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Dipeptidyl peptidase-4 inhibition prevents lung injury in mice under chronic stress via the modulation of oxidative stress and inflammation

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Abstract: Exposure to chronic psychosocial stress is a risk factor for various pulmonary diseases. In view of the essential role of dipeptidyl peptidase 4 (DPP4) in animal and human lung pathobiology, we investigated the role of DPP4 in stress-related lung injury in mice. Eight-week-old male mice were randomly divided into a non-stress group and a 2-week immobilization stress group. Non-stress control mice were left undisturbed. The mice subjected to immobilized stress were randomly assigned to the vehicle or the DPP4 inhibitor anagliptin for 2 weeks. Chronic stress reduced subcutaneous and inguinal adipose volumes and increased blood DPP4 levels. The stressed mice showed increased levels in the lungs of genes and/or proteins related to oxidative stress ($p67^{phox}$, $p47^{phox}$, $p22^{phox}$ and $gp91^{phox}$), inflammation (monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and intracellular adhesion molecule-1), apoptosis (caspase-3, -8, -9), senescence ($p16^{INK4A}$, p21, and p53) and proteolysis (matrix metalloproteinase-2 to -9, cathepsin S/K, and tissue inhibitor of matrix metalloproteinase-1 and -2), and reduced levels of eNOS, Sirt1, and Bcl-2 proteins; and these effects were reversed by genetic and pharmacological inhibitions of DPP4. We then exposed human umbilical vein endothelial cells *in vitro* to hydrogen peroxide; anagliptin treatment was also observed to mitigate oxidative and inflammatory molecules in this setting. Anagliptin can improve lung injury in stressed mice, possibly by mitigating vascular inflammation, oxidative stress production, and proteolysis. DPP4 may become a new therapeutic target for chronic psychological stress-related lung disease in humans and animals.
Key words: apoptosis, chronic psychological stress, inflammation, pulmonary injury

Introduction

Exposure to chronic psychosocial stress (CPS) is a risk factor for many diseases, including asthma, chronic obstructive pulmonary disease, and lung cancer [1–3]. Stress has been shown to increase inflammation-related gene expression in inflammatory leukocytes through a β-adrenergic induction of myelopoiesis [4]. An association between chronic stress and metabolic and inflammatory diseases including cardiovascular disease and pulmonary disease has been demonstrated [5–7]. The incidence and mortality of CPS-related diseases including pulmonary disease are increasing as the population ages [8]. In the present coronavirus disease 2019 (COVID-19) pandemic, patients under moderate or high stress at the time of pulmonary infection have exhibited increased mortality compared to subjects under low levels of stress [9]. It was also reported that vascular endo-

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thelial cells and stem cells are sensitive to various pathological stressors [10–13]. Chronic stress activates both the sympathetic nervous system and the hypothalamus-pituitary-adrenal axis, and the excessive pathophysiological responses that are induced contribute to stress-related pulmonary disease (SRPD) [14, 15]. However, the exact mechanisms underlying CPS-related lung aging and injury remain unclear.

Dipeptidyl peptidase 4 (DPP4) is a member of a complex gene family that acts nonspecifically to cleave a wide variety of structure-related peptides (including growth factors, hormones, chemokines, cytokines, and neuropeptides) [16, 17]. Over last ten years, emerging data have demonstrated unexpected roles for DPP4 in extracellular and intracellular signaling, insulin resistance, oxidative stress production, inflammation, immune activation, cell apoptosis, and lipid metabolism [17–20]. We recently observed that DPP4 inhibition by anagliptin ameliorated iron chloride ($FeCl_3$)-induced thrombosis in mice that were subjected to stress via the modulation of plasminogen activator inhibitor-1 (PAI-1) and ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, member 13) activity [11]. The activation of the endothelial nitric oxide synthase/Sirt1 axis contributed to an anagliptin-mediated amelioration of chronic stress-related vascular aging [10]. It was shown that bone marrow lacking DPP4 or loaded with anagliptin is resistant to bone marrow hematopoietic stem cell chronic stress [13]. Moreover, several studies have indicated that there is a close link between increased plasma DPP4 activity and both atherosclerotic plaque growth and impaired ischemia-induced vascular regeneration capacity in mice under chronic stress conditions, and these harmful effects were rectified by DPP4 deletion and anagliptin treatment. Ikedo and colleagues reported that anagliptin prevented intracranial aneurysm growth by suppressing macrophage infiltration and activation [19]. A recent clinical study demonstrated that anagliptin reduced the serum fatty acid-binding protein-4 concentration independent of the changes in hemoglobin A1c or LDL-C in patients with type 2 diabetes mellitus and dyslipidemia who were on statin therapy [21]. Recent comprehensive review articles pointed out that DPP4 inhibitors (anagliptin, vildagliptin, and sitagliptin) that were developed and marketed for their effects display multipotency in the management of various pulmonary diseases [22, 23]. A clinical study revealed that DPP4 was highly expressed in the lungs of patients both with and without diabetes mellitus [24]. DPP4 is upregulated in metabolic and inflammatory pathological conditions associated with chronic psychological stressor [13]. Although clinical and experimental findings indicate that DPP4 is involved in the occurrence and development of pulmonary fibrosis and embolism [25], little is known about the role of DPP4 in CPS-related lung injury and SRPD.

In the present study, we used DPP4 deficiency ($DPP4^{-/-}$) mice, DPP4 inhibitor anagliptin, and an immobilized stress model to test our hypothesis that DPP4 activity may negatively modulate pulmonary injury in mice that have been subjected to chronic stress, by acting on inflammatory, oxidative, and proteolytic mediators. Given that the vascular endothelial cells were sensitive to the stress in animals under various chronic stress conditions [10–12], we also investigated the expressions of inflammation-, oxidative stress-, and proteolysis-related molecules in cultured endothelial cells in response to oxidative stress.

Materials and Methods

Antibodies and reagents.

The following commercially available antibodies were used: anti-$p16^{INK4A}$ antibody (10883-1-AP, Proteintech, Rosemont, IL); antibodies against Sirt1 (#8469), caspase-3 (#9662), caspase-9 (#9508), p53 (#2524), and Bax (#2772) were from Cell Signaling Technology (Boston, MA); antibodies against Bcl-2 (ab196495), caspase-8 (ab25901), and p21 (ab109199) were from Abcam, (Cambridge, UK); antibodies against β-Actin (sc-47778) and $p22^{phox}$ (sc-271968) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against eNOS (610296) and $gp91^{phox}$ (611415) were from BD Transduction Laboratories (San Jose, CA, USA).

The following commercially available reagents were used: DPP4-Glo assay kit (Promega, Madison, WI, USA); Human umbilical vein endothelial cells (HUVECs), fetal bovine serum (FBS), endothelial basal medium-2 (EBM-2), endothelial growth medium-2 (EGM-2), and SingleQuotes (Clonetics, San Diego, CA, USA); nitrocellulose transfer membrane (Millipore, Boston, MA, USA); RNeasy Micro Kits and SYBR™ Green Master Mix (Qiagen, Hilden, Germany); BCA protein assay kit (PC0020, Solarbio Sciences, Beijing, China); SuperScript III First Strand (Invitrogen, Carlsbad, CA, USA); Amersham ECL Prime Western Blotting Detection kit (Millipore); SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA); urethane (Sinopharm Group, Shanghai, China); and the immobilization stress tube (Cat. 551-BSRR, Natsume Seisakusho, Tokyo, Japan). The anagliptin was a generous gift from Sanwa Kagaku Pharmaceutical (Mie, Japan).

Animals

The male mice (C57BL/6J) used in this study were 8 weeks old and weighed 24–28 g. All animals were provided a standard diet and tap water ad libitum and housed two per cage under standard conditions (50 ± 5% humidity, 23 ± 1°C) with a 12h light/dark cycle (dark beginning at 7:00 pm) in a viral pathogen-free facility at the Animal Center of Yanbian University. The animal study protocol was approved by the Ethics Committee on Animal Research at Yanbian University Medical College [11]. All animal experiments were performed in accordance with the guidelines on animal care of Yanbian University.

Mouse stress protocol and evaluation

Eight-week-old wild-type ($DPP4^{+/+}$) mice were exposed to chronic variable stress for 2 weeks. In brief, to prevent the mice from becoming accustomed to the restraint stress, we administered several different combinations of stressors throughout each week, changing the stress order randomly [26] as previously described.

First, for the examination of the impact of chronic stress on pulmonary injury, eight-week-old male $DPP4^{+/+}$ mice (n=24) fed a standard diet were randomly assigned to the Stress group or the Non-stress group. Restraint stress was conducted as described above [26]. Non-stress control mice were left undisturbed and allowed contact with each other (the Non-stress group), whereas the stressed mice were subjected to a 4h session in an immobilization stress tube once daily (between 9:00 a.m. and 1:00 p.m.) for 2 weeks. We performed separate DPP4 inhibitor experiments to explore the role of DPP4 in stress-related lung injury. $DPP4^{+/+}$ mice were assigned to one of two groups and given (by oral gavage) either vehicle (distilled water, Stress) or a dose of anagliptin (prepared anagliptin aqueous solution with distilled water; 30 mg/kg/d, S-Ana) every day with continued daily immobilization stress for 2 weeks. $DPP4^{+/+}$ and DPP4-deficient ($DPP4^{-/-}$) mice were subjected to chronic variable stress for 2 weeks as described above.

Blood and tissue collections

On the last day of the 2-week stress protocol, the mice (n=42) were anesthetized with an intraperitoneal overdose injection of urethane (2 mg/kg), and the blood samples and the tissues were isolated. The lung tissues were isolated and kept in RNAlater solution for the gene assay or in liquid nitrogen for the protein assay and stored at −20°C for biological analysis.

Western blot analysis

After the extraction of total protein from the tissues and lysates with a RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, Triton-100, and fresh 1× protease inhibitor cocktail; pH 7.4), the protein concentration was evaluated by a BCA protein assay kit. Equal amounts of protein (40 μg/line) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene difluoride membranes and immunoreacted with the following targeted primary antibodies: $p16^{INK4A}$ (1:1,000), p21 (1:1,000), $p22^{phox}$, p53, Sirt1 (1:1,000 for each), β-Actin (1:1,000), $gp91^{phox}$ (1:1,000), eNOS (1:1,000), caspase-3 (1:1,000), caspase-8 (1:1,000), caspase-9 (1:1,000), Bax (1:1,000), and Bcl-2 (1:1,000). These membranes were then incubated with the related secondary antibodies at a 1:2,000–5,000 dilution. SuperSignal™ West Pico PLUS Chemiluminescent Substrate was used for the evaluation of targeted proteins. The levels of the targeted proteins quantified by Western blots were normalized by the loading β-actin levels.

Plasma and tissue DPP4 level analysis

The DPP4 Glo Protease Assay with an amino luciferin substrate was applied to evaluate DPP4 levels as described [13]. In brief, the luminogenic substrate containing the Gly-Pro sequence is cleaved by DPP4. Following DPP4 cleavage, the substrate is released, resulting in a luciferase reaction and the production of light. For the blood DPP4 activity assays, the plasma was isolated using VENOJectII vacuum blood collection tubes containing anticoagulants without serine protease inhibitor and then diluted in 0.1 mM Tris-HCl buffer (pH 8.0) by 30-fold. The diluted plasma (25 μl) and the extracts (lung tissues) were subjected to a DPP4 Glo assay in the presence and absence of the DPP4 inhibitor anagliptin (20 μmol/L). Human recombinant DPP4 was used to derive a standard curve. The luminescence intensity was calculated using a POWERSCAN4 as described [12]. The anagliptin-sensitive value (i.e., the absence value minus the presence value as an absolute value of its DPP4 activity) in relative light units per ml of plasma was calculated with a standard curve to represent the DPP4 level (ng/ml) [12].

Endothelial cell culture and experiments

HUVECs were cultured in EGM-2 containing 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO_2 until confluence. After being cultured in serum-free EBM-2 overnight, the HUVECs were subjected to the following experiments: (1) First, to evaluate the effect of oxidative stress on endothelial cell damage, cells were cultured in the presence and absence of H_2O_2 (0, 200, 400 μmol/L) in EBM-2 medium for 24h and then subjected to an assay of eNOS protein expression. (2) Second, to ex-

plore the inhibitory effect of DPP4 inhibition on oxidative stress-related harmful changes in HUVECs, we incubated cells pretreated for 30 min with various concentrations (0, 20, 40 μmol/L) of anagliptin for 24h in EBM-2 medium in the presence and absence of H_2O_2 (400 μmol/L) and then subjected the cells to the biological analyses, i.e., analyses of the expression of genes and/or proteins related to inflammation, oxidative stress, proteolysis (MMP-2, MMP-9, CatK, and CatS), and senescence ($p16^{INK4A}$ and eNOS). For all cell culture assays, at least three independent experiments were performed in triplicate.

Real-time quantitative polymerase reaction chain (RT-PCR) assay

Whole RNA was harvested from tissues and cell extracts using the RNAeasy Mini Kit according to the recommended protocol. The SuperScript III CellsDirect cDNA Synthesis kit was used to generate cDNA [27]. A quantitative real-time RT-PCR analysis was performed with primers specific for *p67^{phox}*, *p47^{phox}*, *p22^{phox}*, *gp-91^{phox}*, monocyte chemoattractant protein-1 (*MCP-1*), *PAI-1*, endothelial nitric oxide synthase (*eNOS*), vascular cell adhesion molecule-1 (*Vcam-1*), intercellular adhesion molecule-1 (*Icam-1*), cathepsin K (*CatK*), *CatS*, metalloproteinase-2 (*MMP-2*), *MMP-9*, tissue inhibitor of *MMP-2* (*TIMP-2*), and *TIMP-1* with the use of an ABI 7300 PCR System with Graphpad Prism software (the formula equation: 2−ΔΔCt1; Applied Biosystems, Foster City, CA, USA) [11]. The condition was as follows: denaturation 95°C for 10 min followed by amplification by 45 cycles of 95°C for 10 s and 60°C for 1min. After 45 cycles, a melting curve was generated by heating the sample to 95°C followed by cooling down to 60°C for 15 s, and heating to 95°C while the fluorescence was measured continuously. The expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was measured in parallel to that of the genes of interest and was used as an internal standard for the quantitative comparison of mRNA levels. The primer sequences are listed in Supplementary Table 1.

Statistical analyses

Data are presented as the mean ± SEM. A one-way analysis of variance (ANOVA) (for comparisons of three or more groups) followed by Tukey's post hoc tests or Student's *t*-test (for comparisons between groups) were used for statistical analyses. The body weight (BW) data were subjected to a two-way repeated-measures ANOVA and Bonferroni's post hoc tests. All data were calculated by two observers blinded to the experimental groups of the mice. The data that were observed to be in normal distribution were subjected to the subsequent statistical analyses. SPSS software ver. 17.0 (SPSS, Chicago, IL, USA) was used. Probability (*P*)-values <0.05 were considered significant.

Results

Impact of chronic stress on the BW, adipose volumes, and plasma DPP4 levels of the mice

An immobilized stress model (Figs. 1A and B) has often been applied to the study of psychological stress-related metabolic and inflammatory cardiovascular disorders [12, 26]. Here, to test our hypothesis, we created an immobilization model to examine whether chronic stress could influence the levels of DPP4 activity and BW and adipose volumes. The chronic stress-treated mice had decreased BWs and decreased subcutaneous and inguinal adipose tissue and increased plasma DPP4 levels compared to the control $DPP4^{+/+}$ mice (Figs. 1C–F). The stressed lungs also had elevated levels of DPP4 proteins compared to the control lungs (256.3 ± 4.5 vs. 103.9 ± 3.9, ng/ml, $P<0.01$), suggesting an association between pulmonary injury and increased plasma DPP4 activity in mice subjected to chronic stress.

Stress promoted inflammation, oxidative stress production, and proteolysis in the lung

It is well known that inflammation and oxidative processes are involved in various pulmonary diseases [28, 29]. As the second step in studying the relationships between stress and inflammation and oxidative stress, we analyzed the expressions of genes related to oxidative stress (*p47^{phox}*, *p22 ^{phox}*, *p67 ^{phox}*, and *gp91 ^{phox}*) and inflammation (*PAI-1*, *MCP-1*, *Vcam-1*, and *Icam-1*) in the lungs of both experimental and control group mice by conducting a quantitative PCR assay after 14 days of chronic stress. Compared to the non-stressed control $DPP4^{+/+}$ mice, the quantitative PCR using the non-stressed and stressed lung tissues (Figs. 2A–C) showed that the levels of targeted inflammatory and oxidative stress-related genes were higher (2.4–8.3-fold increases of the former genes; 1.6–9.5-fold increases in the latter genes) in the stressed mice than in the non-stressed mice. Conversely, the chronic stress decreased the *eNOS* gene expression in the lungs (Fig. 2C). As anticipated, we also observed that the stressed lung tissues had increased levels of proteolysis-related genes (*MMP-2*, *-9*, *TIMP-1*, *-2*, *CatS*, *CatK*; 1.7–10.4-fold increases) compared to the lung tissues of the non-stressed mice (Figs. 2D–F). An immunoblot analysis using equal amounts of protein from all samples was performed. Quantitative western blotting yielded the same conclusion (Fig. 3). Marked elevations of p22 ^{phox} and gp91 ^{phox} proteins (>1.6-fold

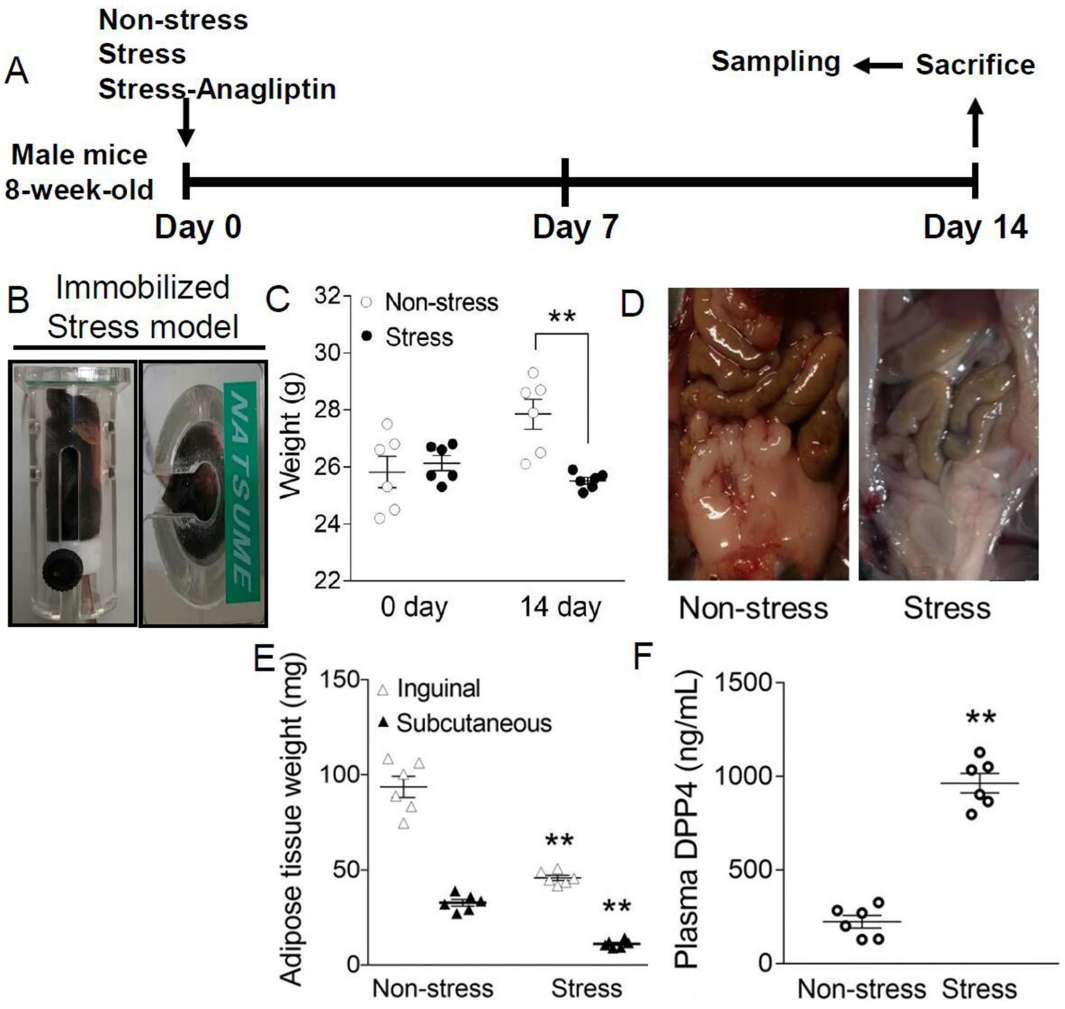

Fig. 1. Chronic stress decreased the body weight and increased plasma levels of DPP4 in DPP4$^{+/+}$ mice. A, B: Schematic diagram of mouse immobilized stress and sampling procedures at the indicated time points. C: Body weights in the non-stress and stress group mice at days 0 and 14 after the induction of restriction stress. D, E: Representative images and combined quantitative data show the abdominal subcutaneous and inguinal adipose. F: The DPP4 Glo Protease Assay showing plasma DPP4 levels in the non-stressed and stressed group mice at 2 weeks of stress. Scale bars: 50 μm. Data are mean ± SEM (n=6–7). ***P*<0.01 vs. the non-stressed group by Student's *t*-test, a two-way repeated-measures ANOVA and Bonferroni's post hoc tests, or a one-way ANOVA followed by Tukey's post hoc tests.

increases) and reductions of eNOS, SIRT1, and BCL-2 proteins (46–64% decreases) were observed in the pulmonary tissues of the stressed mice on day 14 after stress induction (Figs. 3A–C). Likewise, the quantitative data of western blots revealed that the stressed lungs had increased levels of senescence-related proteins (p16^{INK4A}, p53, and p21) and apoptosis-related proteins (caspase-3, -8, -9, Bax) (1.6- to 2.6-fold increases of the former and 1.3- to 2.7-fold increases of the latter) (Figs. 3D and E).

DPP4 inhibition protected against the stress-related pulmonary inflammation and oxidative stress production

We investigated whether pharmacological DPP4 inhibition could prevent pulmonary injury in mice subjected to chronic stress conditions. As anticipated, compared to the control DPP4$^{+/+}$ mice, the DPP4 inhibition by anagliptin decreased the levels of the target inflammatory genes (*MCP-1*, *PAI-1*, *Icam-1*, and *Vcam-1*; 55–88% reductions) and oxidative stress-related genes (*p47^{phox}*, *p22^{phox}*, *p67^{phox}*, and *gp91^{phox}*; 30–93% reductions) in the stressed lungs of S-Ana mice (Figs. 4A–C). We also observed that the administration of anagliptin resulted in decreases in the levels of the targeted proteolysis-related genes (*MMP-2*, *-9*, *TIMP-1*, *-2*, *CatK*, *CatS*; 38–92% reductions) in the lung tissues of the stressed mice (Figs. 4D–F). In contrast, the S-Ana mice had improved levels of *eNOS* gene expression (3.3-fold increase) compared to the control mice (Fig. 4C). As expected, a marked mitigation of the targeted protein

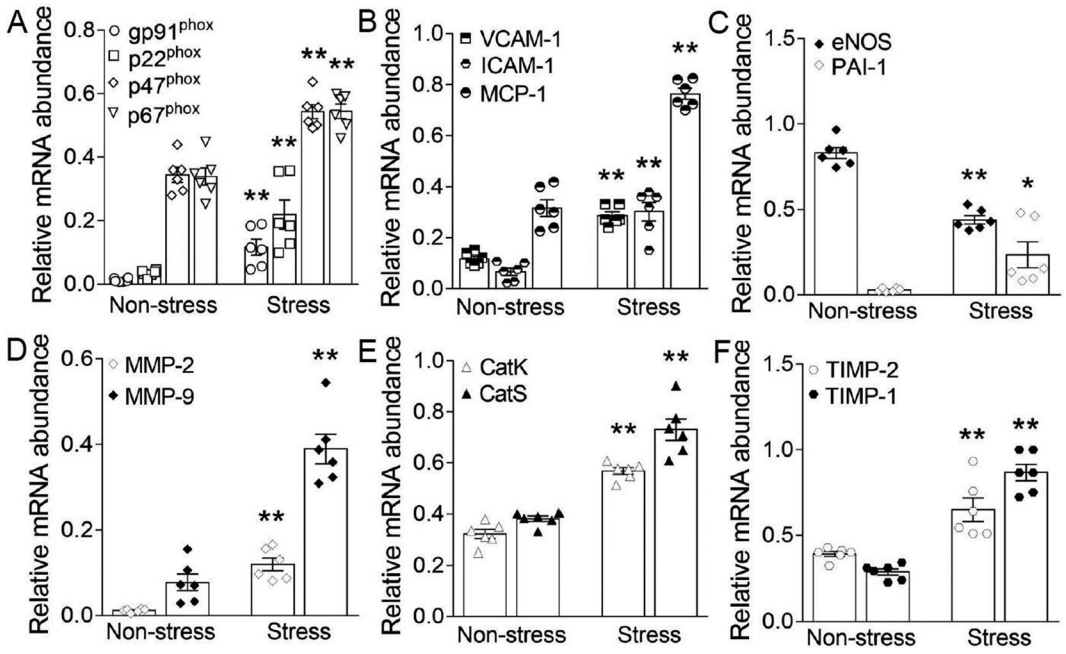

Fig. 2. Two weeks of immobilized stress increased the levels of inflammation-, oxidative stress-, and proteolysis-related genes in the lung tissues of the stressed DPP4$^{+/+}$ mice. A–F: Quantitative real-time PCR data present the expressions of *gp91^{phox}*, *p67^{phox}*, *p22^{phox}*, *p47^{phox}*, *PAI-1*, *Vcam-1*, and *Icam-1*, *MCP-1*, *MMP-2*, *-9*, *TIMP-1*, *-2*, *eNOS*, *CatK*, and *CatS* genes. Results are mean ± SEM (n=6–7). **P*<0.05, ***P*<0.01 vs. non-stressed group by Student's *t*-test or one-way ANOVA followed by Tukey's post hoc tests.

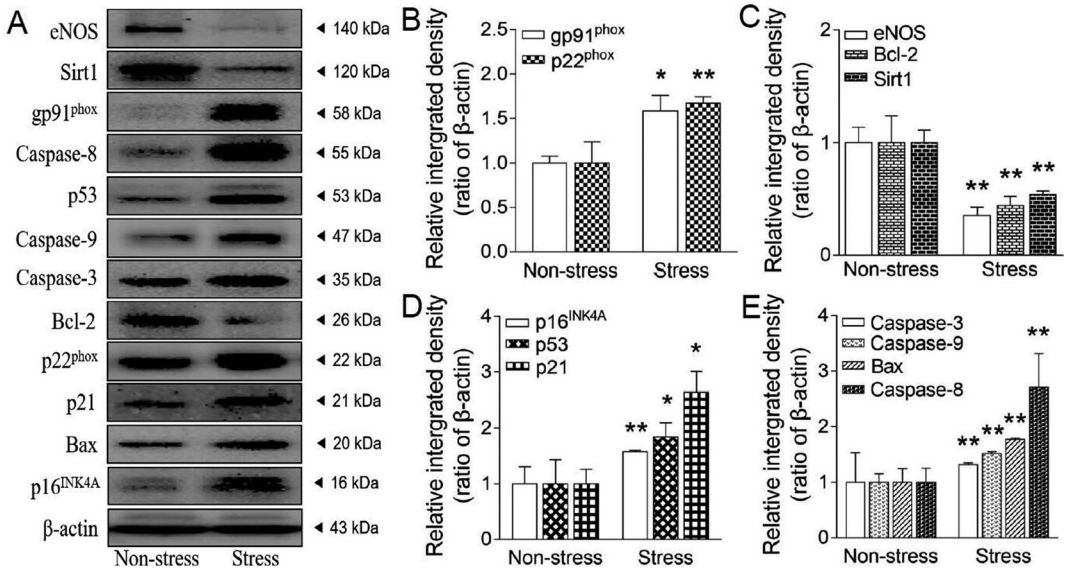

Fig. 3. Two weeks of immobilized stress increased the levels of the inflammation-, oxidative stress-, senescence-, and apoptosis-related protein expression levels in the lung tissues of the stressed DPP4$^{+/+}$ mice. An immunoblot analysis using equal amounts of total proteins from all samples was performed. A–E: Representative western blot images and quantitative data show the levels of p22^{phox}, gp91^{phox}, p53, p21, Sirt1, eNOS, p16^{INK4A}, caspase-3, -8, -9, Bax, and Bcl-2 proteins in the lung tissues of both groups. Results are mean ± SEM (n=4). **P*<0.05, ***P*<0.01 vs. non-stressed group by Student's *t*-test or one-way ANOVA followed by Tukey's post hoc tests.

alterations (eNOS, Sirt1, p22^{phox}, gp91^{phox}, caspase-3, -8, -9, Bcl-2, Bax, p53, p21, and p16^{INK4A}) was observed in the S-Ana mice compared to the stress-only mice (Fig. 5). Consistently, DPP4 deletion also exerted a beneficial effect on the harmful changes (Sirt1, gp91^{phox}, caspase-8, Bcl-2, p53, and p16^{INK4A}) (Fig. 6). As anticipated, the stressed DPP4$^{+/+}$ mice loaded anagliptin had decreased DPP4 levels of the plasma (398.9 ± 9.5 vs. 998.1 ± 13.8,

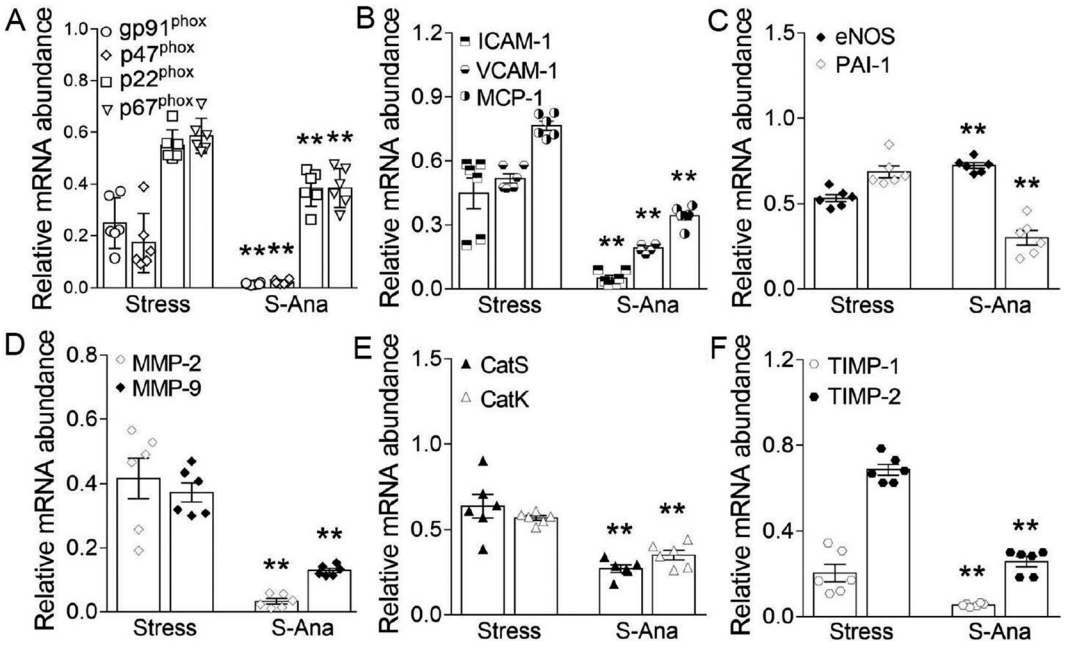

Fig. 4. The administration of anagliptin suppressed the levels of the inflammation-, oxidative stress-, and proteolysis-related mRNAs in stressed DPP4$^{+/+}$ mice. A-F: Quantitative PCR using the lungs tissues of both experimental groups shows the expressions of all targeted mRNAs (*gp91^{phox}*, *p67^{phox}*, *p22^{phox}*, *p47^{phox}*, *PAI-1*, *Vcam-1*, and *Icam-1*, *MCP-1*, *MMP-2*, *-9*, *TIMP-1*, *-2*, *eNOS*, *CatK* and *CatS*). Data are mean ± SEMs (n=6–7). **P*<0.05, ***P*<0.01 vs. the stressed group by one-way ANOVA followed by Tukey's post hoc tests or Student's *t*-test.

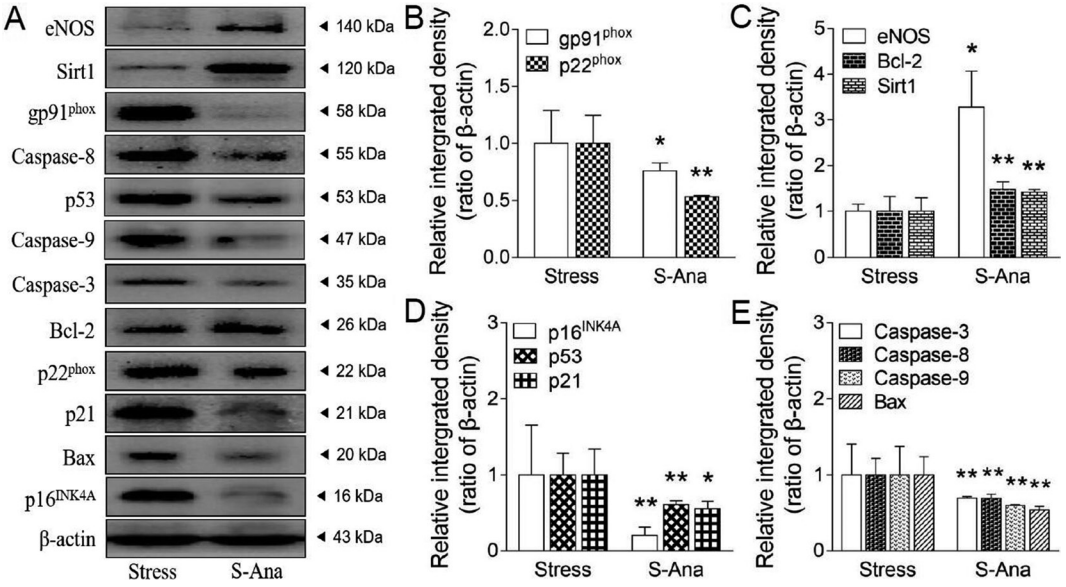

Fig. 5. DPP4 inhibition mitigated the harmful changes in the levels of the inflammation-, oxidative stress-, senescence-, and apoptosis- related protein expression levels in the lung tissues of the stressed DPP4$^{+/+}$ mice at 2 weeks after the induction of stress. A–E: An immunoblot analysis using equal amounts of total proteins from all samples was performed. Representative immunoblots and quantitative data show the levels of p22^{phox}, gp91^{phox}, p53, p21, Sirt1, eNOS, p16^{INK4A}, caspase-3, -8, -9, Bax, and Bcl-2 proteins in the lung tissues of both groups. Data are mean ± SEM (n=4). **P*<0.05, ***P*<0.01 vs. stressed group by Student's *t*-test or one-way ANOVA followed by Tukey's post hoc tests.

ng/ml, *P*<0.01) and lung (145.1 ± 8.1 vs. 309.3 ± 8.5, ng/ml, *P*<0.01) compared with those of the stressed alone control tissues. These observations suggest that DPP4 inhibition might prevent pulmonary aging and injury in mice under our experimental conditions. Additionally, the S-Ana mice had increased BWs and subcutaneous adipose volumes and decreased plasma DPP4 levels (Supplementary Fig. 1).

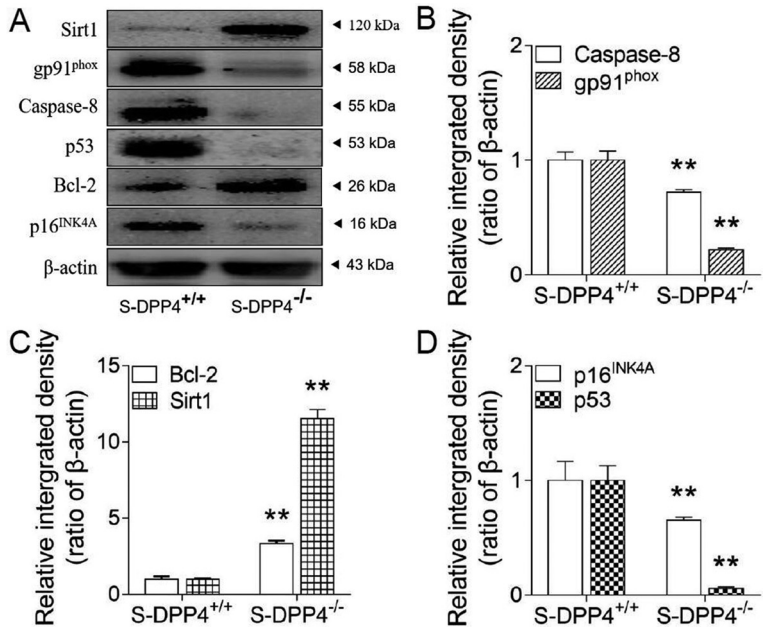

Fig. 6. DPP4 deletion mitigated the harmful changes in the levels of the inflammation-, oxidative stress-, senescence-, and apoptosis- related protein expression levels in the lung tissues of the stressed mice at 2 weeks after the induction of stress. A–D: An immunoblot analysis using equal amounts of total proteins from all samples was performed. Representative immunoblots and quantitative data show the levels of gp91^{phox}, p53, Sirt1, p16^{INK4A}, caspase-8, and Bcl-2 proteins in the lung tissues of both groups. Data are mean ± SEM. (n=4). **P*<0.05, ***P*<0.01 vs. S- DPP4$^{+/+}$ group by Student's *t*-test or one-way ANOVA followed by Tukey's post hoc tests.

Anagliptin prevented oxidative stress-related endothelial cell damage

To further confirm whether DPP4 inhibition protects against oxidative stress-induced pulmonary endothelial cell injury under chronic stress conditions, we first exposed HUVECs to H_2O_2 for 24h at the indicated concentrations, and the eNOS protein level was examined. The expression of eNOS protein was decreased in a dose-dependent manner (Supplementary Fig. 2). Interestingly, pretreatment with anagliptin rectified the reductions of *eNOS* gene expression and the elevations of the *p22^{phox}* and *gp91^{phox}* gene expressions in a dose-dependent manor (Supplementary Fig.S3A). Similar to its effect on oxidative stress genes, anagliptin reversed the increased levels of *MCP-1*, *Icam-1*, *Vcam-1*, *MMP-2*, *MMP-9*, *CatK*, and *CatS* mRNAs in response to H_2O_2 (Supplementary Fig. 3B). Moreover, as shown in Supplementary Figs. 3C and D, DPP4 inhibition mitigated the H_2O_2-induced increases in p22^{phox}, p21, and p16^{INK4A} and enhanced eNOS protein expression in HUVECs. Thus, DPP4 inhibition could exert an inhibitory effect on oxidative stress-induced endothelial injury in cultured HUVECs.

Discussion

As known, anagliptin like other DPP4 inhibitors (vildagliptin and sitagliptin) as a new class of antidiabetic drugs was based on the concept that these agents would enhance systematic and tissue glucagon-like peptide-1 (GLP-1) levels, causing an improvement of the insulinotropic effects of blood sugar. Accumulating evidence from our and other groups indicate anagliptin have a pleiotrophic effect on inflammatory and metabolic disorders. Thus, we studied the inhibitory effect of a DPP4 inhibitor on stress-related lung senescence and injury in mice (both *in vivo* and *in vitro*), with a special focus on inflammation and oxidative stress molecules. Our major findings were as follows: (1) Chronic stress increased pulmonary inflammation and oxidative stress, accompanied by an elevation of plasma DPP4 activity, thereby accelerating lung injury. These changes also caused an increase in the expressions of proteolysis-related targeted MMP and Cat family members as well as apoptosis and senescence-related targeted proteins, producing an inflammatory harmful change; (2) Genetic and pharmacological interventions aganist toward DPP4 ameliorated pulmonary inflammation, oxidative stress production, apoptosis, and proteolysis in mice under our

experimental conditions; (3) *In vitro* experiments, pretreatment with anagliptin mitigated the H_2O_2-induced upregulation of inflammation, oxidative stress, and proteolysis-related gene and/or protein expressions in HUVECs via the induction of eNOS expression.

DPP4 is an important and abundant serine proteolytic enzyme synthesized by blood cells and various tissues, and it is relevant to inflammation-associated metabolic disorders and their sequelae [22–24]. Accumulating evidence from experimental and clinical studies suggests that DPP4 plays important roles in various physiological and pathological conditions [16, 17]. Although those investigations uncovered glucagon-like peptide-1 (GLP-1)-dependent and independent mechanisms underlying DPP4 inhibition-mediated protective effects on inflammatory and metabolic disorders [30–32], the impact of DPP4 on the inception and progression of pulmonary injury and dysfunction in response to chronic stress has not been previously investigated. Several basic and clinical studies from our group and others have revealed that anagliptin ameliorated vascular aging, atherosclerotic plaque growth, thrombosis, and ischemic neovascularization in mice under chronic stress conditions [10–12, 33]. Anagliptin has been shown to mitigate bone marrow hematopoietic stem cell proliferation via the negative modulation of the β3-adrenergic receptor/CXCR4 axis in mice [13]. We showed that mouse lungs lacking DPP4 and those loaded with anagliptin are resistant to chronic variable injury. A comprehensive review described how multiple activities of DPP4 confer a broad range of functions, with implications for potential pathophysiologic roles in the development of pulmonary fibrosis and embolism [25]. Thus, the inhibition of plasma DPP4 activity could represent a common mechanism underlying its pleiotropic effects on inflammatory pulmonary disease in humans and animals subjected to chronic psychosocial stress.

It is clear that inflammation participates in all stages of metabolic pulmonary disease, including initiation, progression, and ultimately, thrombotic complications [29]. Laboratory evidence has revealed that chronic stress can promote the inflammatory process in different tissues, including adipose and vascular tissues [11, 34]. Consistently, we have shown that the lung tissues of stressed mice had increased levels of inflammatory *MCP-1*, *Vcam-1*, *Icam-1*, and *PAI-1* genes [35]. Our present findings demonstrated that anagliptin suppressed these inflammatory actions in the lung tissues of the stressed mice compared to the non-stressed mice. Because DPP4 is up-regulated in lung tissues of stressed mice [13], we propose that DPP4 functions as an important mediator of the inflammatory response to chronic stress during pulmonary injury, at least under our experimental conditions. This notion is further supported by the finding that pretreatment with anagliptin inhibited the expressions of oxidative stress-induced *MCP-1*, *Icam-1*, and *Vcam-1* genes in HUVECs.

It is well established that eNOS can modulate oxidative stress and senescence during the initiation and progression of various diseases [36, 37]. Apoptosis is a phenomenon that affects various biological processes [38]. The ability of chronic stress to increase oxidative stress and apoptosis is likely to contribute to lung aging and injury and dysfunction under experimental conditions. Genetic and pharmacological inhibitions of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity were observed to prevent injury and remodeling of the vascular system [39]. Our present results demonstrate that stress increased the gene and/or protein expressions of NADPH oxidase components (membrane types: $gp91^{phox}$ and $p22^{phox}$; cytoplasmic types: $p47^{phox}$ and $p67^{phox}$) in mouse lungs. The senescence-related proteins (p53, p21, and $p16^{INK4A}$) as well as apoptosis-related proteins (caspase-3, -8, -9, Bax) were also sensitive to chronic stress but stress reductions of eNOS, SIRT1, and BCL-2 proteins expression. Compared to the stress-alone mice, our findings demonstrated significant decrease in the levels of those targeted proteins ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, p53, p21, $p16^{INK4A}$, caspase-3, -8, -9, Bax) and increase the level of eNOS, SIRT1, and BCL-2 proteins expression in the lung tissues of S-Ana mice. Consistently, DPP4 deficiency produces a beneficial effect on lung injury induced by stress. In HUVECs, DPP4 inhibition ameliorated H_2O_2-induced expressions of $p22^{phox}$, eNOS, p21, and $p16^{INK4A}$ proteins and/or mRNAs. Oxidative stress can induce apoptosis and senescence of various cells in pathophysiological conditions [39, 40]. Taken together, these data provide evidence that pulmonary-protective actions of DPP4 inhibition are mediated, at least in part, through the modulation of eNOS-dependent anti-oxidative, anti-apoptotic, and anti-aging signaling pathways. It should be noted that the chronic stress decreased BWs and fat volumes; these alterations in the BWs and subcutaneous adipose volumes were rectified by the pharmacological inhibition of DPP4, suggesting that DPP4 might contribute to the adipogenesis and/or lipolysis. Further study will be needed to investigate this issue.

It has been reported that the synthesis and degradation of the extracellular matrix is critical to the maintenance of pulmonary integrity [29]. The degradation of the extracellular matrix and vascular cell functions are largely dependent on the cathepsin family, especially CatS and CatK [41]. Genetic and pharmacological inhibitions of

CatS and CatK prevented skeletal muscle and vascular remodeling [42–44]. In addition to the cathepsin family, the MMP family, especially MMP-2 and MMP-9, also participates in extracellular matrix protein (elastin and collagen) metabolism and cellular function during the cardiopulmonary disease process [27, 33]. We observed herein that lung tissues of the stressed mice had increased levels of proteolytic enzyme genes (*CatS*, *CatK*, *MMP-2*, *-9*, *TIMP-1*, *-2*) and that these changes were reversed by anagliptin treatment. In HUVECs, anagliptin mitigated the expressions of targeted enzymes in response to oxidative stress. Inflammation has been shown to modulate the expressions of MMPs and cathepsins in various cells under pathological conditions [45]. We thus hypothesized that a DPP4 inhibition-mediated reduction in proteolytic actions has a salutary effect on the pulmonary vasculature under conditions of chronic stress by improving inflammation, thereby preventing pulmonary injury.

One major potential limitation of the present study is that although current studies contains the detail biological data of the genetic and pharmacological interventions targeted toward DPP4 ameliorates chronic stress-related lung injury, we did not conduct morphological analyses regarding the effects of chronic stress and DPP4 inhibition on lung injury. Furthermore, it was included the data of pulmonary epithelial cells and fibroblasts *in vivo* and *in vitro*. Further studies will be needed to investigate these issues.

In conclusion, a chronic stress treatment of experimental mice increased the plasma concentrations of DPP4, which contributes to inflammation, oxidative stress production, apoptosis, and proteolysis, leading to pulmonary senescence and injury. A DPP4 inhibitor-based pharmacological intervention prevented the chronic stress-related pulmonary injury associated with inflammation, oxidative stress production, cell apoptosis, senescence, and proteolysis. This is the first study to demonstrate the beneficial action of anagliptin in CPS-related pulmonary injury, suggesting a potential pharmacological treatment alternative for pulmonary disease in patients with chronic psychosocial stress.

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Authors' Contributions

SZ and XWC designed the study and drafted the manuscript. PL, MX, XJ and LZ were responsible for the collection and analysis of the experimental data. YN and XWC revised the manuscript critically for important intellectual content and were also involved in the conception of the study. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The animal study protocol (YM20013) was approved by the Ethics Committee on Animal Research at Yanbian University Medical College. All experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals of the Chinese Pharmacological Society.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this manuscript.

References

1. Rajpoot A, Garg K, Saini V, Gupta N. Psychological morbidity in interstitial lung disease: a study from India. Monaldi Arch Chest Dis. 2020; 90. [Medline] [CrossRef]
2. Coumoundouros C, von Essen L, Sanderman R, Woodford J. Implementation of e-mental health interventions for informal caregivers of adults with chronic diseases: a protocol for a mixed-methods systematic review with a qualitative comparative analysis. BMJ Open. 2020; 10: e035406. [Medline] [CrossRef]
3. López-Lois B, González-Barcala FJ, Facal D. Application of mindfulness techniques in patients with asthma or COPD. J Asthma. 2020: 1–10. [Medline] [CrossRef]
4. Powell ND, Sloan EK, Bailey MT, Arevalo JM, Miller GE, Chen E, et al. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via β-adrenergic induction of myelopoiesis. Proc Natl Acad Sci USA. 2013; 110: 16574–16579. [Medline] [CrossRef]
5. Osborne MT, Shin LM, Mehta NN, Pitman RK, Fayad ZA, Tawakol A. Disentangling the Links Between Psychosocial Stress and Cardiovascular Disease. Circ Cardiovasc Imaging. 2020; 13: e010931. [Medline] [CrossRef]
6. Burtscher J, Burtscher M, Millet GP. (Indoor) isolation, stress, and physical inactivity: Vicious circles accelerated by COVID-19? Scand J Med Sci Sports. 2020; 30: 1544–1545. [Medline] [CrossRef]
7. Vepa A, Bae JP, Ahmed F, Pareek M, Khunti K. COVID-19 and ethnicity: A novel pathophysiological role for inflammation. Diabetes Metab Syndr. 2020; 14: 1043–1051. [Medline] [CrossRef]
8. Picaza Gorrochategi M, Eiguren Munitis A, Dosil Santamaria M, Ozamiz Etxebarria N. Stress, Anxiety, and Depression in People Aged Over 60 in the COVID-19 Outbreak in a Sample Collected in Northern Spain. Am J Geriatr Psychiatry. 2020; 28: 993–998. [Medline] [CrossRef]

9. Taylor WD, Blackford JU. Mental Health Treatment for Front-Line Clinicians During and After the Coronavirus Disease 2019 (COVID-19) Pandemic: A Plea to the Medical Community. Ann Intern Med. 2020; 173: 574–575. [Medline] [CrossRef]
10. Xin M, Jin X, Cui X, Jin C, Piao L, Wan Y, et al. Dipeptidyl peptidase-4 inhibition prevents vascular aging in mice under chronic stress: Modulation of oxidative stress and inflammation. Chem Biol Interact. 2019; 314: 108842. [Medline] [CrossRef]
11. Jin X, Jin C, Nakamura K, Jin T, Xin M, Wan Y, et al. Increased dipeptidyl peptidase-4 accelerates chronic stress-related thrombosis in a mouse carotid artery model. J Hypertens. 2020; 38: 1504–1513. [Medline] [CrossRef]
12. Piao L, Zhao G, Zhu E, Inoue A, Shibata R, Lei Y, et al. Chronic Psychological Stress Accelerates Vascular Senescence and Impairs Ischemia-Induced Neovascularization: The Role of Dipeptidyl Peptidase-4/Glucagon-Like Peptide-1-Adiponectin Axis. J Am Heart Assoc. 2017; 6: e006421. [Medline] [CrossRef]
13. Zhu E, Hu L, Wu H, Piao L, Zhao G, Inoue A, et al. Dipeptidyl Peptidase-4 Regulates Hematopoietic Stem Cell Activation in Response to Chronic Stress. J Am Heart Assoc. 2017; 6: e006394. [Medline] [CrossRef]
14. Lundh L, Alinaghizadeh H, Törnkvist L, Gilljam H, Galanti MR. A new instrument to predict smoking cessation among patients with chronic obstructive pulmonary disease: an observational longitudinal study of the Trying To Quit smoking questionnaire. NPJ Prim Care Respir Med. 2016; 26: 16013. [Medline] [CrossRef]
15. Dunton G, Dzubur E, Li M, Huh J, Intille S, McConnell R. Momentary Assessment of Psychosocial Stressors, Context, and Asthma Symptoms in Hispanic Adolescents. Behav Modif. 2016; 40: 257–280. [Medline] [CrossRef]
16. Lei Y, Hu L, Yang G, Piao L, Jin M, Cheng X. Dipeptidyl Peptidase-IV Inhibition for the Treatment of Cardiovascular Disease - Recent Insights Focusing on Angiogenesis and Neovascularization. Circ J. 2017; 81: 770–776. [Medline] [CrossRef]
17. Zhong J, Maiseyeu A, Davis SN, Rajagopalan S. DPP4 in cardiometabolic disease: recent insights from the laboratory and clinical trials of DPP4 inhibition. Circ Res. 2015; 116: 1491–1504. [Medline] [CrossRef]
18. Ghorpade DS, Ozcan L, Zheng Z, Nicoloro SM, Shen Y, Chen E, et al. Hepatocyte-secreted DPP4 in obesity promotes adipose inflammation and insulin resistance. Nature. 2018; 555: 673–677. [Medline] [CrossRef]
19. Ikedo T, Minami M, Kataoka H, Hayashi K, Nagata M, Fujikawa R, et al. Dipeptidyl Peptidase-4 Inhibitor Anagliptin Prevents Intracranial Aneurysm Growth by Suppressing Macrophage Infiltration and Activation. J Am Heart Assoc. 2017; 6: e004777. [Medline] [CrossRef]
20. Aoyama M, Kawase H, Bando YK, Monji A, Murohara T. Dipeptidyl Peptidase 4 Inhibition Alleviates Shortage of Circulating Glucagon-Like Peptide-1 in Heart Failure and Mitigates Myocardial Remodeling and Apoptosis via the Exchange Protein Directly Activated by Cyclic AMP 1/Ras-Related Protein 1 Axis. Circ Heart Fail. 2016; 9: e002081. [Medline] [CrossRef]
21. Furuhashi M, Sakuma I, Morimoto T, Higashiura Y, Sakai A, Matsumoto M, et al. Treatment with anagliptin, a DPP-4 inhibitor, decreases FABP4 concentration in patients with type 2 diabetes mellitus at a high risk for cardiovascular disease who are receiving statin therapy. Cardiovasc Diabetol. 2020; 19: 89. [Medline] [CrossRef]
22. Pantanetti P, Cangelosi G, Ambrosio G. Potential role of incretins in diabetes and COVID-19 infection: a hypothesis worth exploring. Intern Emerg Med. 2020; 15: 779–782. [Medline] [CrossRef]
23. Solerte SB, Di Sabatino A, Galli M, Fiorina P. Dipeptidyl peptidase-4 (DPP4) inhibition in COVID-19. Acta Diabetol. 2020; 57: 779–783. [Medline] [CrossRef]
24. Maremanda KP, Sundar IK, Li D, Rahman I. Age-dependent assessment of genes involved in cellular senescence, telomere and mitochondrial pathways in human lung tissue of smokers, COPD and IPF: Associations with SARS-CoV-2 COVID-19 ACE2-TMPRSS2-Furin-DPP4 axis. Front Pharmacol. 2020; 11: 584637. [Medline] [CrossRef]
25. Bassendine MF, Bridge SH, McCaughan GW, Gorrell MD. COVID-19 and comorbidities: A role for dipeptidyl peptidase 4 (DPP4) in disease severity? J Diabetes. 2020; 12: 649–658. [Medline] [CrossRef]
26. Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A, et al. Chronic variable stress activates hematopoietic stem cells. Nat Med. 2014; 20: 754–758. [Medline] [CrossRef]
27. Meng X, Piao L, Wang H, Inoue A, Huang Z, Jiang H, et al. Deficiency of cysteinyl cathepsin K suppresses the development of experimental intimal hyperplasia in response to chronic stress. J Hypertens. 2020; 38: 1514–1524. [Medline] [CrossRef]
28. Archer SL, Marsboom G, Kim GH, Zhang HJ, Toth PT, Svensson EC, et al. Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. Circulation. 2010; 121: 2661–2671. [Medline] [CrossRef]
29. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. Circ Res. 2014; 115: 165–175. [Medline] [CrossRef]
30. Yang G, Lei Y, Inoue A, Piao L, Hu L, Jiang H, et al. Exenatide mitigated diet-induced vascular aging and atherosclerotic plaque growth in ApoE-deficient mice under chronic stress. Atherosclerosis. 2017; 264: 1–10. [Medline] [CrossRef]
31. Qin X, Fang E, Narisawa M, Cheng XW. Alternating P wave morphology. Circulation. 2019; 139: 1225–1227. [Medline] [CrossRef]
32. Krijnen PA, Hahn NE, Kholová I, Baylan U, Sipkens JA, van Alphen FP, et al. Loss of DPP4 activity is related to a prothrombogenic status of endothelial cells: implications for the coronary microvasculature of myocardial infarction patients. Basic Res Cardiol. 2012; 107: 233. [Medline] [CrossRef]
33. Lei Y, Yang G, Hu L, Piao L, Inoue A, Jiang H, et al. FAHA. Increased dipeptidyl peptidase-4 accelerates diet-related vascular aging and atherosclerosis in ApoE-deficient mice under chronic stress. Int J Cardiol. 2017; 243: 413–420. [Medline] [CrossRef]
34. Wang H, Meng X, Piao L, Inoue A, Xu W, Yu C, et al. Cathepsin S Deficiency Mitigated Chronic Stress-Related Neointimal Hyperplasia in Mice. J Am Heart Assoc. 2019; 8: e011994. [Medline] [CrossRef]
35. Piao L, Yu C, Xu W, Inoue A, Shibata R, Li X, et al. Adiponectin/AdiopR1 signal inactivation contributes to impaired angiogenesis in mice of advanced age. Int J Cardiol. 2018; 267: 150–155. [Medline] [CrossRef]
36. Sudhahar V, Okur MN, O'Bryan JP, Minshall RD, Fulton D, Ushio-Fukai M, et al. Caveolin-1 stabilizes ATP7A, a copper transporter for extracellular SOD, in vascular tissue to maintain endothelial function. Am J Physiol Cell Physiol. 2020; 319: C933–C944. [Medline] [CrossRef]
37. Pushpakumar S, Ren L, Juin SK, Majumder S, Kulkarni R, Sen U. Methylation-dependent antioxidant-redox imbalance regulates hypertensive kidney injury in aging. Redox Biol. 2020; 37: 101754. [Medline] [CrossRef]
38. Zhang W, Tian Y, Gao Q, Li X, Li Y, Zhang J, et al. Inhibition of Apoptosis Reduces Diploidization of Haploid Mouse Embryonic Stem Cells during Differentiation. Stem Cell Reports. 2020; 15: 185–197. [Medline] [CrossRef]
39. Juni RP, Duckers HJ, Vanhoutte PM, Virmani R, Moens AL. Oxidative stress and pathological changes after coronary ar-

tery interventions. J Am Coll Cardiol. 2013; 61: 1471–1481. [Medline] [CrossRef]
40. Hu L, Huang Z, Ishii H, Wu H, Suzuki S, Inoue A, et al. PLF-1 (Proliferin-1) Modulates Smooth Muscle Cell Proliferation and Development of Experimental Intimal Hyperplasia. J Am Heart Assoc. 2019; 8: e005886. [Medline] [CrossRef]
41. Wu H, Du Q, Dai Q, Ge J, Cheng X. Cysteine Protease Cathepsins in Atherosclerotic Cardiovascular Diseases. J Atheroscler Thromb. 2018; 25: 111–123. [Medline] [CrossRef]
42. Hu L, Cheng XW, Song H, Inoue A, Jiang H, Li X, et al. Cathepsin K activity controls injury-related vascular repair in mice. Hypertension. 2014; 63: 607–615. [Medline] [CrossRef]
43. Wu H, Cheng XW, Hu L, Takeshita K, Hu C, Du Q, et al. Cathepsin S Activity Controls Injury-Related Vascular Repair in Mice via the TLR2-Mediated p38MAPK and PI3K-Akt/p-HDAC6 Signaling Pathway. Arterioscler Thromb Vasc Biol. 2016; 36: 1549–1557. [Medline] [CrossRef]
44. Ogasawara S, Cheng XW, Inoue A, Hu L, Piao L, Yu C, et al. Cathepsin K activity controls cardiotoxin-induced skeletal muscle repair in mice. J Cachexia Sarcopenia Muscle. 2018; 9: 160–175. [Medline] [CrossRef]
45. Jiang H, Sasaki T, Jin E, Kuzuya M, Cheng XW. Inflammatory Cells and Proteases in Abdominal Aortic Aneurysm and its Complications. Curr Drug Targets. 2018; 19: 1289–1296. [Medline] [CrossRef]