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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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1 **ABSTRACT**

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

21
22 **Key words:** apoptosis, chronic psychological stress, inflammation, pulmonary injury.

1 **Introduction**

2 Exposure to chronic psychosocial stress (CPS) is a risk factor for many diseases, including
3 asthma, chronic obstructive pulmonary disease, and lung cancer [1-3]. Stress has been shown to
4 increase inflammation-related gene expression in inflammatory leukocytes through a β -adrenergic
5 induction of myelopoiesis [4]. An association between chronic stress and metabolic and
6 inflammatory diseases including cardiovascular disease and pulmonary disease has been
7 demonstrated [5-7]. The incidence and mortality of CPS-related diseases including pulmonary
8 disease are increasing as the population ages [8]. In the present coronavirus disease 2019
9 (COVID-19) pandemic, patients under moderate or high stress at the time of pulmonary infection
10 have exhibited increased mortality compared to subjects under low levels of stress [9]. It was also
11 reported that vascular endothelial cells and stem cells are sensitive to various pathological
12 stressors [10-13]. Chronic stress activates both the sympathetic nervous system and the
13 hypothalamus-pituitary-adrenal axis, and the excessive pathophysiological responses that are
14 induced contribute to stress-related pulmonary disease (SRPD) [14, 15]. However, the exact
15 mechanisms underlying CPS-related lung aging and injury remain unclear.

16 Dipeptidyl peptidase 4 (DPP4) is a member of a complex gene family that acts nonspecifically
17 to cleave a wide variety of structure-related peptides (including growth factors, hormones,
18 chemokines, cytokines, and neuropeptides) [16, 17]. Over last ten years, emerging data have
19 demonstrated unexpected roles for DPP4 in extracellular and intracellular signaling, insulin
20 resistance, oxidative stress production, inflammation, immune activation, cell apoptosis, and lipid
21 metabolism [17-20]. We recently observed that DPP4 inhibition by anagliptin ameliorated iron
22 chloride (FeCl_3)-induced thrombosis in mice that were subjected to stress via the modulation of
23 plasminogen activator inhibitor-1 (PAI-1) and ADAMTS13 (a disintegrin-like and

1 metalloproteinase with thrombospondin type 1 motif, member 13) activity [11]. The activation of
2 the endothelial nitric oxide synthase/Sirt1 axis contributed to an anagliptin-mediated
3 amelioration of chronic stress-related vascular aging [10]. It was shown that bone marrow lacking
4 DPP4 or loaded with anagliptin is resistant to bone marrow hematopoietic stem cell chronic stress
5 [13]. Moreover, several studies have indicated that there is a close link between increased plasma
6 DPP4 activity and both atherosclerotic plaque growth and impaired ischemia-induced vascular
7 regeneration capacity in mice under chronic stress conditions, and these harmful effects were
8 rectified by DPP4 deletion and anagliptin treatment. Ikedo and colleagues reported that anagliptin
9 prevented intracranial aneurysm growth by suppressing macrophage infiltration and activation
10 [19]. A recent clinical study demonstrated that anagliptin reduced the serum fatty acid-binding
11 protein-4 concentration independent of the changes in hemoglobin A1c or LDL-C in patients with
12 type 2 diabetes mellitus and dyslipidemia who were on statin therapy [21]. Recent comprehensive
13 review articles pointed out that DPP4 inhibitors (anagliptin, vildagliptin, and sitagliptin) that were
14 developed and marketed for their effects display multipotency in the management of various
15 pulmonary diseases [22, 23]. A clinical study revealed that DPP4 was highly expressed in the lungs
16 of patients both with and without diabetes mellitus [24]. DPP4 is upregulated in metabolic and
17 inflammatory pathological conditions associated with chronic psychological stressor [13].
18 Although clinical and experimental findings indicate that DPP4 is involved in the occurrence and
19 development of pulmonary fibrosis and embolism [25], little is known about the role of DPP4 in
20 CPS-related lung injury and SRPD.

21 In the present study, we used DPP4 deficiency ($\text{DPP4}^{-/-}$) mice, DPP4 inhibitor anagliptin, and
22 an immobilized stress model to test our hypothesis that DPP4 activity may negatively modulate
23 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).

The following commercially available reagents were used: DPP4-Glo assay kit (Promega, Madison, WI); 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); nitrocellulose transfer membrane (Millipore, Boston, MA); RNeasy Micro Kits and SYBRTM Green Master Mix (Qiagen, Hilden, Germany); BCA protein assay kit (PC0020, Solarbio Sciences, Beijing, China); SuperScript III First Strand (Invitrogen, Carlsbad CA); Amersham ECL Prime Western Blotting Detection kit (Millipore); SuperSignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA); urethane (Sinopharm Group, Shanghai, China); and the immobilization stress tube (Cat. 551-BSRR,

Natsume Seisakusho, Tokyo). 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

1 a 1:2,000–5,000 dilution. SuperSignal™ West Pico PLUS Chemiluminescent Substrate was used
2 for the evaluation of targeted proteins. The levels of the targeted proteins quantified by Western
3 blots were normalized by the loading β -actin levels.

4 5 Plasma and tissue DPP4 level analysis

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

18 19 Endothelial cell culture and experiments

20 HUVECs were cultured in EGM-2 containing 10% fetal bovine serum (FBS) and antibiotics at
21 37°C with 5% CO₂ until confluence. After being cultured in serum-free EBM-2 overnight, the
22 HUVECs were subjected to the following experiments: (1) First, to evaluate the effect of
23 oxidative stress on endothelial cell damage, cells were cultured in the presence and absence of

H₂O₂ (0, 200, 400 µmol/L) in EBM-2 medium for 24h and then subjected to an assay of eNOS protein expression. (2) Second, to explore 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 H₂O₂ (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}*, *gp91^{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: $2^{-\Delta\Delta Ct}$; Applied Biosystems, Foster City, CA) [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

1 measured continuously. The expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)
2 was measured in parallel to that of the genes of interest and was used as an internal standard for
3 the quantitative comparison of mRNA levels. The primer sequences are listed in Supplementary
4 Table S1.

5 6 Statistical analyses

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

15 16 Results

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

18 An immobilized stress model (Fig. 1A,B) has often been applied to the study of psychological
19 stress-related metabolic and inflammatory cardiovascular disorders [12, 26]. Here, to test our
20 hypothesis, we created an immobilization model to examine whether chronic stress could
21 influence the levels of DPP4 activity and BW and adipose volumes. The chronic stress-treated
22 mice had decreased BWs and decreased subcutaneous and inguinal adipose tissue and increased
23 plasma DPP4 levels compared to the control DPP4^{+/+} mice (Fig. 1C–F). The stressed lungs also

1 had elevated levels of DPP4 proteins compared to the control lungs (256.3 ± 4.5 vs. 103.9 ± 3.9 ,
2 ng/ml, $p < 0.01$), suggesting an association between pulmonary injury and increased plasma DPP4
3 activity in mice subjected to chronic stress.

4
5 Stress promoted inflammation, oxidative stress production, and proteolysis in the lung.

6 It is well known that inflammation and oxidative processes are involved in various pulmonary
7 diseases [28, 29]. As the second step in studying the relationships between stress and inflammation
8 and oxidative stress, we analyzed the expressions of genes related to oxidative stress ($p47^{phox}$, $p22^{phox}$,
9 $p67^{phox}$, and $gp91^{phox}$) and inflammation (*PAI-1*, *MCP-1*, *Vcam-1*, and *Icam-1*) in the lungs of
10 both experimental and control group mice by conducting a quantitative PCR assay after 14 days of
11 chronic stress. Compared to the non-stressed control DPP4^{+/+} mice, the quantitative PCR using the
12 non-stressed and stressed lung tissues (Fig. 2A–C) showed that the levels of targeted inflammatory
13 and oxidative stress-related genes were higher (2.4–8.3-fold increases of the former genes;
14 1.6–9.5-fold increases in the latter genes) in the stressed mice than in the non-stressed mice.
15 Conversely, the chronic stress decreased the *eNOS* gene expression in the lungs (Fig. 2C). As
16 anticipated, we also observed that the stressed lung tissues had increased levels of
17 proteolysis-related genes (*MMP-2*, *-9*, *TIMP-1*, *-2*, *CatS*, *CatK*; 1.7–10.4-fold increases) compared
18 to the lung tissues of the non-stressed mice (Fig. 2D–F). An immunoblot analysis using equal
19 amounts of protein from all samples was performed. Quantitative western blotting yielded the
20 same conclusion (Fig. 3). Marked elevations of $p22^{phox}$ and $gp91^{phox}$ proteins (>1.6-fold increases)
21 and reductions of *eNOS*, *SIRT1*, and *BCL-2* proteins (46%–64% decreases) were observed in the
22 pulmonary tissues of the stressed mice on day 14 after stress induction (Fig. 3A–C). Likewise, the
23 quantitative data of western blots revealed that the stressed lungs had increased levels of

1 senescence-related proteins (p16^{INK4A}, p53, and p21) and apoptosis-related proteins (caspase-3, -8,
2 -9, Bax) (1.6- to 2.6-fold increases of the former and 1.3- to 2.7-fold increases of the latter) (Fig.
3 3D,E).

4
5 DPP4 inhibition protected against the stress-related pulmonary inflammation and oxidative stress
6 production

7 We investigated whether pharmacological DPP4 inhibition could prevent pulmonary injury in
8 mice subjected to chronic stress conditions. As anticipated, compared to the control DPP4^{+/+} mice,
9 the DPP4 inhibition by anagliptin decreased the levels of the target inflammatory genes (*MCP-1*,
10 *PAI-1*, *Icam-1*, and *Vcam-1*; 55%–88% reductions) and oxidative stress-related genes (*p47^{phox}*, *p22^{phox}*,
11 *p67^{phox}*, and *gp91^{phox}*; 30%–93% reductions) in the stressed lungs of S-Ana mice (Fig. 4A–C).

12 We also observed that the administration of anagliptin resulted in decreases in the levels of the
13 targeted proteolysis-related genes (*MMP-2*, -9, *TIMP-1*, -2, *CatK*, *CatS*; 38%–92% reductions) in
14 the lung tissues of the stressed mice (Fig. 4D–F). In contrast, the S-Ana mice had improved levels
15 of *eNOS* gene expression (3.3-fold increase) compared to the control mice (Fig. 4C). As expected,
16 a marked mitigation of the targeted protein alterations (eNOS, Sirt1, p22^{phox}, gp91^{phox}, caspase-3,
17 -8, -9, Bcl-2, Bax, p53, p21, and p16^{INK4A}) was observed in the S-Ana mice compared to the
18 stress-only mice (Fig. 5). Consistently, DPP4 deletion also exerted a beneficial effect on the
19 harmful changes (Sirt1, gp91^{phox}, caspase-8, Bcl-2, p53, and p16^{INK4A}) (Fig. 6). As anticipated, the
20 stressed DPP4^{+/+} mice loaded anagliptin had decreased DPP4 levels of the plasma (398.9 ± 9.5 vs.
21 998.1 ± 13.8, ng/ml, p< 0.01) and lung (145.1 ± 8.1 vs. 309.3 ± 8.5, ng/ml, p< 0.01) compared with
22 those of the stressed alone control tissues. These observations suggest that DPP4 inhibition might
23 prevent pulmonary aging and injury in mice under our experimental conditions. Additionally, the

1 S-Ana mice had increased BWs and subcutaneous adipose volumes and decreased plasma DPP4
2 levels (Suppl. Fig. S1).

3
4 Anagliptin prevented oxidative stress-related endothelial cell damage.

5 To further confirm whether DPP4 inhibition protects against oxidative stress-induced
6 pulmonary endothelial cell injury under chronic stress conditions, we first exposed HUVECs to
7 H₂O₂ for 24h at the indicated concentrations, and the eNOS protein level was examined. The
8 expression of eNOS protein was decreased in a dose-dependent manner (Suppl. Fig. S2).
9 Interestingly, pretreatment with anagliptin rectified the reductions of *eNOS* gene expression and
10 the elevations of the *p22^{phox}* and *gp91^{phox}* gene expressions in a dose-dependent manor (Suppl. Fig.
11 S3A). Similar to its effect on oxidative stress genes, anagliptin reversed the increased levels of
12 *MCP-1*, *Icam-1*, *Vcam-1*, *MMP-2*, *MMP-9*, *CatK*, and *CatS* mRNAs in response to H₂O₂ (Suppl.
13 Fig. 3B). Moreover, as shown in Supplementary Figure 3C,D, DPP4 inhibition mitigated the
14 H₂O₂-induced increases in p22^{phox}, p21, and p16^{INK4A} and enhanced eNOS protein expression in
15 HUVECs. Thus, DPP4 inhibition could exert an inhibitory effect on oxidative stress-induced
16 endothelial injury in cultured HUVECs.

17 18 **Discussion**

19 As known, anagliptin like other DPP4 inhibitors (vildagliptin and sitagliptin) as a new class of
20 antidiabetic drugs was based on the concept that these agents would enhance systematic and tissue
21 glucagon-like peptide-1 (GLP-1) levels, causing an improvement of the insulinotropic effects of
22 blood sugar. Accumulating evidence from our and other groups indicate anagliptin have a
23 pleiotrophic effect on inflammatory and metabolic disorders. Thus, we studied the inhibitory

1 effect of a DPP4 inhibitor on stress-related lung senescence and injury in mice (both in vivo and in
2 vitro), with a special focus on inflammation and oxidative stress molecules. Our major findings
3 were as follows: (1) Chronic stress increased pulmonary inflammation and oxidative stress,
4 accompanied by an elevation of plasma DPP4 activity, thereby accelerating lung injury. These
5 changes also caused an increase in the expressions of proteolysis-related targeted MMP and Cat
6 family members as well as apoptosis and senescence-related targeted proteins, producing an
7 inflammatory harmful change; (2) Genetic and pharmacological interventions against toward
8 DPP4 ameliorated pulmonary inflammation, oxidative stress production, apoptosis, and
9 proteolysis in mice under our experimental conditions; (3) In vitro experiments, pretreatment with
10 anagliptin mitigated the H₂O₂-induced upregulation of inflammation, oxidative stress, and
11 proteolysis-related gene and/or protein expressions in HUVECs via the induction of eNOS
12 expression.

13 DPP4 is an important and abundant serine proteolytic enzyme synthesized by blood cells and
14 various tissues, and it is relevant to inflammation-associated metabolic disorders and their
15 sequelae [22-24]. Accumulating evidence from experimental and clinical studies suggests that
16 DPP4 plays important roles in various physiological and pathological conditions [16, 17].
17 Although those investigations uncovered glucagon-like peptide-1 (GLP-1)-dependent and
18 independent mechanisms underlying DPP4 inhibition-mediated protective effects on
19 inflammatory and metabolic disorders [30-32], the impact of DPP4 on the inception and
20 progression of pulmonary injury and dysfunction in response to chronic stress has not been
21 previously investigated. Several basic and clinical studies from our group and others have revealed
22 that anagliptin ameliorated vascular aging, atherosclerotic plaque growth, thrombosis, and
23 ischemic neovascularization in mice under chronic stress conditions [10-12, 33]. Anagliptin has

1 been shown to mitigate bone marrow hematopoietic stem cell proliferation via the negative
2 modulation of the β 3-adrenergic receptor/CXCR4 axis in mice [13]. We showed that mouse lungs
3 lacking DPP4 and those loaded with anagliptin are resistant to chronic variable injury. A
4 comprehensive review described how multiple activities of DPP4 confer a broad range of
5 functions, with implications for potential pathophysiologic roles in the development of pulmonary
6 fibrosis and embolism [25]. Thus, the inhibition of plasma DPP4 activity could represent a
7 common mechanism underlying its pleiotropic effects on inflammatory pulmonary disease in
8 humans and animals subjected to chronic psychosocial stress.

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

21 It is well established that eNOS can modulate oxidative stress and senescence during the
22 initiation and progression of various diseases [36, 37]. Apoptosis is a phenomenon that affects
23 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 H₂O₂-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

1 vascular cell functions are largely dependent on the cathepsin family, especially CatS and CatK
2 [41]. Genetic and pharmacological inhibitions of CatS and CatK prevented skeletal muscle and
3 vascular remodeling [42-44]. In addition to the cathepsin family, the MMP family, especially
4 MMP-2 and MMP-9, also participates in extracellular matrix protein (elastin and collagen)
5 metabolism and cellular function during the cardiopulmonary disease process [27, 33]. We
6 observed herein that lung tissues of the stressed mice had increased levels of proteolytic enzyme
7 genes (*CatS*, *CatK*, *MMP-2*, *-9*, *TIMP-1*, *-2*) and that these changes were reversed by anagliptin
8 treatment. In HUVECs, anagliptin mitigated the expressions of targeted enzymes in response to
9 oxidative stress. Inflammation has been shown to modulate the expressions of MMPs and
10 cathepsins in various cells under pathological conditions [45]. We thus hypothesized that a DPP4
11 inhibition-mediated reduction in proteolytic actions has a salutary effect on the pulmonary
12 vasculature under conditions of chronic stress by improving inflammation, thereby preventing
13 pulmonary injury.

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

20 In conclusion, a chronic stress treatment of experimental mice increased the plasma
21 concentrations of DPP4, which contributes to inflammation, oxidative stress production, apoptosis,
22 and proteolysis, leading to pulmonary senescence and injury. A DPP4 inhibitor-based
23 pharmacological intervention prevented the chronic stress-related pulmonary injury associated

1 with inflammation, oxidative stress production, cell apoptosis, senescence, and proteolysis. This is
2 the first study to demonstrate the beneficial action of anagliptin in CPS-related pulmonary injury,
3 suggesting a potential pharmacological treatment alternative for pulmonary disease in patients
4 with chronic psychosocial stress.

Disclosures

The authors have no conflict of interest to declare.

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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.

Competing interests

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

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FIGURE LEGENDS

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 μ m. Data are mean $\pm$ 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.

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 $\pm$ 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 $\pm$ 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.

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.

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 $\pm$ 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.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Original Article: Model animals

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

Suppl. Figs. S1–S3 and Suppl Table S1

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Supplementary Table S1 Primer sequences for mice used for quantitative real-time PCR

Gene	Forward Primer	Reverse Primer
<i>CatK</i>	AGCAGGCTGGAGGACTAAGGT	TTTGTGCATCTCAGTGGAAGACT
<i>CatS</i>	GTGGCCACTA AAGGGCCTG	ACCGCTTTTGTAGAAGAAGAAGGAG
<i>eNOS</i>	TGTCTGCGGCGATGTCACT	CATGCCGCCCTCTGTTG
<i>PAI-1</i>	TCAGAGCAACAAGTTCAACTACACTGAG	CCCACTGTCAAGGCTCCATCACTTGCCCA
<i>MMP-2</i>	CCCCATGAAGCCTTGTTTACC	TTGTAGGAGGTGCCCTGGAA
<i>MMP-9</i>	CCAGACGCTCTTCGA GAACC	GTTATAGAAGTGCGGTTGT
<i>p67^{phox}</i>	AACTACCTGGAGCCAGTTGAG	AATTAGGAGGTGGTGGAATATCGG
<i>p47^{phox}</i>	CTGAGGGTGAAGCCATTGAGG	GCCGGTGATATCCCCTTTCC
<i>p22^{phox}</i>	AACTACCTGGAGCCAGTTGAG	AATTAGGAGGTGGTGGAATATCGG
<i>gp91^{phox}</i>	CTTTCCATAAGATGGTAGCTTGG	GCATTACACACCACTCAACG
<i>TIMP-2</i>	GTCCCATGATCCCTTGCTACA	TGCCCATGATGCTCTTCTCT
<i>TIMP-1</i>	GCCTACACCCAGTCATGGA	GGCCCGTGATGAGAACTCTT
<i>Vcam-1</i>	ACAAAACGATCGCTCAAATCG	GGTGACTCGCAGCCCGTA
<i>Icam-1</i>	CCCCGCAGGTCCAATTC	CCAGAGCGGCAGAGCAA
<i>MCP-1</i>	CCCCACTCACCTGCTGCTACT	CCTGCTGCTGGTGATCCTTGT
<i>Gapdh</i>	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCT

CatK, cathepsin K; *CatS*, cathepsin S; *eNOS*, endothelial nitric oxide synthase; *PAI-1*, plasminogen activator inhibitor type-1; *MMP-2*, matrix metalloproteinase-2; *MMP-9*, matrix metalloproteinase-9; *TIMP-2*, tissue inhibitor of matrix metalloproteinase-2; *TIMP-1*, tissue inhibitor of matrix metalloproteinase-1; *Vcam-1*, vascular cell adhesion molecule-1; *Icam-1*, intercellular adhesion molecule-1; *MCP-1*, monocyte chemoattractant protein-1; *Gapdh*, gluceradehyde-3-phosphate dehydrogenase.

Supplementary Figure Legends

Suppl. Fig. S1. The effect of anagliptin on body weight and plasma DPP4 levels in the stressed mice. **A,B:** The quantitative data show body weights at day 0 and 14 and the abdominal subcutaneous and inguinal adipose weights at day 14 after induction of stress. **C:** The DPP4 Glo Protease Assay show plasma DPP-4 levels in the stress and S-Ana group mice at 2 weeks of stress. * $p < 0.05$, ** $p < 0.01$ vs. the non-stressed group by two-way repeated-measures ANOVA and Bonferroni's post hoc tests or Student's t-test.

Suppl. Fig. S2. H_2O_2 reduced eNOS expression in human umbilical vein endothelial cells (HUVECs). After being cultured in serum-free EBM-2 overnight, the HUVECs were cultured for 24 hrs in EBM-2 medium in the presence of H_2O_2 at the indicated concentrations (200, 400 $\mu\text{mol/L}$), and the lysates were subjected to western blotting assays. Immunoblot analysis using equal amounts of total proteins from all samples was performed. **A, B:** Representative Western blots and quantitative data show the reduction of the eNOS protein in a dose-dependent manner.

Suppl. Fig. S3. Effect of anagliptin on the inflammation-, oxidative stress-, proteolysis-, and senescence-related genes and/or proteins in HUVECs in response to H_2O_2 . Cells pretreated for 30 min with anagliptin at the indicated concentrations (0, 20, 40 $\mu\text{mol/L}$) were incubated for 24 hr in EBM-2 medium in the presence or absence of H_2O_2 (400 $\mu\text{mol/L}$) and then subjected to real-time PCR or western blotting assays, respectively. **A, B:** PCR results showed that anagliptin improved H_2O_2 -induced harmful changes in the levels of targeted (*eNOS*, $p22^{\text{phox}}$, $gp91^{\text{phox}}$, *Vcam-1*, *Icam-1* and *MCP-1*, *MMP-2* and *MMP-9*, *CatK* and *CatS*) mRNA expressions in HUVECs in a dose-dependent manner. **C, D:** Representative Western blots and quantitative data show the anagliptin-mediated mitigation of the targeted protein levels (eNOS, $p22^{\text{phox}}$, $p16^{\text{INK4A}}$ and p21) in HUVECs in response to H_2O_2 at 400 $\mu\text{mol/L}$. Data are expressed as means $\pm$ SEMs (n=3-8). * $p < 0.05$, ** $p < 0.01$ vs. corresponding controls by one-way ANOVA followed by Tukey post hoc tests.

Supp Fig. S1

Supp Fig. S2

Supp Fig. S3