ENDOCANNABINOID SYSTEM EFFECTS ON THE RESPIRATORY SYSTEM. THE ROLE OF TRPV1 RECEPTORS IN PULMONARY FIBROSIS

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Project financed by Beca Fundación Neumosur 14/2010

Abstract

Objectives: to establish a murine model of bleomycin-induced pulmonary fibrosis, analysing the possible protective role of the Endocannabinoid System (ES) against fibrosis.

Methods: wild C5BL/6 and Balb/c mice, as well as the genetically modified strain TRPV1-/- were used. After a single dose of intratracheal bleomycin, the fibrotic response was analysed though histologic studies, the assessment of proinflammatory markers, myeloperoxidase activity, hydroxyproline content, genetic expression of VIP, PACAP, IL-1 β, IL-6, TNF-α and IL-11, as well as MAPK route (phospho-JNK, phospho-ERK), NF-ϰB (p-IϰBα), β-cathenin, TGF-β (GSK-3B), SMAD (p-SMAD2) and pSTAT3, at a protein level.

Results: pulmonary fibrosis was more severe in TRPV1-/- mice compared to C5BL/6 mice. A significant increase in proinflammatory markers such as *TNF-a, IL-1β, IL11 and IL-6*, but not VIP or PACAP, was observed. pIKBα, pSTAT3, pSMAD2 and pJNK, but not pERK, were increased at a protein level in TRPV-/- mice.

Conclusions: the murine model of bleomycin-induced lung fibrosis remains a keystone to pioneer current investigation in lung fibrosis. Modulation of the ES might have a protective role.

Key words: idiopathic pulmonary fibrosis (IPF), bleomycin, murine models, endocannabinoid system (ES)

EFECTOS DEL SISTEMA ENDOCANNABINOIDE EN EL SISTEMA RESPIRATORIO. PAPEL DE LOS RECEPTORES TRPV1 EN LA FIBROGÉNESIS PULMONAR

Resumen:

Objetivos: establecer un modelo murino de fibrosis pulmonar inducida por bleomicina, investigando el posible papel protector del sistema endocannabinoide (SE) frente a la fibrosis.

Métodos: se emplearon ratones salvajes (C5BL/6 y Balb/c) así como la cepa TRPV1-/-. Tras una única dosis intratraqueal de bleomicina, se analizó la respuesta fibrótica mediante un análisis histológico, la determinación de la expresión de marcadores del proceso profibrótico, el estudio de la actividad mieloperoxidasa y del contenido en hidroxiprolina del pulmón, así como el análisis de la expresión génica de VIP, PACAP, IL-1β, IL-6, TNF-α e IL-11, y el estado de activación de las rutas MAPKs (fosfo-JNK, fosfo-ERK) de la ruta de NF-ϰB (p-IϰBα), la ruta de β-catenina y del TGFβ (GSK-3B), la activación de SMAD (p-SMAD2) y pSTAT3, a nivel proteico.

Resultados: la fibrosis pulmonar inducida por bleomicina en los ratones de la cepa TRPV-/- fue más severa que en la cepa salvaje C5BL/6. El contenido en hidroxiprolina y la actividad mieloperoxidasa fue mayor en los ratones TRPV1-/-. Se detectó un incremento significativo en la expresión génica de citoquinas proinflamatorias *(TNF-a, IL-1b, IL11 e IL-6)*, pero no de VIP o PACAP, en la cepa TRPV1-/-. A nivel proteico, la expresión de pIKBα, pSTAT3, pSMAD2 y pJNK, pero no la de pERK, se vio incrementada en los ratones TRPV1-/-.

Conclusiones: el modelo murino de fibrosis pulmonar inducida por bleomicina sigue siendo clave para continuar profundizando los conocimientos acerca de la patogénesis de la FPI. La modulación del SE podría tener un papel protector frente a la fibrosis pulmonar.

Palabras clave: fibrosis pulmonar idiopática (FPI), bleomicina, modelos murinos, sistema endocannabinoide (SE).

Received: November 16, 2015. Accepted: April 6, 2017.

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INTRODUCTION

Idiopathic interstitial pneumonias encompass a heterogeneous group of diseases which are included within what is referred to as idiopathic interstitial lung disease (ILD). Under the umbrella of idiopathic ILD are clinical-pathologicalentities as diverse as idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP), respiratory bronchiolitis associated with interstitial lung disease, cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), desquamative interstitial pneumonia (DIP) and lymphocytic interstitial pneumonia (LIP)[1]. These diverse lung disorders are typically characterized by the presence of inflammation in the pulmonary interstitium, distinguishable by their clinical presentation along with radiological and/or anatomic-pathological findings.

IPF is the chronic inflammatory lung disease model that leads to terminal lung disease and currently constitutes the leading indication for lung transplant.IPF is the most prevalent form of idiopathic interstitial pneumonia and the most aggressive of all general interstitial lung diseases, with an average rate of survival after diagnosis of only 2 to 5 years[2]. Its involvement is limited to the lung parenchyma, with an estimated prevalence of 3 to 20 cases per 100,000 inhabitants.

The treatment used for the last 50 years, consisting of administering corticosteroids and cytotoxic drugs like azathioprine and cyclophosphamide, has not been proven to significantly benefit survival. This panorama changed in 2011 with the approval of pirfenidome, the only specific drug shown to stop disease progression.

Lung transplant is the accepted therapeutic treatment for patients suffering from progressive idiopathic ILD which leads to respiratory failure and is refractory to standard medical treatment at maximum doses[3]. In 2007, the number of lung transplants among patients with IPF surpassed those for COPD patients, thus transforming it into the leading indication for lung transplant in the US.

Research in pulmonary fibrosis has been based on disease modulation with the use of animal models.Among the external agents that can be administered to induce pulmonary fibrosis, we can highlight bleomycin, inorganic particulates (silica, asbestos), radiation, gene transfer (TGF-β, IL-1β, GM-CSF), fluorescein isothiocyanate (FITC), vanadium pentoxide or haptenic agents (trinitrobenzenesulfonic acid).

In the last 3 decades, the model for bleomycin-induced pulmonary fibrosis has been at the head of basic research into the regulation of pulmonary fibrosis. A direct rupture in the DNA chain and the generation of free radicals, thus releasing oxidative stress, is believed to be the mechanism which causes lung damage[4]. The potential of this antineoplastic agent to induce pulmonary fibrosis at the experimental level was first shown in dogs[5]. Later, Snider et al.6popularized the unique intratracheal administration in small animals.

The endocannabinoid system (ECS) is comprised of endogenous ligands, regulating enzymes and central and/or peripheral receptors (CB1, CB2 and TRPV1). Among the known functions of the ECS, we can note regulating an organism's homeostasis, possessing antitumor properties, regulating energy metabolism, regulating the stress response, neuromodulation and immunomodulation effects, analgesia and thermo-regulation. The endocannabinoids identified to date include anandamide (N-arachidonoylethanolamine, AEA), 2-arachidonoylglycerol (2-AG), 2-arachidonyl glyceryl ether (noladin ether), O-arachidonoyl ethanolamine (virodhamine), and N-arachidonoyl dopamine (NADA). The TRPV1 receptor is widely expressed in the respiratory tract.

Given that the role of ECS in pulmonary fibrosis has yet to be explored, the objective of this paper is to analyze the role of the endocannabinoid system in pulmonary fibrosis, developing a murine model for bleomycin-induced pulmonary fibrosis.

MATERIAL AND METHODS

Animals: The study used 10-14 week old male C5BL/6 and Balb/c wild-type strain and genetically-modified TRPV1-KO strain mice weighing about 40 grams. They were kept in optimal housing conditions, established by the Animal Experimentation Department at the University of Cordoba and applicable legislation (R.D. 1201/2005). The animals were kept in sawdust-filled plastic cages, maintaining a 12/12 hour light and dark cycle with ad libitum access to food and water. The C5BL/6 and Balb/c strains were acquired from Harlan Sprague-Dawley Inc. (Indianapolis, IN) (BN), and Charles River Canada Inc. (St. Constant, QC, Canada), respectively. The TRPV1 strain was acquired from The Jackson Lab (USA).

Experimental bleomycin-induced pulmonary fibrosis model: a murine model of bleomycin-induced pulmonary fibrosis was developed. To do so, a total

of 60 mice were used. The mice were randomly assigned to 6 experimental groups, using 10 animals per group. The animals in the control group received intratracheal saline, while those in the treatment group received a single dose of bleomycin through endotracheal intubation. The following were the treatment groups:

a) C5BL/6 control (n=10) and C5BL/6 bleomycin (n=10).

b) Balb/c control (n=10) and Balb/c bleomycin (n=10).

c)TRPV1-KO control (n=10) and TRPV1-KO bleomycin (n=10).

In the experimental model, animals received single doses of bleomycin sulfate (0.06 mg in 50µL saline solution; Bleomicina Mylan 15,000 IU; Mylan Pharmaceuticals, S.L. Barcelona, Spain) or only 50µL of saline solution the first day of treatment (day 0). To do so, they were anesthetized intraperitoneally in advance with ketamine + diazepam. Immediately after instillation, the animals were placed in a forced sitting position to improve the endobronchial distribution of the drug through gravity. The animals were sacrificed on day 14 through thoracic bleeding under general anesthesia. The right lung was frozen at -80ºC in order to extract proteins, RNA, measure myeloperoxidase (MPO) activity and quantify hydroxyproline content. The left lung was preserved in formaldehyde, administered through the main bronchus for paraffin processing and to study the histology and immunohistochemistry.

After administering the treatment, group weight loss and mortality were observed daily. Before the definitive experiments were completed, training in the intratracheal administration technique for suspended substances was carried out, during which correct administration was confirmed in order to ensure uniform distribution in both lungs after intratracheally injecting a colored physiological saline solution (methylene blue).

Morphological examination: lung damage was induced through the intratracheal administration of a single dose of bleomycin sulfate, and findings were compared to the control group for the same strain of mice, who were exclusively administered saline solution. Maximum fibrotic change was seen 14 days after treatment, at which time the animals were sacrificed.

After anesthetic induction and median sterno-laparotomy, a mass was extracted from both lungs. The right lung was explanted after tying and sectioning the main bronchus at the hilum and was preserved at -80ºC. The left lung of each mouse was perfused with formalin, infused through tracheal intubation, and was preserved this way for the next 24 hours. After soaking the lung in paraffin, 5 µM sections were prepared to be stained with hematoxylin and eosin (to complete routine histological studies) and Masson's Trichrome Staining (for mature collagen). The Ashcroft scale[7] was used to quantitatively analyze the fibrotic changes induced through bleomycin administration, from 0 (no fibrosis) to 8 (massive fibrosis in all fields). The severity of fibrotic changes in each section of lung was determined by an average score in all of the observed increased fields, analyzing a minimum of 10 fields of significant increase in each section.After analyzing the entire histological section, the average scores from all analyzed fields were classified as the fibrotic index. Each sample was scored independently by 4 observers. Finally, the average score obtained was considered the final fibrotic index.

Immunohistochemistry: to study the macrophage content of the fibrotic lungs with respect to healthy lungs, the specific anti-macrophage F4/80 antibody was used. The anti-F4/80 monoclonal antibody clearly differentiates the macrophages of fibroblasts in fibrotic tissues, the chosen marker for mice. 5µm sections of tissue were placed on microscope slides and dehydrated and rehydrated successively. After 5 minutes exposed to the DAKO buffer and being treated with 3% H_2O_2 for 5 minutes, they were processed to complete immunohistochemistry studies. Incubation was done at room temperature for 30 minutes.After 10 minutes of goat serum blocking, they were incubated with the specific F4/80 primary antibody. After short rinses, they were first incubated with the secondary biotin antibody, and later with an avidin-biotin-peroxidase complex. After other rinses between incubations, they were developed with 3,3'-diaminobenzidine (DAB; Biomedia, CA), 2 x 5 min. Finally, the stains were dyed with hematoxylin, rehydrated and placed on a cover slip to evaluate the staining with a microscope.The F4/80 macrophage staining was arbitrarily evaluated by counting the number of cells that expressed the marker (percent).

Evaluation of hydroxyproline content: the amount of lung collagen was determined by measuring the hydroxyproline content in the right lung of each animal, both in the treatment and control groups.To do so,

the right lungs from the 6 treatment groups were used (frozen at -80ºC). The objective was to determine the total hydroxyproline expressed per mg of wet lung tissue as a quantitative measure of collagen deposition and fibrosis. Hydroxyproline is an amino acid specifically found in collagen. Each lung was weighed and a 10% homogenate was prepared using frozen saline solution infused through the pulmonary artery, which was then frozen at -80ºC. Finally, the frozen lungs were disaggregated in Ultra-Turrax without Ca^{+2}/Mg^{+2}. The collagen deposition was estimated by determining the total hydroxyproline content of the lung with spectrophotometry.

Determining myeloperoxidase activity: myeloperoxidase activity was decided to be a marker for intensity of inflammatory reaction through spectrophotometric determination. Myeloperoxidase is the most abundant pro-inflammatory enzyme within the azurophilic granules of neutrophils, as well as in monocytes/macrophages. To determine myeloperoxidase activity, whole lungs frozen at -80ºC were used. These experiments were conducted using 96 well plates. Peroxidase activity was measured with 3, 39, 5, 59-Tetramethylbenzidine (TMB, Sigma): 10 ml of sample was mixed with 80 ml 0.75 mM of H2O2 (Sigma) and 110 ml of TMB solution (2.9 mM TMB in 14.5% DMSO [Sigma] and 150 mM sodium phosphate buffer with pH 5.4), incubating the plate at 37ºC for 5 min. The reaction was stopped by adding 50 ml 2 M of H2SO4 (Sigma), and the optical density was measured at 550 nm with a microplate reader. Myeloperoxidase activity was calculated as picomoles per milligram of lung tissue, corrected with a wet-to-dry ratio, for treatment group lungs vs control group lungs. Values were expressed as arbitrary units (averages ± SD), comparing bleomycin vs control and between each strain.

Extraction of mRNA: total RNA was obtained from the right lungs preserved at -80ºC by using the RNeasy Mini Kit (Qiagen). Next, 1ug of total RNA was retrotranscribed into cDNA using the iSCRIPT cDNA Synthesis Kit (Bio-RAD). Quantitative evaluation of RNA expression was carried out using qPCR, using undiluted cDNA in a total volume of 10 uL using the Kit Master SYBR Green I mix. The primers used in the study were VIP, PACAP, IL-1β, IL-6, TNFα and IL-11, using β-Actin as an internal control. Amplification was carried out with the iCycler PCR Detection System (Bio-Rad). Levels of mRNA were expressed as absolute numbers, after normalizing with β-Actin levels, comparing treatment groups with the controls for each strain. The sequences of the primers used are shown in Table 1.

Table 1. The sequences of the primers used are shown

PRIMER	SEQUENCE
IL-1β	forward 5´-GACCTTCCAGGATGAGGACA-3´
	reverse 5´-AGCTCATATGGGTCCGACAG-3´
TNF-α	forward 5´-ACGGCATGGATCTCAAAGAC-3´
	reverse 5´-GTGGGTGAGGAGCACGTAGT-3´
IL-6	forward 5´-GTATGAACAACGATGATGCACTTG-3´
	reverse 5´-ATGGTACTCCAGAAGACCAGAGGA-3´
IL-11	forward 5´-CTGCACAGATGAGAGACAAATTCC-3´
	reverse 5´-GAAGCTGCAAAGATCCCAATG-3
VIP	forward 5´-ATGCTGATGGAGTTTTCACCA-3´
	reverse 5´- TGCAGAATCTCCCTCACTGCTC-3´
PACAP	forward 5´-CTGCTGGTGTATGGGATAAT-3´
	reverse 5´- CTACAAGTATGCTATTCGGC-3´
β-Actina	forward 5'-CCAGCCTTCCTTCTTGGGTAT-3´
	reverse 5'-TGGCATAGAGGTCTTTACGGATGT-3

IL-1β: Interleukin 1β; TNF-α: tumor necrosis factor α; IL-6: Interleukin 6; IL-11: Interleukin 11; VIP: vasoactive intestinal peptide; PACAP: Pituitaryadenylatecyclase-activatingpolypeptide.

Protein extraction and western blot studies: proteins were extracted from a fragment of the right lung (frozen at -80ºC) from both control mice and those in the treatment group (bleomycin).300 ml of the protein extraction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 10% Glycerol, 10% NP-40) containing protease and phosphatase inhibitors were added to each fragment. After homogenizing samples (Ultra-Turrax T10), they were incubated in ice for 15 minutes and then centrifuged for another 15 minutes at 12,000 RPM. The proteins obtained in the supernatant were evaluated with spectrophotometry (Genesis 10uv, Thermo Scientific). Next, a Laemmli sample buffer was added and they were incubated for 2 min at 95ºC. To analyze the marking, western blot was used to study the state of MAPK (JNK, ERK) activation from the NF-ϰB (p-IϰBα) pathway, the β-catenin pathway and from TGFβ (GSK-3B), and the activation of SMAD (p-SMAD2) and pSTAT3, using α-tubulin as a control. To complete the western blot, the membranes were incubated with the primary antibody at 4ºC for a full night. After 3 brief rinses, they were incubated with the secondary antibody. Finally, after another 3 brief rinses, immune complexes were detected using the Alpha Innotech detection system (Alpha Innotech Corp., San Leandro, CA, USA). The intensity of the bands was analyzed using Image J 1.46r software (National Institutes of Health, USA), comparing the expression of each protein in the fibrotic lung, in the healthy lung, and for each strain.

Statistical analysis: the program GraphPad Prism 5 was used for the statistical analysis. The numerical results were expressed as average ± SD. The Mann-Whitney nonparametric test was used to compare numerical values and Fischer's exact test was used to compare categorical variables. For proportional study values, differences observed are shown as percentages. P values less than 0.05 are considered significant.

RESULTS

Histological findings: the histological analysis completed 14 days after bleomycin induction vs saline solution showed the presence of focal fibrotic changes, predominantly subpleural and perivascular, with areas of parenchymal consolidation, loss of normal alveolar architecture as well as prominent inflammatory infiltrate in the alveolar and interstitial spaces (Figure 1).Compared with the C5BL/6 strain (Figure 1A), the fibrotic changes were significantly more severe in the TRPV1-/- strain (Figure 1B). There was thus evidence of sever and intense fibrosis, extending from the subpleural regions to the central areas of the lung, affecting the perivascular and peribronchial areas. An abnormal collagen deposition in the alveolar and interstitial space associated with said areas of fibrosis was detected, as shown by the use of Masson's Trichrome Staining (Figure 1B). In contrast, there was an inferior degree of fibrosis in the C5BL/6 strain, for which fewer subpleural fibrotic lesions were observed in addition to mild inflammatory infiltrate in the central areas (Figure 1A).As expected, the Balb/c strain did not develop significant pulmonary fibrosis.

Quantitative evaluation of the produced histological changes: the fibrotic index was determined using the Ashcroft numerical scale 14 days after administering treatment (bleomycin vs saline solution). A significant correlation was shown between the fibrotic indexes obtained by each of the 4 observers ($r2 = 0.9$; $p = 0.001$) (Figure 1C). The average fibrotic index for TRPV1-/- mice treated with bleomycin was higher than that for the wild-type C5BL/6 strain (4.5 ± 0.2 vs 2.3 ± 0.2). On the other hand, there were no significant fibrotic changes observed in mice from the Balb/c strain, nor those treated with saline solution (control). All of the animal survived through the end of the experiment.

Immunohistochemistry: given that macrophages constitute one of the main stimuli causing TGF-β-mediated pulmonary fibrosis, we decided to use immunohistochemistry to analyze the macrophage content in the wild-type C5BL/6 strain as well as TRPV1-/- mice. As shown in Figure 1D, we saw that the total number of positive cells for the F4/80 marker was significantly higher in the TRPV1-/- mice treated with bleomycin than in the wild-type mice.

Hydroxyproline content: Figure 2 shows the results of the hydroxyproline content analysis in the bleomycin-treated mice versus the controls. The results obtained show that the administration of a single intratracheal dose of bleomycin significantly increased hydroxyproline levels in the lungs at 14 days when compared to the mice who were only administered saline solution (controls). Additionally, hydroxyproline was even greater in the TRPV1-/- treated mice with respect to the controls (2.791 vs 1.951, $p < 0.001$). On the other hand, no significant changes were observed in the Balb/c strain.

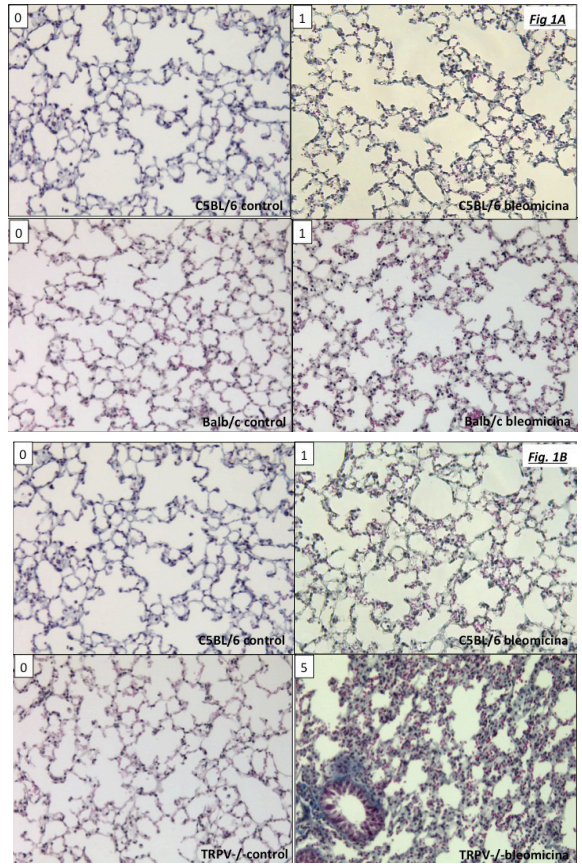


Fig. 1A and 1B: Histological sections from murine lungs after inducing pulmonary fibrosis through intratracheal bleomycin instillation, compared to saline solution (controls). Original magnification 100x. Masson's Trichrome Staining was used.
Fig. 1A: Treatment groups using the C5BL/6 and Balb/c strains. After administering Bleomycin, mild fibrosis was produced in the wild-type C5BL/6 strain (Ashcroft 1), with slight inflammatory infiltrate, a mild thickening of the alveolar or bronchial walls, and minimum collagen fiber deposition. Significant pulmonary fibrosis was not produced in Balb/c strain mice with respect to the controls which were administered saline solution.
Fig. 1B: Treatment groups using the C5BL/6 and TRPV1-/- strains. In TRPV1-/- mice in which pulmonary fibrosis was induced with bleomycin, intense patched fibrosis was produced with severe damage to the lung structure, an increase in alveolar septal cellularity, alveolar collapse, interstitial thickening, and intraalveolar fibrosis, shown as massive collagen fiber deposition with respect to the controls for the same strain which received treatment with saline solution.Mice treated with saline solution (controls) presented normal lung architecture in all cases.

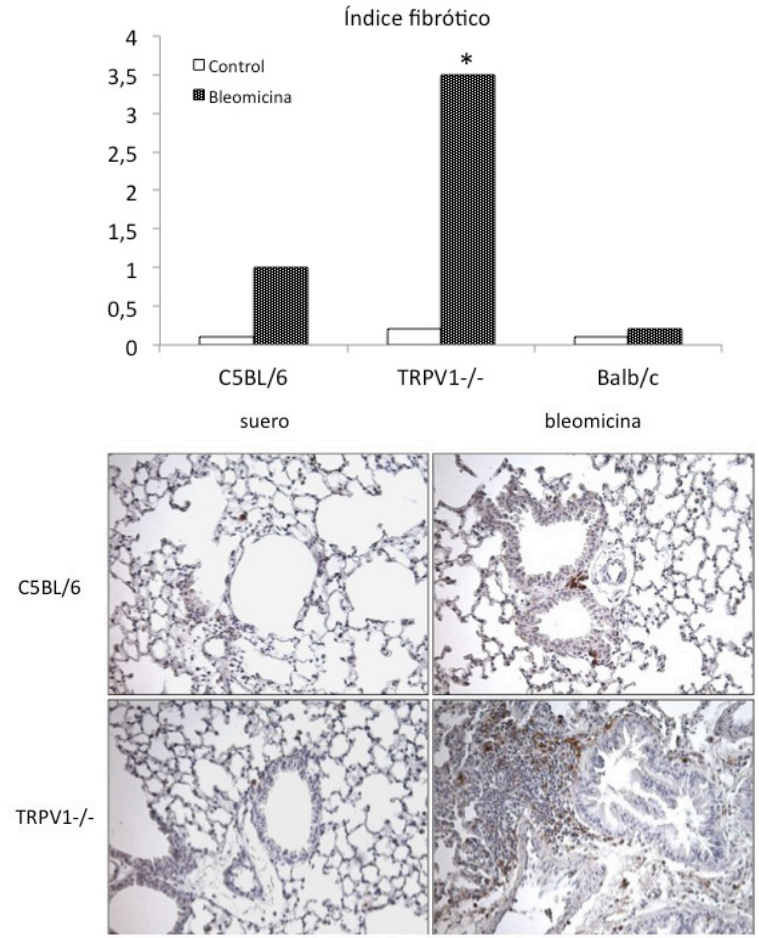


Fig. 1C: The fibrotic index calculated for 5 mice in each experimental group, according to the Ashcroft scale. The average fibrotic index for TRPV1-KO mice treated with bleomycin was higher than that for the wild-type C5BL/6 strain (4.5 ± 0.2 vs 2.3 ± 0.2, $p < 0.05$). On the other hand, there were no significant fibrotic changes observed in mice from the Balb/c strain, nor those treated with saline solution (control).
Fig. 1D: Immunohistochemistry using the F4/80 macrophage marker: original magnification 40x. The presence of the F4/80 marker is shown in lung sections on day 14 after bleomycin administration. The total number of positive cells for the F4/80 maker was significantly higher in the TRPV1-/- mice treated with bleomycin than in the wild-type mice.

Myeloperoxidase activity: there was evidence that MPO levels in the homogenate from lungs frozen at -80ºC were very low in mice that were administered saline solution (non-induction group), compared to the mice that were administered bleomycin, in which a significant increase was detected (Figure 3).This was in line with the leukocyte accumulation seen in the histological analysis.Additionally, the increase in MPO was significantly higher in the TRPV-/- strain treated with bleomycin in comparison to the C5BL/6 mice.

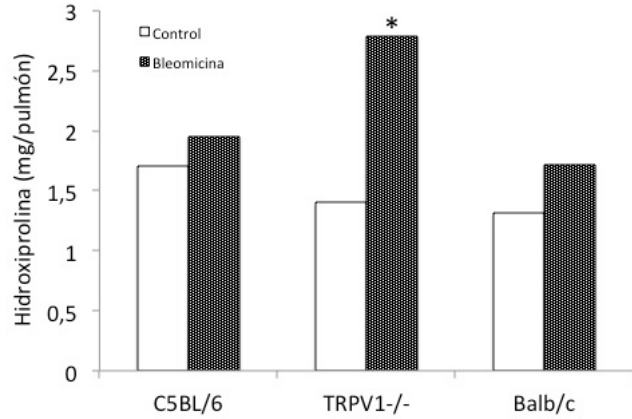


Figure 2. Hydroxyproline levels in mice treated with intratracheal saline solution or bleomycin. Values are expressed as averages (milligrams/lung). Each experiment was conducted in at least 10 animals for each group.In the TRPV1-/- strain treated with bleomycin, the hydroxyproline content was significantly higher (*p <0.05) when compared to the saline solution group.In the C5BL/6 and Balb/c wild-type strains, no significant differences in hydroxyproline content were detected.

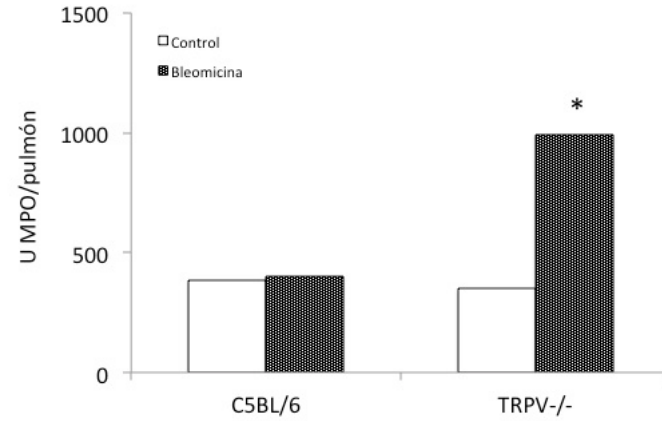
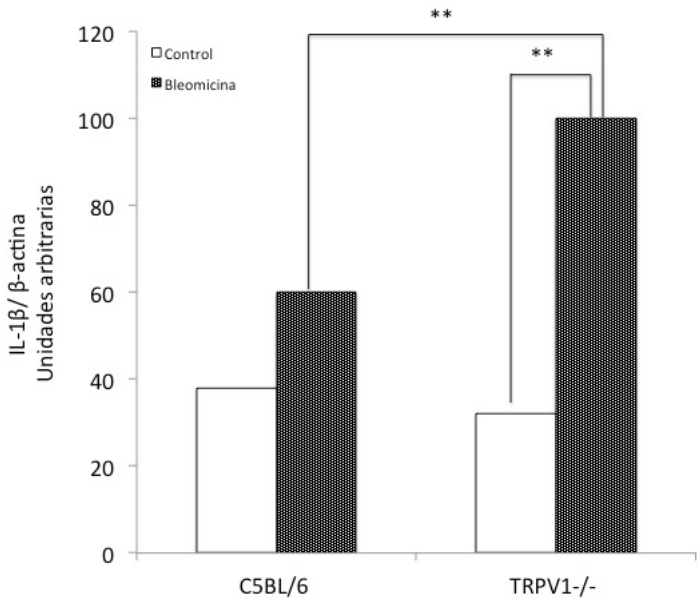


Figure 3. Myeloperoxidase levels were analyzed as a marker for leukocyte inflammatory infiltrate. A significant increase was detected in myeloperoxidase (MPO) levels in the lung homogenate from mice treated with bleomycin with respect to the controls (non-induction group).The increase in MPO was significant in C5BL/6 mice induced with bleomycin with respect to C5BL/6 controls, as well as in TRPV1-/- mice treated with bleomycin compared to TRPV1-/-mice treated with saline solution.Similarly, a significant increase (*p <0.05) was detected in TRPV1-/- mice treated with bleomycin with respect to C5BL/6 mice treated with bleomycin. At least 10 mice were included per group.

Expression of mRNA: the administration of bleomycin translated to the increase in pro-inflammatory genes such as TNF-a, IL-1b, IL-11 and IL-6, both in wild-type mice and the TRPV1-/- strain(Figure 4). This increase was significantly higher in TRPV1-/- mice. IL-6 and IL-11 belong to the same family of pro-inflammatory cytokines, connected to the STAT3 and NF-kB signaling pathways. A significant increase in expression was shown, both for IL-6 and IL-11 at the mRNA level, in C5BL/6 as well as TRPV1-/- mice.In contrast, strong induction in VIP expression at the mRNA level was seen in wild-type C5BL/6 mice treated with bleomycin in comparison with the TRPV1-/- strain.In the case of the PACAP neuropeptide, there was a trend towards increased expression in wild-type C5BL/6 strain mice treated with bleomycin with respect to the TRPV1-/- strain, although this was not statistically significant.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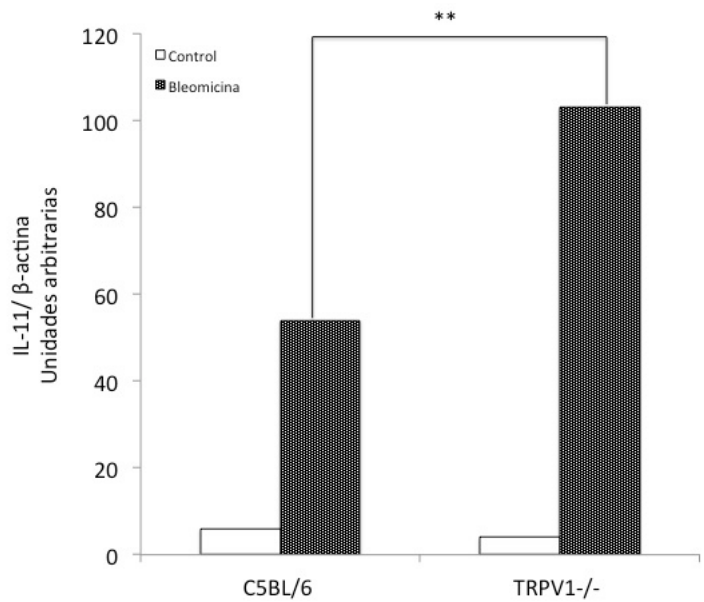
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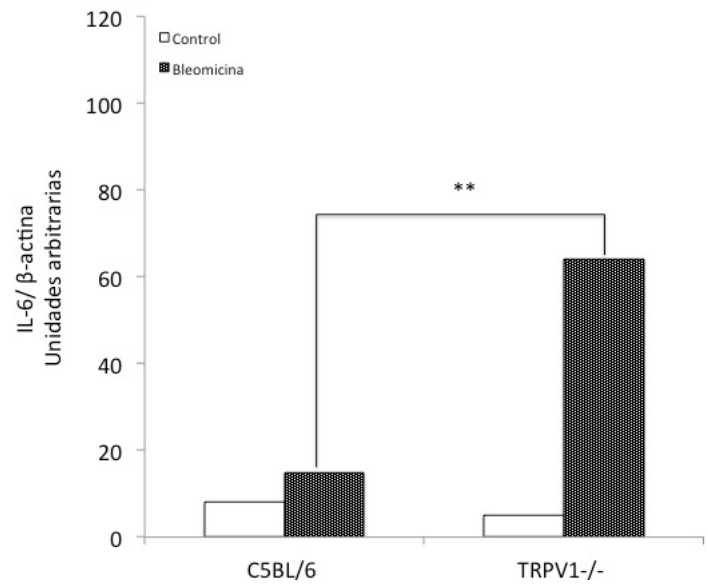
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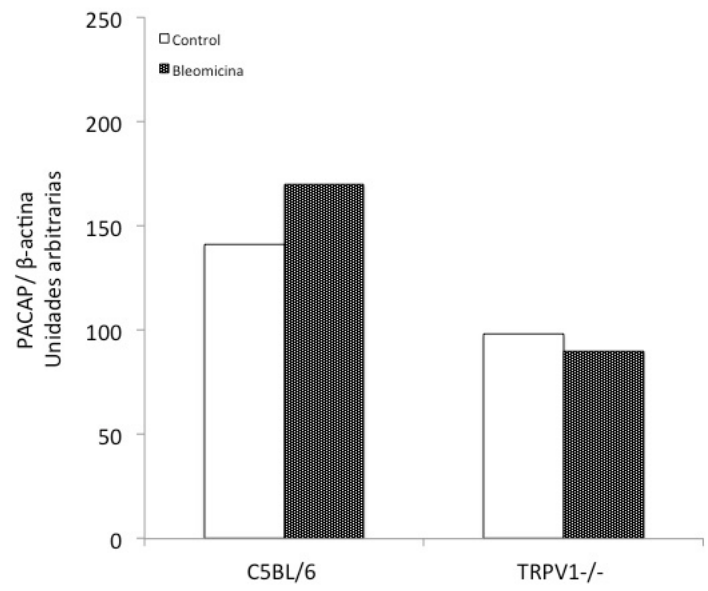
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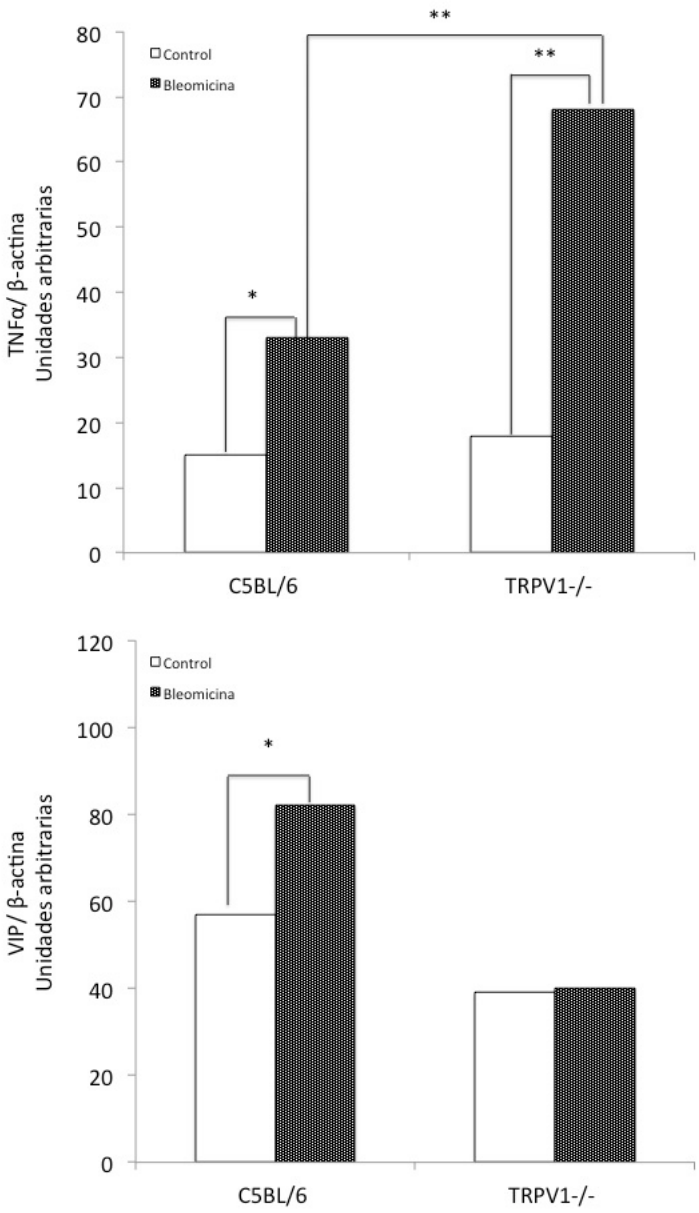

Figure 4. Induction of pro-inflammatory cytokine lung expression (TNF-a, IL-1b, IL-11 and IL-6) in mice treated with intratracheal saline solution or bleomycin. The mRNA levels were expressed as absolute numbers, after normalizing with β-Actin levels, comparing treatment groups with the controls for each strain. The results are shown as averages for each treatment group. P values less than 0.05 (t-student) are considered significant. The absence of TRPV1 receptor expression significantly increased the expression of genes involved in the inflammatory response after bleomycin induction. Histograms showing the relative expression of pro-inflammatory cytokines obtained through RT-PCR from mRNA taken from lung tissue preserved at -80ºC. VIP and PACAP levels were higher in C5BL/6 mice treated with bleomycin than in TRPV1-/- mice.

DISCUSSION

This paper shows the utility of the experimental model, consisting of the intratracheal administration of instilled bleomycin in a single dose in mice for the study of pulmonary fibrosis.In the present study, the role of the endocannabinoid system in pulmonary fibrosis was analyzed, using the TRPV1-/- strain of mice, which does not constitutively express the TRPV1 receptor. Our results show that the absence of the TRPV receptor generated severe fibrotic changes in the lungs of bleomycin-treated mice.Bleomycin sulfate is frequently used in experimental pulmonary fibrosis models, generally in rodents.Bleomycin is a chemotherapy agent whose most feared effect is pulmonary fibrosis[8]. Bleomycin is inactivated by the bleomycin hydrolase enzyme, low amounts of which are produced in the lungs, thus its greater susceptibility to suffer toxic damage from this agent. A direct rupture in the DNA chain and the generation of free radicals, thus releasing oxidative stress, is believed to be the mechanism which causes lung damage[4]. The potential of this antineoplastic agent to induce pulmonary fibrosis at the experimental level was first shown in dogs[5]. Later, Snider et al.6popularized the unique intratracheal administration in small animals.Bleomycin damages the alveolar epithelium, presumably secondary to the damage caused to the DNA, which is followed by an intense inflammatory reaction. Two weeks after administration, the inflammatory and fibroproliferative responses are easily detectable in the tissue, as shown in the model analyzed in this paper.

Pulmonary fibrosis is a chronic disease characterized by the excessive accumulation of extracellular matrix and remodeling of the lung architecture. The diseases that alter the interstitial space, running from the vascular endothelium to the alveolar epithelium, are called interstitial lung diseases[1]. When the alveolar space is occupied by inflammatory cells and/or collagen deposition,the

gas exchange is altered and the number of functioning alveolar units is reduced[9]. IPF is the most frequent and severe form of interstitial lung disease. Despite treatment with a range of drugs, principally corticosteroids and immunosuppressants, there is no effective treatment, thus the average rate of survival after diagnosis is 2 to 5 years[2].

The validity of the bleomycin-induced pulmonary fibrosis experimental model used in this study (through the administration of a single intratracheal dose) has been widely demonstrated. Although numerous animal models have been developed to research pulmonary fibrosis, the most used and best characterized to date is the model based on administering bleomycin to small animals. The advantage of this model is that a single administration of bleomycin produces lung damage which goes on to result in fibrosis[6]. Our work has confirmed that the model for fibrosis induction after a single intratracheal instillation of bleomycin and later sacrifice of the animals after 14 days gives rise to the maximum fibrotic response. Sacrificing the animals 14 days after administering the fibrosing agent showed the presence of severe fibrotic changes along with prominent inflammatory infiltrate in the alveolar and interstitial spaces. These results also confirm that the inflammatory response that takes place in the lungs is characterized by important neutrophil activation, as shown in the significant increase in myeloperoxidase content.

It is important to note that the Balb/c strain did not develop significant pulmonary fibrosis, which corresponds with previous publications in the literature[10]. This strain is thus a very useful control group, in addition to the treatment control groups for each of the strains which were administered saline solution instead of bleomycin.

It is well know that there is an increase in the expression of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α as well as a lower expression of the antifibrotic cytokine IFN-γ in the pathogenesis and progression of pulmonary fibrosis[11,12]. Our results are consistent with these publications. Thus, the increase in the expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-11 and IL-6 may be explained by a stimulus in their production from the alveolar macrophages, given that a significant increase in the expression of the F4/80 macrophage marker was detected. Additionally, the fact that the levels of these cytokines were significantly higher in the TRPV1-/- strain treated with bleomycin may be due to an alteration in the inflammatory response secondary to the absence of the TRPV1 endocannabinoid receptor.

The endocannabinoid system (ECS) is comprised of endogenous ligands, regulating enzymes and central and/or peripheral receptors (CB1, CB2 and TRPV1). Among the known functions of the ECS, we can note regulating an organism's homeostasis, possessing antitumor properties, regulating energy metabolism, regulating the stress response, neuromodulation and immunomodulation effects, analgesia and thermo-regulation. The endocannabinoids identified to date include anandamide (N-arachidonoylethanolamine, AEA), 2-arachidonoylglycerol (2-AG), 2-arachidonyl glyceryl ether (noladin ether), O-arachidonoyl ethanolamine (virodhamine), and N-arachidonoyl dopamine (NADA). Although the neurological effects of cannabinoids have been extensively researched, little is known today about the pharmacological properties of these agents on the respiratory tract. The inhalation of Λ^9 tetrahydrocannabinol, the main active component in marijuana, induces bronchodilation and protects against bronchoconstriction induced by cholinergic agents in the airway of asthmatic patients[13], although it is unknown if these effects are mediated at the central level. The TRPV1 receptor is selectively activated by capsaicin, the main pungent component of cayenne, chili and other spicy peppers, as well as by a variety of toxic respiratory agents, both exogenous and endogenous, including anandamide[14], lipoxygenase products of arachidonic acid[15], H2S[16], ethanol[17], and atmospheric[18,19] acids and pollutants[20].

The role ofthe ECS in pulmonary fibrosis is still unknown, given that it can have both beneficial and damaging effects on the respiratory system. In other organs, it has been proven that endocannabinoids could possess pro-fibrotic or anti-fibrotic properties depending on their interaction with CB1 or CB2 receptors. At the pulmonary level, numerous publications have shown the presence of significant amounts of the TRPV1 receptor in several components of the respiratory system[20-25]. Based on the results shown in this study, it is evident that the absence of TRPV1 receptors translates to the appearance of intense pulmonary fibrosis with respect to the wild-type strain. This assertion is reflected at both the histopathological level and in the quantification of hydroxyproline content, myeloperoxidase activity and the expression of pro-inflammatory cytokines. Thus, we speculate that TRPV1 receptors may be a powerful therapeutic target to prevent the onset of pulmonary fibrosis or halt its evolution after onset.

Among the limitations of these models, we can note the following:

a) On one hand, the variability in the animal model with respect to clinical presentation in humans. The degree of fibrosis varied according to administration route, the strain of mouse used, and there was even different fibrotic response within the same strain using the same dose and administration route.

b) Halting the induction agent in some animal models can result in the regression of the fibrosis, a concept which never occurs in the clinical setting. Pulmonary fibrosis in humans occurs after months/years of aberrant pulmonary remodeling. In the animal models, on the other hand, this sequence is reproduced in a significantly shorter amount of time, generally as a response to a single instance of lung damage. This study did not look into the specific length of pulmonary fibrosis in terms of time, as all animals were sacrificed 2 weeks after receiving treatment.

c) Intratracheal instillation, in spite of successfully inducing fibrosis with a single administered dose, does not resemble the clinical presentation of intravenous administration, which more similarly reproduces the way pulmonary fibrosis results as a side effect to bleomycin administration as a chemotherapy agent.

d) The fibrotic response to bleomycin administration is strain-dependent.Thus, while the C5BL/6 wild-type strain is more susceptible to damage, Balb/c mice are resistant, likely due to their higher bleomycin hydrolase content. Even so, the Balb/c strain was used as a control group in our study, and the mice lacking TRPV1 were those who developed the most intense fibrosis.

In conclusion, this work presents evidence for the first time that altering the endocannabinoid system through the absence of TRPV1 receptor expression leads to the onset of severe pulmonary fibrosis in the murine model of bleomycin-induced pulmonary fibrosis. Our hypothesis is that the TRPV1 receptor could have a protective role in pulmonary fibrosis by modulating the synthesis of pro-inflammatory cytokines, and could be a powerful therapeutic target for this disease.

Acknowledgments: the authors would like to recognize the excellent work done by Lourdes Santaella Jiménez in her invaluable technical assistance and material support for this project.

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