Life Sciences 232 (2019) 116608

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Linalyl acetate prevents three related factors of vascular damage in COPD-like and hypertensive rats

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ARTICLE INFO

Keywords:
Linalyl acetate
COPD
Hypertension
Comorbidity
Vascular damage

ABSTRACT

Preventing vascular damage is considered an effective strategy in patients who suffer from chronic obstructive pulmonary disease (COPD) with hypertension. Here, we investigated vascular damage in COPD-like and hypertensive rats, which demonstrated the presence of the three related factors of COPD with hypertension. These include elevated systolic blood pressure (SBP), serum malondialdehyde (MDA) and serum lactate dehydrogenase (LDH), which are positively correlated with vascular damage in patients. In addition to increases in these three related factors, COPD-like and hypertensive rats exhibited increased levels of pro-inflammatory mediators, such as tumor necrosis factor-α, interleukin-6, and matrix metallopeptidase-9 in bronchoalveolar lavage fluid, and enlargement of alveolar airspaces, recapitulating clinical findings in previous studies of patients. Moreover, the appearance of these related factors was prevented by linalyl acetate. Our results provide novel insight into the potential of LA to prevent vascular damage and elevated SBP, serum MDA and serum LDH in COPD with hypertension, and could lead to an alternative strategy for preventing vascular damage for patients who suffered from COPD with hypertension in a clinical setting.

1. Introduction

Comorbidities are frequent in chronic obstructive pulmonary disease (COPD) patients [1,2], and are also related to decreased quality of life and increased mortality [3]. Cardiovascular disease is the second-highest cause of death in patients with COPD after respiratory problems [4]. One of the risk factors for cardiovascular diseases, hypertension, is the most common comorbidity in COPD [2,5], and its prevalence in COPD patients is higher than that in non-COPD subjects [6]. However, the need to simultaneously prevent COPD and comorbidities is in clinical settings [3].

Vascular damage is an important factor in the development of hypertension [7,8] and COPD [9,10]. Studies have shown that vascular endothelial dysfunction, which also induces vascular damage, is related to the comorbidities of cardiovascular risk, hypertension [11], and COPD [2,5]. Specifically, an increased incidence of hypertension and elevated cardiovascular mortality are now considered degenerative risk factors for COPD [12]. Thus, early detection or prevention of vascular damage is an effective strategy for managing both diseases [10,13,14]. By more accurately establishing disease related factors, it should be possible to implement more efficient preventive strategies in clinical settings.

However, mechanistic links between vascular damage in COPD and hypertension remain to be elucidated. Chronic inflammation [15], oxidative stress [9] and vascular endothelial dysfunction are known hypertension-inducing factors [16] that are associated with COPD and could be instigators of the vascular damage observed in patients with COPD and hypertension [17]. However, disrupted vascular function in this population has received limited research attention. And to date, no studies have sought to identify the related factors of vascular damage in COPD with hypertension, highlighting the importance of understanding the primary mechanistic links among chronic inflammation, oxidative stress, and endothelial dysfunction in preventing the development of elevated incidence and mortality in this population.

Various animal models that take into account the causes of COPD have been developed [18], but there are few animal models of COPD that incorporate comorbidities such as systemic hypertension. Therefore, clarifying the pathophysiological characteristics and related factors of vascular damage in COPD with hypertension and comorbidities requires the development of an appropriate animal model. The purpose of this study is to clarify the pathophysiological characteristics and related factors of vascular damage in COPD-like and hypertensive rats by evaluating hemodynamic factors and related molecular factors.

Linalyl acetate (LA), a natural component of the essential oil of

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https://doi.org/10.1016/j.lfs.2019.116608
Received 2 April 2019; Received in revised form 25 June 2019; Accepted 26 June 2019
Available online 26 June 2019

Citrus bergamia Risso, *Lavandula angustifolia* and *Salvia sclarea*, has been reported to decrease systolic blood pressure (SBP) in hypertensive rats [20] and prevent ischemic injury by reducing oxidative stress induced vascular damage in the aorta of hypertensive rats [20]. LA has also been shown to have anti-inflammatory effects, protecting against vascular endothelial dysfunction in a rat model of inflammation [21] and blocking cardiovascular disease induced endothelial dysfunction by modulating intracellular calcium in endothelial cells [22]. Moreover, LA was reported to decrease inflammatory nuclear factor kappa B (NF-κB) expression in diabetic rats [23]. Despite previous reports of anti-inflammatory and/or anti-hypertensive effects of LA in various animal models, no studies have investigated the possible preventive effects of LA in COPD with hypertension. Accordingly, we here tested the hypothesis that LA mitigates vascular damage in COPD-like and hypertensive rats.

2. Materials and methods

2.1. Experimental animals

Male Sprague-Dawley rats (4 weeks old) were obtained from Samtaco (Osan, Korea) and housed in the laboratory environment for a week. The experimental procedures were conducted in accordance with guidelines relevant to the care of experimental animals, as approved by the Korea University Animal Experiment Ethics Committee (KUIACUC-2016-153). After randomized, rats were assigned to 7 groups: normotensive controls (Control); C COPD-like and hypertensive rats treated with vehicle (Vehicle), 1 mg/kg LA (LA 1), 10 mg/kg LA (LA 10), or 100 mg/kg LA (LA 100); and COPD-like and hypertensive rats treated 2.5 mg/kg dexamethasone (Dexa) or 10 mg/kg nifedipine, used as positive controls.

2.2. COPD-like and hypertensive rats

COPD-like characteristics and hypertension were simultaneously induced by intranasal administration of porcine pancreatic elastase (PPE) and lipopolysaccharide (LPS) [24], with chronic exposure to nicotine and immobilization stress, as previously described [19]. LA, nifedipine, and dexamethasone were intraperitoneally administered 2 h before intranasal administration of PPE 15 units and LPS 87.5 μg. Dexamethasone is well known as the effect of anti-inflammation on COPD treatment in clinical settings [25], and nifedipine has not only antioxidant effect but also antihypertensive effect. Therefore, dexamethasone and nifedipine were used as positive control. Blood and tissue samples were taken under anesthesia after blood pressure measurements were completed. The specific protocol is illustrated in Supplementary Fig. S-1.

2.3. Histopathological analysis of lung tissue

Inflammatory responses in lung tissue were investigated using histopathological analyses. Lung tissues were fixed in 10% paraformaldehyde and embedded in paraffin, after which paraffin blocks were cut into 4-μm–thick sections. Sections were stained with hematoxylin and eosin (H&E) and then imaged at 200× magnification using a Nikon DS-Ri2 fluorescence microscope (Nikon, Japan). The alveolar diameter of each airspace was calculated using NIS Elements imaging software (Nikon, Japan).

2.4. Western blotting

The lung tissue was homogenized and lysed using a Protein Extraction Kit (iNtRON, Korea). Proteins in samples (20 μg/lane) were resolved by SDS-PAGE on 10% gels, then transferred to nitrocellulose membranes. Membranes were probed by first incubating overnight at 4 °C with rabbit polyclonal anti-NF-κB primary antibody (Santa Cruz, USA), then incubated with the anti-rabbit IgG HRP-linked secondary antibody for 1 h at room temperature. Signals were visualized using an ECL Plus Western blot detection kit (Bio-Rad, USA), and analyzed densitometrically using Image J software (National Institutes of Health, USA).

2.5. Preparation of bronchoalveolar lavage (BAL) fluid

BAL fluid was collected from anesthetized rats by delivering 2 ml of 0.9% saline via the trachea using a 22-gauge catheter. After centrifuging samples at 500 ×*g* for 10 min, supernatants were collected and transferred to fresh tubes for experimental preparation.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of cytokine tumor necrosis factor (TNF)-α and interleukin (IL)-6, in BAL fluid were measured using rat TNF-α and IL-6 ELISA kits (Pepro Tech, London, UK). Results were obtained by measuring absorbance at 450 nm using a microplate ELISA reader (BMG Labtech, Germany), according to the manufacturer's instructions.

2.7. Zymographic analysis

Matrix metallopeptidase (MMP-9) protein was measured by zymography using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels containing 1 mg/ml gelatin (Sigma-Aldrich, USA). After electrophoresis, gels were renatured with 2.5% Triton X-100 for 1 h, then incubated for 20 h in 50 mM Tris-Cl buffer (pH 7.4) containing 10 mM $CaCl_2$ and 0.02% NaN_3. Gels were stained for 2 h with 0.5% Coomassie Brilliant Blue (Sigma-Aldrich, USA) in 7.5% acetic acid and 10% isopropyl alcohol, followed by destaining to visualize MMP-9 bands. Relative densities were analyzed using Image J software (National Institutes of Health, USA).

2.8. Measurement of blood pressure

Blood pressure was measured 30 min after intraperitoneal administration of test agents, at 1-week intervals thereafter, and at the end of the experiment, using a CODA-6 non-invasive tail cuff system (Kent Scientific, Torrington, CT, USA).

2.9. Nitrite assay

Blood was collected from the abdominal aorta and incubated at room temperature for 2 h, followed by centrifugation at 1900 ×*g* for 20 min to obtain serum. The level of nitrite was measured by adding 100 μl Griess reagent (0.1% naphthylethylenediamide and 1% sulfanilamide) (Sigma-Aldrich, USA) each to 50-μl serum sample, and then incubated for 10 min at room temperature. The level of nitrite accumulated in serum was determined by measuring absorbance at 540 nm using a microplate ELISA reader (BMG Labtech, Germany).

2.10. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Serum was incubated with DPPH (23.6 μg/ml in ethanol) for 30 min at 37 °C in the presence of different concentrations of LA or ascorbic acid (positive control). Absorbance was measured at 517 nm using an ELISA microplate reader (BMG Labtech, Germany). Total antioxidant capacity was expressed as the percentage of maximum inhibition obtained with ascorbic acid.

2.11. Malondialdehyde (MDA) assay

A 0.5-ml serum sample was mixed with 1 ml of a solution of 0.37% (w/v) thiobarbituric acid and 15% w/v trichloroacetic acid in 0.25 M HCl. Samples were placed in boiling water for 15 min, then centrifuged

at 1000 ×*g* for 10 min. Absorbance was measured at a wavelength of 535 nm at room temperature using an ELISA microplate reader (BMG Labtech, Germany).

2.12. Lactate dehydrogenase (LDH) activity

Lactate dehydrogenase enzymatic activity, used as a marker of cytotoxicity, was determined with a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Co., USA) according to the manufacturer's instructions. Changes in absorbance at 340 nm were measured using an ELISA microplate reader (BMG Labtech, Germany).

2.13. Solutions and chemicals

LA, nifedipine, dexamethasone, polyethylene glycol (PEG) 200, LPS, nicotine, were obtained from Sigma-Aldrich Co. (Saint Louis, Missouri, USA). PPE was purchased from Elastin Products Co. (Owensville, Missouri, USA). LA, nifedipine, and dexamethasone were dissolved in PEG 200, whereas PPE and LPS were dissolved in phosphate-buffered saline.

2.14. Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Blood pressure was analyzed by repeated measures one-way analysis of variance (ANOVA), and other variables were compared by one-way ANOVA followed by an LSD post hoc test using SPSS Statistics 22 version (IBM, USA). A P-value < 0.05 was considered statistically significant.

3. Results

3.1. LA prevents inflammatory responses in COPD-like and hypertensive rats

Histopathological analyses revealed emphysematous destruction in lung tissue from vehicle group compared with the untreated normotensive control group (Fig. 1A). This lung damage was prevented by the highest dose of LA (100 mg/kg), which maintained the lung structure similar to that of the control group. In addition, alveolar diameter was enlarged in the vehicle group; this effect was also prevented by treatment with 100 mg/kg LA (Fig. 1B), which restored alveolar diameter to the normal range. NF-κB expression abnormally increased in lung tissue of vehicle group, which was restored to normal level in the LA 100 group (Fig. 1C).

TNF-α tended to increase in vehicle group compared to control group, although there was no significant difference. The levels of the pro-inflammatory cytokine IL-6, and the degradative enzyme MMP-9 in BAL fluid was significantly elevated in the vehicle group compared with the control group ($p < 0.001$, respectively). Both LA and dexamethasone showed anti-inflammatory effects, which were greater in the LA 100 group for IL-6 and MMP-9 compared with the vehicle group (Fig. 2B–C). These results showed that treatment with LA may prevent increase in IL-6 and MMP-9 in COPD-like and hypertensive rats.

3.2. LA prevents the increase in SBP, serum lipid peroxidation, and vascular cytotoxicity in COPD-like and hypertensive rats

Two-way ANOVA was used to analyze differences in blood pressure over time, with the level of significance of time and group interactions illustrated in Fig. 3A. SBP continuously increased up to day 22 in COPD-like and hypertensive rats treated with vehicle and was then maintained at this elevated level until the end of the experiment (Fig. 3A). SBP was significantly elevated over time in the vehicle group compared with the normotensive control group. The elevation in SBP in the vehicle group was attenuated by treatment with LA or nifedipine, such that SBP was significantly lower over time in the LA 10 group, LA 100 group, and nifedipine group compared with the vehicle group. A plot of blood pressure over time showed that hypertension was maintained in the vehicle group until the end of the experiment, whereas increases in blood pressure were inhibited in the LA and nifedipine groups. These results indicate that LA exerts an antihypertensive effect by preventing the elevation in SBP in COPD-like and hypertensive rats.

Serum levels of MDA and LDH were significantly elevated in the vehicle group compared with the control group. For both indices, LA 100 group exerted a significant reversely effect (Fig. 3B, C). Also, serum levels of LDH was significantly reduced in the LA 1 and LA 10 group compared with the vehicle group. These results indicate that LA may prevent vascular damage in COPD-like and hypertensive rats by blocking lipid peroxidation and cytotoxicity, the latter indicated by LDH levels. These three factors were considered that have relationship with inflammation and vascular function simultaneously. Thus, the dexamethasone and nifedipine were presented as a common positive control.

3.3. Serum nitrite levels and total antioxidant capacity in COPD-like and hypertensive rats

Serum nitrite levels were significantly lower in vehicle group. Although LA did not significantly affect serum nitrite level, LA showed a tendency to normalize serum nitrite compared with the vehicle group (Fig. 4A). Serum DPPH levels, a total antioxidant capacity, was decreased in the vehicle group compared with the control group (Fig. 4B). However, LA did not significantly affect total antioxidant capacity in serum.

4. Discussion

Considering factors that combine chronic inflammatory and systemic hypertensive features, we reviewed the available literature about COPD and hypertension to identify the foremost and common related factors of vascular damage in our study, other animal models of COPD or hypertension, and clinical research. Our findings presented in Fig. 5 indicate that COPD-like and hypertensive rats in the current study reflect the pathophysiological characteristics of COPD patients who suffer from hypertension. Increases in the pro-inflammatory cytokines TNF-α [26,27] and IL-6 [27,28], the degradative enzyme MMP-9 [29,30] and the inflammation-related gene NF-κB [27,31], as well as enlarged alveoli are commonly observed in COPD patients and rat models [32,33]. In addition, decreases in serum nitrite and serum total antioxidant capacity are associated with altered vascular tone in hypertensive rats [34,35] and patients with hypertension [36,37] and coronary heart disease [38] in a clinical setting. An important concurrent finding in our rat model was the significant elevation in SBP, serum MDA and serum LDH, which are similar to observations in patients with COPD [39,40] and hypertension [41,42].

Here, we assessed the potential of the three major related factors of COPD with hypertension—elevated SBP, elevated serum MDA and elevated serum LDH—as predictive biomarkers and therapeutic targets of vascular damage. An elevation in SBP makes a major contribution to increases in systemic inflammation and vascular damage [43]. Previous studies have also reported that SBP is increased in hypertensive rats [19,20,35], a finding that is associated with elevated levels of lipid peroxidation [34,44,45]. Such increases in SBP and lipid peroxidation have been shown to affect mortality and morbidity in patients with COPD [46,47].

Among the various processes involved in hypertension, vascular damage due to oxidative stress is particularly important in patients with hypertension, atherosclerosis, or stroke [48]. Under oxidative stress conditions, reactive oxygen species attack lipids, resulting in lipid peroxidation. Increased serum MDA has been identified in situations in which lipid peroxidation induces atherosclerosis or inflammatory

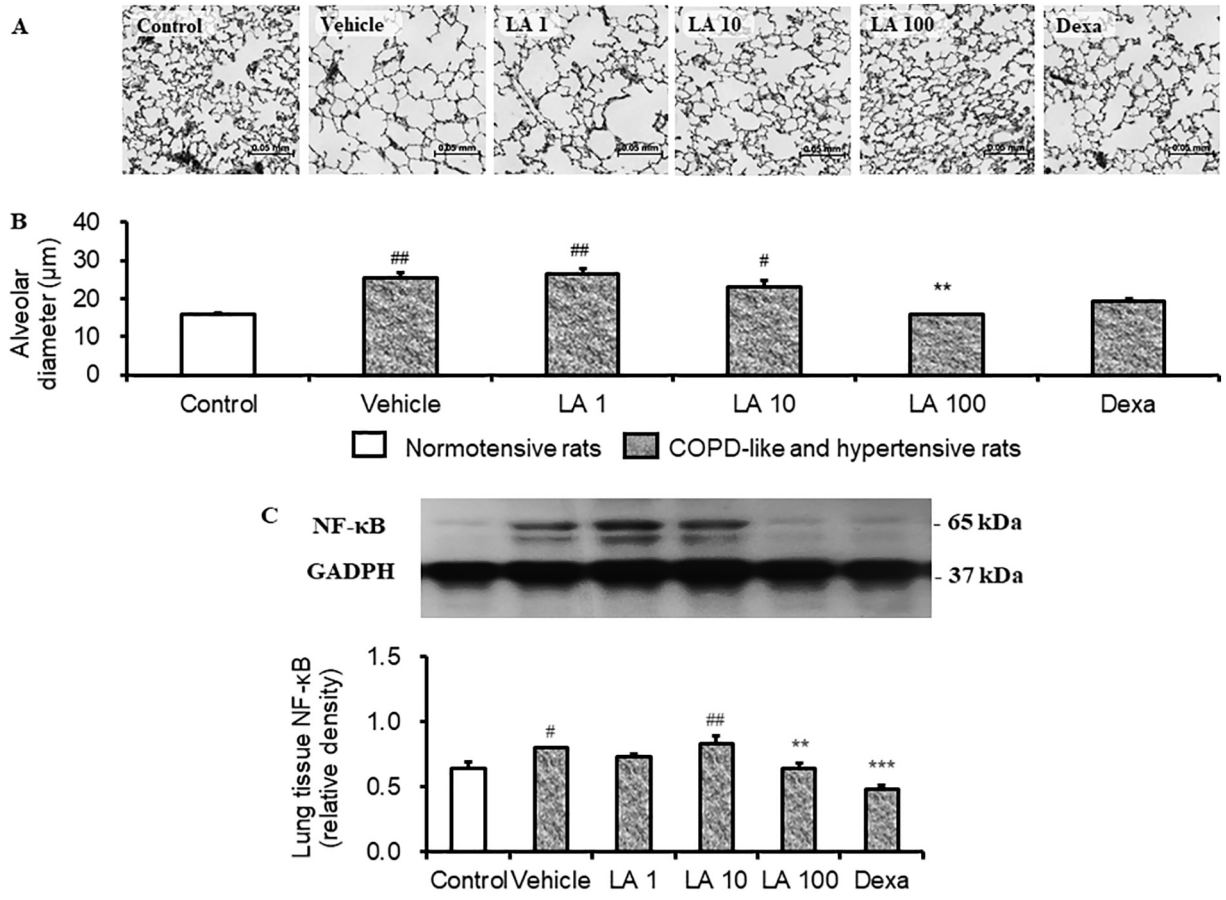

Fig. 1. Linalyl acetate (LA) prevents inflammatory responses in lung tissue in the chronic obstructive pulmonary disease (COPD)-like and hypertensive rats. (A) Histopathological analysis of lung sections by hematoxylin and eosin staining. Scale bars: 50 μm. (B) Lung injury was quantified as mean alveolar diameter in a field of view (n = 2–4/group). (C) Western blot analysis of nuclear factor kappa B (NF-κB) in lung tissue showed as full-length (n = 3–6/group). Data are presented as means ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the control group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the vehicle group.

vascular damage. This phenomenon has also been reported in COPD mouse models [49,50], hypertensive rat models [19,35,51] and patients [41].

Likewise, LDH is a well-known pathologic marker in pulmonary hypertension [52] and cardiac dysfunction [53]. LDH is a cytoplasmic enzyme in all cells of the body that catalyzes the reversible conversion of pyruvate to lactate as a part of the lactic acid cycle [54]. Following cellular injury, LDH is released from damaged cells into serum [55]. Serum LDH is also elevated in patients with COPD [40], hypertensive rats [56], and atherosclerotic mice [57].

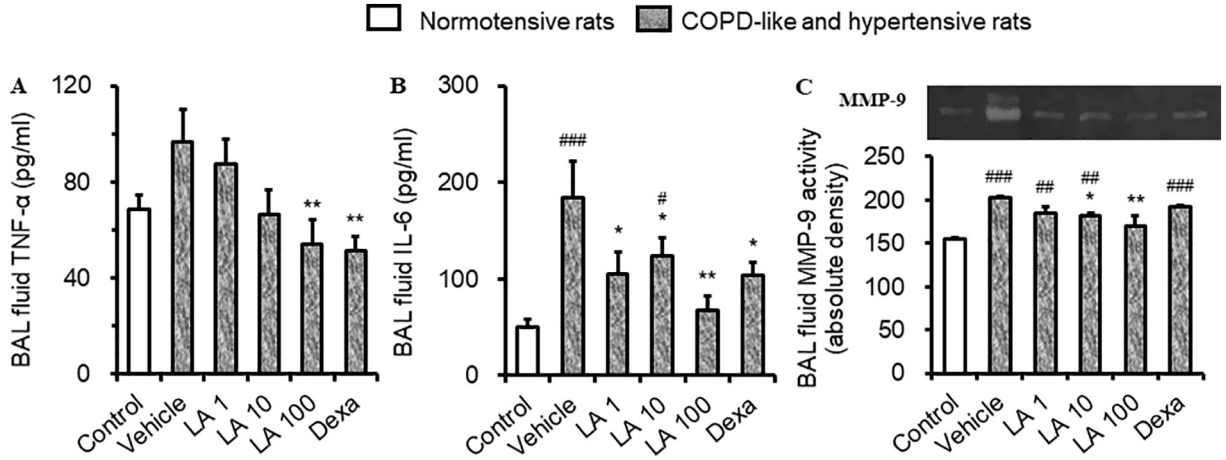

Fig. 2. Linalyl acetate (LA) suppresses the level of pro-inflammatory cytokines and degradative enzymes in bronchoalveolar lavage (BAL) fluid in the chronic obstructive pulmonary disease (COPD)-like and hypertensive rats. (A) Quantification of tumor necrosis factor (TNF)-α and (B) interleukin (IL)-6 in BAL fluid samples by enzyme-linked immunosorbent assay (n = 3–4/group). (C) Zymographic analysis of matrix metallopeptidase (MMP)-9, measured in the same samples showed as full-length gel (n = 3–5/group). Data are presented as means ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the vehicle group.

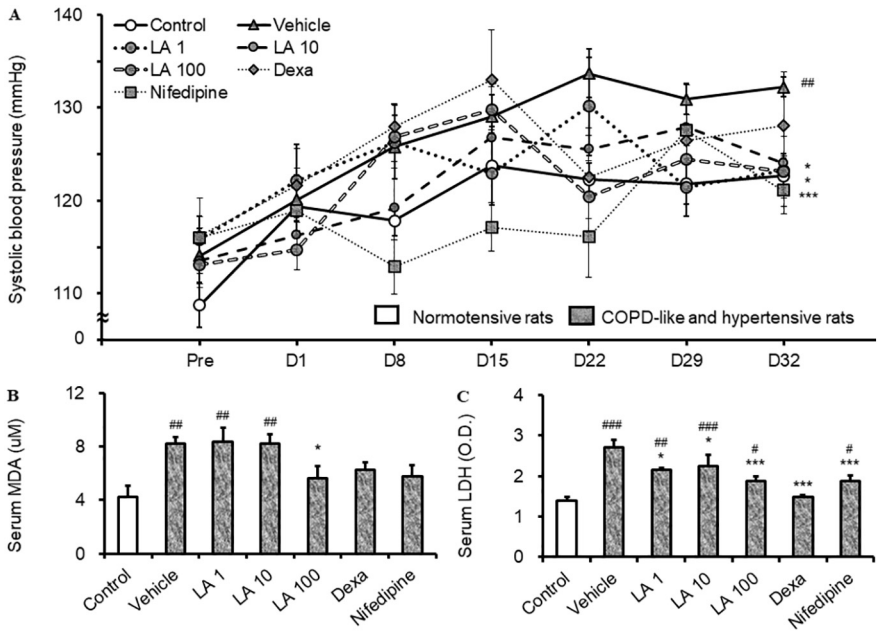

Fig. 3. Linalyl acetate (LA) prevents increases in systolic blood pressure (SBP), serum lipid peroxidation, and vascular cytotoxicity in the chronic obstructive pulmonary disease (COPD)-like and hypertensive rats. (A) SBP was measured before the experiment and at days 1, 8, 15, 22, 29, and 32. Result analyzed by repeated measures one-way analysis of variance (ANOVA) (n = 6–9/group). (B) Malondialdehyde (MDA) (n = 3–7/group) and (C) lactate dehydrogenase (LDH) levels (n = 4–7/group) were measured in serum. Data are presented as means ± SEM. $^{\#\#}p < 0.01$ vs. the control group; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. the vehicle group.

The results of the present study on COPD-like and hypertensive rats confirm literature reports that increases in SBP and serum MDA and LDH levels are major collaborative factors of both COPD [39,40,58] and hypertension [41,42,59]. Thus, our findings suggest that these three related factors have potential predictive and preventive value in patients who suffer from COPD with hypertension.

In the present study, only dexamethasone was used as a positive control for the measurement of inflammatory factors (TNF-a, IL-6, MMP-9). However, nifedipine has antihypertensive as well as anti-inflammatory [60] and antioxidant [61] effects. Although we could not exclude the possibility that the effect of nifedipine was caused by its anti-inflammatory and antioxidant properties as well as its reduction in blood pressure, our findings suggest that LA may have preventive value in COPD with hypertension. Importantly, we demonstrated that LA prevents the manifestation of these three related factors by preventing inflammatory and hypertensive effects, and further exhibits preventive potential by decreasing serum nitrite levels and total antioxidant capacity in COPD-like and hypertensive rats. In previous research, the LA-rich essential oil *Salvia sclarea* was reported to exert a preventive effect on endothelial dysfunction by increasing serum nitrite production in a rat model with immobilization stress-induced hypertension [62]. However, *Citrus bergamia Risso* was demonstrated to exert a protective effect against vascular disorders by regulating the vascular tone of smooth muscle [63], indicating that LA effects may not be mediated exclusively through the NO/cGMP pathway. Although details of the molecular mechanisms underlying the protective effect of LA against vascular damage will require further investigation, this study supports the potential of LA to prevent vascular damage in COPD with hypertension.

Collectively, our results provide novel insight into the action of LA in preventing vascular damage and suggest the potential of LA treatment as a new strategy for preventing vascular damage in COPD with hypertension. In situations where COPD and hypertension occur simultaneously, early detection of these three related factors highlighted here might predict subsequent vascular damage. Notable in this context, LA showed early preventive effects against vascular damage. This study also could provide a descriptive basis for further research on COPD with hypertension that might produce future benefits for patients in a clinical setting.

Supplementary data to this article can be found online at https://

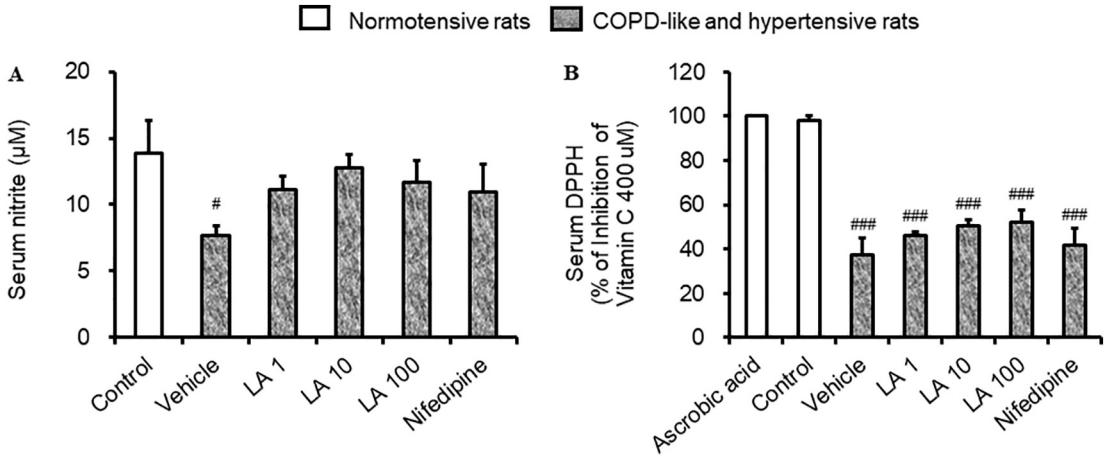

Fig. 4. LA potentially increases serum nitrite levels and total antioxidant capacity in the chronic obstructive pulmonary disease (COPD)-like and hypertensive rats. The levels of (A) nitrite and (B) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (n = 3–8/group) were measured in serum. Data are presented as means ± SEM (n = 3–5/group). $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the control group.

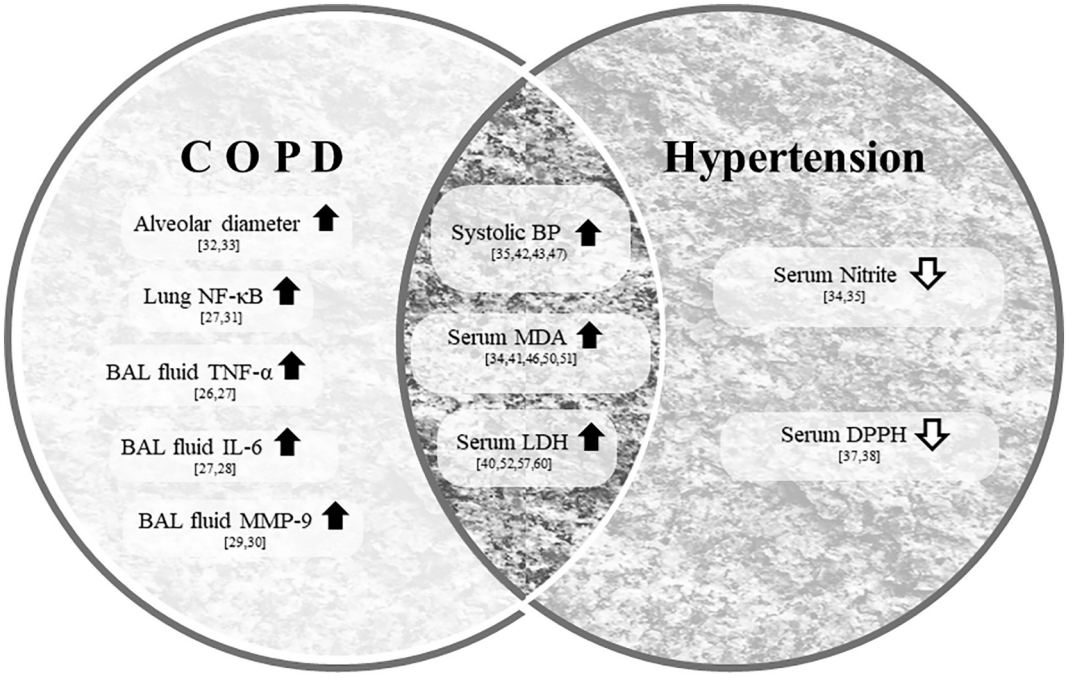

Fig. 5. Venn diagram comparing factors of chronic obstructive pulmonary disease (COPD) (light gray) and hypertension (dark gray), based on published reports. The three main related factors observed in the rat model of COPD with hypertension are indicated in the overlapping regions.

doi.org/10.1016/j.lfs.2019.116608.

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF-2018R1D1A1B07050048) and the Institute of Nursing Research, Korea University Grant.

Declaration of Competing Interest

The authors declare no competing financial interests.

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