

Glucagon induces airway smooth muscle relaxation by nitric oxide and prostaglandin E₂

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Abstract

Glucagon is a hyperglycemic pancreatic hormone that has been shown to provide a beneficial effect against asthmatic bronchospasm. We investigated the role of this hormone on airway smooth muscle contraction and lung inflammation using both *in vitro* and *in vivo* approaches. The action of glucagon on mouse cholinergic tracheal contraction was studied in a conventional organ bath system, and its effect on airway obstruction was also investigated using the whole-body pletysmographic technique in mice. We also tested the effect of glucagon on lipopolysaccharide (LPS)-induced airway hyperreactivity (AHR) and inflammation. The expression of glucagon receptor (GcgR), CREB, phospho-CREB, nitric oxide synthase (NOS)-3, pNOS-3 and cyclooxygenase (COX)-1 was evaluated by western blot, while prostaglandin E₂ (PGE₂) and tumour necrosis factor- α were quantified by enzyme-linked immunoassay and ELISA respectively. Glucagon partially inhibited carbachol-induced tracheal contraction in a mechanism clearly sensitive to des-His1-[Glu9]-glucagon amide, a GcgR antagonist. Remarkably, GcgR was more expressed in the lung and trachea with intact epithelium than in the epithelium-denuded trachea. In addition, the glucagon-mediated impairment of carbachol-induced contraction was prevented by either removing epithelial cells or blocking NOS (L-NAME), COX (indomethacin) or COX-1 (SC-560). In contrast, inhibitors of either heme oxygenase or COX-2 were inactive. Intranasal instillation of glucagon inhibited methacholine-induced airway obstruction by a mechanism sensitive to pretreatment with L-NAME, indomethacin and SC-560. Glucagon induced CREB and NOS-3 phosphorylation and increased PGE₂ levels in the lung tissue without altering COX-1 expression. Glucagon also inhibited LPS-induced AHR and bronchoalveolar inflammation. These findings suggest that glucagon possesses airway-relaxing properties that are mediated by epithelium-NOS-3-NO- and COX-1-PGE₂-dependent mechanisms.

Key Words

- ▶ glucagon
- ▶ nitric oxide
- ▶ prostaglandins
- ▶ lung
- ▶ muscle

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Introduction

Glucagon is a 29-amino-acid peptide hormone secreted during fasting periods by the pancreatic islet α -cells into hepatic portal venous circulation in response to falling glucose levels (Conarello *et al.* 2007, Cryer 2012). Glucagon acts as a counter-regulatory hormone to insulin and the combined action of both hormones maintains glucose homeostasis. While insulin acts primarily in response to low blood glucose levels by stimulating tissue and cellular uptake of glucose and hepatic glycogen synthesis, glucagon increases hepatic glucose production and its release into the bloodstream by stimulating glycogenolysis and gluconeogenesis (Quesada *et al.* 2008, Cryer 2012).

Moreover, glucagon has several effects in extra-hepatic tissues, including positive inotropic effects in the heart, lipolysis in adipose tissue, inducing satiety signals in the CNS and regulatory effects on the glomerular filtration rate in the kidneys. The action of glucagon is correlated with the tissue distribution of its specific receptor, which is highly expressed in the liver and kidney and expressed to a lesser extent in the heart, adipose tissue and cerebral cortex (Authier & Desbuquois 2008, Jones *et al.* 2012). Glucagon receptor (GcgR) is a member of the seven-transmembrane receptors coupled to the G stimulatory (Gs) protein. When glucagon binds to its receptor, the α -subunit of Gs leads to the activation of adenylyl cyclase, with a subsequent increase in the intracellular levels of cAMP (Qureshi *et al.* 2004, Authier & Desbuquois 2008, Altarejos & Montminy 2011).

cAMP regulates several airway smooth muscle (ASM) cell functions, including proliferation, migration, secretion of inflammatory mediators, and production of extracellular matrix components. In the lungs, cAMP has a major effect on ASM contraction (Billington *et al.* 2013). Elevation of intracellular cAMP induces relaxation of ASM through several mechanisms, including reduction in the intracellular Ca^{2+} concentration, decreased Ca^{2+} sensitivity, activation of Ca^{2+} -dependent K^{+} channels, and inhibition of inositol phospholipid hydrolysis, as well as an impaired ability to promote myosin light chain phosphorylation (Oguma *et al.* 2006, Roscioni *et al.* 2011, Olsen *et al.* 2012, Billington *et al.* 2013). β -adrenergic agonists are the drugs of choice for treatment of acute bronchospasm in asthmatic subjects, which remarkably induce airway relaxation by elevating cAMP levels in ASM (Horvat *et al.* 2012). Currently, the literature described that several hormones responsible for regulation of metabolism are able to modulate the contractile response of ASM through induction of contraction, including insulin, or relaxation,

like epinephrine, estrogen and progesterone (Bosse 2014). Since the effect of glucagon on ASM contraction is poorly understood and mouse lungs express GcgR and its activation leads to an increase in the intracellular cAMP (Authier & Desbuquois 2008), we undertook this study to evaluate the putative mechanism underlying the antispasmodic effect of glucagon on ASM.

Materials and methods

Animals

Male A/J mice (18–20 g) were obtained from the Oswaldo Cruz Foundation breeding colony and used in accordance with the guidelines of the Committee on Use of Laboratory Animals of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, license LW 23/11). Mice were housed in groups of five in a temperature-, humidity- and light-controlled (12 h light:12 h darkness cycle) colony room. Mice were given *ad libitum* access to food and water.

Tracheal smooth muscle contraction *in vitro*

Mice were euthanized in a CO_2 chamber before the tracheas were dissected free of adhering fat and connective tissue. Tracheas were then mounted in isolated organ baths filled with 10 ml of aerated (95% O_2 and 5% CO_2) Krebs' buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 24 mM NaHCO_3 and 11 mM glucose) and kept at 37 °C. An initial tension of 1 g was applied to the tracheas for 60 min to obtain a constant resting tension. Then, the response to carbachol (2.5 μM) was recorded to confirm the viability of the preparation. After washout of carbachol and re-establishment of the baseline resting tension, concentration curves were constructed with the cumulative addition of carbachol (10^{-8} – 10^{-4} M) in the presence of glucagon (0.1 and 1 μM) or 0.9% NaCl sterile solution. The treatment with glucagon was carried out 30 min before tissues were re-exposed to carbachol. All responses were expressed as percentages of the response to 2.5 μM carbachol.

In some experiments, epithelial cells were removed mechanically, as described previously (Coelho *et al.* 2008). The contractile response to the cumulative addition of carbachol was measured in intact or denuded epithelium tracheas in the presence or absence of glucagon. To investigate glucagon's antispasmodic mechanisms of action, tracheas were pretreated with a GcgR antagonist (des-his1-[Glu9]-glucagon amide – 1 μM)

(Leibiger *et al.* 2012) and with inhibitors of adenylyl cyclase (SQ 22 536 – 5 μ M), nitric oxide synthase (NOS) (L-NAME – 100 μ M) (Coelho *et al.* 2008), heme oxygenase (HO) (ZnPP IX – 10 μ M) (Undem *et al.* 1996), cyclooxygenase (COX) (indomethacin – 10 μ M) (Coelho *et al.* 2008), COX-1 (SC-560 – 1 μ M) (Ito *et al.* 2006) and COX-2 (NS-398 – 0.03 μ M) (Schlemper *et al.* 2005). Stimulation-induced isometric contractile responses were measured with a force-displacement transducer (Ugo Basile, Comerio, Italy) and the readout used to assess contractility was obtained by isolated organ data acquisition software (Proto 5; Letica Scientific Instruments, Barcelona, Spain). Contractile responses were expressed as a percentage of the maximal contraction induced by 2.5 μ M carbachol.

Non-invasive *in vivo* assessment of airway obstruction

Using barometric whole-body plethysmography (Buxco Research System, Wilmington, NC, USA) as described (Hamelmann *et al.* 1997), we measured the enhanced pause (Penh) responses in conscious, spontaneously breathing mice following appropriate provocations. Aerosolized PBS and increasing methacholine concentrations (6.25, 12.5, 25 and 50 mg/ml) were nebulized through an inlet of the individual chambers for 2.5 min, and Penh readings were recorded for 5 min following each nebulization. Penh averages were obtained at 1, 3 and 6 h after intranasal treatment with glucagon (1 μ g/kg) or 0.9% NaCl sterile solution.

LPS-induced lung inflammation

Male A/J mice were anesthetized with aerosolized isoflurane for stimulation by nasal instillation with LPS (25 μ g/25 μ l) or 0.9% NaCl sterile solution. Treatment with glucagon was intranasally given 1 h before LPS provocation. Untreated mice received intranasal administration of 0.9% NaCl sterile solution.

Measurement of pulmonary inflammation by bronchoalveolar lavage fluid

Eighteen hours after the LPS provocation, mice were euthanized by anesthetic overdose (sodium thiopental, 500 mg/kg, i.p.). Cells were recovered from the airway lumen through bronchoalveolar lavage fluid (BALF). Airways were flushed twice with 0.75 ml PBS containing 10 mM EDTA via a tracheal cannula. BALF was centrifuged (400 g, 10 min, 4 °C) and cell pellets were resuspended in 250 μ l PBS plus EDTA for further enumeration of leukocytes in a Neubauer chamber, by means of a light

microscope (BX40; Olympus), after dilution in Türk solution (2% acetic acid). Differential cell counts were carried out on May–Grunwald–Giemsa-stained cytospin preparations under oil immersion objective to determine the percentage of mononuclear cells and neutrophils.

Invasive assessment of respiratory mechanics

Mice were anesthetized (nembutal 60 mg/kg, i.p.) and subjected to both endotracheal and esophageal intubations for pulmonary function and airway hyperreactivity (AHR) assessment in a FinePointe Buxco Platform (Buxco Electronics, Sharon, CT, USA), as previously described (Serra *et al.* 2012). This assessment was carried out in mechanically ventilated mice under neuromuscular blockade induced by pancuronium bromide (1 mg/kg, i.p.).

Lung resistance (RL) (cm H₂O/ml per s) and dynamic lung compliance (C_{dyn}) (ml/cm H₂O) in each breath cycle were calculated based on airflow and pressure signals. The former was measured using the differential pressure changes in the whole-body plethysmograph as the animal breathed in and out, whereas the latter was measured at the mouth (airway pressure). Airway pressure at the mouth and the esophageal pressure were taken into consideration in order to obtain RL values not masked by the resistance component that comes from the chest wall. Analog signals were digitized using a Buxco Analog/Digital Converter (Buxco Electronics). Mice were allowed to stabilize for 5 min and were stimulated by the inhalation of increasing concentrations of aerosolized methacholine (3, 9 and 27 mg/ml) for 5 min each. Baseline measurements of pulmonary parameters were obtained by exposure to the vehicle (PBS) (Ferreira *et al.* 2013). In all experiments, the animals received glucagon (0.1, 1 or 10 μ g/kg, i.n.) or NaCl sterile solution (0.9%, i.n.) 3 h before the inhalation of aerosolized methacholine. In some experiments, the animals were pretreated intraperitoneally with L-NAME (20 mg/kg) (Coelho *et al.* 2008), indomethacin (10 mg/kg) (Steiner *et al.* 2001) or SC-560 (5 mg/kg) (Steiner *et al.* 2001) 30 min before glucagon administration. The control animals received the same amount of vehicle i.p. (0.9% NaCl or DMSO 0.3 and 1.6% for indomethacin and SC-560 respectively). In another set of experiments, we analyzed the AHR to increasing concentrations of aerosolized methacholine (3, 9 and 27 mg/ml) 18 h after the LPS provocation (25 μ g/25 μ l, i.n.).

Western blot

Lung tissue as well as intact and denuded epithelium tracheas was obtained to evaluate the GcGR expression.

To analyze the time-dependent stimulation or expression of CREB, NOS-3 and COX-1 induced by glucagon, lungs were recovered after instillation with glucagon (1 µg/kg) at several time intervals (5, 10, 15, 30, 60, 120 and 180 min). All tissues were homogenized in cold lysis buffer containing the protease inhibitor cocktail Complete (F. Hoffmann-La Roche Ltd) and 0.1% Triton X-100 in PBS. The lysate was centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant was recovered and protein quantification was performed using the BCA assay (Sigma–Aldrich Corp.). Equal amounts of sample protein (50 µg/lane for GcgR and 100 µg/lane for CREB, phospho-CREB (pCREB), NOS-3, phospho-NOS-3 (pNOS-3) and COX-1 were loaded and separated on a 10% SDS–PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking in 5% (w/v) skimmed milk powder in TBS-T for 1 h, membranes were exposed overnight at 4 °C to either GcgR-, CREB-, pCREB-, NOS-3-, pNOS-3- (1:500; Santa Cruz Biotechnology), COX-1- (1:100; Cayman Chemical, Ann Arbor, MI, USA) specific rabbit or mouse polyclonal antibodies or a β-actin-specific mouse monoclonal antibody (MAB) (1:1000; Santa Cruz Biotechnology) followed by three washes and incubation with a HRP-conjugated secondary antibody (1:10 000; R&D Systems, Minneapolis, MN, USA) and IRDye goat anti-rabbit secondary antibody or IRDye goat anti-mouse secondary antibody (both at 1:10 000; LI-COR Corporate, Lincoln, NE, USA) for 1 h at room temperature. The membranes were washed in TBS-T, and protein expression was detected using ECL (SuperSignal West Dura, Thermo Fisher Scientific, Inc., Rockford, IL, USA) or fluorescence using the Odyssey Image System (LI-COR Corporate). The band intensity was quantified by densitometry using the software Image-Pro Plus 6.2 (Media Cybernetics Inc, Bethesda, MA, USA).

Prostaglandin E₂ measurement

Prostaglandin E₂ (PGE₂) levels were measured in lung tissue obtained at 15, 30, 60, 120 and 180 min after glucagon instillation (1 µg/kg) into mice. PGE₂ was assayed in the homogenized tissue using an enzyme-linked immunoassay (EIA) according to the manufacturer's instructions (Cayman Chemical).

TNF-α quantification

Eighteen hours after intranasal LPS provocation (25 µg/25 µl), the BALF recovered from the airway lumen was centrifuged (400 *g*, 10 min, 4 °C) and the supernatant immediately frozen in liquid nitrogen, and stored at –

80 °C. Tumour necrosis factor-α (TNF-α) was assayed in the BALF using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems).

Drugs

Carbachol, methacholine, L-NAME, indomethacin, SC-560, NS-398, SQ 22 536, glucagon, des-his1-[Glu9]-glucagon amide, LPS and nembutal were purchased from Sigma Chemical Co. Pancuronium bromide, isoflurane and sodium thiopental were purchased from Cristália (São Paulo, Brazil), and ZnPP IX was purchased from Frontier Scientific (Logan, UT, USA). Glucagon, des-his1-[Glu9]-glucagon amide, carbachol, SQ 22 536, L-NAME, LPS, pancuronium bromide, nembutal and sodium thiopental were diluted in 0.9% NaCl sterile solution. Methacholine was diluted in PBS. Indomethacin, ZnPP IX, SC-560 and NS-398 were prepared in DMSO 0.1% for *in vitro* assays. Indomethacin and SC-560 were dissolved in DMSO 0.3 and 1.6% respectively, for *in vivo* experiments. All of the solutions were freshly prepared immediately before use.

Statistical analysis

The data were reported as the mean ± s.e.m. and statistically analyzed using one-way ANOVA followed by Newman–Keuls–Student's *t*-test. Probability values (*P*) of 0.05 or less were considered significant.

Results

Glucagon inhibits ASM contraction induced by carbachol *in vitro* by activating its receptor (GcgR) in the airway epithelium

Administration of increasing concentrations of carbachol (10⁻⁸–10⁻⁴ M) induced a smooth muscle contraction of tracheas obtained from A/J mice. Pretreatment with glucagon (0.1 and 1 µM) for 30 min before the addition of cumulative concentrations of carbachol inhibited tracheal contraction *in vitro* (Fig. 1). To evaluate the involvement of GcgR on the antispasmodic effect of glucagon *in vitro*, we pre-incubated the trachea preparations with an adenylyl cyclase inhibitor (SQ 22 536; 5 µM) or a GcgR antagonist (des-his1-[Glu9]-glucagon amide; 1 µM). Both SQ 22 536 and des-his1-[Glu9]-glucagon amide significantly inhibited the antispasmodic effect of glucagon on the carbachol-induced contractile trachea response (Fig. 2A and B respectively). Unexpectedly, the tracheas that only received SQ 22 536

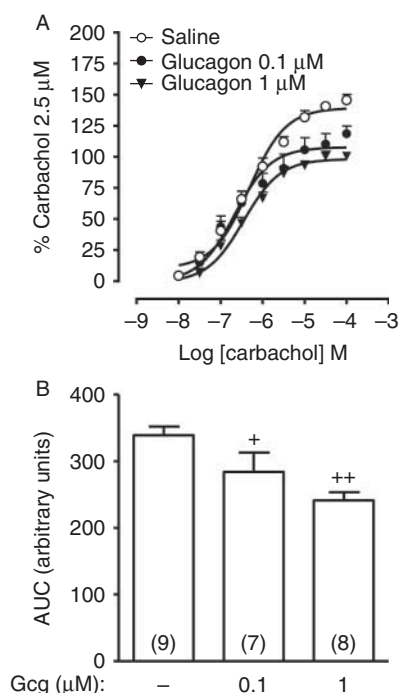


Figure 1

Glucagon prevents the carbachol-stimulated contraction of mouse tracheas *in vitro*. (A) Effect of glucagon on tracheal contraction induced by carbachol (10^{-8} – 10^{-4} M). (B) The AUC was calculated from the concentration–response curves of contraction caused by carbachol. All of the results are expressed as the percentage of contractile responses induced by 2.5 μ M carbachol. Each value represents the mean \pm S.E.M. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. $^+P < 0.05$ compared to untreated group. $^{++}P < 0.01$ compared to untreated group. Gcg, glucagon.

showed an inhibition of smooth muscle contraction induced by carbachol *in vitro* (Fig. 2A).

Then, we assessed the putative role played by the epithelium in the glucagon relaxing effect. As shown in Fig. 2C, the mechanical removal of the epithelial cell layer clearly abrogated the protective effect of glucagon on carbachol-induced tracheal contraction. We further investigated the expression of GcgR in mice tracheas in the presence or absence of epithelium. As observed in Fig. 2D, the GcgR was significantly less expressed in epithelium-denuded tracheas than in tracheas with intact epithelium and the positive controls (lungs).

Inhibition of airway constriction by glucagon *in vitro* depends of nitric oxide and COX-1 products

To study which epithelium-derived factor could be involved in the antispasmodic effect of glucagon *in vitro*, we pre-incubated tracheas with NOS (*L*-NAME;

100 μ M), HO (ZnPP IX; 10 μ M) unselective COX (indomethacin; 10 μ M), selective COX-1 (SC-560; 1 μ M), or selective COX-2 (NS398; 0.03 μ M) inhibitors 30 min before glucagon treatment. As illustrated in Fig. 3, co-incubation with *L*-NAME, indomethacin, or SC-560 clearly prevented the antispasmodic effect of glucagon in tracheas subjected to carbachol-induced contraction *in vitro*, whereas exposure to ZnPP IX or NS-398 was inactive.

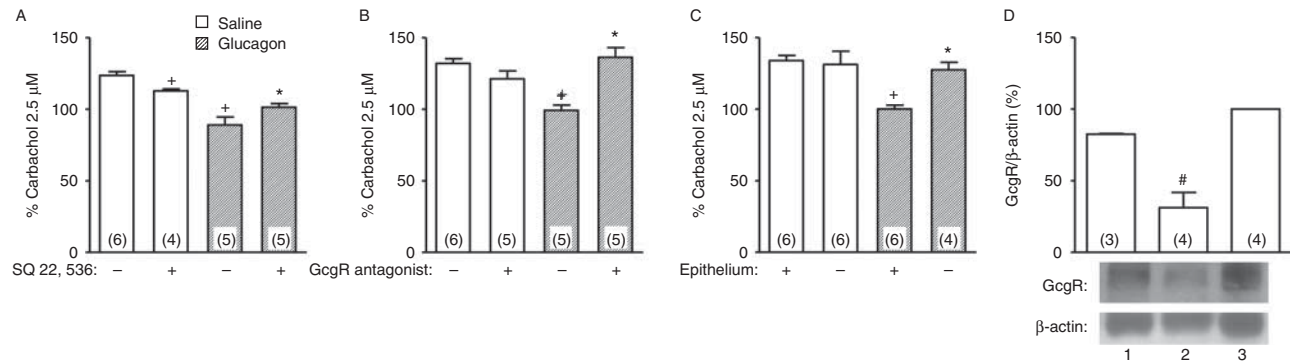
Glucagon prevents methacholine-induced ASM contraction *in vivo*

While using the non-invasive barometric plethysmography, we observed that glucagon (1 μ g/kg, *i.n.*) significantly inhibited methacholine-induced bronchospasm in mice. The best blockade of the cholinergic bronchoconstrictive response was noted when the treatment was performed 3 h before methacholine. Specifically, for methacholine concentrations of 0, 6.25, 12.5, 25 and 50 mg/ml, the Penh values decreased from 0.58 ± 0.03 , 1.00 ± 0.11 , 1.93 ± 0.29 , 3.61 ± 0.84 and 5.25 ± 0.87 (mean \pm S.E.M., $n = 7$) respectively, to 0.52 ± 0.02 , 0.61 ± 0.016 , 0.74 ± 0.057 , 1.59 ± 0.20 and 3.41 ± 0.60 (mean \pm S.E.M., $n = 8$) respectively. The values for the area under the curve (AUC) were analyzed by a range of concentrations between 0 and 50 mg/ml. The AUC values of animals treated with glucagon decreased from 159.7 ± 28.3 (mean \pm S.E.M., $n = 7$) to 85.1 ± 8.7 (mean \pm S.E.M., $n = 8$), which correspond to a blockade of $46.7 \pm 5.4\%$ (mean \pm S.E.M., $n = 8$) as compared to the vehicle-treated animals. For treatments occurring at 1 h or 6 h before, the inhibition levels were of 32.8 ± 4.9 and $20.8 \pm 8.7\%$ (mean \pm S.E.M., $n = 8$) respectively.

In another setting of experiments, we evaluated the effect of intranasal glucagon upon methacholine-induced changes in increased RL (Fig. 4A and B) and decreased Cdyn in A/J mice (Fig. 4C and D). Treatment with glucagon (0.1, 1 and 10 μ g/kg, *i.n.*) 3 h before provocation inhibited the methacholine-induced increase in RL (Fig. 4A and B) and reduction in Cdyn (Fig. 4C and D). In both lung function parameters, the dose of 1 μ g/kg glucagon was more effective, and significantly increased the baseline values of Cdyn.

NO and COX-1 products are key factors in the antispasmodic effect of glucagon on methacholine-evoked airways spasm *in vivo*

To investigate the possible mechanism involved in the antispasmodic action of glucagon *in vivo*, *L*-NAME (20 mg/kg), indomethacin (10 mg/kg) or SC-560 (5 mg/kg) were administered intraperitoneally 30 min before glucagon

**Figure 2**

The antispasmodic effect of glucagon on tracheal contraction induced by carbachol *in vitro* is mediated by the activation of the GcgR expressed in airway epithelium. Adenylyl cyclase inhibitor (SQ 22 536; 5 μ M) (A), GcgR antagonist (des-his1-[Glu9]-glucagon amide; 1 μ M) (B), and epithelium removal (C) blocked the antispasmodic effect of glucagon (1 μ M) on the tracheal contraction induced by carbachol (10^{-8} – 10^{-4} M) *in vitro*. (D) Expression of GcgR in tracheas from AJ mice was determined by western blot. Lanes 1, 2 and 3 are tracheas with intact epithelium, tracheas

with denuded epithelium and positive control (lung) respectively. The data were normalized to β -actin and represented as the ratio between the expressions of GcgR: β -actin relative to the control. Pooled samples of six animals were used in lanes 1 and 2. Each value represents the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. $^+P < 0.05$ compared to untreated group. $^*P < 0.05$ compared to glucagon-treated group. $^{\#}P < 0.05$ compared to intact epithelium trachea.

(1 μ g/kg, i.n.). All these treatments abrogated the glucagon protective effect concerning methacholine-induced changes in RL and Cdyn (Figs 5 and 6).

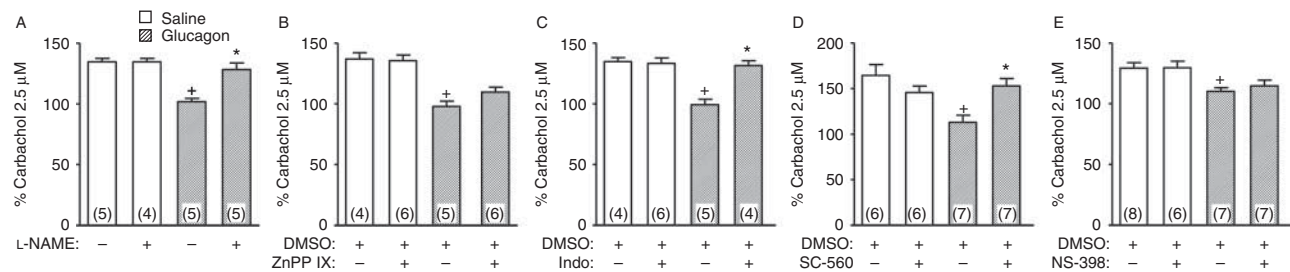
Glucagon administration induces CREB, NOS-3 and COX-1 activation in lungs of mice *in vivo*

To access the effect of glucagon on CREB, NOS-3 and COX-1 expression and activation, we performed a western blot and EIA analysis of lungs of mice instilled with glucagon (1 μ g/kg, i.n.) for different periods of time (5, 10, 15, 30, 60, 120 and 180 min). Glucagon phosphorylated CREB 15–30 min after its administration (Fig. 7A), while NOS-3 phosphorylation started at 30 min and remained detectable

for at least 180 min (Fig. 7B). Glucagon was followed by PGE₂ production from 30 to 180 min after instillation (Fig. 7D), without changes in COX-1 expression except a reduction noted at 180 min post-treatment (Fig. 7C).

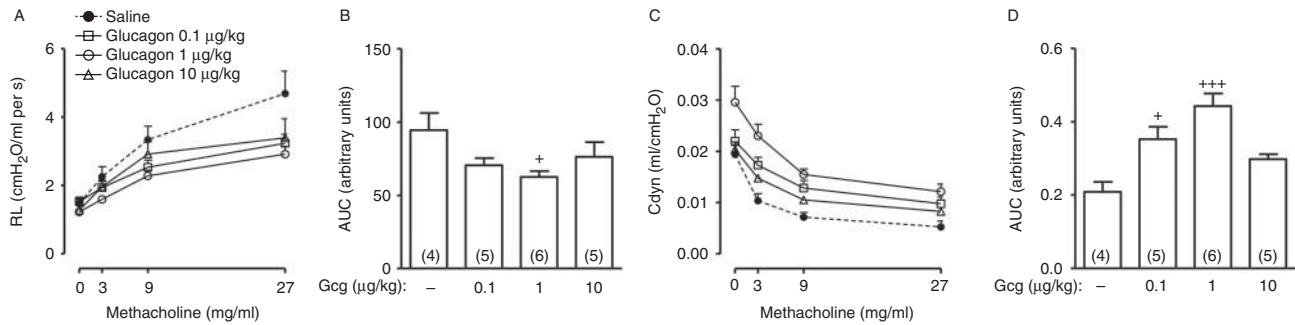
Glucagon inhibits inflammation and AHR induced by LPS

Finally, we assessed whether the antispasmodic effect of glucagon might be kept in the context of inflammation. As compared with control mice challenged with saline, LPS intranasal provocation caused a significant increase in total leukocyte numbers detected in BALF, which was accounted for by elevations in the numbers of neutrophils and mononuclear cells (Fig. 8A) 18 h after provocation.

**Figure 3**

The antispasmodic effect of glucagon on the tracheal contraction induced by carbachol *in vitro* requires NOS and COX-1 metabolites, but not HO and COX-2 products. NOS (L-NAME; 100 μ M) (A), non-selective COX (indomethacin; 10 μ M) (C) or selective COX-1 (SC-560; 1 μ M) inhibitors (D) abolished the antispasmodic effect of glucagon (1 μ M) on tracheal contraction induced by carbachol (10^{-8} – 10^{-4} M) *in vitro*. HO (ZnPP IX; 10 μ M) (B) or selective COX-2 (NS-398; 0.03 μ M) inhibitors (E) had no effect

on the glucagon-mediated (1 μ M) inhibition of the tracheal contraction induced by carbachol (10^{-8} – 10^{-4} M) *in vitro*. Each value represents the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. $^+P < 0.05$ compared to untreated group. $^*P < 0.05$ compared to glucagon-treated group. Indo, indomethacin. DMSO (0.1%).

**Figure 4**

Glucagon administration inhibits the airway obstruction provoked by methacholine *in vivo*. The dose–response analysis of the antispasmodic effect of glucagon (0.1, 1 and 10 µg/kg, *i.n.*) on increased RL (A) and decreased Cdyn (C) induced by methacholine (0–27 mg/ml) *in vivo*. AUC was calculated from the concentration–response curves of RL (B) and Cdyn (D)

following exposure to methacholine (range: 0–27 mg/ml). Each value represents the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. $^+P < 0.05$ compared to untreated group. $^{+++}P < 0.001$ compared to untreated group. Gcg, glucagon.

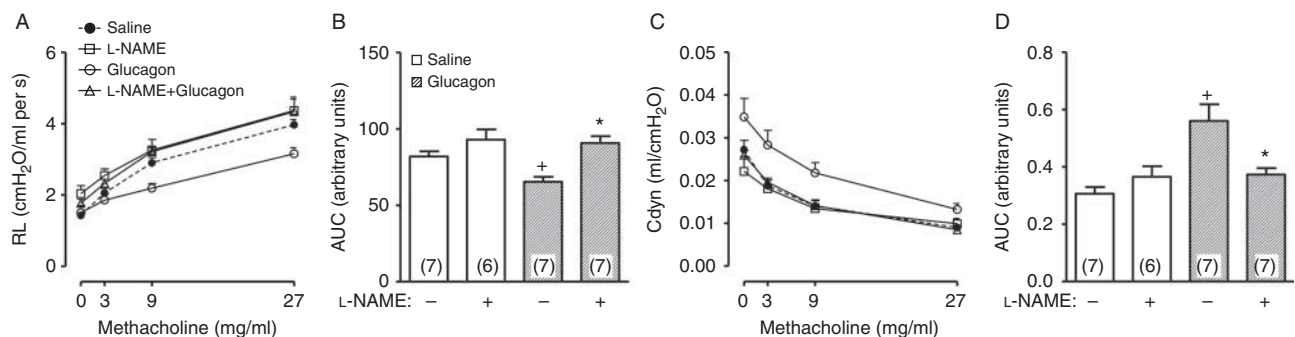
The LPS-induced leukocyte infiltrating response occurred in parallel with increased levels of TNF- α in lung tissue samples (Fig. 8B). All these inflammatory changes, except elevation in mononuclear cell counts, were clearly sensitive to glucagon (1 µg/kg, *i.n.*) given 1 h before LPS. Remarkably, the glucagon treatment clearly abrogated LPS-induced AHR regarding both RL changes (Fig. 8C and D) as well as Cdyn (Fig. 8E and F).

Discussion and conclusion

In this study, we showed that glucagon prevents ASM contraction *in vitro* and *in vivo* induced by cholinergic agents in A/J mice. We showed that the activation of GcgR was essential in this effect, as both the adenylyl cyclase inhibitor and GcgR antagonist suppressed the

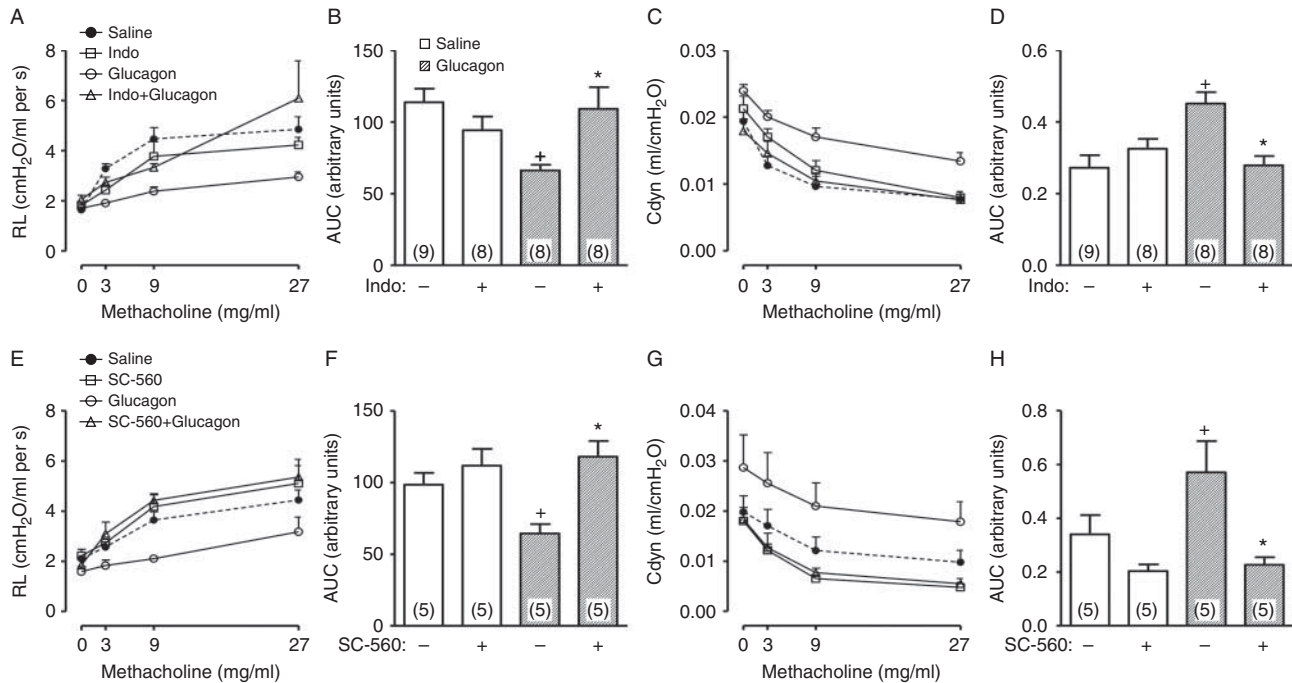
antispasmodic action of glucagon. The effect of glucagon on carbachol-induced trachea contraction was abrogated by removal of the epithelium and treatment with the NOS inhibitor, unselective COX inhibitor, or selective COX-1 inhibitor. Nasal instillation of glucagon induced CREB, NOS-3 and COX-1 activation in lung tissue. Furthermore, treatment with glucagon inhibited LPS-induced AHR and airway inflammation. Our findings indicate that glucagon prevents ASM contraction, acting through a synergic induction of NO and PGE₂ by airway epithelial cells.

Several studies revealed a controversy surrounding the benefits of glucagon therapy in asthmatic patients. While some authors have shown an improvement in lung function after glucagon inhalation (Sherman *et al.* 1988, Melanson *et al.* 1998), others did not find any effect from

**Figure 5**

The antispasmodic effect of glucagon on methacholine-induced reduction in respiratory function *in vivo* depends on NO production. L-NAME (20 mg/kg, *i.p.*) reversed the antispasmodic effect of glucagon (1 µg/kg, *i.n.*) on increased RL (A) and decreased Cdyn (C) induced by methacholine (0–27 mg/ml) *in vivo*. AUC was calculated from the

concentration–response curves of RL (B) and Cdyn (D) following exposure to methacholine (range: 0–27 mg/ml). Each value represents the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. $^+P < 0.05$ compared to untreated group. $^*P < 0.05$ compared to glucagon-treated group.

**Figure 6**

COX-1 derived products are involved in the antispasmodic effect of glucagon on methacholine-induced reduction in respiratory function *in vivo*. Indomethacin (10 mg/kg, i.p.) (A and C) and SC-560 (5 mg/kg, i.p.) (E and G) abolished the antispasmodic effect of glucagon (1 µg/kg, i.n.) on increased RL (A and E) and decreased Cdyn (C and G) induced by methacholine (0–27 mg/ml) *in vivo*. Following methacholine exposure (range: 0–27 mg/ml), the AUC was calculated from the concentration-

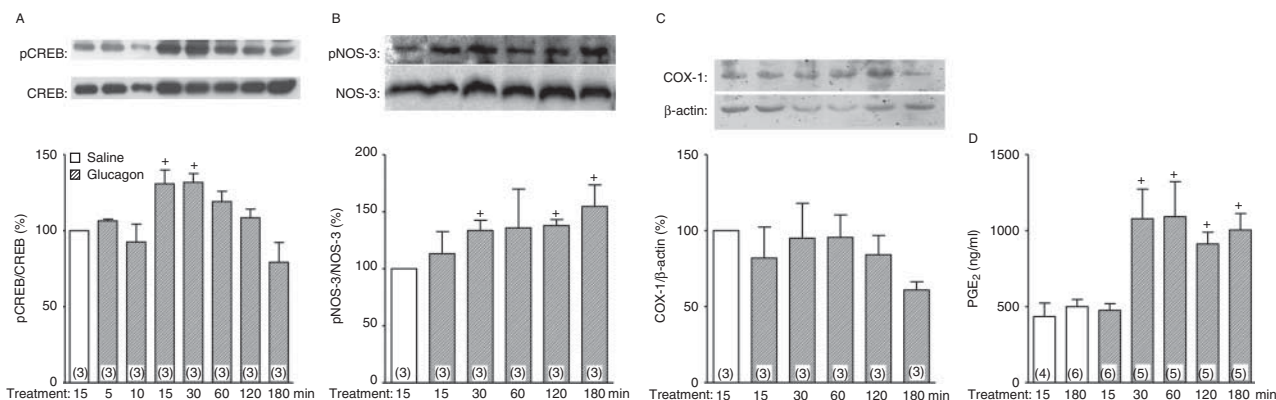
response curves of the RL (B and F) and Cdyn (D and H) of animals pre-treated with indomethacin (B and D) or SC-560 (F and H). Each value represents the mean ± s.e.m. The statistical analysis was performed by ANOVA followed by Newman-Keuls-Student's *t*-test. **P* < 0.05 compared to untreated group. +*P* < 0.05 compared to glucagon-treated group. Indo, indomethacin. DMSO (0.3 and 1.6% for indomethacin and SC-560 respectively).

the i.v. injection of this hormone (Imbruce *et al.* 1975, Wilber *et al.* 2000). Under our conditions, glucagon inhibited the carbachol-induced contractile response of tracheas in mice. These findings are in line with those of Blumenthal & Brody (1969), in which glucagon relaxed the bronchial smooth muscle in guinea pigs. In addition, vasoactive intestinal peptide and pituitary adenylyl cyclase-activating polypeptide, which are peptides that have high homology with glucagon amino acid sequence (Mentlein 2009), also have the ability to relax ASMs *in vitro* (Sherman *et al.* 1988, Melanson *et al.* 1998).

In another set of experiments, we showed that the inhibition of adenylyl cyclase with SQ 22 536 reduced the antispasmodic activity of glucagon on carbachol-evoked contraction, indicating that this effect depends on the activation of adenylyl cyclase. Notably, GcgR is coupled to a Gs protein that activates adenylyl cyclase to produce cAMP (Habegger *et al.* 2010). We confirmed this hypothesis by showing that the GcgR antagonist des-his1-[Glu9]-glucagon amide abolished the effects of glucagon on tracheal smooth muscle contraction. Surprisingly, the antispasmodic

activity of glucagon on carbachol-evoked contraction was unapparent after removal of the tracheal epithelium, clearly showing the crucial role of epithelial cells in this protective effect. This result was unexpected, once the elevation of intracellular cAMP levels leads to a relaxation of ASM (Zieba *et al.* 2011, Billington *et al.* 2013). To better explain this finding, we evaluated the expression of GcgR on both tracheal and lung tissue in mice from the A/J strain. We noted that GcgR was much more expressed in tracheas with intact epithelium and lung tissues than in denuded tracheas. Despite the presence of GcgR in tracheas without epithelium, glucagon cannot exert its effect when the epithelium is removed. This happens perhaps because the amount of receptors in the trachea without epithelium is so small that it is not sufficient to induce a strong enough signal to relax tracheal smooth muscle. Thereby, these results show that the effect of glucagon occurs through its receptor activation in epithelial cells and not in smooth muscle cells directly.

The observation that the integrity of the airway epithelial layer is essential for the antispasmodic activity of glucagon *in vitro* shows that epithelial cells

**Figure 7**

Glucagon treatment increases CREB, NOS-3 and COX-1 activity in the lungs of *A/J* mice. Representative samples of pCREB (A), pNOS-3 (B) and COX-1 (C) expression in the lungs of glucagon-treated (1 μ g/kg, i.n.) animals. The expression of pCREB, pNOS-3 and COX-1 was determined by western blot. Data were normalized to CREB, NOS-3 and β -actin respectively.

(D) Quantification of PGE₂ levels in lungs from glucagon-treated animals was realized by EIA. Data are expressed as the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman-Keuls-Student's *t*-test. ⁺*P* < 0.05 compared to untreated animals.

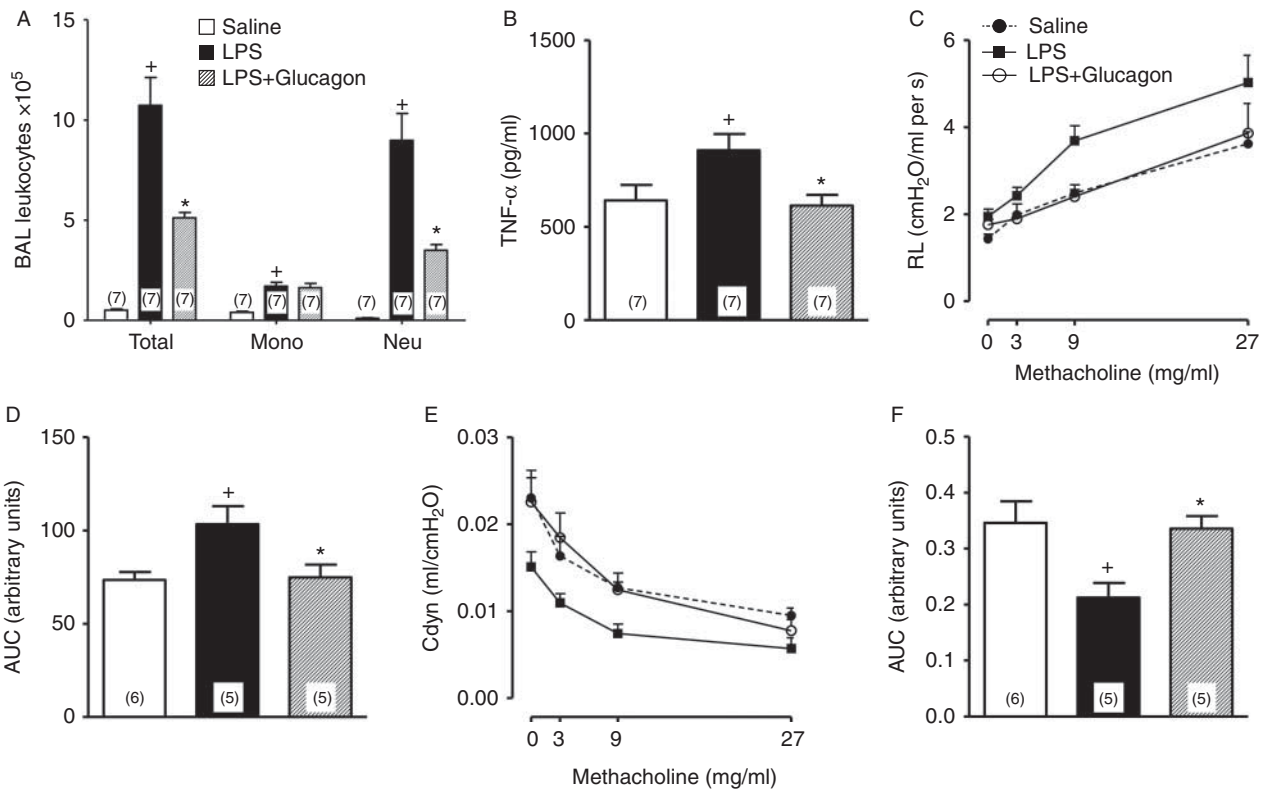
not only provide a crucial physical barrier that protects sensory nerves and smooth muscle cells from inhaled irritants (Folkerts & Nijkamp 1998, Schlemper *et al.* 2005) but also actively regulate the smooth muscle contractile machinery. In fact, the epithelium capacity to provide factors to relax the smooth muscle is firmly established. These factors include nitric oxide (NO), carbon monoxide, PGE₂ and prostacyclin, which protect the airway from excessive bronchoconstriction (Barnes 1998, de Sousa *et al.* 2010, Billington *et al.* 2013, Vieira *et al.* 2013). We tested several inhibitors to identify which factors produced by epithelial cells could be involved in the antispasmodic activity of glucagon *in vitro*. We observed that the protective effect of glucagon on the carbachol-induced contraction of mice tracheal was neither modified by HO inhibitor ZnPP IX nor by selective COX-2 inhibitor NS-398, precluding the involvement of carbon monoxide and COX-2 derived factors in this effect. Furthermore, we showed that the antispasmodic activity of glucagon upon carbachol-induced contraction was sensitive to pretreatment with the NOS inhibitor L-NAME, unselective COX inhibitor indomethacin and selective COX-1 inhibitor SC-560 *in vitro*. These results suggest that both NO and COX-1 metabolites are products derived from epithelial cells that are involved in the antispasmodic effect of glucagon *in vitro*.

To gain insight in the putative therapeutic application of the glucagon hormone, we used barometric plethysmography to assess the effectiveness of glucagon in airway contraction *in vivo*. We used two systems to measure methacholine-induced airway obstruction in mice

subjected to intranasal glucagon treatment. First, we used a non-invasive system and showed that glucagon significantly reduced methacholine-induced bronchoconstriction 3 h post-treatment. In agreement with this data, glucagon (1 μ g/kg, i.n.) yielded a significant blockade of RL as well as improved Cdyn as measured by an invasive assessment of lung function. One potential explanation for the lack of activity of 10 μ g/kg glucagon is the recognized ability of this hormone to cause GcGR desensitization (Krilov *et al.* 2011).

While trying to clarify whether the molecular mechanisms attributed to the glucagon effect *in vitro* are also relevant *in vivo*, we showed that L-NAME, indomethacin and SC-560 inhibited the protective effect of glucagon on the increase in RL and reduction of Cdyn caused by methacholine. These results confirmed the putative participation of NO and products of COX-1 in the antispasmodic effect of glucagon *in vivo*. Furthermore, we showed that glucagon promoted CREB phosphorylation in the lungs 15 and 30 min after its instillation. Once activation of GcGR increases intracellular levels of cAMP (Habegger *et al.* 2010) and CREB is phosphorylated in response to this second messenger (Altarejos & Montminy 2011), this results confirm *in vivo* our data obtained *in vitro* that showed that antispasmodic effect of glucagon depends on its receptor and adenylyl cyclase activation.

Because NOS-3 and COX-1 are constitutively expressed in respiratory epithelial cells (Maarsingh *et al.* 2005, Radi *et al.* 2010, Strapkova & Antosova 2011), and are also sensitive to expressional up-regulation (Forstermann *et al.* 1998, Molloy *et al.* 2008), we evaluated

**Figure 8**

LPS induced inflammation and AHR in the airways is sensitive to glucagon treatment. Glucagon inhibited leukocyte accumulation (total and differential cell counts) (A) and TNF- α levels (B) in BALF, 18 h after the provocation with LPS (25 μ g/25 μ l) or saline. Analysis of the antispasmodic effect of glucagon (1 μ g/kg, i.n.) on increased RL (C) and decreased Cdyn (E) induced by methacholine (0–27 mg/ml) in *A/J* mice provoked with

LPS (25 μ g/25 μ l) or saline. AUC was calculated from the concentration-response curves of RL (D) and Cdyn (F) following exposure to methacholine (range: 0–27 mg/ml). Each value represents the mean \pm s.e.m. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's *t*-test. ⁺*P* < 0.05 compared to saline-provoked group. **P* < 0.05 compared to LPS-provoked group.

the activation and/or expression of NOS-3 and COX-1 after an intranasal glucagon treatment. We found that glucagon effectively increased the activation of NOS-3 and COX-1, evidenced by an increase of pNOS-3 and production of PGE₂ in the lung tissue from the time of 30 until 180 min after its administration, although glucagon did not enhance the COX-1 expression. These results showed that glucagon probably induces activation of NOS-3 and COX-1 through cAMP pathway, once CREB is phosphorylated before activation of both enzymes; likewise, cAMP can activate NOS and COX (Zhang & Hintze 2006, Han *et al.* 2010). In our study, both NOS-3 phosphorylation and PGE₂ production occurred from 30 to 180 min after glucagon treatment; thereby, we believed that NO and PGE₂ act synergistically. Furthermore, the presence of both pro-relaxation mediators all this time in the lungs explains why the antispasmodic effect of glucagon on methacholine-provoked airway obstruction is better 3 h post-instillation.

Finally, we investigated whether the glucagon effectiveness is maintained in the context of inflammation. Therefore, we assessed the impact of glucagon treatment on LPS-induced inflammatory response marked by activation of NOS-2 and production of high amounts of NO as previously reported (Hsia *et al.* 2012). Our results revealed that LPS provocation yielded a state of AHR, as determined by exacerbation on RL and Cdyn changes triggered by aerosolized methacholine. Moreover, we noted an intense leukocyte infiltration demonstrated by the accumulation of mononuclear cells and neutrophils in the BALF in association with increased levels of TNF- α . Remarkably, local glucagon treatment inhibited airway hyperreactivity, neutrophils accumulation and TNF- α production, supporting the interpretation that this hormone has also marked anti-inflammatory properties and can maintain its antispasmodic effect even in the presence of pro-inflammatory signals and overproduction of NO. It is likely that, as observed in the smooth muscle relaxing

effect, the anti-inflammatory effect of glucagon is also associated, in a causative manner, with NO and prostanoid generation. In fact, it is well established that PGE₂ can prevent airway inflammation by inhibiting the recruitment of inflammatory cells into the lungs (Vancheri *et al.* 2004). Furthermore, NOS-3-derived NO has been associated with airway inflammation blockade by inhibiting the expression of NOS-2 and production of inflammatory cytokines, including TNF- α (Thomassen *et al.* 1997, Cook *et al.* 2003, Ten Broeke *et al.* 2006).

The activation of ASM is classically considered to be regulated only through a paracrine signaling, but several pieces of evidence pointed out that hormones released by different organs, including adrenals, gonads, adipose tissue and pancreas, can affect ASM contractility. This endocrine regulation of ASM is important, since changes in the circulating levels of such hormones can inhibit or exacerbate the contraction of ASM in asthmatics (Bosse 2014). For instance, patients with type 1 diabetes who have high circulating levels of glucagon (Aronoff *et al.* 2004) had a lower incidence of asthma (Caffarelli *et al.* 2004, Karlstad *et al.* 2012). Our results could help explain, at least in part, a possible mechanism associated with this phenomenon.

In conclusion, our findings demonstrate that glucagon inhibits both smooth muscle spasms caused by cholinergic stimuli and AHR and lung inflammatory response induced by LPS. These effects seem to be associated with increased NOS-3 and COX-1 activation, with consequent production of NO and PGE₂ by epithelial cells. Taken together, these findings suggest glucagon as a possible new treatment for limited airflow resulting from human lung diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D B R I performed the research; analyzed the data; wrote the paper. J B D performed the research; analyzed the data. L P C performed the research;

analyzed the data. A R S performed the research; contributed essential reagents or tools; analyzed the data. P M R S designed the research study; contributed essential reagents or tools. M A M contributed essential reagents or tools; wrote the paper. V F C designed the research study; contributed essential reagents or tools; wrote the paper.

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References

- Altarejos JY & Montminy M 2011 CREB and the CREB co-activators: sensors for hormonal and metabolic signals. *Nature Reviews. Molecular Cell Biology* **12** 141–151. (doi:10.1038/nrm3072)
- Aronoff SL, Berkowitz K, Shreiner B & Want L 2004 Glucose metabolism and regulation: beyond insulin and glucagon. *Diabetes Spectrum* **17** 183–190. (doi:10.2337/diaspect.17.3.183)
- Authier F & Desbuquois B 2008 Glucagon receptors. *Cellular and Molecular Life Sciences* **65** 1880–1899. (doi:10.1007/s00018-008-7479-6)
- Barnes PJ 1998 Pharmacology of airway smooth muscle. *American Journal of Respiratory and Critical Care Medicine* **158** S123–S132. (doi:10.1164/ajrccm.158.supplement_2.13tac800)
- Billington CK, Ojo OO, Penn RB & Ito S 2013 cAMP regulation of airway smooth muscle function. *Pulmonary Pharmacology & Therapeutics* **26** 112–120. (doi:10.1016/j.pupt.2012.05.007)
- Blumenthal MN & Brody TM 1969 Studies on the mechanism of drug-induced bronchiolar relaxation in the guinea pig. *Journal of Allergy* **44** 63–69. (doi:10.1016/0021-8707(69)90001-X)
- Bosse Y 2014 Endocrine regulation of airway contractility is overlooked. *Journal of Endocrinology* **222** R61–R73. (doi:10.1530/JOE-14-0220)
- Caffarelli C, Cavagni G, Pierdomenico R, Chiari G, Spattini A & Vanelli M 2004 Coexistence of IgE-mediated allergy and type 1 diabetes in childhood. *International Archives of Allergy and Immunology* **134** 288–294. (doi:10.1159/000079166)
- Coelho LP, Serra MF, Pires AL, Cordeiro RS, Rodrigues e Silva PM, dos Santos MH & Martins MA 2008 7-Epiclusianone, a tetraprenylated benzophenone, relaxes airway smooth muscle through activation of the nitric oxide-cGMP pathway. *Journal of Pharmacology and Experimental Therapeutics* **327** 206–214. (doi:10.1124/jpet.108.138032)
- Conarello SL, Jiang G, Mu J, Li Z, Woods J, Zycband E, Ronan J, Liu F, Roy RS, Zhu L *et al.* 2007 Glucagon receptor knockout mice are resistant to diet-induced obesity and streptozotocin-mediated β cell loss and hyperglycaemia. *Diabetologia* **50** 142–150. (doi:10.1007/s00125-006-0481-3)
- Cook S, Vollenweider P, Menard B, Egli M, Nicod P & Scherrer U 2003 Increased eNO and pulmonary iNOS expression in eNOS null mice. *European Respiratory Journal* **21** 770–773. (doi:10.1183/09031936.03.00121203)
- Cryer PE 2012 Minireview: glucagon in the pathogenesis of hypoglycemia and hyperglycemia in diabetes. *Endocrinology* **153** 1039–1048. (doi:10.1210/en.2011-1499)
- Ferreira TP, de Arantes AC, do Nascimento CV, Olsen PC, Trentin PG, Rocco PR, Hogaboam CM, Puri RK, Martins MA & Silva PM 2013 IL-13 immunotoxin accelerates resolution of lung pathological changes triggered by silica particles in mice. *Journal of Immunology* **191** 5220–5229. (doi:10.4049/jimmunol.1203551)
- Folkerts G & Nijkamp FP 1998 Airway epithelium: more than just a barrier!. *Trends in Pharmacological Sciences* **19** 334–341. (doi:10.1016/S0165-6147(98)01232-2)

- Forstermann U, Boissel JP & Kleinert H 1998 Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB Journal* **12** 773–790.
- Habegger KM, Heppner KM, Geary N, Bartness TJ, DiMarchi R & Tschop MH 2010 The metabolic actions of glucagon revisited. *Nature Reviews. Endocrinology* **6** 689–697. (doi:10.1038/nrendo.2010.187)
- Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG & Gelfand EW 1997 Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *American Journal of Respiratory and Critical Care Medicine* **156** 766–775. (doi:10.1164/ajrccm.156.3.9606031)
- Han EH, Kim HG, Hwang YP, Choi JH, Im JH, Park B, Yang JH, Jeong TC & Jeong HG 2010 The role of cyclooxygenase-2-dependent signaling via cyclic AMP response element activation on aromatase up-regulation by α , β -DDT in human breast cancer cells. *Toxicology Letters* **198** 331–341. (doi:10.1016/j.toxlet.2010.07.015)
- Horvat SJ, Deshpande DA, Yan H, Panettieri RA, Codina J, DuBose TD Jr, Xin W, Rich TC & Penn RB 2012 A-kinase anchoring proteins regulate compartmentalized cAMP signaling in airway smooth muscle. *FASEB Journal* **26** 3670–3679. (doi:10.1096/fj.11-201020)
- Hsia BJ, Pastva AM, Giamberardino CD, Potts-Kant EN, Foster WM, Que LG, Abraham SN, Wright JR & Zaas DW 2012 Increased nitric oxide production prevents airway hyperresponsiveness in caveolin-1 deficient mice following endotoxin exposure. *Journal of Allergy & Therapy* (Supplement 1(4)) 1–18. (doi:10.4172/2155-6121.S1-004)
- Imbruce R, Goldfedder A, Maguire W, Briscoe W & Nair S 1975 The effect of glucagon on airway resistance. *Journal of Clinical Pharmacology* **15** 680–684. (doi:10.1002/j.1552-4604.1975.tb05920.x)
- Ito S, Kume H, Oguma T, Ito Y, Kondo M, Shimokata K, Suki B & Naruse K 2006 Roles of stretch-activated cation channel and Rho-kinase in the spontaneous contraction of airway smooth muscle. *European Journal of Pharmacology* **552** 135–142. (doi:10.1016/j.ejphar.2006.08.067)
- Jones BJ, Tan T & Bloom SR 2012 Minireview: glucagon in stress and energy homeostasis. *Endocrinology* **153** 1049–1054. (doi:10.1210/en.2011-1979)
- Karlstad O, Nafstad P, Tverdal A, Skurtveit S & Furu K 2012 Comorbidities in an asthma population 8–29 years old: a study from the Norwegian Prescription Database. *Pharmacoeconomics and Drug Safety* **21** 1045–1052. (doi:10.1002/pds.2233)
- Krilov L, Nguyen A, Miyazaki T, Unson CG, Williams R, Lee NH, Ceryak S & Bouscarel B 2011 Dual mode of glucagon receptor internalization: role of PKC α , GRKs and β -arrestins. *Experimental Cell Research* **317** 2981–2994. (doi:10.1016/j.yexcr.2011.10.001)
- Leibiger B, Moede T, Muhandiramlage TP, Kaiser D, Vaca Sanchez P, Leibiger IB & Berggren PO 2012 Glucagon regulates its own synthesis by autocrine signaling. *PNAS* **109** 20925–20930. (doi:10.1073/pnas.1212870110)
- Maarsingh H, Tio MA, Zaagsma J & Meurs H 2005 Arginase attenuates inhibitory nonadrenergic noncholinergic nerve-induced nitric oxide generation and airway smooth muscle relaxation. *Respiratory Research* **6** 23. (doi:10.1186/1465-9921-6-23)
- Melanson SW, Bonfante G & Heller MB 1998 Nebulized glucagon in the treatment of bronchospasm in asthmatic patients. *American Journal of Emergency Medicine* **16** 272–275. (doi:10.1016/S0735-6757(98)90100-0)
- Mentlein R 2009 Mechanisms underlying the rapid degradation and elimination of the incretin hormones GLP-1 and GIP. *Best Practice & Research. Clinical Endocrinology & Metabolism* **23** 443–452. (doi:10.1016/j.beem.2009.03.005)
- Molloy ES, Morgan MP, Doherty GA, McDonnell B, Hilliard M, O'Byrne J, Fitzgerald DJ & McCarthy GM 2008 Mechanism of basic calcium phosphate crystal-stimulated cyclo-oxygenase-1 up-regulation in osteoarthritic synovial fibroblasts. *Rheumatology* **47** 965–971. (doi:10.1093/rheumatology/ken144)
- Oguma T, Kume H, Ito S, Takeda N, Honjo H, Kodama I, Shimokata K & Kamiya K 2006 Involvement of reduced sensitivity to Ca in β -adrenergic action on airway smooth muscle. *Clinical and Experimental Allergy* **36** 183–191. (doi:10.1111/j.1365-2222.2006.02412.x)
- Olsen PC, Coelho LP, da Costa JC, Cordeiro RS, Silva PM & Martins MA 2012 Two for one: cyclic AMP mediates the anti-inflammatory and anti-spasmodic properties of the non-anesthetic lidocaine analog JMF2-1. *European Journal of Pharmacology* **680** 102–107. (doi:10.1016/j.ejphar.2012.01.040)
- Quesada I, Tuduri E, Ripoll C & Nadal A 2008 Physiology of the pancreatic α -cell and glucagon secretion: role in glucose homeostasis and diabetes. *Journal of Endocrinology* **199** 5–19. (doi:10.1677/JOE-08-0290)
- Qureshi SA, Rios Candelore M, Xie D, Yang X, Tota LM, Ding VD, Li Z, Bansal A, Miller C, Cohen SM *et al.* 2004 A novel glucagon receptor antagonist inhibits glucagon-mediated biological effects. *Diabetes* **53** 3267–3273. (doi:10.2337/diabetes.53.12.3267)
- Radi ZA, Meyerholz DK & Ackermann MR 2010 Pulmonary cyclooxygenase-1 (COX-1) and COX-2 cellular expression and distribution after respiratory syncytial virus and parainfluenza virus infection. *Viral Immunology* **23** 43–48. (doi:10.1089/vim.2009.0042)
- Rosciioni SS, Maarsingh H, Elzinga CR, Schuur J, Menzen M, Halayko AJ, Meurs H & Schmidt M 2011 Epac as a novel effector of airway smooth muscle relaxation. *Journal of Cellular and Molecular Medicine* **15** 1551–1563. (doi:10.1111/j.1582-4934.2010.01150.x)
- Schlemper V, Medeiros R, Ferreira J, Campos MM & Calixto JB 2005 Mechanisms underlying the relaxation response induced by bradykinin in the epithelium-intact guinea-pig trachea *in vitro*. *British Journal of Pharmacology* **145** 740–750. (doi:10.1038/sj.bjp.0706222)
- Serra MF, Anjos-Valotta EA, Olsen PC, Couto GC, Jurgilas PB, Cotias AC, Pao CR, Ferreira TP, Arantes AC, Pires AL *et al.* 2012 Nebulized lidocaine prevents airway inflammation, peribronchial fibrosis, and mucus production in a murine model of asthma. *Anesthesiology* **117** 580–591. (doi:10.1097/ALN.0b013e31826687d5)
- Sherman MS, Lazar EJ & Eichacker P 1988 A bronchodilator action of glucagon. *Journal of Allergy and Clinical Immunology* **81** 908–911. (doi:10.1016/0091-6749(88)90949-9)
- de Sousa AA, Soares PM, de Almeida AN, Maia AR, de Souza EP & Assreuy AM 2010 Antispasmodic effect of Mentha piperita essential oil on tracheal smooth muscle of rats. *Journal of Ethnopharmacology* **130** 433–436. (doi:10.1016/j.jep.2010.05.012)
- Steiner AA, Li S, Llanos QJ & Blatteis CM 2001 Differential inhibition by nimesulide of the early and late phases of intravenous- and intracerebroventricular-LPS-induced fever in guinea pigs. *Neuroimmunomodulation* **9** 263–275. (doi:10.1159/000054289)
- Strapkova A & Antosova M 2011 Competition of NO synthases and arginase in the airways hyperreactivity. *General Physiology and Biophysics* **30** 75–83. (doi:10.4149/gpb.2011_01_75)
- Ten Broeke R, De Crom R, Van Haperen R, Verweij V, Leusink-Muis T, Van Ark I, De Clerck F, Nijkamp FP & Folkerts G 2006 Overexpression of endothelial nitric oxide synthase suppresses features of allergic asthma in mice. *Respiratory Research* **7** 58. (doi:10.1186/1465-9921-7-58)
- Thomassen MJ, Buhrow LT, Connors MJ, Kaneko FT, Erzurum SC & Kavuru MS 1997 Nitric oxide inhibits inflammatory cytokine production by human alveolar macrophages. *American Journal of Respiratory Cell and Molecular Biology* **17** 279–283. (doi:10.1165/ajrcmb.17.3.2998m)
- Udem BJ, Ellis JL, Meeker S, Fischer A & Canning BJ 1996 Inhibition by zinc protoporphyrin-IX of vasoactive intestinal peptide-induced relaxations of guinea pig isolated trachea. *Journal of Pharmacology and Experimental Therapeutics* **278** 964–970.
- Vancheri C, Mastruzzo C, Sortino MA & Crimi N 2004 The lung as a privileged site for the beneficial actions of PGE₂. *Trends in Immunology* **25** 40–46. (doi:10.1016/j.it.2003.11.001)
- Vieira AB, Coelho LP, Insuela DB, Carvalho VF, Dos Santos MH, Silva PM & Martins MA 2013 Mangiferin prevents guinea pig tracheal contraction via activation of the nitric oxide-cyclic GMP pathway. *PLoS ONE* **8** e71759. (doi:10.1371/journal.pone.0071759)
- Wilber ST, Wilson JE, Blanda M, Gerson LW, Meerbaum SO & Janas G 2000 The bronchodilator effect of intravenous glucagon in asthma

exacerbation: a randomized, controlled trial. *Annals of Emergency Medicine* **36** 427–431. (doi:10.1016/S0196-0644(00)44407-0)

Zhang XP & Hintze TH 2006 cAMP signal transduction induces eNOS activation by promoting PKB phosphorylation. *American Journal of Physiology. Heart and Circulatory Physiology* **290** H2376–H2384. (doi:10.1152/ajpheart.00614.2005)

Zieba BJ, Artamonov MV, Jin L, Momotani K, Ho R, Franke AS, Nepl RL, Stevenson AS, Khromov AS, Chrzanowska-Wodnicka M *et al.* 2011 The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *Journal of Biological Chemistry* **286** 16681–16692. (doi:10.1074/jbc.M110.205062)

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