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The role of endothelin pathway in modulation of airway reactivity to methacholine in C57Bl/6 and BALB/c mice

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ABSTRACT

Endothelin peptides have been shown to increase cholinergic neurotransmission in the airway. Genetic differences in airway responsiveness to methacholine where reported in mice. The present study compared the airway reactivity to methacholine in C57Bl/6 and BALB/c mice, the involvement of endothelin on this reactivity and endothelin levels in lung homogenates. Whole airway reactivity was analyzed by means of an isolated lung preparation where lungs were perfused through the trachea with warm gassed Krebs solution at 5 ml/min, and changes in perfusion pressure triggered by methacholine at increasing bolus doses (0.1–100 μg) were recorded. We found that the maximal airway response to methacholine was much greater in C57Bl/6 than in BALB/c (Emax 34±2 vs 12±1 cmH_2O, respectively). Bosentan (mixed endothelin A/B receptor antagonist; 10 mg/kg, i.p., 30 min before sacrifice) reduced lung responsiveness to methacholine in C57Bl/6 (58% at EC50 level) but had no effect in BALB/c mouse strain. This effect seems to be mediated by the endothelin ET_A receptor since it was significantly reduced by the selective endothelin ET_A receptor antagonist, BQ 123. Immunoreactive endothelin levels were higher in C57Bl/6 than in BALB/c lungs (43±5 vs 19±5 pg/g of tissue). In conclusion, airway reactivity to methacholine and lung endothelins content varies markedly between C57Bl/6 and BALB/c strains. Endothelins upregulate lung responsiveness to methacholine only in C57Bl/6, an effect achieved through the endothelin ET_A receptor.

1. Introduction

Endothelins are a family of peptides (i.e., ET-1, ET-2, ET-3) with a variety of biological activities in both vascular and non-vascular tissues. One of the important properties of endothelins is smooth muscle contraction which allows them to play important roles in the pathogenesis of airway diseases (Polikepahad et al., 2006). The levels of endothelins in the lung tissue are particularly high compared to other tissues. Within the lung endothelins are synthesized by the epithelial cells of the airway, endothelial cells and inflammatory cells. Receptors for endothelins are found throughout the respiratory tract in humans and animals, in general localized in pulmonary blood vessels, airway smooth muscle, and sensory nerves (reviewed by Filep et al., 1993; Goldie and Henry, 1999).

Several studies conducted by Fernandes, Henry and Goldie some years ago, clearly demonstrated that endothelins modulate cholinergic neurotransmission in human, rat and mouse airway tissues (Henry and Goldie, 1995; Fernandes, 1996). In these studies, airway cholinergic responses induced by electric field stimulation were markedly increased by addition of endothelin-1 (Fernandes et al., 1999). This effect was mediated by enhanced acetylcholine release by endothelin-1, at least in the trachea (Knott et al., 1996). It is discussed that pre-junctional endothelin receptors are responsible for the enhanced cholinergic airway response to electric field stimulation.

It has been suggested that the baseline tone of the airways is dependent on the activity of cholinergic nerves. Genetic differences among mouse strains relative to basal lung functions and airway responsiveness were reported in mice (Held and Uhlig, 2000).

In the present study we compared the lung responsiveness to methacholine, the contribution of endogenous endothelins to this response and the basal lung levels of endothelins in C57Bl6 and BALB/c mouse strains.

2. Materials and methods

2.1. Animals

Male C57Bl/6 and BALB/c mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with 12 h light–dark cycle with water and food *ad libitum*. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA)

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and approved by the Biomedical Sciences Institute/USP—Ethical Committee for Animal Research (CEEA). The animals were killed by injection of ketamine/xylasine (50 µl of a 100 mg/ml solution, i.p.).

2.2. Determination of immunoreactive endothelin

Lungs were homogenized with a Polytron homogenizer (20 s) in 2 ml of phosphate buffer pH 7.2 and the samples were centrifuged (3000 ×*g*, 15 min). The concentration of endothelin in lung tissue was measured by specific ELISA—(Endothelin-1 EIA kit, Cayman Chem., USA) after extraction. The antiserum had 100% cross-reactivity with endothelin-1, endothelin-2 and endothelin-3, 0.1% cross-reactivity with human big-endothelin-1. The extraction of endothelins was performed using SepPak C18 cartridges as follows: the SepPak columns were equilibrated with 1 ml methanol and 1 ml water. To each ml of the sample 0.25 ml HCl (2 M) was added and the acidified samples were then applied to the cartridges. After washing with 10 ml aqueous trifluoroacetic acid (TFA; 0.1%,v/v), the endothelin was eluted with 3 ml methanol:water:TFA (90:10:0.1). After that the samples were evaporated under nitrogen and re-suspended in EIA buffer.

2.3. Evaluation of airway reactivity

Whole airway reactivity was measured as previously described (Landgraf et al., 2003). Mice received injection of ketamine/xylasine (50 µl of a 100 mg/ml solution, i.p.), the peritoneal cavity was cut open and animals were exsanguinated by section of the abdominal aorta. The thoracic cavity was then opened; the pulmonary artery was cannulated and perfused with 10 ml Kreb's solution at 10 ml/min. A cannula was then inserted in the trachea; the lungs were removed carefully and perfused (5 ml/min) through the trachea with Kreb's (37 °C, 95% O_2 and 5% CO_2) solution. A small incision was made in the lower end of each lobe to permit the outflow of the solution. The perfusion pressure was recorded in a Beckman R511A using Gould P23DB pressure transducers. Increases over basal levels of perfusion pressure following bolus injection of methacholine were taken as a measure of constriction of the airways. Increase in perfusion pressure (cmH_2O) as a function of the dose of methacholine (µg) was measured for the entire recording period. Areas under the curve were calculated and results were expressed as mean area under the curve (mm^2).

2.4. Drug treatments

The antagonists used in this study were injected intraperitoneally 30 min before the mice were killed and lungs removed. Bosentan (mixed endothelin ET_A and ET_B receptor antagonist) was dissolved in double distilled water and used at a dose of 10 mg/kg. The endothelin ET_A receptor antagonist, BQ 123 (Cyclo(D-Asp-Pro-D-Val-Leu-D-Trp, Na)) was dissolved in PBS with 20% DMSO to obtain a 10 mg/ml stock solution and used at 1 mg/kg. The endothelin ET_B receptor antagonist, BQ 788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-1-methoxycarbonyl-Trp-D-Nle) was dissolved in PBS with 5% DMSO to obtain a solution of 1 mg/ml and used at 1 mg/kg. DMSO has to be used because these antagonists are insoluble in aqueous solution at this concentration according to Brochu et al. (2002). The concentration of the antagonists and the time of pre-treatment were chosen from previous studies (Ihara et al., 1992; Bazil et al., 1992; Ishikawa et al., 1994; Teixeira et al., 2004).

2.5. Drugs and reagents

Physiological solutions were prepared with salts of analytical grade in double distilled water. Endothelin-1 and BQ 788 were purchased from American Peptide (Sunnyvale, CA, USA); Endothelin-1 EIA kit from Cayman Chemical (Ann Arbor, MI, USA); Trifluoroacetic acid from Burdick & Jackson (Muskegon, MI, USA); SepPak Cartridges from Waters Corporation (Milford, MA, USA) and methacholine from Sigma (St. Louis, MO, USA). BQ 123 was synthesized in the Department of Pharmacology, University of Sherbrooke, Canada and kindly supplied by Dr. Pedro D'Orléans Juste. Bosentan, a non-selective endothelin receptor antagonist was supplied by Dr. Giles A. Rae.

2.6. Statistical analysis

Data are expressed as the means ± S.E.M. Statistical analysis of the data was carried out by analysis of variance (ANOVA) and sequential analysis of differences among means was done by Tukey's contrast analysis. A P value lower than 0.05 was considered to be significant.

3. Results

3.1. Airway reactivity to methacholine and role of endothelins

To measure airway reactivity, lungs of mice were removed, perfused with physiologic solution through the trachea and a small incision was made in the lower end of each lobe to permit the outflow of the solution. The airway reactivity was measured as increases in perfusion pressure over basal levels. The basal perfusion pressure of C57Bl/6 (10.80 ± 0.33) was similar than that of BALB/c (10.28 ± 0.57).

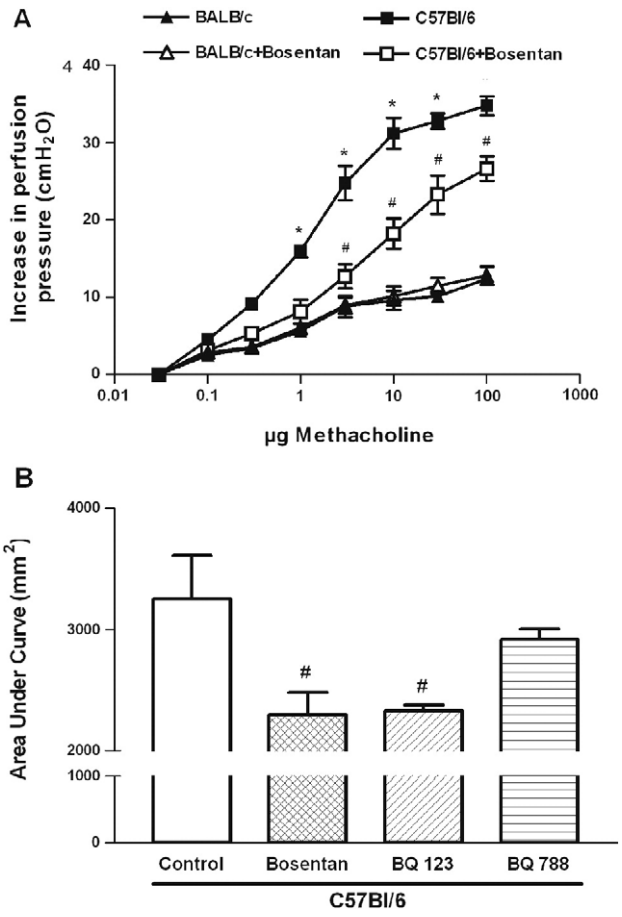

Fig. 1. Reactivity to methacholine of C57Bl/6 and BALB airways. The lungs were removed, perfused via the trachea and increases in perfusion pressure to bolus injection of methacholine were recorded. In A is shown the effect of treatment with Bosentan (10 mg/kg) for 30 min on the dose–response curve to methacholine. Results are expressed as increase in perfusion pressure ± S.E.M. In B is shown the effect of treatment of C57Bl/6 mice with BQ132 or BQ788 (1 mg/kg, i.p., 30 min before the animals were killed). The results were recorded as increase in perfusion pressure and expressed as mean area under the curve ± S.E.M). $n = 5–8$ animals/group. * $P < 0.001$ comparing C57Bl/6 with BALB/c and # $P < 0.05$ comparing the treated group with the non-treated groups of each strain.

Injection of methacholine (0.1 to 100 μg) into the airway increased dose-dependently the perfusion pressure.

Fig. 1A shows that the reactivity to methacholine of the C57Bl/6 mouse airway was significantly higher than that of the BALB/c mouse (around 203% higher comparing areas under the curves). The maximal response of C57Bl/6 lungs to methacholine was much greater than that of BALB/c lungs (Emax 34±2 vs 12±1 cmH_2O, respectively).

This figure also shows that the airway reactivity to methacholine was significantly reduced (58% at the EC50 level) in C57Bl/6 mice that were pre-treated i.p. with the non-selective antagonist of endothelin receptors (Bosentan, 10 mg/kg, 30 min) whereas in BALB/c mice this treatment had no effect on airway reactivity to methacholine.

We then treated C57Bl/6 mice (i.p. for 30 min) with the selective endothelin ET_A receptor antagonist, BQ-123 (1 mg/kg) or with the endothelin ET_B receptor antagonist, BQ-788 (1 mg/kg). In Fig. 1B the results of the dose–response curves are expressed as area under the curve (mm^2). We can see that the response to methacholine was significantly reduced (56% at the EC50 level) in the group treated with BQ-123 compared with the non-treated group (3035 vs 2330 mm^2). Pre-treatment with the BQ-788 did not significantly affect the airway reactivity to methacholine.

3.2. Endothelin levels in lung tissue

The concentration of endothelin in homogenates of lung tissue was measured by ELISA as described in Materials and methods. Fig. 2 shows that the concentration of endothelin was significantly higher in C57Bl/6 than in BALB/c mice (42.9 vs 18.6 pg endothelin/g lung). Perfusion of the airway with methacholine had no effect on the basal lung levels of endothelin. Pre-treatment of the mice with the non-selective antagonist of endothelin receptors (Bosentan, 10 mg/kg), for 30 min significantly reduced (56%) the concentration of endothelin in the lung of C57Bl/6 mice but not in BALB/c mice. These results suggest a turnover of endothelin synthesis and degradation in the lung, where endothelins exert a positive feedback on their synthesis in C57Bl/6 mice.

3.3. Airway reactivity to endothelin-1 and effect of endothelin receptor antagonists

We then measured the responsiveness of C57Bl/6 airway to endothelin-1. Fig. 3 show that a bolus injection of a single dose of endothelin-1 (50 μg) induced airway contraction as assessed by the increase in perfusion pressure. The response to this dose of endothelin-1

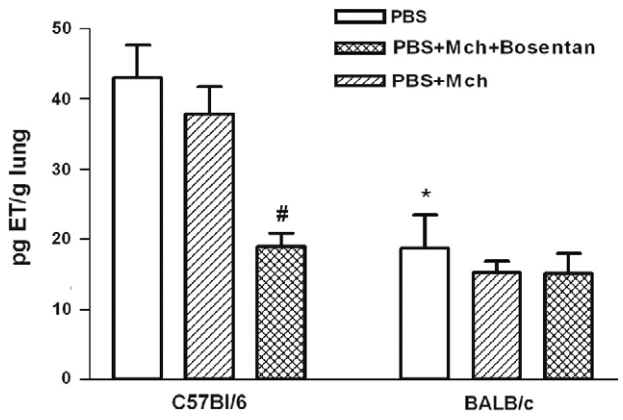

Fig. 2. Concentration of endothelin in lung homogenates. The concentration of endothelin in whole lung homogenates from C57Bl/6 and BALB/c mice was measured by ELISA, 30 min after addition of methacholine (10 mg/ml) or methacholine (10 mg/ml) plus Bosentan (10 mg/kg, i.p., 30 min before killing). Results are expressed as pg endothelin/g lung±S.E.M of 5–8 animals group.* $P<0.05$ comparing C57Bl/6 and BALB/c, # $P<0.05$ comparing the non-treated with the Bosentan treated groups of each strain.

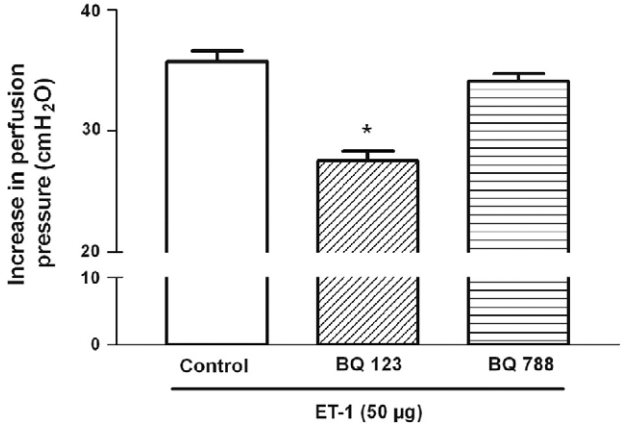

Fig. 3. Airway reactivity to endothelin–1. Groups of C57Bl/6 mice were treated with the selective endothelin ET_A (BQ 123) or endothelin ET_B (BQ 788) receptor antagonists (1 mg/kg, i.p., 30 min before the animals were killed). The lungs were removed, perfused via the trachea and increases in perfusion pressure to bolus injection of endothelin (50 μg) were recorded. Results are expressed as increase in perfusion pressure (cmH_2O)±S.E.M of 5–8 animals group. * $P<0.05$ comparing the mouse treated group with the non-treated group.

was equivalent to 100 μg of methacholine. Groups of mice were treated *in vivo* with BQ 123 or BQ 788, for 30 min before lung isolation. The selective endothelin ET_A receptor r antagonist, BQ 123, decreased significantly the airway response to endothelin-1 (from 35.7 to 27.5 cmH_2O). However, the selective endothelin ET_B receptor antagonist, BQ 788, had no significant effect on endothelin-1 induced airway contraction.

4. Discussion

This study showed that the basal airway response to methacholine was much greater in C57Bl/6 than in BALB/c mice. Treatment of mice with Bosentan (mixed endothelin A and B receptor antagonist) reduced the lung responsiveness in C57Bl/6 without affecting the BALB/c. The airway responsiveness to methacholine in C57Bl/6 mice is partially mediated by endothelin ET_A receptor since it was significantly reduced by the selective endothelin A antagonist and not by the endothelin B antagonist. The airway of C57Bl/6 mouse responds to endothelin-1 and this response is through endothelin ET_A receptor. The concentration of endothelin in homogenates of lung tissue from C57Bl/6 is significantly higher than in lungs from BALB/c mice.

Genetic differences among mouse strains relative to basal lung functions and airway responsiveness have been previously reported (Held and Uhlig, 2000). However, strain differences in endothelin basal lung levels were not reported before. It is noteworthy that treatment of mice with the mixed endothelin ET_A and ET_B receptor antagonist Bosentan, reduced the lung concentration of endothelin in C57Bl/6 but not in BALB/c. This result is indicative of a continuous turnover of endothelins synthesis/degradation in lungs which is regulated by endothelins. This only occurs in the strain presenting high levels of endothelin, suggesting that a certain level of endothelin is required for this feedback to occur.

The response of the airway to methacholine was also strikingly different among the mouse strains; C57Bl/6 showed significantly higher responsiveness than BALB/c. This contrasts with the data presented by Held and Uhlig (2000) where C57Bl/6 was less responsive to methacholine than BALB/c. However, the experimental conditions employed in their study were much different from ours: they measured pulmonary insufflation pressure in anesthetized and mechanically ventilated mice whereas we measured the perfusion pressure in isolated lungs perfused through the trachea. We have used this technique in previous publications as a measure of airway hyperreactivity in an asthma model (Landgraf et al., 2003, 2004).

The response of C57Bl/6 to methacholine was clearly reduced by Bosentan suggesting that methacholine induces endothelin release which then act on endothelin receptors in the lung amplifying the response to methacholine. This was not observed in BALB/c mice. It has been demonstrated that endothelins, acting on pre-junctional receptors present in nerves, induces acetylcholine release upregulating the cholinergic nerve-mediated contraction of the airway (Henry and Goldie, 1995; Fernandes et al., 1999). In their study, addition of endothelin-1 upregulated the cholinergic contraction of the airway induced by electric field stimulation in several species including mouse. Thus we speculate that this phenomenon is responsible for the increased responsiveness to methacholine observed in C57Bl/6 mice. Richter et al. (2007) have described recently that endothelin-1 increases airway reactivity to carbachol in mouse isolated lung preparations which is in accordance with our results. Our results show in addition that the upregulation of airway responsiveness to methacholine is partially mediated by endothelin ET_A receptor since it was significantly reduced by BQ 123 but was not affected by the endothelin ET_B antagonist. Infusion of ET-1 into the airway caused contraction also mediated via endothelin ET_A receptor. The concentration of the ET_A and ET_B antagonists and the time of pre-treatment were chosen from previous studies (Ihara et al., 1992; Bazil et al., 1992; Ishikawa et al., 1994) The concentration of Bosentan was taken from previous work from our laboratory where this dose was effective to inhibit the lung hemorrhage induced by immune complexes in rats (Teixeira et al., 2004).

We speculate that perfusion of the airways with methacholine will induced endothelins release by the epithelial cells of the airway mucosa, and some will diffuse through the sub-mucosa and stimulate endothelin receptors in cholinergic nerves to release acetylcholine. The combination of this effect with the contraction induced by methacholine acting on its receptors in smooth muscle would account for the increased airway responsiveness observed in C57Bl/6 mice. However, the fact that the endothelin antagonists did not reduce the contraction down to the level observed in BALB/c, indicates that the difference between the two strains is only partially dependent on endothelin. Furthermore, in our experiments infusion of methacholine did not increase the concentration of endothelins over the basal levels. The basal levels were measured in whole lung homogenates and we think that with the infusion of methacholine into the airways even small amounts of endothelins released close to the cholinergic nerves would be sufficient to upregulate the cholinergic transmission.

It has been suggested that in asthmatics there is an increase in cholinergic drive which contributes to airway obstruction (Ward et al., 1981; Sheppard et al., 1982). This was attributed to dysfunctional M2 cholinergic receptor in asthmatics which is responsible for the negative feedback on acetylcholine release. Fernandes et al. (1999) have suggested that endothelins increase cholinergic contraction in human bronchus (induced by electric field stimulation) acting on pre-junctional endothelin receptors.

The two mouse strains have been extensively used in asthma research because they differ in the intensity of some asthma symptoms. The increased responsiveness to methacholine that develop in sensitized mice upon antigen challenge, is markedly strain dependent (reviewed by De Sanctis et al., 2001). We showed here that these mice also differ in airways reactivity to methacholine under non-disease conditions as well as in basal lung endothelin levels. We discussed here that endothelins modulate cholinergic neurotransmission under physiological conditions to maintain the baseline tonus of the airway. It is plausible to speculate that in situations of lung diseases such as in asthma, chronic lung inflammation could interfere with these homeostatic interactions facilitating airway obstruction.

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