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The effect of cannabinoids on dinitrofluorobenzene-induced experimental asthma in mice

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ABSTRACT

Cannabinoids have anti-inflammatory effects and can produce bronchodilation in the airways. We have investigated the effects of cannabinoids on tracheal hyperreactivity and airway inflammation in dinitrofluorobenzene (DNFB)-induced experimental non-atopic asthma in mice. 5-hydroxytryptamine (5-HT)-induced contraction response was enhanced while carbachol- and electrical field stimulation-induced contractions, and isoprenaline-induced relaxation responses were remained unchanged in DNFB group. The increased 5-HT-induced contractions were inhibited by incubation with either atropine or tetrodotoxin. DNFB application resulted in increased macrophage number in the bronchoalveolar lavage fluid (BALF). *In vivo* ACEA (CB_1 agonist) treatment prevented the increase in 5-HT contractions, while JWH133 (CB_2 agonist) had no effect. However, neither ACEA nor JWH133 prevented the increase in macrophage number in BALF. *In vitro* ACEA incubation also inhibited the increase in 5-HT contraction in DNFB group. These results show that cannabinoid CB_1 receptor agonist can prevent tracheal hyperreactivity to 5-HT in DNFB-induced non-atopic asthma in mice.

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1. Introduction

Asthma is a chronic airway disease associated with bronchospasm and airway hyperreactivity, which mostly occurs due to inflammation (Bousquet et al., 2000; Groot Kormelink et al., 2009). Asthma has multiple phenotypes such as atopic and non-atopic asthma with different pathophysiologic and clinical characteristics. In the respiratory research, the interest is mainly focused in atopic asthma although a significant population of asthmatic patients are non-atopic. Non-atopic asthmatic patients give negative results in skin prick test for common allergens and they do not have allergen-specific IgE in their serums (Humbert et al., 1999; Walker, 1993). Non-atopic asthma generally starts following a lower airway infection, and its triggers such as exercise, cold air, air pollution and inhaled irritants are the same as those in atopic asthma despite allergens (Barnes, 2009). The clinical symptoms are also similar to allergic asthma and are alleviated with bronchodilators, but higher doses of corticosteroids are needed in the treatment (Barnes, 2009). Eosinophil, mucosal mast cell and T cell numbers (Barnes, 2008; Humbert et al., 1999) and also T-helper-2 (Th2) type cytokines IL-4, IL-5, IL-9 and IL-13 are increased in both atopic and non-atopic asthma (Ying et al., 1999). On the other hand, it has been shown that the macrophage or neutrophil numbers are increased only in non-atopic asthma (Bentley et al., 1992; Groot Kormelink et al., 2009; Humbert et al., 1996).

The experimental models used in non-atopic asthma are very limited. In some studies, it has been shown that low molecular weight substances like picril chloride, toluen diisocyanide and dinitrofluorobenzene (DNFB) can be used to develop experimental non-atopic asthma in mice (Buckley and Nijkamp, 1994; Garssen et al., 1991, 1989; Scheerens et al., 1996). Following skin sensitization, local application of these substances into the airways produces a hapten-induced pulmonary hypersensitivity reaction which is also a type IV delayed hypersensitivity reaction. In DNFB-induced experimental model, acute bronchoconstriction, pulmonary oedema, mononuclear cell infiltration, *in vitro* tracheal hyperreactivity and *in vivo* airway hyperresponsiveness were demonstrated in mice. Furthermore these reactions were not associated with an increase in serum hapten-specific IgE levels. Most of these findings reflect the characteristics of human non-

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atopic asthma (Dearman and Kimber, 1991; Garssen et al., 1991; Kraneveld et al., 2002; Scheerens et al., 1996).

Cannabinoids are biologically active substances, which are found in the plant *cannabis sativa*. There are also endogeneous cannabinoids in mammalian tissues that contribute to various physiological functions. The effects of cannabinoids are mediated by two G protein-coupled seven trans-membrane receptors namely CB_1 and CB_2 (Pertwee et al., 2010). Although both receptors are expressed in the central nervous system and peripheral tissues, CB_1 receptors are mainly located in the neurons where CB_2 receptors are mostly found in the immune related organs like tonsils, spleen, thymus and bone marrow (Braun et al., 2011; Matsuda et al., 1990; Onaivi et al., 2008). The majority of cannabinoid research has been focused on their effects in the central nervous system and immune system. However, cannabinoids have also significant effects in the airways. Earlier studies have shown that smoking marihuana or ingestion of 9-tetrahidrocannabinol (THC) leads to bronchodilation in human airways (Tashkin et al., 1974, 1975). The bronchodilator effect of THC was observed in both healthy volunteers and asthmatic patients at doses that did not affect central nervous system (Hartley et al., 1978; Tashkin et al., 1973; Vachon et al., 1973). In ovalbumin-induced atopic mice models of experimental allergic asthma, cannabinoids have also been shown to exert an inhibitory effect in airway inflammation along with reduced cell count in the bronchoalveolar lavage fluid (BALF) (Braun et al., 2011; Jan et al., 2003).

Cannabinoids have been shown to possess inhibitory effects on inflammatory cell expression in atopic asthma however no data is available for their effects in non-atopic asthma models. Therefore, in the present study, the effect of cannabinoids on airway inflammation was investigated in an experimental non-atopic asthma model. For this purpose tracheal hyperreactivity and BALF cell count were evaluated in DNFB-treated mice.

2. Methods

20–25 g female CD1 mice were used in the present study. All procedures performed in animals were approved by Hacettepe University Animal Research Ethics Committee.

2.1. DNFB-induced experimental asthma model

DNFB was used to induce experimental non-atopic asthma model in mice (Houtman et al., 2003; Kraneveld et al., 2005, 2002; van der Kleij et al., 2003, 2004). For this purpose, at the beginning (day 0) 0.5% DNFB dissolved in acetone:olive oil (4:1) was applied to the shaved thorax of mice and to each paw epicutaneously in a volume of 50 μl under light ketamine/xylazine anesthesia. At day 1, a second epicutaneous application was made only to thorax, and at day 5, 0.6% dinitrobenzene sulphonic acid (DNS), the water soluble hapten of DNFB which was dissolved in PBS (pH:7.2), was applied intranasally in a volume of 50 μl to mice. The control group received vehicle (acetone:olive oil (4:1)) on days 0 and 1, and challenged with intranasal DNS at day 5. 48 h after DNS application mice were killed by cervical dislocation, and their tracheas and lungs were removed in order to evaluate tracheal reactivity and histopathological changes.

2.2. In vivo *cannabinoid agonist treatment*

In order to evaluate the effects of systemic cannabinoid treatment, control and DNFB groups of mice were treated intraperitoneally with either CB_1 receptor agonist ACEA (7.5 mg/kg/day) (n = 7), CB_2 agonist JWH133 (5 mg/kg/day) (n = 6) or their vehicle (n = 6). The treatments were started one hour before DNS application and repeated after 24 h.

2.3. In vitro *tracheal reactivity*

In vitro tracheal reactivity was evaluated in four groups of mice which were control (n = 6), DNFB (n = 7), ACEA-treated DNFB (n = 7) and JWH-treated DNFB (n = 6) groups.

After isolation of the tracheas, the connective tissues were removed and tracheal rings were prepared. The preparations were mounted in organ baths containing Krebs-Heinseleit solution at 37 °C and gassed with 95% O_2-5% CO_2. The change in tension was measured with an isometric force transducer.

The tissues were equilibrated for 1 h under a resting tension of 1 g by washing every 15 min, and cumulative carbachol (10^{-8} to 10^{-4} M) and 5-HT (10^{-8} to 10^{-4} M)-induced contraction responses, and isoprenaline-induced relaxation in precontracted (10^{-7} M carbachol) tracheas were elicited, respectively. One hour of washout period was applied between each response.

In order to evaluate the contribution of cholinergic nerve stimulation and the release of endogeneous acetylcholine in 5-HT-induced contractions, 5-HT response was examined in the presence of muscarinic receptor antagonist atropine (10^{-6} M) (n = 7 for control; n = 8 for DNFB group) and neuronal sodium channel blocker tetrodotoxin (10^{-6} M) (n = 6 for control; n = 7 for DNFB group). 5-HT-induced contraction responses in the presence of antagonists were elicited in separate tracheal ring preparations due to the tachyphylaxis.

In vitro effects of cannabinoid agonists on 5-HT-induced contractions were also tested by incubation the tracheas with either CB_1 agonist ACEA (10^{-5} M) (n = 7) or CB_2 agonist JWH133 (10^{-5} M) (n = 6) in isolated organ baths. After 30 min of incubation with the agonists, the concentration-dependent contraction response to 5-HT was elicited. 5-HT responses in the presence of cannabinoid agonists were obtained in separate tracheal ring preparations due to tachyphylaxis.

Electrical field stimulation (EFS)-induced contraction responses were also elicited in isolated tracheas in order to assess the cholinergic nerve function in DNFB-induced non atopic asthma model in mice. For this purpose EFS (10 mV, 1 ms, 60 s)-induced contractions were evaluated in a frequency-dependent (0.25, 0.5, 1, 5, 10, 20 Hz) manner in control (n = 6) and DNFB (n = 6) group of mice.

2.4. Histopathology

Isolated tracheas and lungs were fixed in 10% formaldehyte for at least 48 h and stained with hematoxylene/eosin. The histopathological changes were evaluated thereafter.

2.5. Leukocyte accumulation in BALF

Bronchoalveolar lavage fluid (BALF) was collected from all four groups (control (n = 5), DNFB (n = 6), ACEA-treated DNFB (n = 6) and JWH-treated DNFB (n = 5) group of mice) in order to examine the inflammatory cell count. 48 h after DNS application mice were anesthetized with ketamin/xylazine (100/10 mg/kg). Then, the trachea was cannulated and lungs were washed gently three times with 1 ml PBS (37 °C). The samples were kept in 4 °C until cytospin preparations were made and leukocyte (neutrophil, eosinophil, mononuclear cell) cell count was performed by randomly counting 300 cells from each sample.

2.6. Statistical analysis

Carbachol-induced contraction response was represented as absolute value and 5-HT contraction was as the percentage of

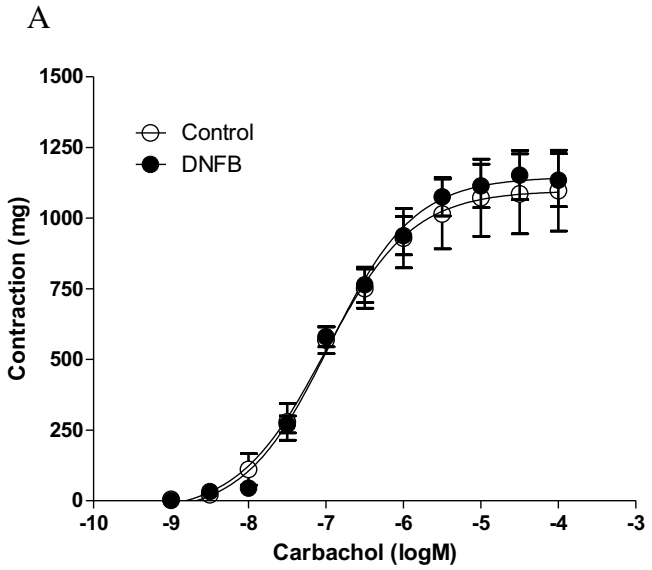

Fig 1. (A) Concentration-dependent contraction responses elicited with carbachol in tracheas isolated from control and DNFB groups of mice. (B) Concentration-dependent contraction response elicited with 5-HT in the absence and presence of atropine in tracheas isolated from control and DNFB groups of mice. Carbachol-induced contractions are expressed as the absolute values and 5-HT-induced contractions were expressed as the percentage of maximum carbachol contraction and represented as mean ± SEM. (*, significantly different from control group, $P < 0.05$).

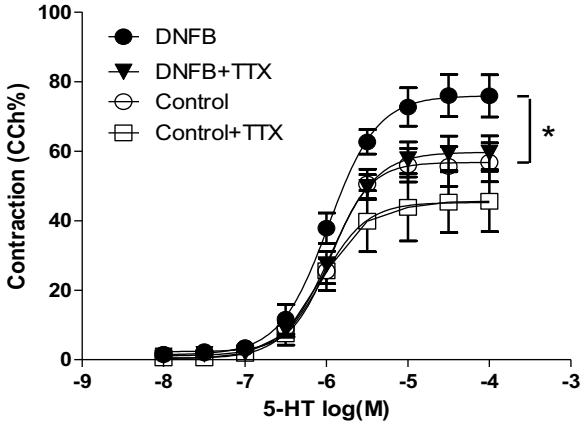

Fig. 2. Concentration-dependent contraction responses elicited with 5-HT after *in vitro* incubation with tetrodotoxin (10^{-6} M) (TTX) in tracheas isolated from control and DNFB groups of mice. Data are expressed as the percentage of maximum carbachol contraction and represented as mean ± SEM. (*, significantly different from control group, $P < 0.05$).

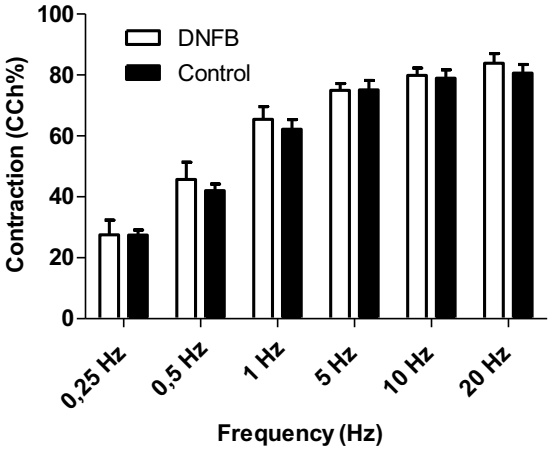

Fig. 3. Frequency-dependent electrical field stimulation-induced contraction responses in tracheas isolated from control and DNFB groups of mice. Data are expressed as the percentage of maximum carbachol contraction and represented as mean ± SEM.

maximum carbachol-induced contraction response. Isoprenaline-induced relaxation response was given as the percentage of 10^{-7} M carbachol-induced precontraction level. All values were represented as mean ± s.e.m. and statistical analysis were performed by using ANOVA/Bonferroni and Student's *t* test. $P < 0.05$ is considered significant.

3. Results

Carbachol (10^{-9} to 10^{-4} M) elicited concentration-dependent contraction responses in tracheal ring preparations isolated from control and DNFB groups of mice which were not significantly different from each other (Fig. 1A). However, 5-HT (10^{-9}-= to 10^{-4} M) – induced contraction response was enhanced in the DNFB group with an E_{max} of 66.79 ± 3.88 when compared to that obtained in the control group (E_{max} = 51.86 ± 3.76) ($P < 0.05$) (Fig. 1B). Incubation with atropine (10^{-6} M) caused a significant decrease in the contractile response to 5-HT in the control group (E_{max} = 29.48 ± 5.43) and inhibited the enhanced contraction in the DNFB group (E_{max} = 31.25 ± 6.54) to the control level (Fig. 1B).

In order to evaluate the neuronal component in the 5-HT response, the effect of sodium channel blocker tetrodotoxin was tested in isolated tracheas. Incubation with tetrodotoxin (10^{-6} M) prevented the enhancement of 5-HT-induced contraction in the DNFB group while causing a slight inhibition of that in the control group (Fig. 2). EFS (10 mV, 1 ms, 60 s)-induced contraction responses were also examined to identify the contribution of cholinergic nerve function in tracheas isolated from DNFB-treated mice. We have demonstrated that frequency-dependent (0.25, 0.5, 1, 5, 10, 20 Hz) contraction responses to EFS were similar in tracheas isolated from both control and DNFB groups of mice (Fig. 3).

Isoprenaline (10^{-9} to 10^{-5} M) induced concentration-dependent relaxation response in carbachol-(10^{-7} M) precontracted tracheal rings isolated from control and DNFB groups of mice and the concentration-response curves elicited were not significantly different from each other (Fig. 4).

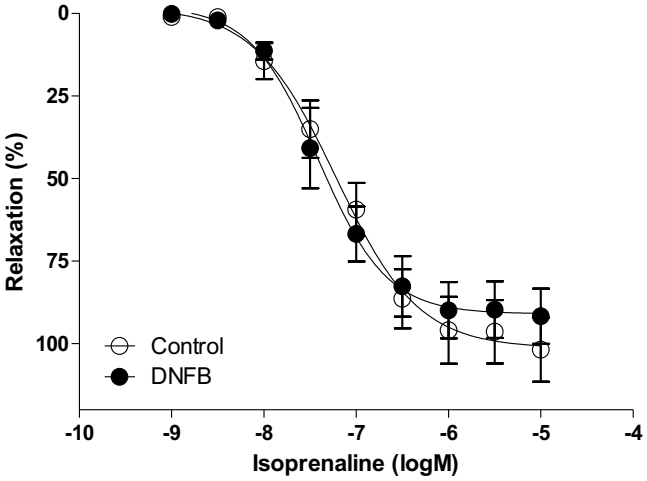

Fig. 4. Concentration-dependent relaxation responses elicited with isoprenaline in tracheas isolated from control and DNFB groups of mice. Data are expressed as the percentage of carbachol (10^{-7} M)-induced precontraction and represented as mean ± SEM.

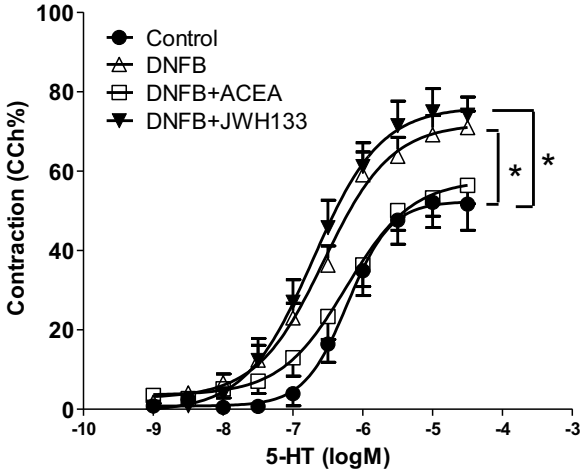

Fig. 5. The effect of *in vivo* ACEA (7.5 mg/kg/day) and JWH133 (5 mg/kg/day) treatment on concentration-dependent contraction responses elicited with 5-HT in tracheas isolated from DNFB group of mice. Data are expressed as the percentage of maximum carbachol contraction and represented as mean ± SEM. (*, significantly different from control group, $P < 0.05$).

The effects of systemic treatment with selective cannabinoid receptor agonists on tracheal reactivity were evaluated after intraperitoneal administration of CB_1 receptor agonist ACEA (7.5 mg/kg/day), CB_2 receptor agonist JWH133 (5 mg/kg/day) and their vehicle to the mice. Treatment with ACEA prevented the increase in 5-HT-induced contraction response in tracheas isolated from DNFB group of mice (Fig. 5). However, JWH133 or vehicle treatment had no effect on the enhanced 5-HT response in the DNFB group (Fig. 5). ACEA and JWH133 treatments did not alter the 5-HT contractions in the control group (data not shown).

The *in vitro* effects of cannabinoid agonists on 5-HT-induced contractions were also examined after incubation of the tracheas with either ACEA or JWH133 in isolated organ baths. Incubation with ACEA (10^{-5} M) inhibited the increase in 5-HT-induced contractions while incubation with JWH133 (10^{-5} M) had no effect in the DNFB group (Fig. 6). 5-HT responses were not affected by incubation with ACEA or JWH133 in the control group (data not shown).

The number of macrophages was increased significantly in the bronchoalveolar lavage fluid obtained from the DNFB group of mice

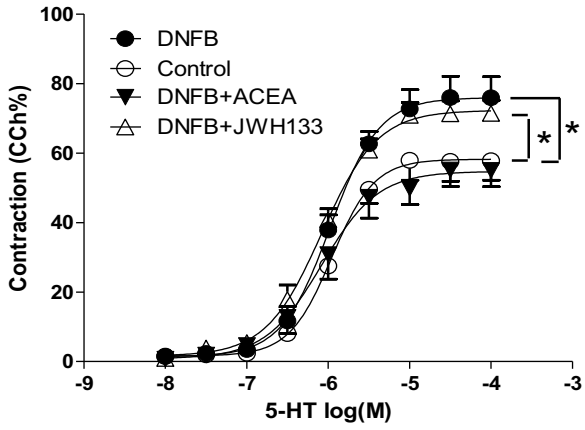

Fig. 6. Concentration-dependent contraction responses elicited with 5-HT after *in vitro* incubation with ACEA (10^{-5} M) and JWH133 (10^{-5} M) in tracheas isolated from DNFB group of mice. Data are expressed as the percentage of maximum carbachol contraction and represented as mean ± SEM. (*, significantly different from control group, $P < 0.05$).

when compared to that of control group (Fig. 7). Furthermore, slight histopathologic changes were observed along with accumulation of inflammatory cells around bronchioli and pulmonary blood vessels in the DNFB group. However, ACEA or JWH133 treatments did not affect the increase in macrophage numbers and histopathologic changes in the DNFB group (Fig. 7).

4. Discussion

Cannabinoids have significant immunomodulatory effects which emphasize their role in inflammation (Berdyshev, 2000; Klein et al., 2000). In the present study, the effects of cannabinoid receptor agonists on airway inflammation and tracheal hyperreactivity were investigated in an experimental model of non-atopic asthma in mice and DNFB model was used for this purpose. DNFB-induced non-atopic asthma has been described in previous studies and shown to produce several features of non-atopic asthma (Bloemen et al., 1996; Dearman and Kimber, 1991; Garssen et al., 1991; Groot Kormelink et al., 2009; Houtman et al., 2003; Kraneveld et al., 2005, 2002; van der Kleij et al., 2003, 2004; van Houwelingen et al., 2002). In the present study, DNFB administration to mice resulted in enhanced contractile response to 5-HT in isolated tracheas and an increase in macrophage number in the BAL fluid. There was also a slight accumulation of inflammatory cells around bronchioli and pulmonary blood vessels of lung sections. However, carbachol-induced contractions remained unchanged in tracheas isolated from DNFB group of mice. This data suggest that DNFB-induced non-atopic asthma causes tracheal hyperreactivity to 5-HT without affecting muscarinic receptor-mediated contractions, and leads to airway inflammation.

It has been reported that 5-HT levels are increased in the blood of asthmatic patients that correlates with the severity of asthma (Lechin et al., 1996). Moreover, it is well established that 5-HT is an important mediator in the airways especially in rodents and causes a significant contraction response (Adner et al., 2002; De Bie et al., 1998; De Swert et al., 2007; Eum et al., 1999; Fernandez-Rodriguez et al., 2010; Zhang et al., 2007). Inflammatory stimuli can affect the responses elicited with 5-HT in the airways of guinea-pig and mouse as it has been shown that incubation of the isolated tracheas with TNF-α or IL-1β up-regulates the 5-HT-induced contractions (Makwana et al., 2012; Zhang et al., 2007). Thus, these findings indicate the role of 5-HT in inflammation-induced airway hyperreactivity. 5-HT is also one of the major mediators in mice mast cells. The significant contribution of mast cells to airway hyperre-

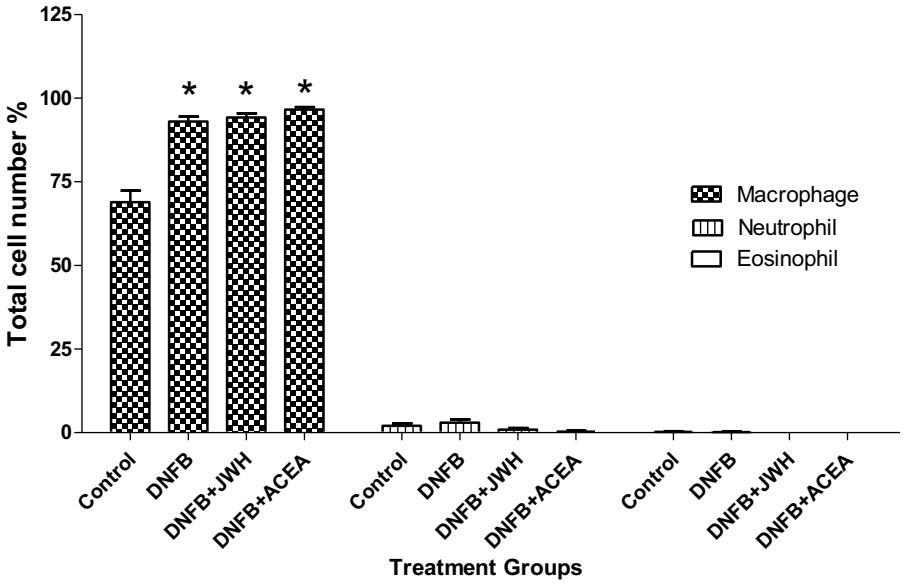

Fig. 7. The effect of *in vivo* ACEA (7.5 mg/kg/day) and JWH133 (5 mg/kg/day) treatment on macrophage, neutrophil and eosinophil numbers in BALF of DNFB group of mice. Data are expressed as the percentage of total cell number and represented as mean ± SEM. (*, significantly different from control group, $P < 0.05$).

sponsiveness and hyperreactivity in DNFB-induced asthma model has also been reported (Kraneveld et al., 2002; van der Kleij et al., 2004; van Houwelingen et al., 2002). Therefore, we suggest that tracheal hyperreactivity to 5-HT in DNFB model of non-atopic asthma is in parallel with the previous studies.

5-HT-induced contraction response has direct and indirect components. The indirect response is shown to be mediated by postganglionic cholinergic nerves in the mouse airways (Eum et al., 1999; Szarek et al., 1995; Van Oosterhout et al., 1991; Weigand et al., 2009). However, Moffatt et al. (2004) also suggests the contribution of epithelial acetycholine release by 5-HT. 5-HT produces its direct effect through 5-HT_{2A} receptors located in the airway smooth muscle whereas 5-HT receptors in the neurons and airway epithelium are mainly 5-HT_{2A} and 5-HT_3 subtypes, and stimulation of these receptors leads to acetylcholine release to elicit contraction response by an indirect effect (De Swert et al., 2007; Eum et al., 1999; Moffatt et al., 2004). In the present study, our finding suggest that enhanced contraction to 5-HT in the DNFB group occurs through its indirect effect since incubation of the tracheal rings with atropine, resulted in the inhibition of 5-HT response to the level elicited in the control group. We have tested the effect of sodium channel blocker tetrodotoxin to further evaluate the neuronal contribution in the 5-HT-induced contractile responses. The inhibitory effect of tetrodotoxin on 5-HT-induced contractions has been demonstrated in mouse trachea previously (De Swert et al., 2007). In our study, tetrodotoxin prevented the enhancement of 5-HT-induced contractions in the DNFB group. Therefore, this data suggest that the neurons in the tracheas of mice from the DNFB group respond more to 5-HT, thus leading to an increase in the acetylcholine release and causing a hyperreactivity to 5-HT. In order to differentiate whether DNFB-induced inflammation leads to a specific up-regulation of 5-HT receptor signaling or to an up-regulation in cholinergic nerve function, frequency-dependent EFS-induced contraction responses were performed in the control and DNFB groups. Our results showed that EFS-induced contractions were similar in both groups and this finding indicated that DNFB-induced inflammation leads to a specific up-regulation of 5-HT receptor signaling rather than an up-regulation in cholinergic nerve function.

In the present study, the effect of cannabinoid treatment on airway inflammation and airway hyperreactivity was evaluated by using selective cannabinoid CB_1 and CB_2 receptor agonists. We showed that *in vivo* treatment with the CB_1 selective agonist ACEA prevented the hyperreactivity to 5-HT in tracheas isolated from DNFB group of mice. However, ACEA treatment did not affect the increase in macrophage number in BAL and inflammation-induced histopathogical changes in lung tissue. CB_2 agonist JWH133 treatment did not alter 5-HT hyperreactivity, increased macrophage number in BAL and histopathogical changes. Our data suggest that selective stimulation of cannabinoid CB_1 receptors prevent the tracheal hypereactivity to 5-HT without altering inflammatory cell count in DNFB-induced non-atopic asthma in mice. Cannabinoids are shown to inhibit airway inflammation and reduce mononuclear cell count in the bronchoalveolar lavage in ovalbumin-induced experimental allergic asthma in mice (Braun et al., 2011; Jan et al., 2003). However, it is not known which receptors are responsible from this effect. Studies in guinea pigs also indicate an anti-inflammatory effect of cannabinoids which seems to be mediated by CB_2 receptors in neurogenic inflammation models (Belvisi et al., 2008; Fukuda et al., 2010; Yoshihara et al., 2005). In the present study the failure of CB_2 agonist treatment to prevent airway inflammation is more likely due to the differences in species used in these studies as well as the differences in experimental airway inflammation models.

The increase in TNF-α level in BALF has been shown in DNFB-induced asthma in mice and has been suggested as the main trigger of the inflammatory airway response and airway hyperreactivity in this model (Kraneveld et al., 2002; van der Kleij et al., 2004). Therefore, in the present study, it seems that *in vivo* ACEA treatment prevented some of the DNFB-induced inflammatory changes related to the initiation of tracheal hyperreactivity to 5-HT without altering the increase in macrophage numbers in BALF.

We have also examined the *in vitro* effects of cannabinoid ligands on 5-HT-induced contractions in tracheas isolated from either control or DNFB group of mice. Incubation with ACEA prevented the enhancement of 5-HT-induced contraction response in a similar manner as it did after *in vivo* treatment in the DNFB group of mice. Cannabinoids are shown to inhibit nerve mediated smooth muscle contractions in different tissues. It has been reported that neurogenic contractions in isolated rat trachea (Yousif and Oriowo, 1999) and isolated gastrointestinal smooth muscle preparations (Hinds et al., 2006; Mule et al., 2007) were inhibited by cannabinoid lig-

ands. In a recent study, it has been also shown that CB_1 receptor stimulation inhibited the nerve mediated cholinergic contractions in isolated human airways (Grassin-Delyle et al., 2014). The prevention of enhanced 5-HT contraction by ACEA like tetrodotoxin and atropine in DNFB-induced asthma in our study is in accordance with these data in the literature, and thus, suggests a presynaptic cholinergic inhibitory effect of cannabinoids through CB_1 receptors.

In summary, our data suggests that DNFB-induced experimental model of non-atopic asthma in mice leads to 5-HT-induced tracheal hyperreactivity which is probably due to the increased neuronal acetylcholine release, and is resulted in an increase in macrophage number in BALF. Among the selective cannabinoid agonists used, *in vivo* treatment with CB_1 agonist ACEA prevented the tracheal hyperreactivity, and neither ACEA nor CB_2 agonist JWH133 affected the increase in macrophage number in BALF. The information about the effects of cannabinoids in airway inflammation and asthma is very limited, and there is even no data for their effect in non-atopic asthma in the literature. Therefore, depending on our data, we can conclude that CB_1 receptor agonists may be a new therapeutic agent in the treatment of airway hyperreactivity in non-atopic asthma.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

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