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Inhibition of NF-κB activation by diethylcarbamazine prevents alcohol-induced liver injury in C57BL/6 mice

Bruna Santos da Silva*, Gabriel Barros Rodrigues, Sura Wanessa Santos Rocha, Edlene Lima Ribeiro, Fabiana Oliveira dos Santos Gomes, Amanda Karolina Soares e Silva, Christina Alves Peixoto

Laboratório de Ultraestrutura, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, PE, Brazil

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ABSTRACT

Induction of NF-κB-mediated gene expression has been identified in the pathogenesis of alcoholic liver disease (ALD). Diethylcarbamazine (DEC) is a piperazine derivative drug with anti-inflammatory properties. The present study was designed to evaluate the effect of DEC on NF-κB pathways in mice undergoing alcoholism induced hepatic inflammation. Forty male C57BL/6 mice were divided equally into four groups: control group (C); DEC-treated group, which received 50 mg/kg (DEC50); alcoholic group (EtOH), submitted to chronic alcohol consumption and the alcohol-DEC treated group (EtOH50), submitted to chronic alcoholism consumption plus DEC treatment. Histological analysis of the alcoholic group showed evident hepatocellular damage which was reduced in EtOH50 group. Immunohistochemistry and western blot results showed elevated expression of inflammatory markers such as MDA, TNF-α, IL-1β, COX-2 and iNOS in hepatocytes of EtOH group. However, low immunopositivity for these markers was detected following DEC treatment. In the EtOH group the activation of NF-κB was observed by an increase in the expression of both NF-κB and pNF-κB in hepatocytes. This expression was significantly reduced in livers of EtOH50 group. Protein expression of Iκβα was measured to determine whether activation of NF-κB might be the result of Iκβα degradation. It was observed that expression of this protein was low in EtOH group, while animals treated with DEC had a high expression of Iκβα. The results of the present study indicate that DEC alleviates alcoholic liver injury, in part by the inhibiting activation of NF-κB and by suppressing the induction of NF-κB-dependent genes.

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1. Introduction

The hazardous and harmful use of alcohol is a major contributing global factor to death, disease and injury. Alcohol consumption is directly associated with liver disease mortality and leads to elevated social and economic costs (Bruha et al., 2012). The most commonly recognized symptoms of alcohol consumption are associated with chronic alcoholism, and it is a causal or risk factor in 60 types of diseases and injuries. These and other effects of alcohol consumption have made alcohol the third leading global risk factor for disease and disability (WHO, 2011).

The toxic effects of alcohol have an impact on multiple organs; however, the liver, as the primary site of alcohol metabolism, is a major injury target (Lieber, 2000, 2005; Karinch et al., 2008). Increasingly, alcoholic liver disease (ALD) is considered to be a complex and multifaceted pathological process, involving oxidative stress, inflammation and excessive fatty acid synthesis (Mandrekar, 2007). The progression of the disease involves various pro-inflammatory molecules such as interleukins, cytokines, adhesion molecules and nuclear factor-kappa B (NF-κB) (Achur et al., 2010; Ballas et al., 2012; Santos-Rocha et al., 2012).

NF-κB is a transcription factor involved in inflammation and immune response (Baeuerle and Baltimore, 1996) and is activated by oxidants and cytokines such as interleukin 1β (IL-1β) and cytokine tumor necrosis factor-alpha (TNF-α) (Barnes and Karin, 1997), which play important roles in inflammation and the development of ALD. IL-1β and TNF-α trigger the degradation and phosphorylation of IκB proteins, thus permitting the entry of

Abbreviations: ALD, alcoholic liver disease; DEC, diethylcarbamazine; MDA, malondialdehyde; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1-β; COX-2, cyclooxygenase-2; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; WB, Western blot; NO, nitric oxide; TXB, thromboxanes; TGFβ, transforming growth factor-β.

* Corresponding author at: Laboratório de Ultraestrutura, Centro de Pesquisas Aggeu Magalhães (CPqAM-FIOCRUZ), Av. Moraes Rego s/n, Cidade Universitária, CEP: 50670-420 Recife, PE, Brazil. Tel.: +55 81 21012583; fax: +55 81 21012500.
E-mail address: silvabrunasantos@hotmail.com (B.S. da Silva).

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NF-κB p65/p50 into the nucleus, where NF-κB p65/p50 activates the transcription of various genes including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), both considered important mediators in the recruitment of inflammatory cells (Bhaskaran et al., 2010; Arias-Salvatierra et al., 2011).

Since 1947 diethylcarbamazine (DEC) is the most widely used drug for the treatment of lymphatic filariasis (Hewitt et al., 1947). As a result of its interference with arachidonic acid metabolism, DEC has anti-inflammatory properties, including lipoxygenase (LOX) and cyclooxygenase (COX) enzymes (Maizels and Denham, 1992; McGarry et al., 2005). Queto et al. (2010) demonstrated that DEC has important role in blocking pulmonary eosinophilic inflammation in mice sensitized with ovalbumin, effectively preventing the effects of subsequent airway resistance, Th1/Th2 cytokine production, pulmonary eosinophil accumulation and *in vivo* and *ex vivo* eosinophilopoiesis. Additionally, DEC directly suppressed IL-5-dependent eosinophilopoiesis in naive bone marrow.

Recently, Santos-Rocha et al. (2012) demonstrated that chronic consumption of ethanol increases NF-κB levels and targets several proinflammatory cytokines, chemokines and oxidases. The administration of DEC inhibits hepatic injury and decreases inflammatory markers, suggesting potential therapeutical use in chronic inflammation induced by alcoholism. However, the inhibition of the nuclear transcription factors of the κB family pathways needs to be clarified. Therefore, the aim of the present study was to investigate the role of DEC on NF-κB pathways in hepatic inflammation induced by alcoholism. The following points were examined: (1) hepatic injury (histology), (2) detection of TNF-α, IL-1β, malondialdehyde (MDA), COX-2 and NF-κB (immunohistochemistry), (3) expression of IL-1β, COX-2, TNF-α, IκB and NF-κB (western blot).

2. Materials and methods

2.1. Animals

Forty male C57BL/6 mice aged 5 weeks and weighting 15–17 g were used in all experiments. The health of the mice was examined and they were acclimatized to the laboratory environment of 22 °C and 12 h light: 12 dark photoperiods. The animals were housed in metal cages and fed a standard diet and water *ad libitum*. The Animal Ethics Committee of Oswaldo Cruz Institute approved all the experiments reported (CEUA LW-32/10).

2.2. Diethylcarbamazine solutions

The solutions were compound of distilled water and DEC (Farmanguinhos, FIOCRUZ, Brazil) adjusted according to the body weight of each animal. The treated groups received 50 mg/kg of DEC for twelve days through gavage (200 μl) (Santos-Rocha et al., 2012; Rocha et al., 2012). The control group received distilled water only, administered in the same way.

2.3. Experimental groups

After a week of acclimation, 30 day old C57BL/6 mice were separated into four groups ($n = 10$ each): control group (C) that received distilled water by gavage, DEC-treated group (DEC50) that received 50 mg/kg DEC for twelve days by gavage, alcoholic group (EtOH) which received ethanol and the alcoholic plus 50 mg/kg DEC group (EtOH50). Ethanol was provided in drinking water at 10% (v/v) for 2 days, 15% for 5 days, and 20% for 5 weeks (Ballas et al., 2012; Cook et al., 2004). According to Cook et al. (2004), innate immune system of mice presents significant changes after 20% ethanol consumption for three weeks. Solutions were changed daily and the remaining liquid in the bottles was measured and then discarded. After five weeks of alcoholism induction, mice received alcohol plus treatment for 12 days with a solution of DEC administered orally. The alcoholic group received alcohol plus water by gavage for 12 days.

2.4. Histopathology

Liver fragments were fixed in formalin (10%) and paraffin embeddeded. Sections (4–5 μm) were cut and placed on glass slides and were stained with hematoxylin-eosin (HE) following evaluation with an inverted microscope (Observer Z1, Zeiss MicroImaging GmbH) equipped with a camera and 4.7.4 Image Analysis Program (AxionCam MRm Zeiss) at a magnification of 400×.

2.5. Immunohistochemical assays

Five sections (5 μm) of each group were cut and adhered to slides treated with 3-amino-propyl-trietoxi-silane (APES [Sigma, USA]). Briefly, the sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). Sections were incubated for 30 min in a sodium citrate buffer (0.01 M, pH 6.0). Slides were treated with 0.3% (v/v) H_2O_2 in water for 5 min. Sections were washed with 0.01 M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1 h at room temperature. Sections were then incubated with antibody against NF-κB-p65 (1:50, Santa Cruz Biotechnology, CA), pNF-κB (1:100, Abcam, Cambridge, UK), IκBα (1:100, Santa Cruz Biotechnology, CA), TNF-α (1:50, Abcam, Cambridge, UK), MDA (1:50, Abcam Cambridge, UK), IL-1β (1:250, Abcam, Cambridge, UK), COX-2 (1:400; Abcam, Cambridge, UK), iNOS (1:50 Abcam, Cambridge, UK) for 12 hours at 4 °C, separately. The antigen-antibody reaction was visualized with (Dako) Universal LSAB®+ Kit, Peroxidase using 3.3-diaminobenzidine as chromogn. Slides were counterstained with hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but in the absence of the first antibody. Five pictures at the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.6. Total protein extraction

Livers were quickly dissected and then homogenized in a Wheaton Overhead Stirrer (No. 903475) in an extraction cocktail (10 mM ethylenediamine tetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate ($NaVO_4$), 10 mg of aprotinin and 100 mM tris(hydroxymethyl)aminomethane, pH 7.4). Homogenates were centrifuged at 3000 × *g* for 10 min and the supernatant was collected and stored at −80° C until use for IL-1≡ and COX-2 immunoblotting.

2.7. Cytosolic and nuclear protein extraction

Liver cytosolic and nuclear proteins were isolated using Cayman's Nuclear Extraction kit (Item No. 10009277, Cayman Chemical Company, Ann Arbor, MI, USA). Liver fragments were briefly homogenized in a hypotonic buffer supplemented with DTT and Nonidet P-40 per gram of tissue. Livers were centrifuged and resuspended by adding specified assay reagents as instructed. The cytosolic and nuclear fractions were stored in pre-chilled vials at −80 °C until further analysis. Livers cytosols were used to determine amounts of IκBα in immunoblotting, and nuclear fractions were used for NF-κB immunoblotting.

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