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Neuroscience Letters 288 (2000) 41–44

Neuroscience Letters

www.elsevier.com/locate/neulet

Oral administration of the anti-inflammatory substance triflusal results in the downregulation of constitutive transcription factor NF-κB in the postnatal rat brain

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Received 7 April 2000; received in revised form 17 May 2000; accepted 17 May 2000

Abstract

In this study we have evaluated the in vivo ability of triflusal (2-acetoxy-4-tri-fluoromethylbenzoic acid) to inhibit constitutive nuclear factor-kappa B (NF-κB) activation in the brain of postnatal rats. One week old Long–Evans black hooded rat pups received three oral administrations of triflusal (30 mg/kg) and were sacrificed at 9 days of age. After fixation, brains were cut in a cryostat and processed immunocytochemically for the demonstration of NF-κB. In control postnatal rats, NF-κB is constitutively present in some neuronal populations and in glial cells of white matter tracts. In contrast, triflusal treated rats showed a drastic downregulation of neuronal and glial NF-κB, both in the number of labelled cells and in the intensity of staining. The inhibition of NF-κB activation could be an important step in the modulation of inflammatory processes occurring after several pathological conditions. © 2000 Published by Elsevier Science Ireland Ltd.

Keywords: Inflammation; Non-steroid anti-inflammatory drugs; Glial; Transcription factor

Non-steroid anti-inflammatory drugs (NSAID) are potent inhibitors of inflammatory processes, which act by blocking the enzyme cyclooxygenase (COX) and inhibiting prostaglandin synthesis [18]. Classically, these drugs have been used as analgesics and fever reducers. However, in the last years, epidemiological data have suggested that NSAIDs could play an important role in protecting against certain cancers and Alzheimer's disease, due to their anti-proliferative and anti-inflammatory properties, respectively [15].

Triflusal (2-acetoxy-4-tri-fluoromethylbenzoic acid) is a NSAID structurally related to the salicylate group of compounds, with a characterized pharmacological profile [13]. Triflusal has an antiaggregant effect on platelets and has been largely used for the prevention and/or treatment of vascular thromboembolisms [13]. In vitro studies have recently shown that triflusal blocks the activation of the inflammatory related transcription factor nuclear factor kappa B (NF-κB) more effectively than aspirin [6], inducing a decrease in the expression of different NF-κB regulated genes, such as the inflammatory enzyme cyclooxygenase-2 (COX-2) [8] and the vascular cell adhesion molecule-1 [6]. In this sense, the increasing hypothesis that inflammatory processes may play a key role in the development of neurodegenerative diseases and other neuropathological situations may suggest a putative role of this drug in obstructing neuroinflammation. However, there are no available in vivo studies on the ability of this drug to reach the brain when administered peripherally. Accordingly, the aim of the present study was to evaluate the effects of orally administered triflusal in the cerebral expression of NF-κB.

Long–Evans black-hooded rat pups received three oral administrations of triflusal (2-acetoxy-4-tri-fluoromethylbenzoic acid) supplied by Uriach & cia. at a dose of 30 mg/kg, using a gastric probe. Administrations were made at 7, 8 and 9 days of age, and animals were sacrificed at 6 h ($n = 6$), 12 h ($n = 6$) and 24 h ($n = 6$) after the last administration. Moreover, non-administered 9 day old ($n = 4$) pups were used as controls. All rat pups were anaesthetized by ether inhalation and perfused intracardially for 10 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). This experimental procedure was approved by the ethical commission of Autonomous University of Barcelona,

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0304-3940/00/$ - see front matter
PII: S0304-3940(00)01202-7

and efforts were made to minimize animal suffering in all steps. After perfusion, brains were immersed in the same fixative for 4 h and were quickly frozen with dry CO_2 after being cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer. Frozen coronal sections (30–40 μm thick) obtained in a cryostat were processed free-floating. Parallel sections were mounted on slides and Nissl stained for routine histological examination. For the immunocytochemical demonstration of NF-κB, frozen free floating sections were treated with buffer blocking (BB) [10% fetal-calf serumin Tris–buffered saline(TBS) + 1% Triton X–100] for 30 min, and incubated with rabbit anti-human NF-κB (p65) primary antibody (Santacruz Biotechnology, sc-109) (36 h at 4°C) diluted to 1:100 in BB. After rinsing, the sections were incubated at room temperature for 1 h with a biotinylated anti-rabbit secondary antibody (Amersham, RPN-1004) diluted to 1:200, rinsed again and incubated with avidin–peroxidase (Dakopatts, P-364) at a 1:400 dilu-

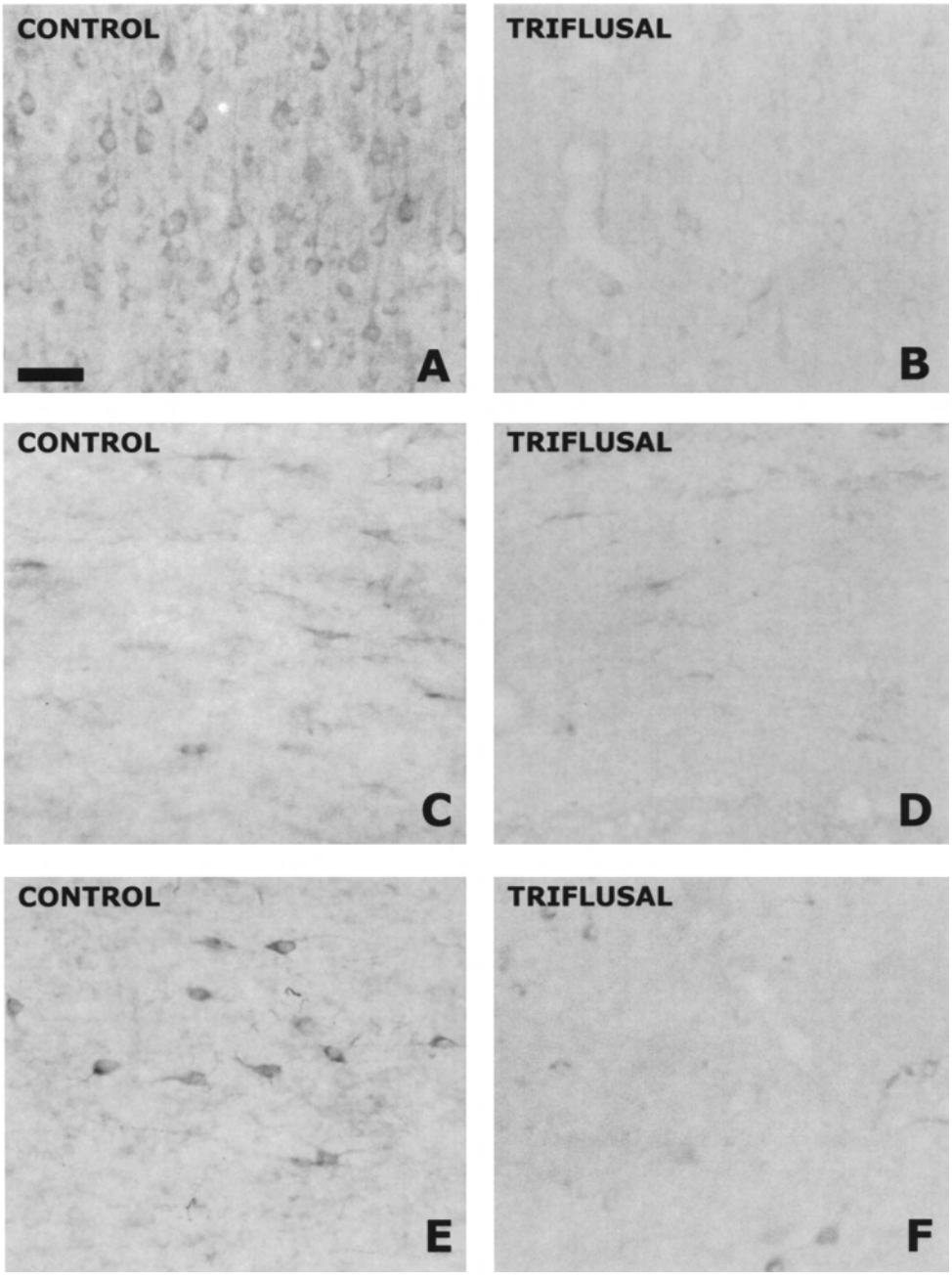

Fig. 1. NF-κB immunoreactivity in control postnatal day 9 rat brains (A,C,E) and in triflusal treated littermates (B,D,F). Neuronal NFκB is mainly observed in the neocortex (A) and in the hypothalamus (E), and is strongly downregulated by triflusal administration (B and F, respectively). Glial NFκB is observed in the corpus callosum of control postnatal brain (C) and is also decreased by triflusal (D). Scale bar (A–F) = 30μm.

tion in BB. Finally, sections were washed, and the peroxidase reaction product was visualized in 100 ml of Tris buffer containing 50 mg diaminobenzidine and 33 μl hydrogen peroxide. As negative controls, sections were incubated in media lacking the primary antibody.

In control postnatal rats, the transcription factor NF-κB was constitutively present throughout the brain. Immunoreactivity for NF-κB was observed in neuronal cells, mainly cytoplasmatically, and especially in populations of the dorsal and ventral hypothalamus (Fig. 1E), stratum oriens of the CA1 hippocampal region and neocortical layers III, V and VI (Fig. 1A). In addition, some glial cells of white matter tracts such as the corpus callosum also showed constitutive NF-κB immunoreactivity (Fig. 1C).

Histological analysis of the brain of triflusal treated animals showed no tisular alterations or cellular morphological changes in comparison with the non-treated control littermates (data not shown). However, all animals that had received oral administration of triflusal, showed a strong inhibition of constitutive neuronal and glial NF-κB. In these animals, we observed a decrease both in the number of positive neuronal cells as well as in their intensity of labelling (Fig. 1B). Only mild NF-κB immunoreactivity was detected in neuronal cells of the retrosplenial cortical area, cingulum and hypothalamus (Fig. 1F), in contrast with the strong staining observed in these neurons in controls. Constitutive presence of glial NF-κB was also affected by triflusal. Thus, very few NF-κB positive glial cells were observed in the corpus callosum of treated animals, and these cells showed milder immunoreactivity than glial cells of control brains (Fig. 1D). No discernible differences have been found when comparing treated animals that had received the last triflusal administration 6, 12 or 24 h prior to sacrifice.

In this study we have demonstrated that orally administered triflusal is capable of inhibiting the constitutive presence of NF-κB in the brain of postnatal rats. Therefore, this work is describing for the first time an in vivo effect of this drug in downregulating this transcription factor, which is implicated in a growing number of cellular processes. Interestingly, NF-κB downregulation equally occurs in animals administered 6–24 h prior to sacrifice. It has been shown that in adult rats, orally administered triflusal has a short half life of less than 10 min in blood [17], and is rapidly transformed to its main deacetylated metabolite HTB (2-hydroxy-4-trifluoromethyl benzoic acid), which is similarly pharmacologically active [6] and has a half life of 24 h in blood [17]. In this sense, it seems likely that HTB is responsible for the inhibitory effect observed. The mechanism by which peripheral HTB targets cerebral NF-κB has not been specifically studied, although its trifluoromethyl group makes this compound more lipophilic than salycilates, suggesting its putative ability to cross the blood–brain barrier and to act directly on central nervous system parenchymal cells.

It has previously been reported that the transcription factor NF-κB is ubiquitously present in the brain, both during development [1,2] and in adulthood [11,16]. In this study, constitutive NF-κB is observed mainly cytoplasmatically, although we have previously shown by electron microscopy mild staining in the nucleus [2]. Other authors have mainly described a nuclear appearance of NF-κB in neuronal cells [11].

The constitutive presence of this transcription factor suggests its participation in normal brain function, probably reflecting a certain condition of cellular activity or state of differentiation of the particular NF-κB positive populations [14]. Actually, several well known NF-κB inducers are present during normal cerebral activity and are involved in important mechanisms of neural cell development and basal activity: NF-κB activation is triggered by the physiological stimulation of glutamate ionotropic receptors in neuronal cell cultures [10], by nerve growth factor binding [7], and by tumor necrosis factor alpha (TNFα)-mediated regulation of hippocampal synaptic plasticity [3].

As we could observe in our histological examination, it seems that the pharmacological downregulation of constitutive NF-κB during postnatal development does not apparently affect normal brain morphology. However, it may play an important role in preventing the induction of several genes modulated by NF-κB under appropriate stimuli. In general, NF-κB is activated by oxidative stress and cytokine or growth factor binding to their specific receptors [5,14]. Activated NF-κB regulates the expression of several proinflammatory proteins like cytokines, major histocompatibility complexes, adhesion molecules, COX-2 and inducible nitric oxide synthase (see [5,14] for review), but it also seems to trigger the transcription of anti-apoptotic genes [4], being the basis of the neuroprotection achieved by low doses of amyloid β-treatment in neuronal cultures [9], and clearly suggesting a dual role of this transcription factor [12]. Accordingly, NF-κB downregulation could protect brain cells against inflammatory processes, but it may also increase susceptibility to cell death processes. In this sense, further studies on the effects of NF-κB inhibition by triflusal in pathological conditions are warranted.

We would like to thank M.A. Martil for his outstanding technical help. The work was supported by the DGICYT project PB98-0892.

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