# Chemerin influences endothelin- and serotonin-induced pulmonary artery vasoconstriction in rats

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## ABSTRACT

*Aims:* Chemerin has been recently identified as a vasoactive adipokine implicated in blood pressure regulation. In this context, we evaluated whether chemerin could influence pulmonary vasoreactive response.

*Materials and methods:* Vascular reactivity to chemerin and to phenylephrine, serotonin and endothelin-1 after chemerin pretreatment was evaluated in rat isolated pulmonary artery *versus* thoracic aorta with and without endothelium. Vasoreactivity to acetylcholine in presence of nitric oxide (NO)-synthase inhibitor (L-NAME) and to NO donor sodium nitroprusside (SNP) was evaluated in chemerin-pretreated pulmonary artery *versus* thoracic aorta with endothelium. Pretreatment with ODQ, a soluble guanylate cyclase inhibitor and apocynin, a ROS production inhibitor, were also tested. Arteries and lung tissue were harvested for pathobiological evaluation.

*Key findings:* Chemerin contracted endothelium-denuded pulmonary artery, while no response was observed in arteries with endothelium. Chemerin potentiated phenylephrine-, endothelin-1- and serotonin-induced vasoconstriction, which was further enhanced by endothelium removal. Chemerin decreased acetylcholine-induced vasorelaxation in arteries with endothelium, while it did not affect SNP-induced relaxation. In presence of L-NAME, there remained a vasorelaxation in chemerin-pretreated arteries. Chemerin or ODQ alone partly decreased acetylcholine-induced vasorelaxation in pulmonary artery and thoracic aorta, while combined chemerin and ODQ incubation abolished it. Treatment with apocynin partly or totally reversed chemerin effects. In both types of arteries, chemerin reduced acetylcholine-induced NO production, as well as endothelial and inducible NO-synthase expression.

*Significance:* Chemerin potentiates vascular responses to vasoconstrictors in pulmonary artery and thoracic aorta and, impairs acetylcholine-induced pulmonary artery vasodilatation, by mechanisms involving at least partly NO signaling and oxidative stress.

## 1. Introduction

The adipokine chemerin, which has been initially described as regulator of glucolipid metabolic processes and inflammatory/immune responses [1,2], was recently described as a key molecule influencing systemic vascular homeostasis and tone, and contributing to the pathogenesis of hypertension [3]. In patients with hypertension, elevated circulating levels of chemerin have been strongly correlated to systolic and diastolic blood pressures, but also with body mass index and insulin resistance [4,5]. In isolated systemic arteries, chemerin caused a concentration-dependent contraction [through its main receptor, the chemokine-like receptor 1 (CMKLR1)] [3] and potentiated contractile responses of other mediators, such as phenylephrine and prostaglandins [3,6,7]. Additionally, chronic 6-week infusion of chemerin increased systolic blood pressure in mice [8]. Elevated plasma chemerin levels have also been reported in chronic inflammatory diseases and states, not necessarily related to obesity [9,10] and correlated to systemic inflammatory markers, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and C reactive protein levels [11,12].

Morbid obesity may contribute to the development of pulmonary hypertension associated to heart failure (also called group 2 pulmonary hypertension) [13]. In heart failure with preserved ejection fraction,

obese patients presented with more severe right ventricular dysfunction and reduced pulmonary artery vasodilator reserve compared with non-obese [14]. Moreover, there are few experimental data connecting all these pathognomonic features of obesity to alterations of the pulmonary circulation observed in pulmonary hypertension. Vasoactive adipokines produced by adipose tissue in contact with heart and blood vessels, could play a role [15]. Experimental data suggest that vasoactive adipokines may alter pulmonary artery vasomotion and contribute to pulmonary hypertension. Indeed, an increased pulmonary artery pressure associated to decreased endothelial nitric oxide (NO) bioavailability was observed in adiponectin-deficient mice [16]. Moreover, leptin receptor-deficient rats were partially protected against hypoxia-induced pulmonary hypertension [17].

In this context, we evaluated the expression of chemerin and its 3 receptors CMKLR1, G protein-coupled receptor 1 (GPR1) and C-C chemokine receptor-like 2 (CCRL2) in rat pulmonary artery and thoracic aorta, and investigated the effects of chemerin on pulmonary vasoreactivity alone or in association with other pulmonary vasoactive agents, including phenylephrine, serotonin and endothelin-1.

## 2. Materials and methods

All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the Faculty of Medicine of the *Université Libre de Bruxelles* (Brussels, Belgium; protocol number: 561N) at which the studies were conducted. All applicable international, national and/or institutional guidelines for the care and the use of animals, in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996) were followed.

### 2.1. Animals and sample preparation

After a one-week-acclimatization period in a temperature (21 °C)-, relative humidity (60%)- and light/dark cycles (12 h each)-controlled room, male Wistar rats weighting 504 ± 9 g (Janvier, Le Genest Saint-Isle, France) were euthanized with carbon dioxide. Female rats were excluded to avoid variations due to hormonal cycle, which has been shown to influence vasomotion [18]. Pulmonary artery (taken after the first branch point of the common pulmonary trunk) and thoracic aorta were carefully excised and cleaned of blood. After removal of adhesive fat and connective tissue, artery sections were immediately harvested for *ex vivo* vasoreactivity experiments and for NO production and real time quantitative polymerase reaction (RTQ-PCR) experiments after chemerin treatment or in snap-frozen and stored at $-80$ °C samples for RTQ-PCR experiments. Artery samples and pulmonary tissue were also harvested and, after 24-hour fixation in 10%-neutral buffered formalin, embedded in paraffin for immunohistochemistry.

### 2.2. Immunohistochemistry

Three-micrometer pulmonary artery and thoracic aortic sections, and pulmonary tissue sections were dewaxed and progressively rehydrated. To block endogenous peroxidase activity, artery and lung tissue sections were immersed in methanol containing hydrogen peroxide (3%) during 30 min. Nonspecific binding sites were blocked by a 20-minute incubation period with normal goat serum (5%) in an avidin/biotin blocking kit (Vector Laboratories, Burlingame, California, USA). After overnight incubation at 4 °C with polyclonal rabbit anti-rat chemerin [1:100 diluted in phosphate buffered saline (PBS); Bioss Antibodies, Aachen, Germany], polyclonal rabbit anti-rat CMKLR1 (1:100 diluted in PBS; Bioss Antibodies) primary antibodies, or rabbit IgG (1:10000 diluted in PBS; Vector Laboratories; used as negative control), sections were incubated for 1 h with biotinylated goat anti-rabbit IgG secondary antibody for colorimetric staining (1:200 diluted

in PBS; Vector Laboratories) or with Cy™ 3-conjugated donkey anti-rabbit IgG secondary antibody for fluorometric staining (1:200 diluted in PBS; Jackson Immuno-Research Laboratories, Baltimore, Maryland, USA). Colorimetric immunostaining was performed using the biotin-streptavidin immunoperoxidase method (Vector Laboratories) with 3,3-diaminobenzidine (Vector Laboratories) as a chromogen, according to manufacturer's instructions. Nuclei were counterstained with methyl-green or DAPI. Liver and spleen sections were used as positive controls for chemerin and CMKLR1 immunostaining respectively. Histological sections were read with a visible light and fluorescence microscope (Leica DM2000, Leica Microsystems, Wetzlar, Germany) and images were acquired by Leica Application Suite X software.

### 2.3. Ex vivo evaluation of vascular reactivity

Experiments of vasoreactivity were performed as previously described [19]. Briefly, pulmonary artery and thoracic aorta were cut into 3 mm-length segments, with special care taken to conserve their endothelial layer. In half of the rings, the endothelium was removed by rubbing the inner intimal surface with a surgical steel rod to obtain artery rings without endothelium. Pulmonary artery and thoracic aortic rings were mounted on two stainless steel hooks in 5 mL-organ chambers filled with Krebs-Henseleit solution (118 mmol/L NaCl; 4.7 mmol/L KCl; 1.2 mmol/L MgSO₄; 1.2 mmol/L KH₂PO₄; 2.5 mmol/L CaCl₂; 25 mmol/L NaHCO₃; 5.1 mmol/L glucose; Merck, Darmstadt, Germany) bubbled with 95% $O_2$ and 5% $CO_2$ and maintained at 37 °C for continuous isometric tension recording (EMKA Technologies, Paris, France). Pulmonary artery and thoracic aortic rings were placed under a resting tension of 600 mg and 1000 mg respectively, as previously used [19,31].

After a 60-minute equilibration period, contraction capacity was assessed with $8.10^{-2}$ mol/L potassium chloride (KCl; Merck). After a washout period, endothelial integrity was functionally confirmed with acetylcholine chloride ($10^{-4}$ mol/L; Sigma-Aldrich, Sᵗ Louis, Missouri, USA) in phenylephrine hydrochloride (PHE; $10^{-6}$ mol/L; Sigma-Aldrich)-pre-contracted rings. Artery rings exhibiting a vasodilator response to acetylcholine > 80% and < 20% of the phenylephrine-induced contraction were considered as "with endothelium" or "without endothelium" respectively. Artery segments that did not match with any of these criteria were excluded. After a washout period allowing the artery rings to return to their basal vascular tone, three protocols were separately tested on different artery rings. Firstly, increasing concentrations ($10^{-16}$ to $10^{-9}$ mol/L) of recombinant mouse chemerin (aa 17-156; R&D Systems, Minneapolis, Minnesota, USA) were tested in pulmonary artery and thoracic aortic rings with and without endothelium. After, increasing concentrations of different vasoconstrictors, including phenylephrine hydrochloride ($10^{-10}$ to $10^{-5}$ mol/L), serotonin ($10^{-8}$ to $10^{-3}$ mol/L; Sigma-Aldrich) and endothelin-1 ($10^{-10}$ to $10^{-7}$ mol/L; Sigma-Aldrich) were successively tested pulmonary artery and thoracic aortic rings with and without endothelium pre-incubated or not during 1 h with chemerin ($10^{-8}$ mol/L). Secondarily, after one-hour pre-incubation with chemerin ($10^{-8}$ mol/L) or vehicle, increasing concentrations of acetylcholine chloride ($10^{-10}$ to $10^{-4}$ mol/L) or a NO donor, the sodium nitroprusside ($10^{-9}$ to $10^{-5}$ mol/L; Sigma-Aldrich) were performed in pulmonary artery and thoracic aorta rings with endothelium pre-contracted with phenylephrine hydrochloride ($10^{-6}$ mol/L). The same protocol used to build the concentration-response curve to acetylcholine hydrochloride, was repeated after a 30-min pre-incubation period with a NO-synthase inhibitor, the NG-nitro-L-arginine methyl ester hydrochloride (L-NAME; $2.5.10^{-4}$ mol/L; Sigma-Aldrich). Thirdly, after one-hour pretreatment with chemerin ($10^{-8}$ mol/L) or vehicle and 30-min pretreatment with an inhibitor of soluble guanylate cyclase (sGC), the 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ; $10^{-6}$ mol/L; Sigma-Aldrich) or an inhibitor of NADPH oxidase activity, the 4′-hydroxy-3′-methoxyacetophenone or apocynin ($10^{-4}$ mol/L; Sigma-Aldrich), we

performed the same concentration-response curves to acetylcholine hydrochloride.

The three different sets of experiments were performed on new freshly dissected arteries from different animals. In each of the 3 protocols, washout and stabilization periods were performed between each dose-response curve allowing the artery rings to return to their basal tone.

### 2.4. Quantification of NO metabolite (nitrite) production

Directly after dissection, other standardized segments (4 mm-length) of pulmonary artery and thoracic aorta with endothelium were incubated during 1 h with or without recombinant mouse chemerin ($10^{-8}$ mol/L) in a 24-well cell culture plate. Five minutes before the end of chemerin incubation, half of the segments were treated with acetylcholine hydrochloride ($10^{-4}$ mol/L). Supernatants were collected and the levels of NO were measured indirectly by the determination the nitrite levels, using the Measure-iT™ High-Sensitivity Nitrite Assay kit (Molecular Probes, Eugene, Oregon, USA) according the manufacturer's instructions.

Briefly, collected supernatants were ultra-filtered through a 10000-molecular weight cut-off filter to eliminate proteins (VWR; Leuven, Belgium). Fluorescence was measured with a microplate reader at 365/450 nm. Nitrite concentrations were obtained by referring to a standard curve realized in parallel and expressed in mol/L. Results are expressed as the mean values of three separate measurements performed for each condition.

### 2.5. Real-time quantitative polymerase chain reaction (RTQ-PCR) experiments

Directly after dissection, other segments of pulmonary artery and thoracic aorta with endothelium were incubated with or without recombinant mouse chemerin ($10^{-8}$ mol/L) in a 24-well cell culture plate. After a five-hour incubation, artery segments were rapidly snap-frozen in liquid nitrogen and stored at $-80\,°C$ until use. Total RNA was extracted using TRIzol reagent (Invitrogen, Merelbeke, Belgium) followed by a chloroform/ethanol extraction and a final purification using RNeasy® Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration was determined with a standard spectrophotometer Nanodrop® ND-1000 (Isogen life science, De Meern, The Netherlands) and RNA integrity was assessed by visual inspection of GelRed (Biotium, Hayward, California, USA) -stained agarose gels. Reverse transcription was carried out using SuperScript™ II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions.

For RTQ-PCR, sense and anti-sense primers were designed, using Primer3 program, for *Rattus norvegicus* chemerin (also called RARRES2), CMKLR1 (also called ChemR23), CCRL2, GPR1, endothelial NO-synthase (eNOS), inducible NO-synthase (iNOS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) 1 mRNA sequence amplification (Table 1). Intron-spanning primers were selected when exon sequences were known and a BLAST analysis was run to check if primer pairs were only matching the sequence of interest. For each sample, amplification reaction was performed in triplicate using SYBRGreen PCR Master Mix (Quanta Biosciences, Gaithersburg, Maryland, USA), specific primers and diluted template cDNA. Result analysis was performed using an iCycler system (BioRad Laboratories, Nazareth Eke, Belgium). Relative quantification was achieved with Pfaffl method [20] by normalization with the housekeeping genes, GAPDH and HPRT1.

### 2.6. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Contractile concentration-response curves to chemerin, phenylephrine, serotonin and endothelin-1 were presented as the percentage of the contractile response developed by $8.10^{-2}$ mol/L KCl. Relaxations to acetylcholine or sodium nitroprusside were expressed as the percentage of the maximal contractile response developed by phenylephrine. ED50-values were obtained using Prism 8 (Graphpad, San Diego, California, USA). Data were analyzed with a two-factor (groups, doses) analysis of variance (ANOVA) for missing data with repeated measurements on doses and interaction, using the algorithm described by Winer and associates [21].

Inter-group differences of RT-QPCR and NO metabolite (nitrite) production results were tested with one-way ANOVA. When the F ratio of this analysis reached a critical p-value < 0.05, comparisons were made with a parametric Student *t*-test. A value of p < 0.05 was considered as statistically significant; n represents the number of individual data.

## 3. Results

### 3.1. Expression of chemerin and its receptors in rat pulmonary artery and thoracic aorta

As illustrated in Fig. 1, chemerin and its three receptors CMKLR1, CCRL2 and GPR1 were all detected in rat pulmonary artery and thoracic aorta. Relative gene expressions of CMKLR1 and GPR1 were higher in thoracic aorta compared to pulmonary artery, while CCRL2 expression was higher in pulmonary artery (Fig. 1a). In pulmonary artery and thoracic aorta, gene expression of CMKLR1 was higher than expressions of CCRL2 and GPR1 (Fig. 1a). Similar gene expression of chemerin was observed in pulmonary artery and thoracic aorta (data not shown).

To identify vascular cells expressing chemerin and its mainly expressed receptor CMKLR1, we performed immunofluorescence staining for these two proteins in isolated rat pulmonary artery and thoracic aorta. Chemerin (Fig. 1b) and CMKLR1 (Fig. 1c) were localized in vascular intimal and medial layers in both pulmonary and thoracic arteries. In the lungs, immunohistochemical analyses showed that chemerin (Fig. 1b) and CMKLR1 (Fig. 1c) were present in vascular intimal and medial cells, as well as in bronchial smooth muscle and epithelial cells (Fig. 1b and c).

### 3.2. Vascular reactivity of rat pulmonary artery and thoracic aorta: Effects of chemerin alone

Chemerin (tested from $10^{-16}$ to $10^{-9}$ mol/L) did not alter vascular tone in pulmonary artery and thoracic aortic rings with endothelium (Fig. 2a and b). In pulmonary artery without endothelium, chemerin induced a concentration-dependent contraction (Fig. 2a), while it did not in thoracic aorta without endothelium (Fig. 2b).

### 3.3. Vascular reactivity of rat pulmonary artery and thoracic aorta: Effects of chemerin in presence of vasoconstrictors and vasodilators

Phenylephrine (tested from $10^{-10}$ to $10^{-5}$ mol/L; Fig. 3a and b), endothelin-1 (tested from $10^{-8}$ to $10^{-3}$ mol/L; Fig. 3c, d) and serotonin (tested from $10^{-8}$ to $10^{-3}$ mol/L; Fig. 3e and f) induced a concentration-dependent contraction in pulmonary artery and thoracic aortic rings. These contractile responses were higher in endothelium-denuded compared to artery rings with endothelium (Fig. 3). Pretreatment with chemerin ($10^{-8}$ mol/L) potentiated these concentration-dependent vasocontractions in pulmonary artery and thoracic aortic rings with and without endothelium (Fig. 3), except in pulmonary artery with endothelium in which chemerin did not alter the contractile response induced by serotonin (Fig. 3e).

After phenylephrine pre-contraction, acetylcholine (tested from $10^{-10}$ to $10^{-4}$ mol/L) induced a concentration-dependent vasorelaxation, which was steeper in thoracic aortic compared to pulmonary artery rings with endothelium (Fig. 4a). Treatment with chemerin

**Table 1**

Primers used for real-time quantitative polymerase chain reaction in *Rattus norvegicus* thoracic aorta and pulmonary arteries.

| Genes | | Sequences |
|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase | Sense | 5′ - AAGATGGTGAAGGTCGGTGT - 3′ |
| (**GAPDH**) | Antisense | 5′ - ATGAAGGGGTCGTTGATGG - 3′ |
| Hypoxanthine phosphoribosyltransferase 1 | Sense | 5′ - ACAGGCCAGACTTTGTTGGA - 3′ |
| (**HPRT1**) | Antisense | 5′ - TCCACTTTCGCTGATGACAC - 3′ |
| **Chemerin** | Sense | 5′ - AAGGACTGGAAAAAGCCAGAG - 3′ |
| (RARRES2) | Antisense | 5′ - TCCGGCCTAGAACTTTACCC - 3′ |
| Chemokine-like receptor 1 | Sense | 5′ - GACCGGATTAGAACCCCAGT - 3′ |
| (**CMKRL1**) | Antisense | 5′ - AAAACCCCAAACCCATTAGC - 3′ |
| C-C chemokine receptor-like 2 | Sense | 5′ - ACCACGCTGTTTTAGGCAGT - 3′ |
| (**CCRL2**) | Antisense | 5′ - TGGGTCTGTCCCACTGTTGTC - 3′ |
| G protein-coupled receptor 1 | Sense | 5′ - GGAGCTCAGCATTCATCACA - 3′ |
| (**GPR1**) | Antisense | 5′ - GACAGGCTCTTGGTTTCAGC - 3′ |
| Endothelial nitric oxide synthase | Sense | 5′ - GGTATTTGATGCTCGGGACT - 3′ |
| (**eNOS**) | Antisense | 5′ - TGATGGCTGAACGAAGATTG - 3′ |
| Inducible nitric oxide synthase | Sense | 5′ - GTTTCCCCCAGTTCCTCACT - 3′ |
| (**iNOS**) | Antisense | 5′ - CTCTCCATTGCCCCAGTTT - 3′ |

($10^{-8}$ mol/L) prior to phenylephrine ($10^{-6}$ mol/L) contraction, reduced acetylcholine-induced vasorelaxation in both pulmonary artery and thoracic aortic rings with endothelium (Fig. 4a). However, ED50-values were similar between the four groups (data not shown), suggesting that acetylcholine concentration necessary to decrease maximal contraction was not influenced by chemerin pretreatment or vessel type. To assess if the decreased vasorelaxation induced by chemerin was dependent on NO, we tested a NO donor, the sodium nitroprusside (tested from $10^{-9}$ to $10^{-5}$ mol/L) after or not pretreatment with chemerin (Fig. 4b). In both pulmonary artery and thoracic aortic rings, sodium nitroprusside induced a concentration-dependent vasorelaxation, which was similar after chemerin pretreatment suggesting that chemerin did not alter the activity of NO (Fig. 4b). Thereafter, acetylcholine-induced vasorelaxation was tested after pretreatments with a NO-synthase inhibitor, the L-NAME ($2.5.10^{-4}$ mol/L) and with

chemerin ($10^{-8}$ mol/L). In vehicle-pretreated pulmonary artery and thoracic aortic rings, the blockade of NO production abolished acetylcholine-induced vasorelaxation (Fig. 4c), while it partly abolished after chemerin pretreatment. This remaining small vasodilatory response in presence of chemerin suggested that chemerin could potentiate another vasodilating pathway in both types of vessels.

### 3.4. Chemerin decreases acetylcholine-induced vasodilatation through NO/sGC/cGMP and ROS signaling pathways

After, we evaluated the implication of the NO/sGC/cGMP signaling in acetylcholine-induced vasorelaxation after incubation with a soluble guanylate cyclase inhibitor ODQ. As illustrated in Fig. 5a and b, acetylcholine-induced relaxation was reduced after ODQ ($10^{-6}$ mol/L) or chemerin ($10^{-8}$ mol/L) pretreatments in pulmonary artery and thoracic
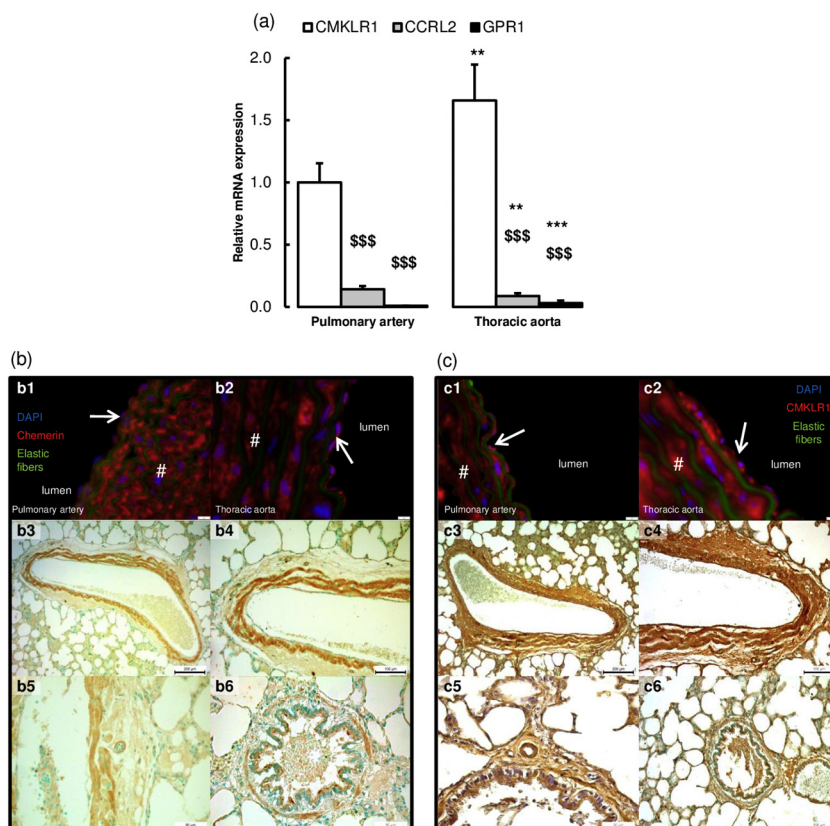


**Fig. 1.** Expression and localization of chemerin and its three receptors. (a) Relative gene expressions of chemerin receptors, the chemokine-like receptor 1 (CMKLR1, also called ChemR23; white bars), the C−C chemokine receptor-like 2 (CCRL2; grey bars) and the G protein-coupled receptor 1 (GPR1; black bars) in pulmonary artery (n = 13) and thoracic aorta (n = 10) from Wistar rats. Results were expressed as means ± SEM. **p < 0.01, ***p < 0.001 compared to the expression of the same receptor in pulmonary artery; $^{\$\$\$}$p < 0.001 compared to the expression of CMKLR1 in the same vessel type. Representative images (b, c) of immunofluorescence staining of (b1 and b2) chemerin (red) and (c1 and c2) CMKLR1 (red) in pulmonary artery and thoracic aorta (positive for elastic fibers in green) from Wistar rats. Chemerin and CMKLR1 were expressed in vascular intimal and medial layers indicated by arrow and # respectively, in both types of vessels. Scale bar = 8 μm (in sections b1-2 and c1-2). Immunostainings of chemerin (b3-6) and CMKLR1 (c3-6) in lung sections from Wistar rats, showing expression of chemerin and CMKLR1 in vascular intimal and medial cells, as well as in bronchial smooth muscle and epithelial cells. Scale bars = 200 μm (in sections b3 and c3), 100 μm (in sections b4, c4 and c6) and 50 μm (in sections b5, b6 and c5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
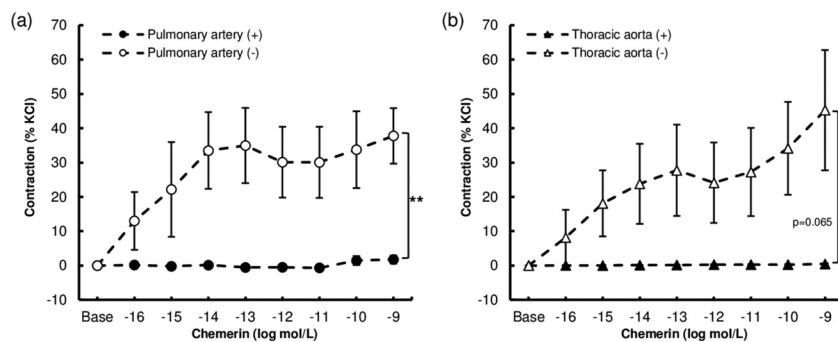
**Fig. 2.** Concentration-response curves to chemerin ($10^{-16}$ to $10^{-9}$ mol/L) in pulmonary artery (n = 7) and thoracic aorta (n = 7) with ($+$) and without endothelium ($-$). Contraction responses were expressed as the percentages of the maximal tension response obtained with $8.10^{-2}$ mol/L KCl. Results are presented as means ± SEM. **p < 0.01.

aorta with endothelium. However, when we combined pretreatment with both ODQ and chemerin, acetylcholine-induced vasorelaxation was abolished. This strongly suggests that chemerin acts through NO/sGC/cGMP signaling to counteract acetylcholine-induced relaxation in pulmonary artery and in thoracic aorta, but also through other mechanisms.

To assess if the decreased vasorelaxation induced by chemerin was dependent on ROS production, we tested a NADPH oxidase inhibitor, the apocynin ($10^{-4}$ mol/L) in presence or not of chemerin ($10^{-8}$ mol/L). A NADPH oxidase inhibitor (apocynin) alone did not alter acetylcholine-induced relaxation in both pulmonary artery and thoracic aorta with endothelium (Fig. 5c and d). However, apocynin partly or totally reversed the effects of chemerin on acetylcholine-induced relaxation in pulmonary artery and thoracic aorta respectively (Fig. 5c and d). This shows that chemerin reduced acetylcholine-induced relaxation through ROS production.

### 3.5. NO production in rat pulmonary artery and thoracic aorta: Effects of chemerin

As illustrated in Fig. 6a and b, one-hour treatment with chemerin ($10^{-8}$ mol/L) had no effect on basal NO metabolite concentration in supernatants of both pulmonary artery and thoracic aorta with endothelium. After five-minute stimulation with acetylcholine, there was a two-fold increase in NO metabolite concentration in supernatants from both types of vessels (Fig. 6a and b). This increase was abolished after chemerin ($10^{-8}$ mol/L) pretreatment.

To understand better the mechanisms underlying these variations in NO production after chemerin treatment, we evaluated gene expressions of two enzymes implicated in NO production, the endothelial and inducible NO-synthases in whole tissue homogenates of pulmonary artery and thoracic aorta with endothelium. Five-hour treatment with $10^{-8}$ mol/L chemerin decreased gene expressions of endothelial and inducible NO-synthases in pulmonary artery, while, in thoracic aorta, it significantly decreased the expression of inducible NO-synthase (Fig. 6c and d).

### 4. Discussion

The present study shows that chemerin increases pulmonary vasoconstriction, potentiating the action of vasoconstrictors (phenylephrine, endothelin-1 and serotonin) and impairs the effects of the vasodilating agent (acetylcholine), by mechanisms involving at least partly NO/sGC/cGMP signaling and oxidative stress. The effects observed on the pulmonary circulation are similar as those observed in the systemic thoracic aorta.

Here, we showed that chemerin expression was localized to the intimal and medial layers of rat pulmonary artery and thoracic aorta, suggesting that it could act as a locally released vascular mediator. This staining is consistent with other literature, which found that chemerin was expressed by dermal microvascular endothelial cells [22] and smooth muscle medial layer of systemic arteries [23]. We know that

chemerin is released as biologically inactive pro-chemerin and must be cleaved at the C-terminus for activation, by enzymes present in the vasculature to become active [24]. However, the chemerin antibody that we used in the present study was designed to detect expression of all chemerin isoforms. So, we were not able to identify the expression of active chemerin within the rat artery wall. We also showed that CMKLR1 was widely expressed in the tunica media and the intima, suggesting that chemerin-CMKLR1 signaling could have an important role in modulation of pulmonary vascular tone by acting on endothelial cells or on contractile cells directly. This is consistent with previous findings of CMKLR1 expression on smooth muscle and endothelial cells in human [23] and rat [3] vessels.

In the present study, we showed for the first time, that chemerin can modulate vascular reactivity of pulmonary artery *ex vivo*. Vascular reactivity is one of the most important determinants of vascular tone and even more so in vascular pathologies. Previous studies have shown that adipokines may affect vasoreactivity of systemic arteries [25]. Leptin, adiponectin, visfatin, nesfatin and omentin have been shown to present vasorelaxing action, while resistin and angiotensin II have been identified as vasoconstrictor agents. In pathological conditions, such as obesity which is characterized by a dysfunctional adipose tissue, there is overproduction of pro-inflammatory adipokines and under-production of anti-inflammatory adipokines which contributes to systemic vascular dysfunction [25]. Recently, adipokines have been incriminated in the pathogenesis of pulmonary hypertension [17,26]. Gene deletion of adiponectin aggravated experimental pulmonary hypertension induced by inflammation [27] or by chronic hypoxia exposure [28]. Adiponectin overexpression or chronic infusion of omentin inhibited the development of experimental pulmonary hypertension in rats [29,30]. Finally, an impaired vasorelaxation to leptin has been demonstrated in the pulmonary circulation of spontaneous hypertensive rats [19,31]. These studies indicate that vasoactive adipokines may alter pulmonary artery vasomotion and could be therefore implicated in the pathogenesis of pulmonary hypertension.

Epidemiologic studies suggest that obesity may be a risk factor for pulmonary hypertension [26,32]. Inflammation and dysimmunity are now increasingly recognized to play crucial roles in the pathogenesis of pulmonary hypertension [33]. We also know that circulating chemerin was increased in obese subjects and tightly correlated to their inflammatory vascular phenotype [34]. CMKLR1 expression was up-regulated in endothelial cells by inflammatory cytokines including TNF-α, IL-1β, and IL-6 [35], whose circulating and pulmonary artery expressions were shown to be elevated in pulmonary arterial hypertension [36]. Moreover, CMKLR1 is expressed in various leukocyte populations including monocytes, macrophages and dendritic cells [24], which were incriminated in the pathogenesis of pulmonary arterial hypertension [36]. These observations lead us to suggest that chemerin may be a candidate connecting fat deposits and/or inflammation to the pathogenesis of pulmonary hypertension.

In the present study, we observed that chemerin alone had no effect on basal contractility of rat vessels with endothelium, but induced contraction of pulmonary artery without endothelium with the same
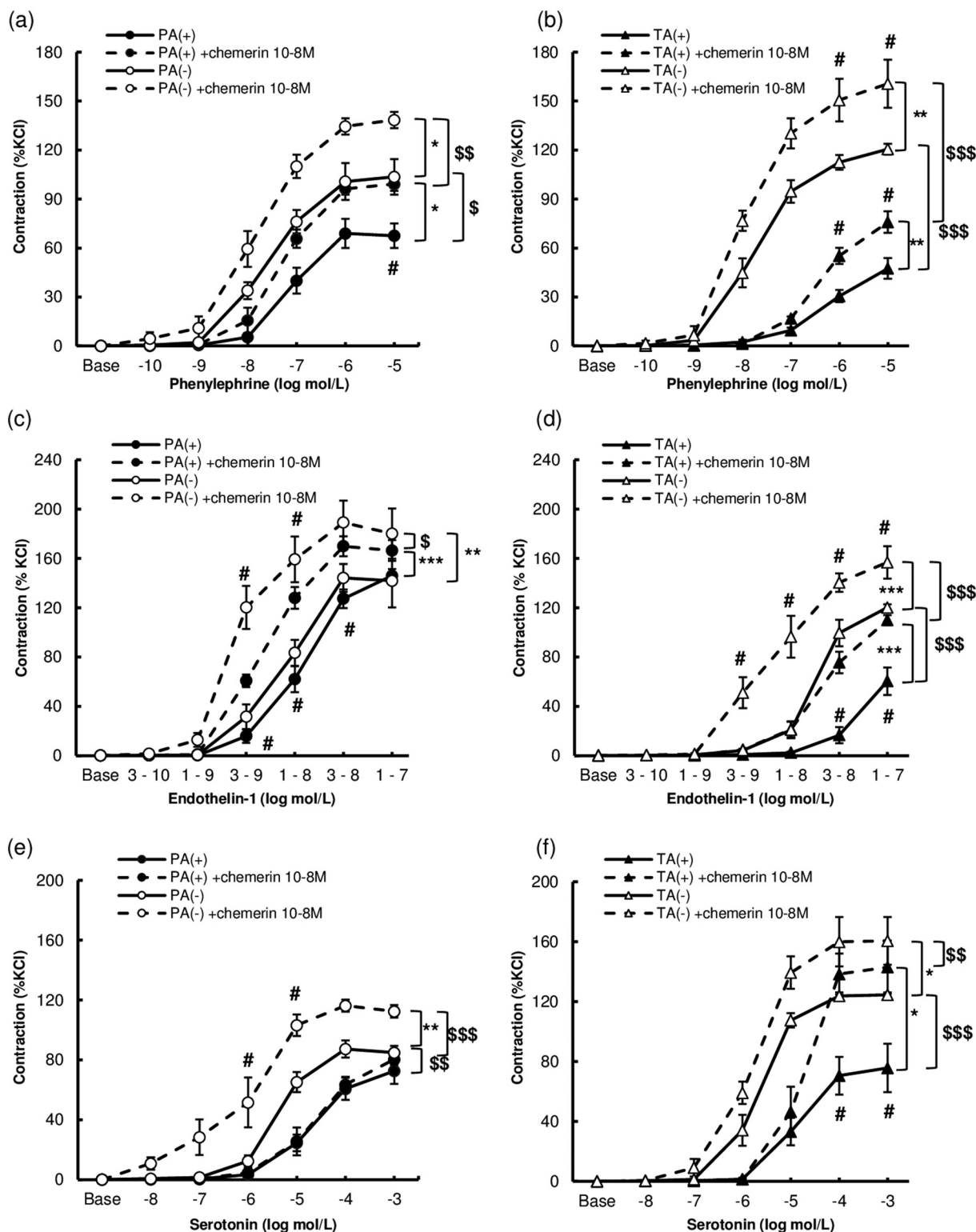
**Fig. 3.** Concentration-response curves to phenylephrine (tested from $10^{-10}$ to $10^{-5}$ mol/L) (a, b), **endothelin-1** (tested from $10^{-8}$ to $10^{-3}$ mol/L) (c, d) and **serotonin** (tested from $10^{-8}$ to $10^{-3}$ mol/L) (e, f) in pulmonary artery (n = 7) and thoracic aorta (n = 7) with (+) and without endothelium (−) pretreated during 1 h with chemerin ($10^{-8}$ mol/L (M)) or vehicle. Contraction responses were expressed as the percentages of the maximal tension response obtained with $8.10^{-2}$ mol/L KCl. Results are presented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the same vessel pretreated with vehicle (global curve analysis). #p < 0.05 compared to the same vessel pretreated with vehicle (point-by-point analysis). $p < 0.05, $$p < 0.01, $$$p < 0.001 compared to the same type of vessel without endothelium.

trend observed in thoracic aorta without endothelium. Previous studies have already shown that chemerin induced vasocontraction in systemic arteries [3,37]. Here, chemerin-induced contraction was only observed

in vessels without endothelium. This is consistent with previous data showing that contraction was amplified when the vascular endothelial function was pharmacologically blocked (through NO-synthase
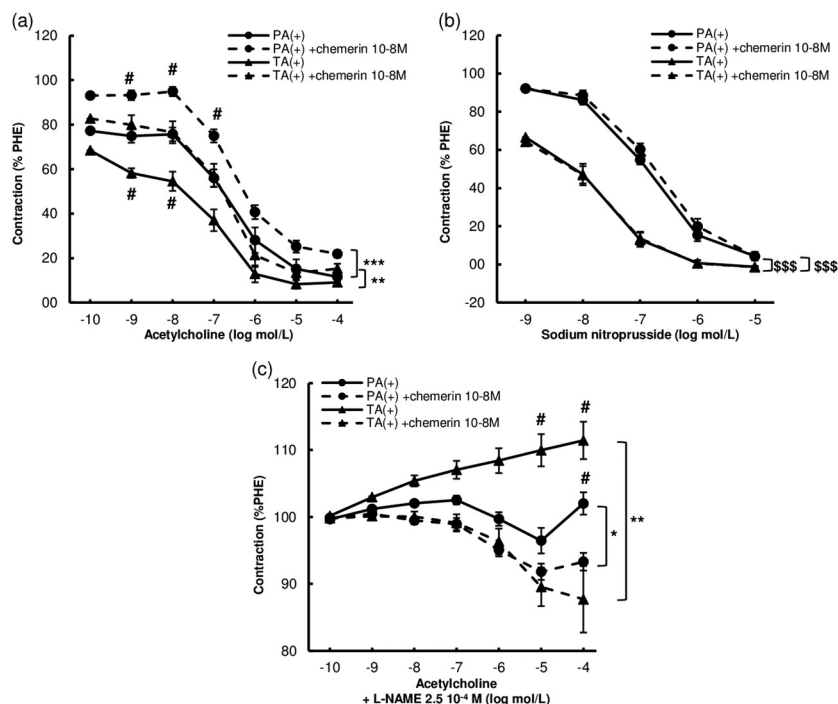
**Fig. 4.** Concentration-response curves to acetylcholine (tested from $10^{-10}$ to $10^{-4}$ mol/L; a), a nitric oxide donor, the sodium nitroprusside (tested from $10^{-9}$ to $10^{-5}$ mol/L; b) acetylcholine (tested from $10^{-10}$ to $10^{-4}$ mol/L; c) in presence of an inhibitor of nitric oxide-synthase, the L-NAME ($2.5.10^{-4}$ mol/L) in pulmonary artery (n = 11) and thoracic aorta (n = 10) with endothelium (+) pretreated during 1 h with chemerin ($10^{-8}$ mol/L) or vehicle. Relaxation responses were expressed as the percentages of the maximal contraction obtained with phenylephrine ($10^{-6}$ mol/L). Results were presented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the same vessel pretreated with vehicle (global curve analysis); #p < 0.05 compared to the same vessel pretreated with vehicle (point-by-point analysis); $$$p < 0.001 compared to thoracic aorta.

inhibition) or altered (*i.e.* in obese or hypertensive strains of rats) [3]. The intrinsic contractile effect of chemerin was highlighted by removal of the endothelial layer, which is known to moderate vasoconstriction (through the release of vasodilating agents) [38]. Here, we showed that chemerin was not able to alter NO production at basal level, while it strongly reduced NO release after stimulation by acetylcholine. This suggests the role of chemerin as an aggravating agent in stimulated or pathological conditions. In addition, chemerin has been shown to initiate a calcium influx in smooth muscle cells [6], acting thus on the smooth muscle layer. Here, when we combined the blockage of soluble

guanylate cyclase activity with chemerin treatment in smooth muscle cells, we totally blocked acetylcholine-induced relaxation, suggesting the implication of vasoactive factors (other than endothelial NO) mediating the effects of chemerin.

Here, we showed that chemerin potentiated phenylephrine- and endothelin-1-induced vasoconstriction; this effect was not specific on the vessel type and not dependent of the presence of endothelium. This is consistent with previous data showing that chemerin augmented phenylephrine- and endothelin-1-induced vasoconstriction in rat thoracic aorta without endothelium *via* activation of ERK1/2 signaling
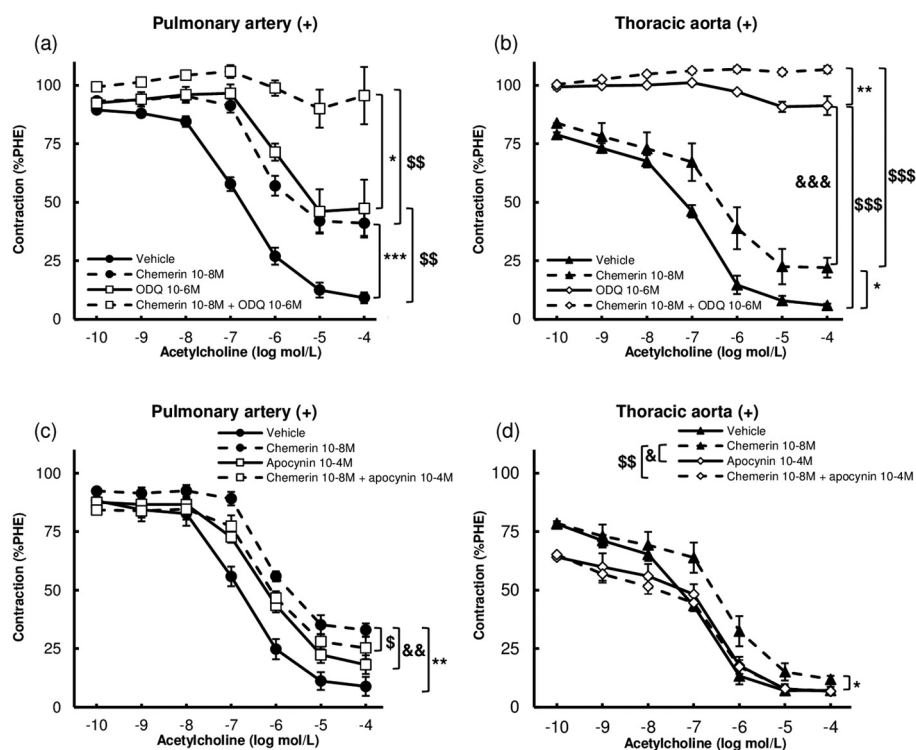


**Fig. 5.** Concentration-response curves to acetylcholine (tested from $10^{-10}$ to $10^{-4}$ mol/L) in presence of ODQ, an inhibitor of soluble guanylyl cyclase ($10^{-6}$ mol/L; a and b) or apocynin, an inhibitor of NADPH oxidase ($10^{-4}$ mol/L; c and d) in pulmonary artery (n = 6; a and c) and thoracic aorta (n = 6; b and d) with endothelium (+) pretreated during 1 h with chemerin ($10^{-8}$ mol/L) or vehicle. Relaxation responses were expressed as the percentages of the maximal contraction obtained with phenylephrine ($10^{-6}$ mol/L). Results were presented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 chemerin pretreated vessel compared to pretreated vessel with vehicle with or without inhibitor (global curve analysis); &p < 0.05, &&p < 0.01, &&&p < 0.001 chemerin pretreated vessel compared to inhibitor pretreated vessel (global curve analysis); $p < 0.05, $$p < 0.01, $$$p < 0.001 chemerin pretreated vessel or vehicle pretreated vessel compared to the same vessel pretreated with inhibitor (global curve analysis).
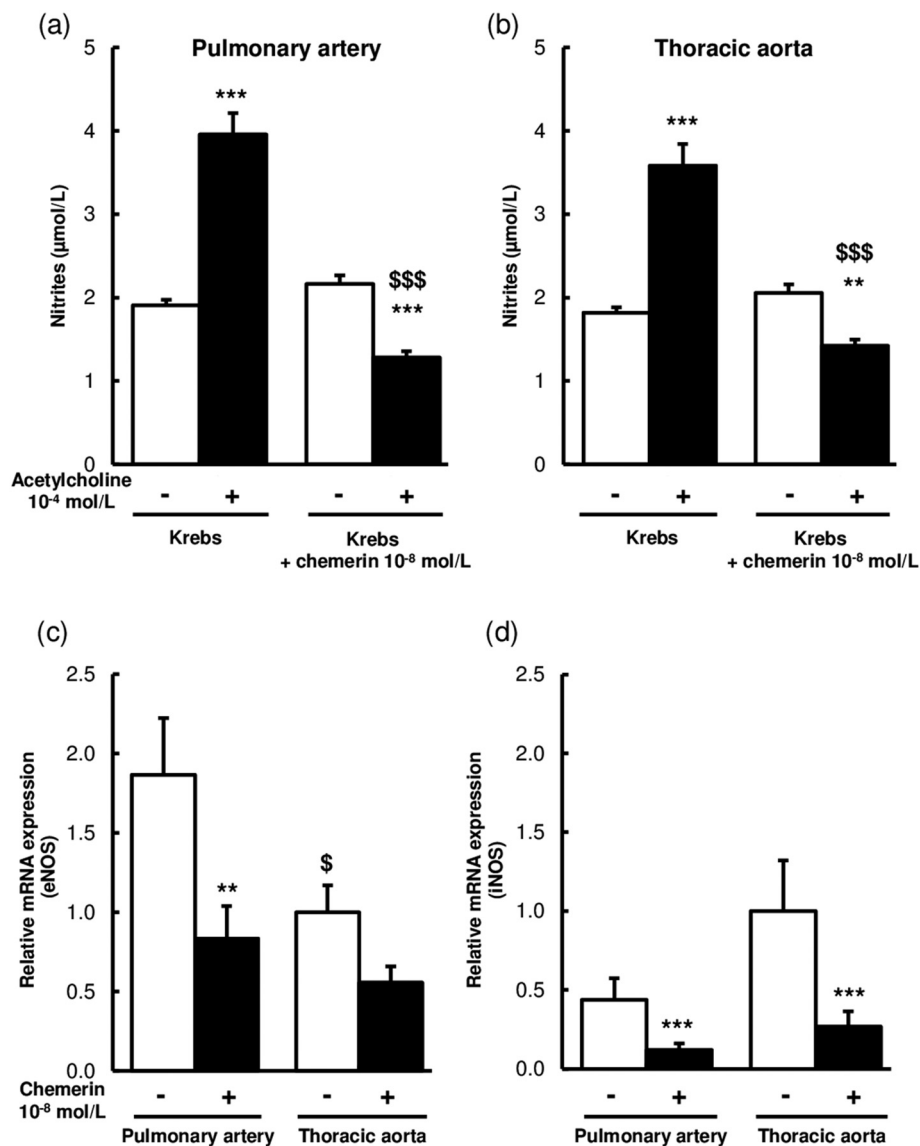
Fig. 6. Effects of chemerin on nitric oxide signaling. (a, b) Concentration of nitrites (more stable nitric oxide metabolites) in supernatants of standardized 3 mm-length pulmonary artery (n = 7; a) and thoracic aortic (n = 6; b) rings with endothelium incubated during 1 h with chemerin ($10^{-8}$ mol/L) or vehicle followed or not by five-minute incubation with acetylcholine ($10^{-4}$ mol/L). Results were expressed as means ± SEM. **p < 0.01, ***p < 0.001 compared to corresponding rings not treated with acetylcholine; $^{\$\$\$}$p < 0.001 compared to corresponding rings not treated with chemerin. (c, d) Expression of genes implicated in nitric oxide synthesis, the endothelial (eNOS, c) and inducible (iNOS, d) nitric oxide synthases in rat pulmonary artery (n = 13–16) and thoracic aorta (n = 12–15) treated during 1 h with chemerin ($10^{-8}$ mol/L) or vehicle. Results were expressed as means ± SEM. **p < 0.01, ***p < 0.001 compared to corresponding vessel not treated with chemerin; $^{\$}$p < 0.05 compared to the same condition in pulmonary artery.

pathway [7]. However, in contrast to Lobato et al. [7], we observed the same effect on vessels with endothelium. This may be explained by the fact that despite the use of similar concentrations of phenylephrine and endothelin-1, they used a 300-fold lower concentration of chemerin. Taken together, these observations led us to suggest that at a concentration of $10^{-8}$ mol/L, chemerin was acting directly on vascular smooth muscle cells to increase vascular tone. Endothelin-1 is a potent vasoconstrictor [39], which has been largely incriminated in the pathogenesis of pulmonary arterial hypertension [40]. Endothelin receptor antagonists are approved drugs for treatment of this condition [41]. Our results showed that chemerin was able to potentiate endothelin-1-induced vasoconstriction, participating therefore to increased pulmonary vascular resistance. In the present study, concentrations of endothelin-1 that we used, were higher than circulating endothelin-1 levels in healthy subjects and in patients with pulmonary arterial hypertension [42]. However, we know that endothelial cells release > 80% of endothelin-1 abluminally and endothelin-1 acts primarily as a local autocrine and paracrine mediator rather than as an endocrine hormone [43].

Serotonin is a vasoconstrictor which has been incriminated in the pathogenesis of pulmonary arterial hypertension, contributing to increased pulmonary vascular tone and remodeling [44,45]. In the present study, we showed that chemerin potentiated serotonin-induced

vasoconstriction in both thoracic aorta with and without endothelium, while in pulmonary artery, chemerin potentiated serotonin-induced vasoconstriction in rings without endothelium only. It has already been shown that the regulation of vascular tone may be different between pulmonary and systemic arteries [46]. Chemerin could contribute to serotonin-induced increased pulmonary vascular tone, when the pulmonary artery endothelium is dysfunctional, which has been tremendously demonstrated in pulmonary arterial hypertension [47].

Endothelial cells are the primary source of relaxing factors, such as NO [48]. Endothelial cell damage or dysfunction, which leads to decrease NO production and release, is a key initial event in the development of pulmonary arterial hypertension [49]. In the present study, we found that chemerin impaired acetylcholine-induced relaxation in pulmonary artery and thoracic aorta with endothelium. This is consistent with our results concerning NO/sGC/cGMP signaling and a previous study showing that chemerin decreased aortic relaxation, reducing the vascular NO/endothelial NO-synthase/cyclic guanylate cyclase signaling [50,51]. Considering that signaling pathways involved in NO synthesis are the primary mediators of the effects of chemerin on acetylcholine-induced relaxation, a NO donor sodium nitroprusside was used. This showed that sodium nitroprusside-induced relaxation remained unchanged after chemerin incubation, which suggested that the underlying mechanism could be related to a decrease in acetylcholine-

stimulated NO production. Indeed, in the present study, chemerin decreased endothelial and inducible NO-synthase expression and abolished acetylcholine-induced NO release in both pulmonary artery and thoracic aorta. In endothelial cells, chemerin has been shown to increase the generation of mitochondrial reactive oxygen species (ROS), thereby perpetuating a cycle of events that potentially underlie endothelial dysfunction. Moreover, chemerin-induced ROS generation in vascular smooth muscle cells might contribute to impair endothelial NO production and bioavailability, indicating that the effects of chemerin on the endothelium may be potentiated by its harmful action in vascular smooth muscles [50,51]. This is consistent with our results showing that chemerin reduced acetylcholine-induced relaxation through ROS production.

In addition to NO, endothelial cells produce other vasorelaxing agents, such as prostanoïds and endothelium-derived hyperpolarizing factor (EDHF) [38,52]. To determine their contribution in acetylcholine-induced vasodilation, we blocked NO production by L-NAME. L-NAME totally abolished acetylcholine-induced vasodilation in vehicle-pretreated vessels. However, a small remaining vasorelaxing effect was observed after chemerin treatment. Moreover, acetylcholine-induced relaxation was totally abolished by combined incubation with chemerin and soluble guanylate cyclase inhibitor in both pulmonary artery and thoracic aorta. Altogether, this strongly suggested that chemerin could potentiate other vasodilating pathways, such as the prostanoïd and/or EDHF pathways. However, we were not able to identify them in the present study. This clearly needs further studies.

## 5. Conclusion

In conclusion, the present study demonstrates that chemerin increases vascular tone in rat pulmonary artery without endothelium and potentiates vasoconstriction induced by phenylephrine, as well as by endothelin-1 and serotonin. In addition, chemerin impairs acetylcholine-induced pulmonary artery vasodilatation, through at least partly reduced NO production and oxidative stress. Altogether, this suggests that chemerin potentiates pulmonary vasoconstriction, which could therefore reinforce the actions of mediators implicated in the pathogenesis of pulmonary hypertension, such as endothelin-1 and serotonin.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

## Authors contributions

AH, PJ, LD and KMC: conceptualization of study; AH, PJ and GV: investigation; AH, PJ, GND, JYS, PL, LD and KMC: analyzed data; AH, PJ, GV, GND, LD and KMC: methodology and models; LD and KMC: supervision and validation; AH, LD and KMC: writing original draft. All authors have given approval to the final version of the manuscript.

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