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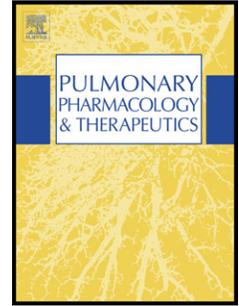
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**LOCAL AND SYSTEMIC EFFECTS OF ANGIOTENSIN RECEPTOR BLOCKADE
IN AN EMPHYSEMA MOUSE MODEL**

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Running title: ARB in an emphysema mouse model

Abstract

Objectives: COPD with emphysema causes marked neurohumoral activation. Angiotensin II receptors are highly expressed within the lung and interfere with mechanisms involved in the progression of emphysema. This study examined the effects of an angiotensin II receptor blocker (ARB) on pulmonary and systemic manifestations of emphysema in a mouse model.

Methods: Female NMRI mice received five intratracheal instillations of porcine pancreatic elastase (emphysema; n=11) or phosphate-buffered saline (PBS; n=4). Emphysema severity was quantified histologically by mean linear intercept, exercise tolerance by treadmill running distance, and lung biomechanics by compliance. Following emphysema induction, 6 mice were treated with the ARB irbesartan for 8 weeks, while 5 mice receiving standard food served as controls.

Results: Following emphysema induction, mean linear intercept was higher in elastase-treated than in PBS-treated lungs ($103.0 \pm 6.2 \mu\text{m}$ vs. $35.0 \pm 0.6 \mu\text{m}$; $p = 0.043$) while running distance was shorter in emphysema mice ($418.6 \pm 83.5 \text{ m}$ vs. $906.6 \pm 244.6 \text{ m}$, $p = 0.028$). Irbesartan-treated emphysema mice showed a lower mean linear intercept ($90.8 \pm 3.8 \mu\text{m}$ vs. $121.5 \pm 8.1 \mu\text{m}$; $p = 0.005$), improved compliance ($163.6 \pm 55.9 \mu\text{l/cmH}_2\text{O}$ vs. $354.4 \pm 72.5 \mu\text{l/cmH}_2\text{O}$; $p = 0.063$) and greater running distance (p ANOVA = 0.015) compared to emphysema mice receiving standard food.

Conclusions: The ARB irbesartan elicits encouraging beneficial effects on emphysema severity, lung biomechanics and exercise capacity in an emphysema mouse model. These findings might help to understand the corresponding positive effects of angiotensin II receptor blockade noticed in patients with COPD.

Key words: emphysema, animal model, angiotensin, neurohumoral activation

1. Introduction

Several interacting mechanisms contribute to the pathogenesis of chronic-obstructive pulmonary disease (COPD) [1]. While the inhalation of cigarette smoke causes an influx of inflammatory cells, pathophysiological processes include an imbalance between proteolytic and anti-proteolytic activity within the lung [2]. Recent perspectives [1, 3] highlight the dysbalance of apoptosis and replenishment in the destruction of lung tissue leading to progressive emphysema. Emphysema is characterized by abnormal, permanent enlargement of airspaces distal to the terminal bronchioles, accompanied by destruction of their walls [4]. Far more smokers develop emphysema than symptomatic COPD [5]. In fact, in patients with normal initial forced expiratory volume in one second (FEV_1) values, lung hyper-inflation as detected by quantitative CT scanning is a strong predictor of a rapid subsequent FEV_1 decline [6].

Although COPD and emphysema primarily affect the lungs, it has become clear that systemic effects of the disease significantly contribute to its severity and mortality [2, 7]. These systemic effects are related to tobacco smoke, chronic inflammation, oxidative stress as well as muscle and cardiovascular dysfunction [7-9]. Additionally, recent human and animal data demonstrate that COPD causes marked activation of the renin-angiotensin system and the sympathetic nervous system [10-14]. This maladaptive activation may well have negative consequences such as impaired exercise tolerance or cachexia in COPD [13, 15].

Interestingly, angiotensin II receptors are highly expressed within the lungs [16] and control alveolar epithelial cell apoptosis [17] and lung fibroblast growth [18]. Angiotensin II appears to be a crucial mediator of lung injury and apoptosis [7, 19]. Angiotensin receptor blockers (ARB) not only mitigate the cellular effects of Angiotensin II, but also reduce sympathetic activity [20, 21].

Preliminary clinical data point towards positive effects of ARB in patients with COPD, but the underlying mechanisms are poorly understood [7, 15, 22, 23]. A recent 4-week pilot study reported that treatment of stable COPD patients with the angiotensin converting enzyme (ACE) inhibitor enalapril moderately increased maximum work rate and peak oxygen pulse while the ventilatory response to exercise was unaltered [24]. We recently developed an emphysema mouse model exhibiting important systemic manifestations of the disease [14]. We thus set up a pilot study using this model to evaluate the effects of ARB on emphysema, lung compliance, running distance, weight loss and neurohumoral activation.

2. Methods

2.1 Study protocol

The study comprised two phases: In the first phase (day 1 to day 48; 'induction phase'), emphysema was induced by repetitive intratracheal instillation of porcine elastase (emphysema group; n = 11); animals in the control group received phosphate-buffered saline solution (control group, n = 4). As in our previous study [14], we used five instillations, each separated by a week of recovery. The last instillation was followed by a three-week recovery period after which the study entered its second phase ('treatment' phase). Starting on day 49, animals in the control group and half of the emphysema group (n = 5) continued to receive a standard diet. The remaining 6 animals in the emphysema group were provided with food containing irbesartan to achieve a dose of 50 mg/kg body weight per day. The two study phases comprised a total study duration of four months. Functional measurements were carried out on day 105 (i.e. week 8 of the treatment phase), and mice were sacrificed and phenotyped thereafter. The study protocol is outlined in **Figure 1**. Ethics approval for this study was obtained from The Bezirksregierung Braunschweig, Dezernat 604 - Tierschutz (Proposal no. 33.42502-061/06G30.02).

2.2 Experimental animals and emphysema induction

As previously described [14], female NMRI mice were used (body weight 20-25 g, Harlan-Winkelmann, Borcheln, Germany), assuming this strain would exhibit better tolerance toward the acute toxic effects of intratracheal elastase administration as compared to, e.g., mice from the C57 background [25]. For elastase instillation, mice were anaesthetized with isoflurane, intubated orotracheally and ventilated via a ventilator/respirator (Mini-Vent Small Animal Ventilators, Harvard Apparatus/Hugo Sachs Elektronik, March-Hugstätten, Germany). Experimental animals received 3.3 U/100 g body weight porcine pancreatic elastase (Sigma-Aldrich, Taufkirchen, Germany) dissolved in 50 µl phosphate-buffered saline solution (PBS). Healthy control animals received 50 µl of PBS. The respective solution was administered intratracheally via a tube followed by 200 µl of air for an even distribution of the liquid throughout both lungs.

2.3 Food preparation

Irbesartan is an angiotensin II receptor blocker which is currently approved for the treatment of arterial hypertension. Drug supply for the present study was provided by an unrestricted grant from Sanofi-Aventis (formerly Sanofi-Synthelabo), Paris, France. Before starting the experiments, average daily food consumption of the 15 experimental animals was determined to be 4.5 g per mouse. In order to achieve a daily uptake of 50 mg irbesartan per kg (i.e. 1.5 mg per 30 g), irbesartan concentration needed to be 1.5 mg per 4.5 g standard food. Food preparation involved macerating pellets weighing 900 g in plain water after which 300 mg irbesartan were added. Before drying, the homogenised food mass was broken up in small pellets.

2.4 Body weight, norepinephrine concentration, and exercise tolerance

Body weight was determined daily throughout the induction and treatment phase until day 105. At baseline, at the end of the induction phase as well as at the end of the treatment phase, each mouse was placed in a metabolic cage (Tecniplast GmbH, Wilhelmshaven, Germany) for 24 hours, provided with food and water ad libitum. Urine was collected, acidified and stored at -70°C until determination of norepinephrine (NE) concentration [14]. NE was measured by high-performance liquid chromatography and normalised to urinary creatinine.

For the quantification of exercise tolerance, treadmill tests were performed using a motorized rodent treadmill equipped with a shock-plate incentive (Exer-6M Open Treadmill, Columbus Instruments, Columbus, Ohio, USA) as previously described [14]. Exercise tests were performed at baseline, at the end of the induction phase as well as eight weeks after starting irbesartan and control food, respectively.

2.5 Organ preparation and assessment of functional lung parameters

At the end of the second study phase, organ preparation and measurement of lung function parameters for the calculation of lung compliance were performed. Mice were anaesthetized deeply and their chest opened. Blood was collected by cardiac puncture in order to avoid congestion of the lungs. A tracheal incision was performed facilitating the insertion and fixation of a blunted cannula which was used to determine lung compliance as previously described [14]. Briefly, lungs were inflated via the cannula twice at a rate of 15 ml/h to a maximum airway pressure. We used a maximum airway pressure of only 12.5 cm H₂O, since

higher pressure lead to over distension of the lung and a substantial amount of elastic hysteresis. The tubing system was then opened to the atmosphere. Volumes and pressures were digitally recorded and served as basis for the calculation of lung compliance ($\mu\text{l}/\text{cmH}_2\text{O}$) and elastic recoil.

The time constant τ [tau] of spontaneous deflation of the lungs against a constant afterload was determined. For this purpose, the lungs were inflated to a maximum pressure of 12.5 cm H_2O and the tubing system was suddenly opened to the atmosphere, with the resistance of a 26-G injection cannula (12.5 mm in length) serving as afterload. The pressure vs time data was fitted with a first-order exponential decay function following the equation ($P = P_0 e^{-(t-t_0)/\tau}$), with P representing current pressure, P_0 initial pressure, t current time, t_0 initial time, and τ the time constant for the pressure decline. Higher values of τ indicate a reduced elastic recoil. Afterwards, the right lung was kept in 10% formalin fixation liquid at a constant pressure of 12,5 cm H_2O for at least 24 hours, followed by histological assessment.

2.6 Histological analysis

The fixed lungs were embedded in paraffin and 5- μm sections were cut as described before [14]. Tissue sections were stained with haematoxylin and eosin. A computerized microscope equipped with a high-resolution videocamera (BX 51, Olympus, Tokyo, Japan, magnification 10x) was used for morphometric analysis. In each histological slice, 10 sagittal sections spanning the entire lung and containing apical as well as basal areas of the organ were randomly selected. The mean linear intercept (Lm) as indicator of air space size was calculated from counting lines of defined length as previously described [26]. Briefly, the lines were randomly placed on every of the 10 lung sections and the number of intercepts crossing the lines counted. The mean linear intercept is calculated from the length of the lines multiplied by the number of the lines divided by the sum of all counted intercepts.

2.7 Gene expression analysis

In order to demonstrate effective suppression of angiotensin II signalling by irbesartan, renal renin mRNA expression was determined [27]. For this purpose, kidneys were frozen at the time of organ preparation. Subsequently, total RNA was isolated from the frozen kidneys as described by Chomczynski and Sacchi [28]. Real-time PCR was performed using a Light Cycler Instrument (Roche Diagnostics Corp.) and the QuantiTect SYBR Green PCR kit

(Qiagen), with β -actin as a control. For each mouse kidney, the ratio of renin mRNA to β -actin mRNA was calculated.

2.8 Statistical analysis

Analysis was performed by authors (LL, TR, CD, HK) blinded to the animal group and time-point of data collection. All variables are given as mean \pm SEM if normally distributed. For comparisons between different groups as well as for longitudinal comparisons within groups, unpaired and paired T-tests were carried out, respectively. Repeated-measures analysis of variance (ANOVA) with time as within-groups factor and the time-by-treatment interaction as indicator of differential changes in two groups was used to analyze the effects of elastase instillation (phase one; comparison between control and emphysema animals) and irbesartan (phase two; comparison within the emphysema group). All tests were two-tailed; significance levels were set to 5%.

3. Results

3.1 Induction phase – functional parameters

Despite repetitive elastase instillations, mice in the emphysema group ($n = 11$) experienced a significant weight gain (from 23.8 ± 0.8 to 29.5 ± 1.1 g; $p < 0.001$). This was comparable to the weight gain observed in the control group ($n = 4$; from 23.0 ± 0.1 to 27.1 ± 0.5 g; $p = 0.004$). ANOVA revealed that there was no significant difference between weight change over time in both groups ($p = 0.153$).

Similarly, a decrease in running distance was noted in both groups. However, this did not reach statistical significance in the control group (from 1358.5 ± 167.7 to 906.6 ± 244.6 m; $p = 0.101$) whereas it was significant in the emphysema group (from 1380.9 ± 167.1 to 418.6 ± 83.5 m; $p < 0.001$). ANOVA showed a significant difference between the two groups ($p = 0.046$).

Catecholamine concentration decreased in the control group (from 24.1 ± 3.1 to 17.1 ± 1.8 $\mu\text{g/l/mg}/100$ ml creatinine, $p = 0.139$) but increased in the emphysema group (from 16.9 ± 1.5 to 27.2 ± 5.2 $\mu\text{g/l/mg}/100$ ml creatinine, $p = 0.086$), but both changes were non-significant. However, time-treatment interaction as evaluated by ANOVA showed a borderline significant effect ($p = 0.053$).

3.2 Treatment phase – renin expression

The renin/ β -actin mRNA ratio was significantly elevated in mice treated with irbesartan (0.98 ± 0.21) as compared to emphysema mice receiving control food (0.10 ± 0.02 ; $p = 0.004$).

3.3 Treatment phase – functional parameters

During the second phase of the study, body weight continued to increase in both the emphysema-control ($n = 5$) and the emphysema-irbesartan ($n = 6$) group. This weight change was more pronounced in the irbesartan group (3.2 ± 0.9 vs. 1.3 ± 0.5 g), although the difference was not significant (p ANOVA = 0.127).

A significant difference in exercise tolerance between the two groups was observed during the treatment phase (see **Figure 2**): While running distance increased in both groups, mice receiving control food showed but a minor and non-significant change ($p = 0.296$). Irbesartan treatment elicited a greater and significant improvement ($p = 0.002$). In fact, after eight weeks, mice receiving irbesartan achieved the same running distance as control mice without emphysema induction ($n = 4$) who had received control food throughout the treatment phase (977.6 ± 110.6 vs. 965.3 ± 176.4 m; $p = 0.951$). Results for maximal running speed resembled the results for running distance (data not shown).

Finally, there was no significant change in urine norepinephrine excretion normalized to urine creatinine in either group (control food: from 33.1 ± 10.5 to 22.9 ± 1.1 ($p = 0.362$); irbesartan food: from 23.7 ± 5.8 to 17.9 ± 3.3 ($p = 0.413$); control mice group (no emphysema): from 17.1 ± 1.8 to 23.4 ± 5.4 $\mu\text{g/l/mg}/100$ ml creatinine; $p = 0.432$).

3.4 Histology and lung function

As shown in **Figure 3**, there were large and significant differences between healthy control mice, emphysema mice receiving control food and emphysema mice receiving irbesartan: Mean linear intercept was lowest in the control group and highest in the emphysema group without irbesartan treatment. While the lungs of mice in the latter group displayed significantly increased lung compliance as compared to healthy control mice ($p = 0.007$),

there was only a trend between emphysema mice receiving irbesartan and healthy control mice ($p = 0.063$). Accordingly, emphysematous lungs without irbesartan treatment displayed decreased elastic recoil as evidenced by a significantly increased time constant as compared to both healthy controls ($p = 0.008$) and emphysema mice treated with irbesartan ($p = 0.034$). There was no difference between the two latter groups ($p = 0.240$).

4. Discussion

We demonstrated a positive effect of ARB on emphysema as evaluated by the mean linear intercept in an elastase mouse model. These novel histological findings were accompanied by corresponding positive effects of ARB on exercise capacity and ex vivo lung function. Renin mRNA levels confirmed an effective suppression of angiotensin-II effects comparable to previous studies.

4.1 Running distance

Exercise intolerance is a central symptom in COPD patients and closely relates to quality of life and prognosis [29, 30]. However, there are only scarce data on running distance or exercise capacity in emphysema or COPD animal models [14]. The current data of the induction phase corroborate earlier work of our group demonstrating reduced running distance in emphysematous mice [14].

Running distance as a measure of exercise capacity integrates abnormalities of pulmonary, cardiovascular and muscle function in a single measurement. Thus, the observed beneficial effects of ARB on emphysema severity and lung function may underly the 2.5-fold increase in running distance. Additionally, effects on autonomic nervous system activity, although insignificant in the present study, might have contributed [14]. These results are in line with a recent report of improved maximum work rate and peak oxygen pulse in COPD patients after 4 weeks of treatment with the ACE inhibitor enalapril [24]. In this study, however, no difference in lung mechanics or arterial blood gases was observed, and diffusion capacity even mildly decreased. Although it is possible that a four-week treatment period in human patients is not sufficiently long to cause clinically relevant structural alterations, this study indicates that additional factors affected by the renin-angiotensin-aldosterone system may contribute to improved exercise tolerance.

4.2 Emphysema and lung function

Elastase instillation models are rather crude models inducing massive lung injury with a single or repetitive administration of the protease. This does not reflect the continuous low grade inflammatory process, which is believed to underlie the genesis of smoking-related emphysema [31-33]. However, the elastase-induced emphysema model is well suited to study the systemic sequel of emphysema such as exercise limitation [14]. Furthermore, the model is useful for studying mechanisms and therapeutic strategies in established emphysema following an insult [31, 33, 34]. It has been known for many years that the changes following elastase application progress for many months following the initial insult [31, 35-37]. The positive feedback loop characterizing progressive emphysema includes mechanical forces, matrix metalloproteinases, proteolysis, thus resembling the pathophysiology of ageing in general [1, 31, 38-41].

Since we have no longitudinal data on mean linear intercept or lung function, we are unable to comment on the time course of emphysema during the treatment phase. However, we believe it to be unlikely that ARB elicits lung repair. Rather we assume that ARB interacts with mechanisms related to the evolution of emphysema following the initial event [31, 35, 38].

COPD causes marked activation of the renin-angiotensin system and the sympathetic nervous system [12-14]. Angiotensin II receptors are highly expressed within the lung [16] and control alveolar epithelial cell apoptosis [17] and lung fibroblast growth [18]. Local Angiotensin II appears to be a crucial mediator of lung injury and apoptosis [7, 19]. There is evidence both in culture and in situ within human pathological specimens and animal models demonstrating that Angiotensin II leads to apoptosis of lung epithelial cells via the receptor subtype 1 [17, 42, 43] and bronchial constriction [44]. Given these actions of Angiotensin II, it is conceivable albeit speculative that ARB interacts with the evolution of emphysema in our mouse model.

There is recent progress in the evaluation of lung function in mice [45]. Generally non-invasive methods are capable of detecting bronchial constriction in intact mice, but invasive methods give more precise results [45, 46]. Loss of elastic recoil in the lung is a hallmark of emphysema and is well captured by lung compliance. Thus the increased compliance noticed in our emphysema mice is a typical finding in various animal models of emphysema [46, 47]. We also evaluated the time constant during passive deflation as a complementary measure of elastic recoil. The results for the ex vivo compliance and elastic recoil measures were remarkable similar and corroborate the positive effects of ARB on the mean linear intercept as a measure of emphysema.

4.3 Limitations

In this study, we found a robust effect of ARB on mean linear intercept that was consistent with complementary data on lung function and running distance. Nevertheless, mean linear intercept is a simple measure of emphysema subject to pitfalls of lung morphometry [48]. The fact that both control and emphysema mice gained weight over a 3.5-month period might be explained by the fact that these animals were not fully grown-up at study onset and thus had potential to gain weight over the course of the study.

In this study, we did not investigate ARB effects on inflammation, muscle function or anabolic / catabolic imbalance, and the number of studied animals was limited. Further studies are necessary to better delineate the positive effects of ARB in severe emphysema not only on the lungs but also on other organ systems involved in gas exchange during exercise. Since this study aimed at eliciting the effects of a pre-defined treatment period with irbesartan on lung histology, we are unable to comment on longitudinal effects of ARB treatment for elastase-induced emphysema. A different experimental approach would be needed to address this question.

Our findings in an elastase model of emphysema are in line with current animal and human respiratory research [1, 31, 40]. However, as pointed out above, emphysema elicited by elastase instillation only partially mimics smoke-induced emphysema. In order to expand our knowledge on the role of neurohumoral activation in the progression and/or regeneration of smoke-induced lung injury, longitudinally examining the effects of ARBs in mice with lung emphysema due to cigarette smoke inhalation would be an interesting goal for future investigations [49].

4.4 Conclusions

The ARB irbesartan has distinct positive effects on emphysema and running distance in a mouse model. These findings might help to explain the positive effects of ARB on lung overinflation observed in a clinical trial [22] and on mortality in epidemiological surveys [15, 23].

5. Conflict of interest statement

The authors declare that they have no competing interests.

6. Funding

This study was supported by an unrestricted grant from Sanofi-Aventis (formerly Sanofi-Synthelabo), Paris, France. The sponsor had no role in the study design, data collection or writing of the report.

Ethics Statement

As stated in the Methods section, ethics approval for this study was obtained from The Bezirksregierung Braunschweig, Dezernat 604 - Tierschutz (Proposal no. 33.42502-061/06G30.02).

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Figure legends

Figure 1: Study protocol. E/U, dates of exercise testing and urine collection for catecholamine measurements.

Figure 2: Impact of control food (grey columns, n = 5) and irbesartan food (black columns, n = 6) on running distance in mice with elastase-induced lung emphysema. Running distances achieved by healthy control mice receiving standard food are displayed as white columns. p ANOVA refers to the comparison between irbesartan and control food in emphysema mice.

Figure 3: Histological and functional parameters in healthy controls (white columns), emphysema mice receiving control food (grey) and emphysema mice treated with irbesartan (black). A, mean linear intercept; B, static compliance; C, time constant. * p<0.05 compared to healthy controls; § p<0.05 compared to emphysema-controls

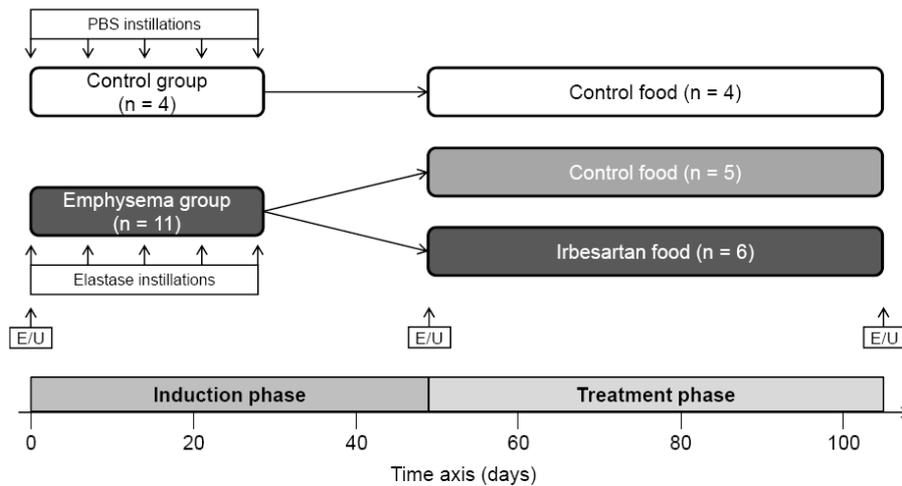


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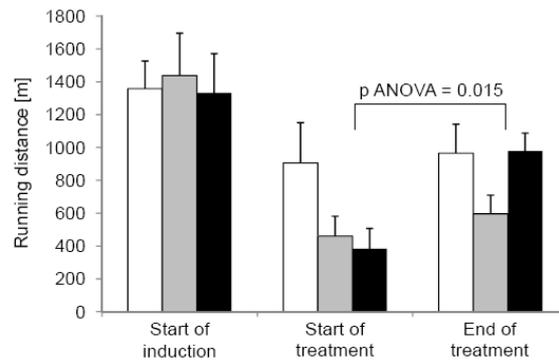


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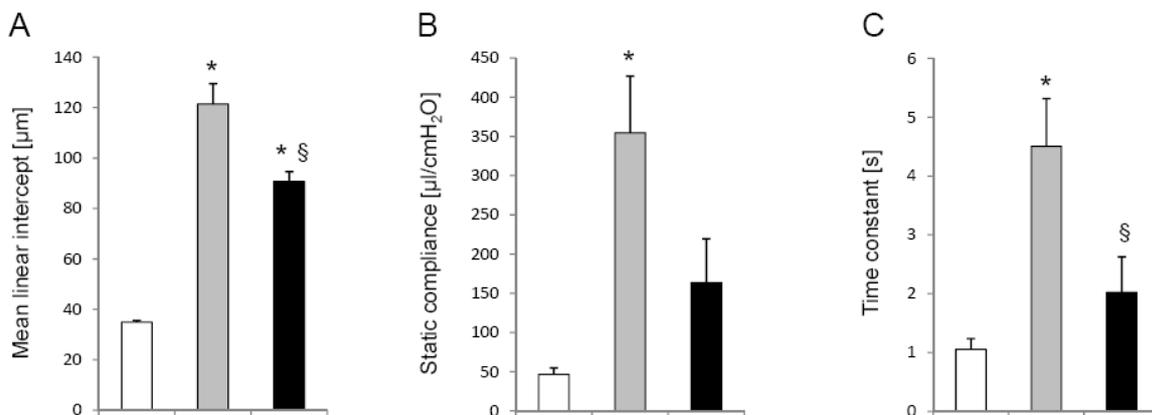


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