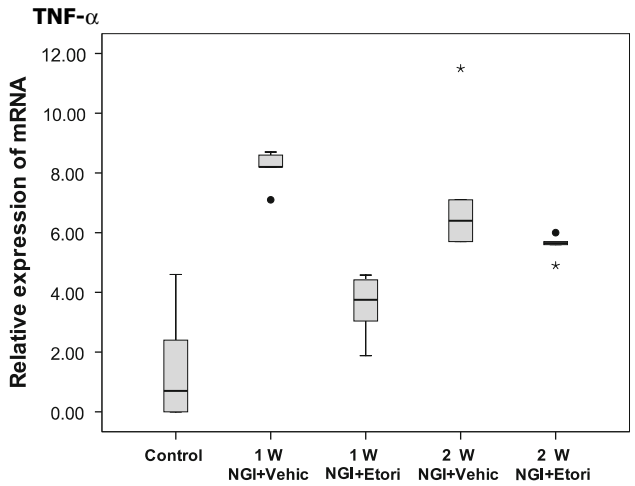

Fig. 4 Effect of Etoricoxib treatment upon the relative mRNA expression of TNF-α after 1 and 2 weeks of NGI. The selective COX-2 inhibition decreased the TNF-α expression induced by NGI after 1 week of treatment, mimicking the control levels, but not after 2 weeks. All values are normalized to β-actin (reference gene). Values shown as mean ± SD. *Asterisks* and *dots* represent outliers. Statistical significances are not presented for clarity of the figure, but are specified in "Results". Relative expression of target mRNA was calculated using dCT (change in cycling threshold) [20]

(one-way ANOVA, $p < 0.0001$) (Fig. 6a) and in the number of COX-2-IR cells by stereological procedures (one-way ANOVA, $p < 0.01$) (Figs. 6b, 7, 8). In what concerns laryngeal mRNA levels of COX-2, no expression was detected in the CONT group (Fig. 6a). After 1 week of NGI the values increased significantly (NGI + VEHIC$_{1w}$ × CONT, $p < 0.01$, Tukey test), with the Etoricoxib treatment being effective in reducing mRNA levels (NGI + ETORI$_{1w}$ × NGI + VEHIC$_{1w}$, $p < 0.047$, Tukey test) to the negligible levels of controls (Fig. 6a). After 2 weeks of treatment, there was a tendency for reduction of the increased COX-2 mRNA levels observed in the NGI + VEHIC group, although it did not reach statistical significance (Fig. 6a). Even though no interaction between time and treatment was present, there was a significant effect with both time (time effect, $p < 0.001$, two-way ANOVA) and treatment (treatment effect, $p < 0.001$, two-way ANOVA).

Concerning the presence of laryngeal cells expressing COX-2, there was an increased number after the first week of NGI when compared to non-intubated animals (NGI + VEHIC$_{1w}$ × CONT, $p < 0.009$, Tukey test) (Figs. 6b, 7a–d), which was also reverted at the end of first week of treatment with Etoricoxib (Figs. 6b, 7a–c, e, f), but not after the second week (NGI + ETORI$_{1w}$ × NGI + VEHIC$_{1w}$, $p = 0.01$; NGI + ETORI$_{2w}$ × NGI + VEHIC$_{2w}$, $p > 0.05$, Tukey test) (Figs. 6b, 8). Additionally, there was a significant interaction between time and treatment (time × treatment effect, $p < 0.002$, two-way ANOVA).

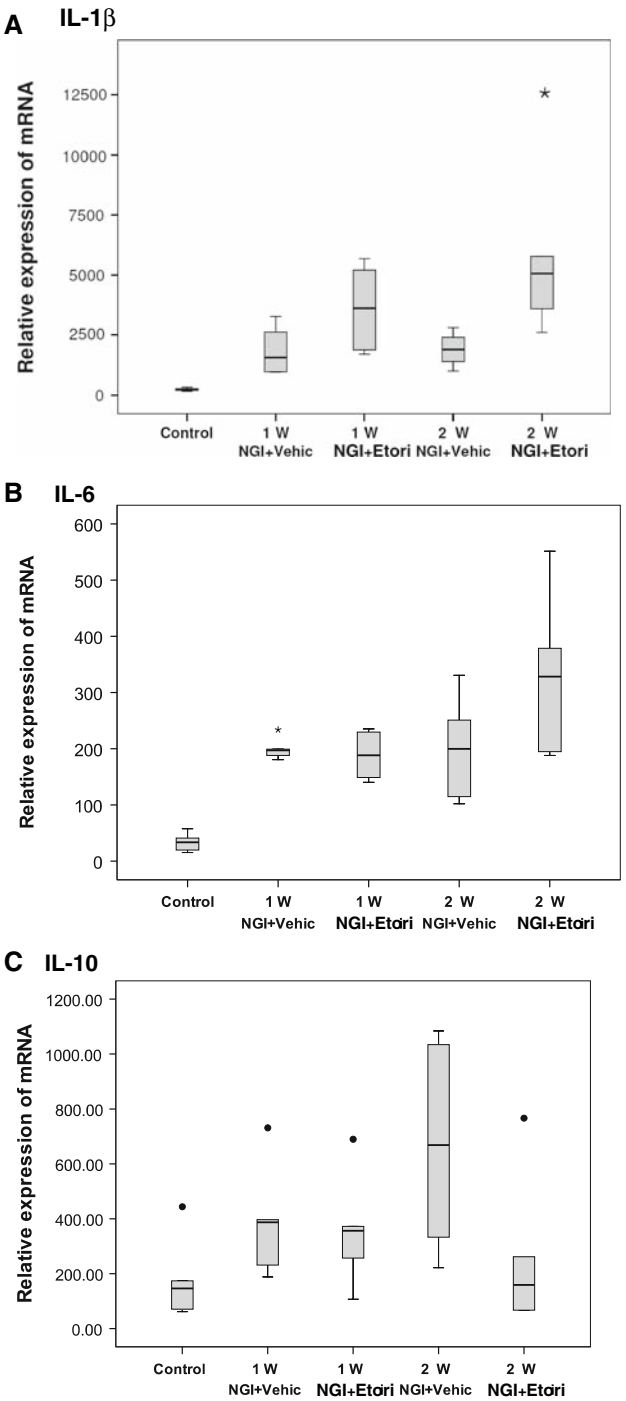

Fig. 5 Effect of Etoricoxib treatment upon the relative mRNA expression of IL-1β (**a**), IL-6 (**b**) and IL-10 (**c**) in the laryngeal mucosa, after 1 and 2 weeks of NGI. Animals treated with Etoricoxib showed a tendency for higher levels of IL-1β and IL-6 and lower levels of IL-10 than NGI plus vehicle after 2 weeks of nasogastric intubation. All values are normalized to β-actin (reference gene). Values shown as mean ± SD. *Asterisks* and *dots* represent outliers. Statistical significances are not presented for clarity of the figure, but are specified in "Results". Relative expression of target mRNA was calculated using dCT (change in cycling threshold) [20]

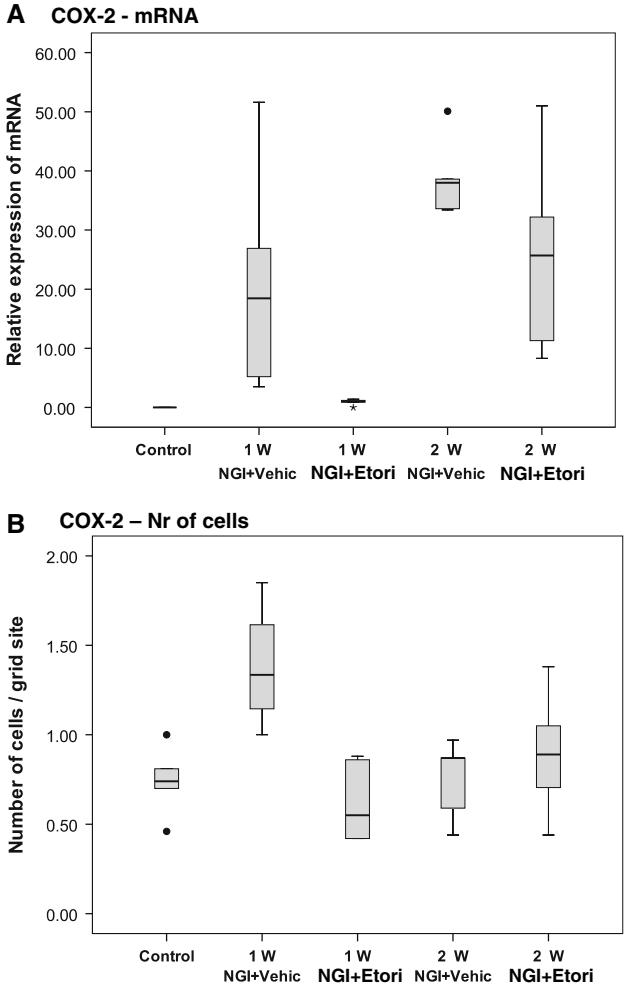

Fig. 6 Effect of Etoricoxib treatment upon laryngeal inflammation measured by COX-2 levels of mRNA (**a**) and COX-2-immunoreactive cells (**b**) present in the mucosa after 1 and 2 weeks of NGI. Note that the drug was effective only at the first week by reducing both the levels of mRNA and the number of COX-2-IR cells to control levels. Values shown as mean ± SD. *Asterisks* and *dots* represent outliers. Statistical significances are not presented for clarity of the figure, but are specified in "Results". Relative expression of target mRNA was calculated using dCT (change in cycling threshold) [20]

Discussion

Classical NSAIDs are the most commonly used drugs for the treatment of pain and inflammation in a wide range of diseases, including ear, nose and throat conditions [21–23]. These drugs act primarily by blocking the synthesis of prostaglandins by cyclooxygenase (COX) enzymes [13, 21]. Their therapeutic effects are related to the inhibition of inflammation-induced COX-2, whereas their most common side effects are related to the inhibition of the constitutive COX-1 enzyme [21, 24, 25]. Interestingly, aerodigestive and cutaneous adverse events related to the use of NSAIDs are known to be one of the most common in medicine [21].

Contrarily to the classical NSAIDs that inhibit both isoforms of COX enzymes, the selective COX-2 inhibitors like Etoricoxib, mainly block the activity of the inducible COX-2 enzyme [13, 14, 21, 24, 25]. This may result in a better tolerability profile, with less associated side effects. However, to the best of our knowledge, there are no indications in clinical practice to the use of the latter drugs in laryngitis, probably because the expression of the COX-2 enzyme in the larynx and the potential therapeutic effects of these drugs have not been explored in detail. In clinics and in experimental studies, NGI induced laryngitis and COX-2 overexpression in inflammatory laryngeal cells have already been shown [9, 11]. In the present study, by showing decreased levels of COX-2 and TNF-α and reversion of depletion of the neurogenic CGRP peptide at the end of the first week of treatment, NGI animals treated with a selective COX-2 inhibitor Etoricoxib revealed an anti-inflammatory effect of this drug during the first week of laryngitis.

Animals submitted to 1 week of nasogastric intubation (NGI + $VEHIC_{1w}$ group) showed increased COX-2, TNF-α and IL-6 mRNA levels, as well as an increased number of laryngeal cells immunoreactive to COX-2. These data are in accordance with studies including other inflammatory conditions, where these molecules were also significantly increased [26, 27]. TNF-α and IL-6 induce COX-2 expression [28] and are known to induce the release of the pro-inflammatory neuropeptides SP and CGRP in different tissues [26, 29]. On the other hand, SP and CGRP are also potent inducers of the production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α [4, 5]. In the group submitted to NGI and treated simultaneously with Etoricoxib (NGI + $ETORI_{1w}$), COX-2 and TNF-α mRNA levels decreased, as well as the number of laryngeal cells immunoreactive to COX-2. This is indicative of an anti-inflammatory action of Etoricoxib in the larynx of intubated rats. However, no changes were observed in the IL-6, IL-1β and IL-10 mRNA levels. This could be conflicting data because Etoricoxib inhibits the nuclear factor kappa B (NF-κB) [24, 25, 27, 30, 31], an important mediator in many inflammatory conditions, and thus the levels of these interleukins were expected to be altered. These results may be explained by the occurrence of alternative pathways of cytokine production, possibly by alterations of the activity of cellular kinases, such as Erk, p38 MAPK or Cdks [32, 33]. Another possible explanation is the presence of a counterbalanced production of interleukins, as other selective COX-2 inhibitors have shown the capacity to induce IL-6 production instead of reducing it [24, 25, 34].

Concerning the period of 2 weeks of NGI, compared to controls, there was an increase in the levels of IL-6, TNF-α, IL-10 and COX-2 mRNA expression in the NGI + $VEHIC_{2w}$ group, confirming the presence of a marked

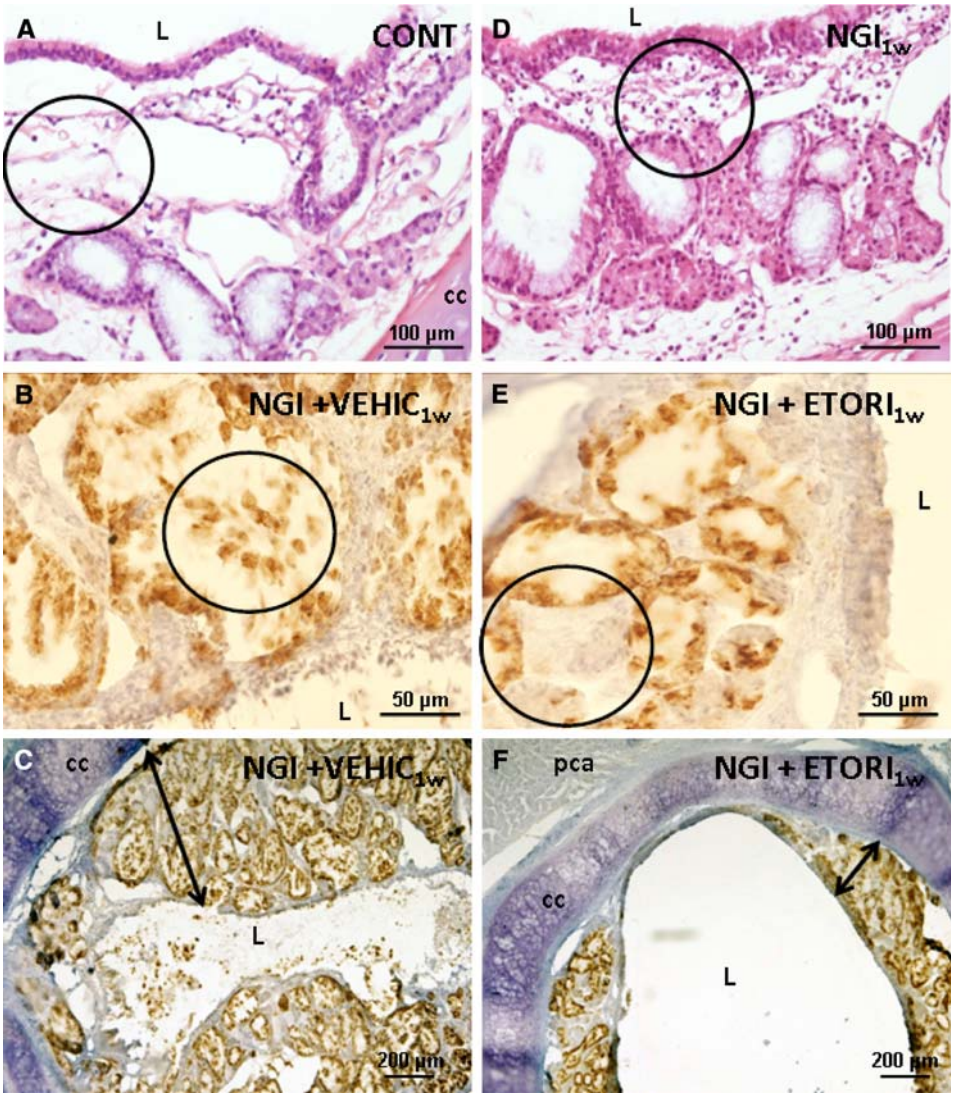

Fig. 7 Histological changes in the connective tissue of the laryngeal mucosa induced by nasogastric intubation (NGI). The presence of an inflammatory infiltrate after NGI as compared to CONT is evident (*circles* **a**, **d**). Inflammatory signs are also present in intubated animals submitted to vehicle for 1 week (NGI + $VEHIC_{1w}$), namely inflammatory cells (*circle* **b**), edema and hypertrophy of the mucosa (*arrow* **c**). Note that these inflammatory signs are reduced by a 1-week administration of Etoricoxib (NGI + $ETORI_{1w}$) (*circle* **e** and *arrow* **f**). *cc* cricoid cartilage, *pca* posterior cricoarytenoid muscle, *L* Laryngeal lumen. **a**, **d** Hematoxylin–eosin staining; **b**, **c**, **e**, **f** immunohistochemistry for COX-2 using DAB as chromogen

inflammatory process in these animals. The treatment with Etoricoxib after 2 weeks of intubation (NGI + $ETORI_{2w}$) induced an increase in IL-1β and IL-6 mRNA levels and a decrease in IL-10 expression, which did not occur in the NGI + $VEHIC_{2w}$ group. The increased levels of IL-10 mRNA in the NGI + VEHIC group can be seen as an immunologic anti-inflammatory response [35, 36] that is aborted by Etoricoxib, whereas the increase in IL-6 can be explained by the capacity of selective COX-2 inhibitors to induce IL-6 production [24, 25, 36]. It is also possible that COX-2 inhibitors can induce IL-1β and IL-6 production and a decrease in the levels of IL-10 by the loss in the activity of protective cellular molecules like hepatocyte growth factor (HGF). Since HGF decreases IL-1β and IL-6 and increases IL-10 through the upregulation of COX-2 [37], a prolonged COX-2 inhibition might lead to a blockade of HGF function, which would induce IL-1β and IL-6 overexpression and a decrease of IL-10 levels. Further studies are needed to elucidate this possibility. Concerning the TNF-α and COX-2 expression, which were decreased by Etoricoxib after 1 week of NGI, the values obtained following 2 weeks of treatment were similar to the NGI + $VEHIC_{2w}$ group, which seems to indicate a loss of therapeutic effect of the drug after long periods of administration [15, 33].

Regarding neuropeptides, in this model of laryngitis, Etoricoxib attenuated the depletion of CGRP from laryngeal primary afferents after 1 week of NGI, but SP-IR was not altered. In our previous studies, we observed less SP-IR fibres than CGRP-IR fibres in the larynx [10]. This finding suggests that CGRP is the most important neuropeptide present in the laryngeal epithelium [9, 10]. Accordingly, the most significant neurogenic anti-inflammatory action of COX-2 inhibitors shown in the present work seems to occur through a CGRP-mediated mechanism. CGRP receptors are co-localized with prostaglandin

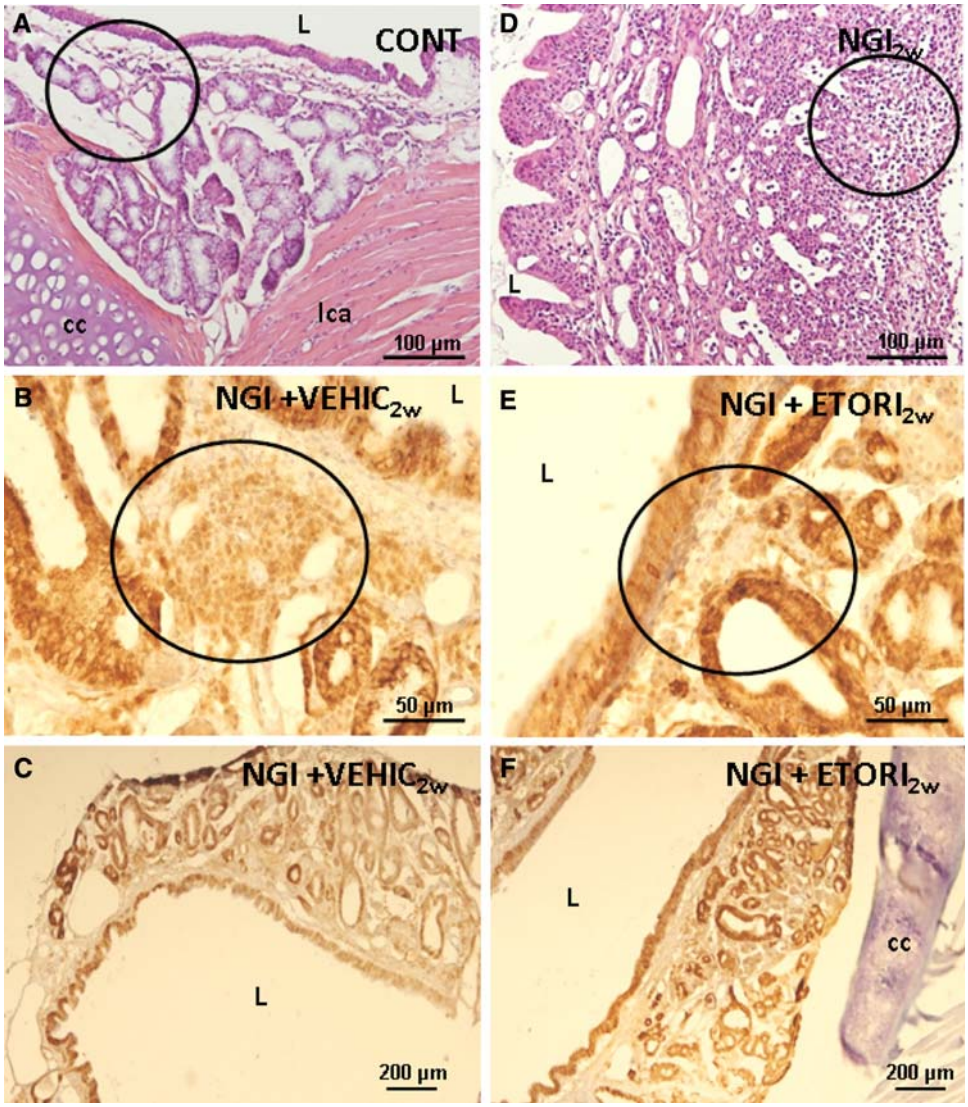

Fig. 8 Histological changes in the connective tissue of the laryngeal mucosa induced by nasogastric intubation (NGI) for 2 weeks. The presence of a significant inflammatory infiltrate after NGI as compared to CONT is evident (*circles* **a**, **d**). Inflammatory signs are also present in intubated animals subjected to vehicle for 2 weeks (NGI + $VEHIC_{2w}$), namely inflammatory cells (*circle* **b**), edema and hypertrophy of the mucosa (**c**). After 2 weeks of NGI, these inflammatory signs are not reduced by administration of Etoricoxib (NGI + $ETORI_{2w}$) (*circle* **e**, **f**). *cc* cricoid cartilage, *lca* lateral cricoarytenoid muscle, *L* Laryngeal lumen. **a**, **d** Hematoxylin–eosin staining; **b**, **c**, **e**, **f** immunohistochemistry for COX-2 using DAB as chromogen

receptor (EP4 receptor-like immunoreactivity) at thin nerve terminals and Prostaglandin E2 (PGE2) enhances CGRP release [38]. It is also known that activation of mechano-sensory nerves by COX-2 results from increasing levels of Prostaglandin E2 (PGE2) [29]. Thus, in the larynx, COX-2 inhibitors may predominantly block PGE2 production and CGRP depletion. At the end of the second week of NGI Etoricoxib becomes ineffective in preventing CGRP (and SP) depletion, which is in accordance with the loss of efficacy already seen for the anti-inflammatory effects observed for cytokine and COX-2 expression.

After this experimental therapeutic trial, we conclude that NGI induces neurogenic laryngeal inflammation through the release of neuropeptides SP and CGRP by laryngeal sensitive fibres and, simultaneously, the overexpression of other inflammatory markers. The attenuation of laryngitis induced by the selective COX-2 inhibitor Etoricoxib at the first week of treatment indicates that this drug can be administered during a limited period of time. Moreover, our data suggest that Etoricoxib should be evaluated in the future as an alternative drug option to current treatment of laryngitis due to reduced side effects comparatively to non-selective NSAIDs and corticosteroids currently used in clinical practice. In support of this hypothesis, recent clinical studies involving another selective COX-2 inhibitor, Valdecoxib, demonstrated efficacy with no significant side effects in a throat pain clinical model of tonsillo-pharyngitis [17]. However, it should be noted that prolonged treatment with Etoricoxib may be unfavourable due to its pro-inflammatory reaction profile, as shown in the present NGI model.

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