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Insulin decreases expression of the proinflammatory receptor proteinase-activated receptor-2 on human airway epithelial cells



To the Editor:

Insulin has anti-inflammatory properties,¹ which are abrogated in the presence of insulin resistance, a phenomenon caused by blockade in insulin signaling, decreased expression of insulin receptor, or both. Insulin resistance is associated with allergic sensitization and allergic asthma in human subjects^{2,3} and animal models.⁴ Recent evidence shows decreased insulin receptor expression in the airway epithelium of asthmatic patients,⁵ suggesting the presence of insulin resistance in the asthmatic epithelium. Therefore airway inflammation in asthmatic patients could be, at least in part, the result of decrease in insulin-mediated anti-inflammatory mechanisms in the airways.

Proteinase-activated receptor-2 (PAR-2) is a G protein-coupled receptor involved in the pathogenesis of a number of inflammatory conditions,^{E1-E6} including allergic asthma,^{6,7} a condition with increased PAR-2 expression on airway epithelial cells (AECs).⁸ Based on a previous observation that PAR-2 expression and PAR-2-mediated inflammation increase in insulin-resistant adipose tissue,^{E7-E9} we hypothesized that insulin regulates PAR-2 expression also in the airways. Here we studied the effects of insulin on PAR-2 expression in whole lungs and AECs by using a mouse model and primary cultures of human bronchial AECs, respectively. Detailed methods are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Because high-fat diet (HFD)-induced insulin resistance modulates PAR-2 expression in adipose tissue,^{E7-E9} we studied PAR-2 expression in the lungs of HFD-fed mice. HFD-fed mice had higher plasma insulin levels compared with control diet-fed mice ([Fig 1, A](#)), as expected, but had no significant difference in downstream insulin signaling,^{9,E10} as measured based on phospho-Akt levels in whole lungs ([Fig 1, B](#), and see

[Fig E1](#) in this article's Online Repository at www.jacionline.org). PAR-2 expression was decreased in lungs of HFD-fed mice compared with control diet-fed mice ([Fig 1, C](#)). We concluded that increased circulating insulin levels suppressed PAR-2 expression in lungs of HFD-fed mice. Based on this *in vivo* observation, we hypothesized that insulin regulates PAR-2 expression in AECs. This hypothesis might explain the observed PAR-2 upregulation on AECs of asthmatic patients⁸ in a background of decreased insulin signaling.⁵

To study directly the effects of insulin on PAR-2 expression in AECs, we cultured primary human AECs in serum free media with growth supplements (media with insulin) or media with all the growth supplements except insulin (insulin-deficient media). Insulin deficiency resulted in PAR-2 mRNA upregulation compared with that seen in cells cultured in media with insulin ([Fig 1, D](#), and see [Fig E2, A-C](#), in this article's Online Repository at www.jacionline.org). Addition of insulin reversed insulin deficiency-induced PAR-2 upregulation ([Fig 1, D](#)), indicating that this is a dynamic change in PAR-2 expression and not the effect of permanent cellular alterations caused by insulin deficiency. Insulin deficiency also increased PAR-2 mRNA expression in differentiated air-liquid interface (ALI) cultures of AECs (see [Fig E2, D-F](#)).

Insulin deficiency did not alter PAR-2 mRNA stability (see [Fig E3, A](#), in this article's Online Repository at www.jacionline.org) but increased PAR-2 promoter activity (see [Fig E3, B and C](#)) in AECs, indicating that insulin can directly regulate PAR-2 gene transcription. Insulin, in the absence of all other growth supplements, dose-dependently downregulated PAR-2 expression ([Fig 1, E](#)), which supports our *in vivo* observation that greater levels of circulating insulin decreased PAR-2 expression in lungs of HFD-fed mice.

Insulin deficiency also increased PAR-2 protein expression in AECs ([Fig 1, F and G](#)). Increased PAR-2 expression leads to increased PAR-2-mediated airway inflammation.⁶ PAR-2-mediated AEC activation induces release of inflammatory mediators relevant in asthma pathogenesis.^{E11-E15} In many cases PAR-2-mediated inflammatory mediator release from AECs depends on PAR-2-mediated increase in intracellular Ca⁺⁺ levels.^{E16} PAR-2-mediated activation of AECs cultured in insulin-deficient media led to increased intracellular Ca⁺⁺ levels compared with activation of cells cultured in media with insulin ([Fig 1, H and I](#), and see [Fig E4](#) in this article's Online Repository at www.jacionline.org), indicating that insulin-mediated regulation of PAR-2 expression might affect PAR-2-mediated activation of AECs and inflammatory mediator release.

Insulin mediates many of its effects by activating the phosphoinositide 3-kinase (PI3K)-Akt pathway.⁹ PI3K inhibition, using wortmannin ([Fig 2, A](#)) or LY294002 (see [Fig E5, A](#), in this article's Online Repository at www.jacionline.org), in the presence of insulin resulted in PAR-2 expression similar with that in insulin-deficient media. Furthermore, PI3K inhibition in cells cultured for 24 hours in insulin-deficient media prevented PAR-2 downregulation induced by the addition of insulin ([Fig 2, B](#), and see [Fig E5, B](#)). These results indicate that insulin regulates PAR-2 through the PI3K-Akt pathway.

Through Akt, insulin phosphorylates and inhibits the transcription factor forkhead box protein O1 (FOXO-1).^{E17,E18} We hypothesized that FOXO-1 becomes activated in AECs during insulin deficiency and induces PAR-2 transcription. In support of this hypothesis, our *in silico* analysis of the PAR-2 promoter

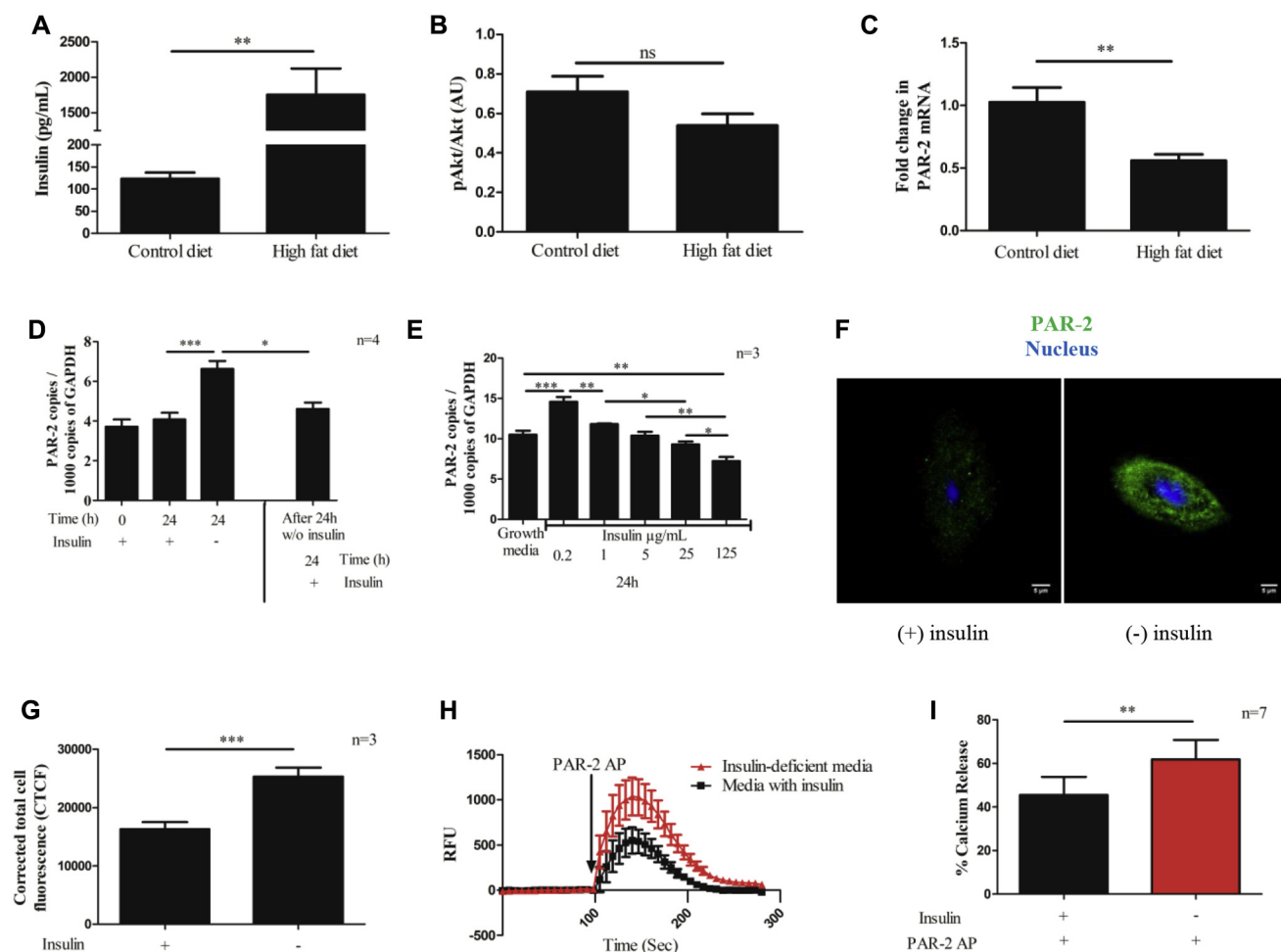


FIG 1. Insulin regulates PAR-2 expression and function in AECs. **A–C**, Plasma insulin levels (Fig 1, A), densitometric analysis of phospho-Akt/total Akt lung content (Fig 1, B), and whole lung PAR-2 mRNA expression (Fig 1, C) in control diet-fed ($n = 5$) and HFD-fed ($n = 9$) mice. **D**, PAR-2 expression determined by using quantitative RT-PCR in AECs grown in media with insulin (Time 0) and cells cultured in media with insulin or insulin-deficient media for another 24 hours (left part of graph), as well as cells cultured in insulin-deficient media for 24 hours, followed by culture in media with insulin for the next 24 hours (right part of graph). **E**, PAR-2 mRNA expression in AECs cultured with various insulin concentrations and no other growth supplements. **F** and **G**, Representative images of PAR-2 expression (green) and nuclear staining (blue) in AECs cultured in media with insulin (left) or insulin-deficient media (right) for 24 hours (Fig 1, F) and corrected total cell fluorescence (CTCF) representing PAR-2 expression in these cells (Fig 1, G). **H**, Time course of PAR-2-mediated intracellular Ca⁺⁺ levels in cells cultured in media with insulin (black traces) and insulin-deficient media (red traces) for 24 hours. **I**, PAR-2-mediated Ca⁺⁺ release was quantified as percentage of thapsigargin-induced Ca⁺⁺ release. Data are presented as means $\pm$ SEMs. ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$ between the groups indicated by horizontal lines.

indicated that the PAR-2 promoter contains FOXO-1-binding sites (data not shown). Addition of insulin to AECs cultured in insulin-deficient media phosphorylated endogenous FOXO-1 (Fig 2, C and D). We then transfected cells with a FOXO-1-green fluorescent protein (GFP) plasmid. During insulin deficiency, FOXO-1-GFP localized to AEC nuclei (Fig 2, E, left image), indicating activation of FOXO-1 and addition of insulin-induced nuclear exclusion of FOXO-1-GFP (Fig 2, E, right image, and see Video E1 in this article's Online Repository at www.jacionline.org). These results indicate that insulin inhibits FOXO-1 activity in primary AECs. Transfection of AECs with constitutively active FOXO-1 (FOXO-1 CA) upregulated PAR-2 expression in cells cultured in the presence of insulin

(Fig 2, F), indicating that FOXO-1 can mediate PAR-2 expression. Finally, the FOXO-1 inhibitor AS1842856 prevented insulin deficiency-induced PAR-2 upregulation in submerged (Fig 2, G) and ALI cultures of AECs (Fig 2, H).

This is the first report of PAR-2 regulation by insulin, a hormone with anti-inflammatory properties. Our results suggest that alteration in the insulin pathway can lead to PAR-2 upregulation on epithelial cells in asthmatic patients, an effect that might exacerbate proteinase-mediated airway inflammation. Altered insulin signaling might be the result of decreased insulin receptor expression⁵ or localized insulin resistance in the airway epithelium because levels of cytokines known to mediate insulin resistance,^{E19} such as TNF, IL-6, and IL-1 β , are also increased in

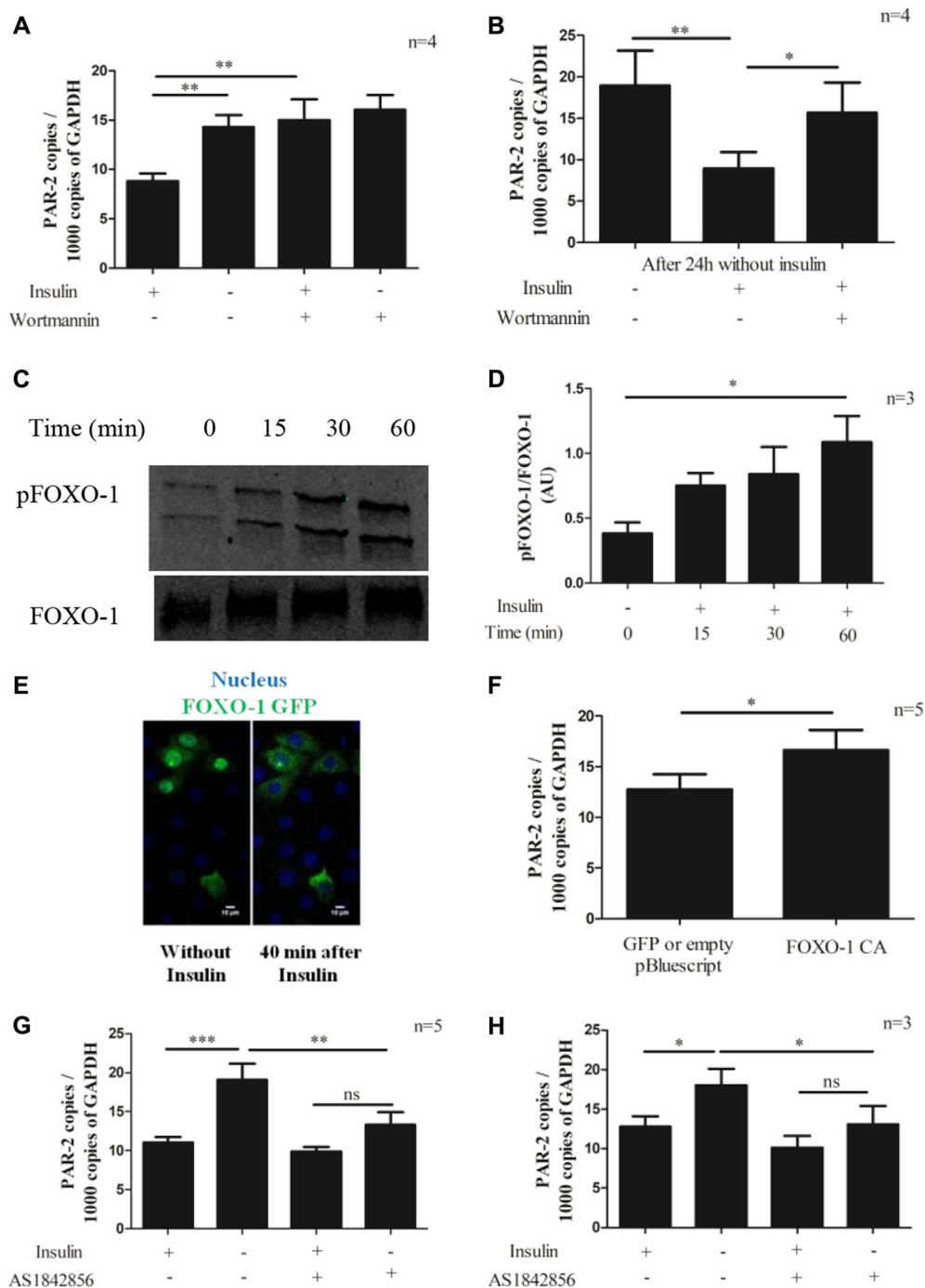


FIG 2. Insulin regulates PAR-2 expression through the PI3K-Akt-FOXO-1 pathway in submerged and differentiated cultures of primary human AECs. **A** and **B**, PAR-2 mRNA expression in cells cultured in the presence or absence of insulin with or without the PI3K inhibitor wortmannin for 24 hours (Fig 2, **A**) and in cells first cultured in the absence of insulin for 24 hours and then further cultured in the absence or presence of insulin or in the presence of insulin with wortmannin for another 24 hours (Fig 2, **B**). **C** and **D**, Representative blot (Fig 2, **C**) and densitometric analysis (Fig 2, **D**) of phospho-FOXO-1/total FOXO-1 in AECs cultured in insulin-deficient media for 4 hours, followed by addition of insulin for the indicated time periods. **E**, Representative images of FOXO1 localization. FOXO-1-GFP (green)-transfected AECs were cultured without (left image) or with insulin (right image). **F**, PAR-2 mRNA expression in AECs nucleofected with FOXO-1 CA or pmaxGFP/empty pBluescript and cultured in the presence of insulin for 24 hours. **G** and **H**, PAR-2 mRNA expression in submerged (Fig 2, **G**) and differentiated (Fig 2, **H**) AECs cultured in the presence or absence of insulin with or without the FOXO-1 inhibitor AS1842856 for 24 hours. Data are presented as means $\pm$ SEMs. ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$ between the groups indicated by horizontal lines.

the lungs of asthmatic patients.^{E20-E23} Whether insulin resistance underlies the increased PAR-2 expression seen on the airway epithelium in asthmatic patients requires further study, as described in the Discussion section in this article's Online Repository at www.jacionline.org.

Improved understanding of insulin-mediated regulation of PAR-2 expression in various cells in patients with asthma and other inflammatory diseases with increased PAR-2 expression^{E2,E5,E24-E26} might uncover common dysregulatory mechanisms across inflamed tissues and lead to the development of new approaches to regulate PAR-2 expression and PAR-2-mediated inflammation.

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Midostaurin in patients with indolent systemic mastocytosis: An open-label phase 2 trial



To the Editor:

Indolent systemic mastocytosis (ISM) is the most prevalent form of mastocytosis and characterized by mast cell infiltration of, in particular, the bone marrow, gastrointestinal tract and skin but by definition no resulting organ dysfunction, setting it apart from advanced systemic mastocytosis (SM).¹ For most patients with ISM, mast cell burden will remain stable throughout a near-normal life expectancy.² More than 70% of patients with ISM experience an impaired quality of life because of refractory symptoms caused by mast cell mediator release, illustrating the large unmet therapeutic need.³

Midostaurin is a multikinase inhibitor capable of inhibiting the kinase activity of both wild-type and D816V mutated *KIT*.⁴ Studies in patients with advanced SM demonstrated a rapid response of mast cell mediator symptoms, suggesting a potential use in patients with ISM.⁵ Therefore we conducted an open-label, non-randomized, single-center, phase 2 trial to study the efficacy and safety of midostaurin in 20 patients with ISM with severe mast cell activation symptoms refractory to antihistamine medication.

Patients 18 years of age and older were eligible if a diagnosis of *KIT* D816V⁺ ISM or smoldering SM (according to the World Health Organization criteria¹) was present. Excluded were pregnant or nursing women, patients with a diagnosis of advanced SM, and patients with a diagnosis of another malignancy unless they were disease free for at least 5 years or had a diagnosis of nonmelanoma skin cancer. Finally, patients with serious comorbidity expected to interfere with therapy and follow-up compliance were excluded.

This study, which is registered with ClinicalTrials.gov as NCT01920204, was approved by the University Medical Center Groningen Institutional Review Board. All patients provided written informed consent before study entry.

All mediator symptom-suppressing drugs that patients were taking before the study were continued. Midostaurin was supplied by Novartis (Basel, Switzerland) as capsules of 25 mg and had to be taken orally at the dose of 100 mg twice a day. Patients received midostaurin continuously from day 1. All patients took premedication with antiemetic 5-HT₃ receptor antagonists 2 hours before midostaurin intake, which was adjusted after 2 weeks according to symptoms of nausea and vomiting.

Starting in June 2012, 248 patients with ISM or smoldering systemic mastocytosis were screened on symptom severity by using the Mastocytosis Symptom Assessment Form (MSAF), which is available for download online.³ A detailed patient accrual and trial profile is presented in Fig E1 in this article's Online Repository at www.jacionline.org. Between September 2013 and August 2015, 20 patients with ISM were included. The mean age of included patients was 49 ± 12 years, with an almost even male/female proportion (45% to 55%), and all but 5 patients had urticaria pigmentosa (75%). There were an insufficient number of patients included with episodic vascular collapse to study an effect on anaphylaxis-like symptoms. During the trial (before week 12), 3 patients dropped out because of nausea-related adverse events (AEs).

The primary objective of the study was the symptomatic response rate according to MSAF score. After 12 weeks of

METHODS

Animal study

C57BL6 (Jackson Labs, Bar Harbor, Me) male mice (6–7 weeks old) were maintained on an HFD (60% kcal fat; Research Diets, New Brunswick, NJ) or standard chow diet (4% kcal fat) *ad libitum* for 30 weeks. Mice were killed with an overdose of sodium pentobarbital, and blood was collected by means of cardiac puncture. Lungs were snap-frozen in liquid nitrogen. Plasma and lung tissue were stored at -80°C until further processing. Experiments were approved by the University of Alberta Health Sciences Laboratory Animal Ethics committee.

Insulin measurement

Insulin levels in mouse plasma were measured by using the Rodent Insulin Chemiluminescence ELISAKit (ALPCO, Salem, NH).

Cell culture

Primary AECs (normal human bronchial epithelial cells) from 5 donors were purchased from Lonza (Walkersville, Md) and used at passages 3 to 5 after receipt. Cells were cultured on tissue-culture flasks or plates (Falcon; Corning, Corning, NY) coated with 30 $\mu\text{g}/\text{mL}$ bovine collagen type I (Life Technologies, Burlington, Ontario, Canada) dissolved in bronchial epithelial basal media (BEBM; Lonza). Cells were cultured with bronchial epithelial growth media (BEGM; Lonza); ie, BEBM supplemented with bovine pituitary extract, hydrocortisone, epidermal growth factor, epinephrine, insulin, transferrin, triiodothyronine, retinoic acid, gentamicin-amphotericin-B, and penicillin-streptomycin (Life Technologies, Grand Island, NY). AEC ALI cultures were performed on inserts (12-mm diameter and 0.4- μm pore size; Corning), as previously described.^{E27}

AECs were treated with the PI3K inhibitors LY294002 (20 $\mu\text{mol}/\text{L}$; Tocris, Minneapolis, Minn)^{E28,E29} and wortmannin (5 $\mu\text{mol}/\text{L}$; Cayman, Ann Arbor, Mich)^{E30} or the FOXO-1 inhibitor AS1842856 (1 $\mu\text{mol}/\text{L}$; Calbiochem, San Diego, Calif)^{E31} to inhibit the insulin-signaling pathway. All inhibitors were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo). The final concentration of dimethyl sulfoxide added to cells was less than 0.1%, which had no effect on PAR-2 expression (data not shown).

BEAS-2B cells were purchased from ATCC (Manassas, Va) and cultured on tissue-culture flasks or plates coated with a mixture of 2 $\mu\text{g}/\text{mL}$ fibronectin (Life Technologies), 30 $\mu\text{g}/\text{mL}$ bovine collagen type I, and 10 $\mu\text{g}/\text{mL}$ BSA (Sigma-Aldrich) dissolved in BEBM. BEGM media without gentamicin-amphotericin and retinoic acid was used to culture cells.

Cells were cultured at 37°C in 5% CO_2 at 90% humidity. All cell cultures maintained in our laboratory were tested monthly for *Mycoplasma* species by using the MycoAlert Mycoplasma Detection Kit (Lonza), and only negative cultures were used.

RNA extraction, reverse transcription, and quantitative RT-PCR

RNA extraction was performed with the RNeasy Kit (Qiagen, Germantown, Md); 0.5 μg of RNA was reverse transcribed with 0.5 μg of Oligo (dt) 12–18 (Life Technologies) and 200U M-MLV reverse transcriptase (Life Technologies) in a 20- μL final volume.

Human PAR-2 mRNA was quantified by using a probe-based quantitative RT-PCR protocol we developed.^{E32} Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was used as an internal control; data are presented as PAR-2 copies/1000 *GAPDH* copies. PAR-2 mRNA was also quantified by using TaqMan gene expression assays (all from Thermo Fisher Scientific, Waltham, Mass) for human PAR-2 (Hs00608346_m1) or mouse PAR-2 (Mm0043360_m1); cyclophilin A (*PPIA*; Hs04194521_s1) or hypoxanthine phosphoribosyltransferase (*HPRT*; Mm03024075_m1) were used as internal controls, respectively.

Confocal microscopy

AECs were cultured under various experimental conditions on collagen-coated glass coverslips. Cells were then fixed with 4% paraformaldehyde-PBS

and permeabilized with 0.5% Triton X100–PBS. After blocking with 1% BSA–PBS, cells were incubated with 2 μg of anti-PAR-2 primary mAb (SAM-11; Santa Cruz Biotechnology, Dallas, Tex) overnight at 4°C , followed by 4 μg of Alexa Fluor 488 anti-mouse IgG_{2a} secondary antibody (Life Technologies) for 90 minutes at room temperature. Cells were then incubated with 5 $\mu\text{mol}/\text{L}$ 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, Ore). Cells stained with only secondary antibody were used as a negative control. Images were obtained by using a $\times 40$ objective on a Leica SP5 confocal microscope (Leica, Wetzlar, Germany). Thirty cells per experiment were analyzed with ImageJ software (National Institutes of Health, Bethesda, Md) to calculate corrected total cell fluorescence (CTCF), as follows:

$\text{CTCF} = \text{Integrated density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background reading})$.

In the case of cell transfection with FOXO-1–GFP, live imaging was performed for up to 40 minutes by using a $\times 20$ objective on a WaveFX spinning disk confocal microscope (Quorum Technologies, Lewes, United Kingdom).

Ca^{++} release assay

AECs were cultured in media with insulin or insulin-deficient media for 24 hours in 96-well plates (Corning Clear Bottom Black plates). Cells were then transferred in calcium- and magnesium-free HBSS buffer (Fisher Scientific), and PAR-2–mediated Ca^{++} release from the endoplasmic reticulum was measured with the Fluo-4 NW Calcium Assay Kit (Life Technologies), according to the manufacturer's instructions. After recording baseline fluorescence (representing baseline cytoplasmic Ca^{++} levels), 50 $\mu\text{mol}/\text{L}$ PAR-2 activating peptide (PAR-2 AP; sequence: SLIGRL-NH₂) or control peptide (PAR-2 CP; sequence: LSLGRL-NH₂ or LRGILS-NH₂)^{E33} was added, and fluorescence was recorded. Subsequently, 1 $\mu\text{mol}/\text{L}$ thapsigargin (Sigma-Aldrich) was added to all wells to induce complete Ca^{++} release from endoplasmic reticulum,^{E34} and fluorescence was measured. PAR-2–mediated Ca^{++} release is shown as the percentage of thapsigargin-induced Ca^{++} release.

Reporter plasmid to study PAR-2 promoter activity

Using PCR, we amplified a 670-bp fragment that covers the 284-bp exon-1 and the adjacent upstream region of the gene encoding PAR-2 by using human genomic DNA as a template, forward primer (5'-CTCCTCTGCAGGGTC CACC-3') and reverse primer (5'-AGAGCCCTCTTGCCAGGT-3'). The PCR product was first ligated into the TOPO vector (Life Technologies) to confirm its orientation and then recovered and ligated into the pGL3-Basic vector upstream of the firefly luciferase gene. The plasmid DNA was purified by using the Plasmid Maxi Kit (Qiagen). The plasmid concentration was measured with Nanodrop 2000c (Thermo Scientific).

Transfection of primary AECs and the BEAS-2B cell line

A pBluescript plasmid containing the coding sequence for a constitutively active form of FOXO-1 (FOXO-1 CA) was used.^{E17} AECs were nucleofected with 2 to 4 μg of FOXO-1 CA or GFP-encoding plasmid pmaxGFP (Lonza) or empty pBluescript by using the Amaxa Basic Nucleofector Kit for Primary Mammalian Epithelial cells (Lonza) or the Ingenio Electroporation Kit (Mirus Bio, Madison, Wis) and Amaxa Nucleofector 2s (W-005 program, Lonza).

AECs were cultured in tissue-culture dishes (Falcon) and transfected with a previously described FOXO-1–GFP plasmid^{E17} by using Mirus X2 reagent, according to the manufacturer's instructions. The next day, transfected cells were incubated with 0.5 $\mu\text{g}/\text{mL}$ Hoechst stain to stain the nuclei. Cells were then cultured in media with all the growth supplements except insulin (insulin-deficient media) for 4 hours to allow nuclear localization of FOXO-1–GFP. Insulin (5 $\mu\text{g}/\text{mL}$) was then added, and the movement of FOXO-1–GFP was studied by using live imaging, as described in the “Confocal microscopy” section.

We used the BEAS-2B cell line to achieve greater transfection efficiency^{E35} to study the effect of insulin on PAR-2 promoter activity. BEAS-2B cells were seeded in 12-well plates (Falcon) and cultured until 85% confluence in BEGM media. On the day of transfection, BEGM media were replaced with BEBM media. Transfection complexes were formed by incubating 1 μ g of PAR-2 reporter plasmid DNA or empty pGL3-Basic vector (negative control), 5 ng of Renilla plasmid DNA, and 2 μ g of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif) in BEBM for 30 minutes at room temperature and then added to the cells dropwise and incubated for 5 hours. Transfection complexes were then removed, and growth media were added for 24 hours. The next day, cells were cultured in media with insulin or insulin-deficient media for 24 hours to study the effect of insulin on PAR-2 promoter, and promoter activity was assessed by using the luciferase reporter assay.

Luciferase reporter assay

Cells were lysed with passive lysis buffer (Promega, Madison, Wis). Lysates were assayed for luciferase activity by using the Dual-Luciferase Reporter Assay Kit (Promega), and the signal was measured with a Turner BioSystems 20/20ⁿ Luminometer (Turner BioSystems, Sunnyvale, Calif). Measured luciferase activity is proportional to the amount of produced firefly luciferase enzyme and thus proportional to the promoter activity of the cloned region. Renilla is a constitutive active luciferase, and thus the Renilla luciferase value is used as a control for transfection efficiency. The firefly luciferase value of each sample was normalized by Renilla luciferase of the same sample (firefly luciferase/Renilla luciferase), which gave us the promoter activity of the PAR-2 promoter construct. Results were expressed as fold change in promoter activity of cells transfected with PAR-2 promoter construct over empty plasmid vector.

Western blotting

To study Akt phosphorylation, we used rabbit anti-Akt and anti-phospho-Akt (Ser473; Cell Signaling, Danvers, Mass) primary antibodies, followed by horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG, Santa Cruz Biotechnology); membranes were developed with ECL reagents (Thermo Fisher Scientific). To study FOXO-1 phosphorylation, we used rabbit anti-FOXO-1 and anti-phospho-FOXO-1 (Ser256; Cell Signaling) primary antibodies, followed by IRDye-conjugated goat anti-rabbit IgG (LI-COR, Lincoln, Neb); membranes were imaged with the Odyssey Infrared Imager (LI-COR). Images were analyzed by using Image Studio Lite software (LI-COR); results were expressed as a ratio of phospho-Akt/total Akt and phospho-FOXO-1/total FOXO-1.

Statistics

Results are expressed as means $\pm$ SEMs. For comparisons between 2 groups, the 2-tailed paired *t* test was performed for AEC experiments, and the 2-tailed Mann-Whitney test was performed for mouse experiments. In case of 3 or more groups, repeated-measures ANOVA with the Tukey multiple comparison test or 2-way repeated-measures ANOVA with the Bonferroni posttest were used depending on the experimental set-up. A *P* value of less than .05 was considered statistically significant.

DISCUSSION

Insulin has anti-inflammatory properties, such as regulating expression of chemokine receptor and cell adhesion molecules.^{E36} Previous studies have suggested a role for insulin in AEC barrier function^{E37} but not in expression of proinflammatory receptors of the epithelium. Our results suggest an anti-inflammatory effect of insulin on AECs through inhibition of PAR-2 expression. If upregulated PAR-2 expression in AECs in asthmatic patients is due to altered insulin signaling in airway epithelium, then an important question is whether altered *in vivo* insulin signaling in asthmatic patients is only the result of

decreased receptor expression^{E38} or whether there is also an element of localized insulin resistance.

There are 2 basic animal models to study the effect of insulin *in vivo*: insulin-deficient mice (after ablation of pancreatic β cells) and insulin-resistant mice. PAR-2-mediated acute paw inflammation is higher in insulin-deficient mice.^{E39} Although alterations in PAR-2 expression were not studied in these mice, the effect described could be the result of increased PAR-2 expression in the absence of insulin. HFD-induced obesity induces insulin resistance in mouse adipose tissue and has been associated with increased PAR-2 expression in adipose tissue.^{E7-E9} However, the authors did not study whether there is a direct pathway between insulin resistance and PAR-2 expression or whether increased PAR-2 expression is the result of alterations in other inflammatory pathways in HFD-fed mice. Using a similar model of HFD-induced obesity, we did not detect evidence of altered baseline insulin signaling in lung tissue (Fig 1, B), whereas PAR-2 expression in lung tissue was decreased. We attributed this decreased PAR-2 expression to hyperinsulinemia, a conclusion supported by our *in vitro* data that the higher the concentration of insulin in the media, the lower the PAR-2 expression in AECs. However, we cannot exclude the possibility that HFD-fed mice have overt insulin resistance in the airways after allergic inflammation, an effect that might increase PAR-2 expression and exacerbate protease-mediated inflammation.

Overall, our results predict a correlation between PAR-2 expression in the airway epithelium and alterations in insulin levels or signaling. This is an intriguing hypothesis that should be followed experimentally. Study of airway mucosal biopsy specimens from asthmatic patients or mice with allergic airway inflammation after HFD diet-induced obesity for the presence of insulin resistance markers,^{E40-E44} along with the development of *in vitro* models of insulin resistance, might allow us to better understand this phenomenon.

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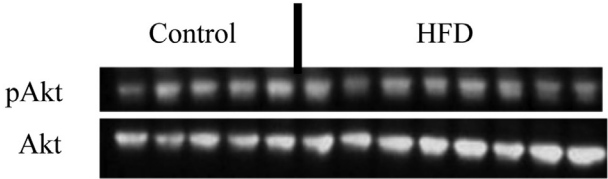


FIG E1. Lung lysates from all control diet- or HFD-fed (one is not shown) mice were analyzed by using Western blotting for the presence of phospho-Akt (*top*) or total Akt (*bottom*) on the same gel.

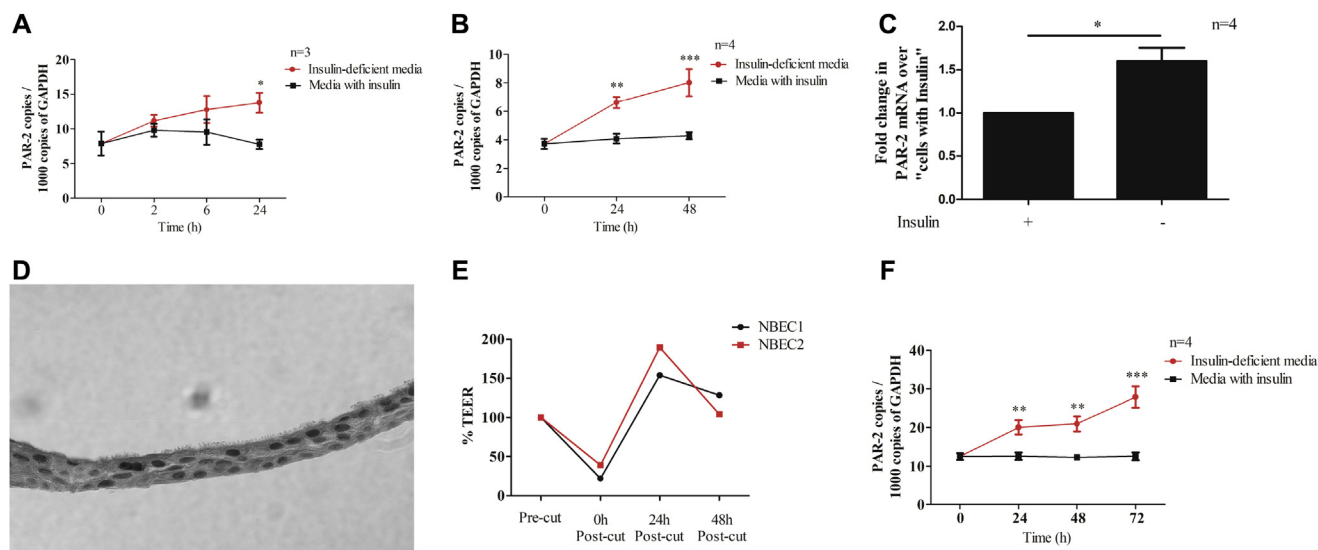


FIG E2. Insulin deficiency upregulates PAR-2 mRNA expression in submerged and differentiated AEC cultures. **A** and **B**, AECs were cultured in media with insulin until they reached 85% confluency (*Time 0*). Cells were then cultured in media with insulin or insulin-deficient media, and PAR-2 expression was studied by using quantitative RT-PCR. Insulin deficiency upregulated PAR-2 mRNA expression at 24 hours (Fig E2, **A**) and 24 and 48 hours (Fig E2, **B**). In both cases PAR-2 expression was normalized to *GAPDH*. **C**, PAR-2 expression was normalized to a second housekeeping gene (*PPIA*) to confirm increased PAR-2 expression in the absence of insulin. **D** and **E**, ALI cultures of AECs were subjected to hematoxylin and eosin staining and transepithelial electrical resistance (TEER) measurements before (*Pre-cut*) and after (*Post-cut*) mechanical wounding. The *Pre-cut* TEER values were considered as 100%, and the *Post-cut* TEER values were expressed as a percentage of *Pre-cut* TEER value. Ciliated AECs (Fig E2, **D**) and wound-healing property (Fig E2, **E**) demonstrate the presence of differentiated AECs. **F**, Insulin deficiency increased PAR-2 expression in AECs cultured in ALI compared with media with insulin. Data are presented as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$ at an indicated time point between 2 groups or between the groups indicated by horizontal lines.

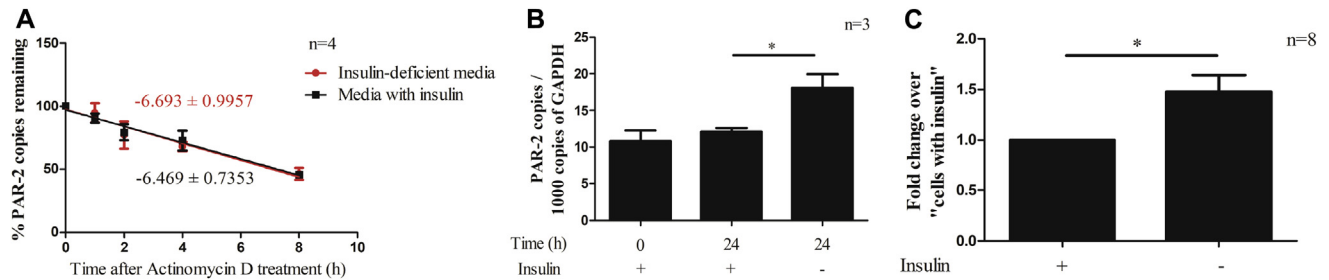


FIG E3. Insulin transcriptionally regulates PAR-2 mRNA expression in AECs. **A**, Absence of insulin did not alter PAR-2 mRNA stability. AECs were cultured in media with insulin or insulin-deficient media for 24 hours (*Time 0*), followed by further culture in the same media in the presence of actinomycin D for the indicated times. PAR-2 levels at various time points (as assessed by using quantitative RT-PCR) were expressed as percentage PAR-2 expression compared with expression at time 0. The rate of PAR-2 mRNA degradation was similar in cells cultured in media with insulin (*black values*) or insulin-deficient media (*red values*). **B**, Insulin deficiency increased PAR-2 mRNA expression in BEAS-2B cells. Cells were cultured in media with insulin until they reached 80% confluency (*Time 0*), followed by 24 hours of culture in media with insulin or insulin-deficient media, and PAR-2 expression was studied by using quantitative RT-PCR. **C**, Insulin can directly regulate PAR-2 gene transcription. BEAS-2B cells transfected with PAR-2 reporter plasmid were cultured in media with insulin or insulin-deficient media for 24 hours. Insulin deficiency increased PAR-2 promoter activity. Data are presented as means $\pm$ SEMs. *ns*, Not significant, $*P < .05$, $**P < .01$, and $***P < .001$ between the groups indicated by horizontal lines.

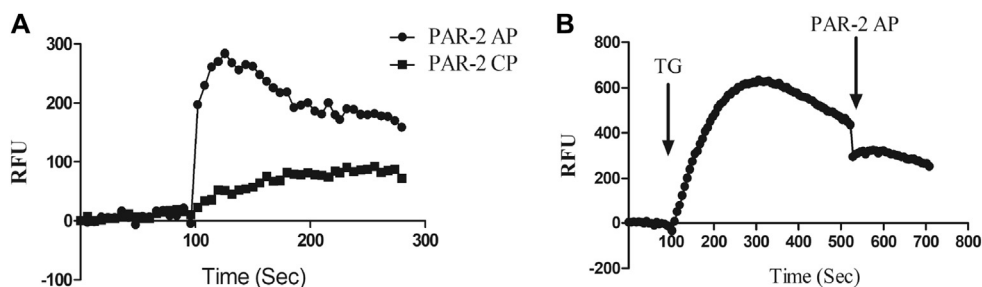


FIG E4. PAR-2-mediated activation of AECs induces intracellular Ca^{++} release from endoplasmic reticulum. AECs were cultured in 96-well plates, and Ca^{++} levels were measured. **A**, Treatment of cells with PAR-2 AP induced Ca^{++} release, which was absent in PAR-2 CP-treated cells. **B**, Treatment of cells with thapsigargin (TG) resulted in Ca^{++} release from intracellular endoplasmic reticulum stores. Subsequent addition of PAR-2 AP did not induce Ca^{++} release, indicating PAR-2 activation results in intracellular Ca^{++} release from endoplasmic reticulum.

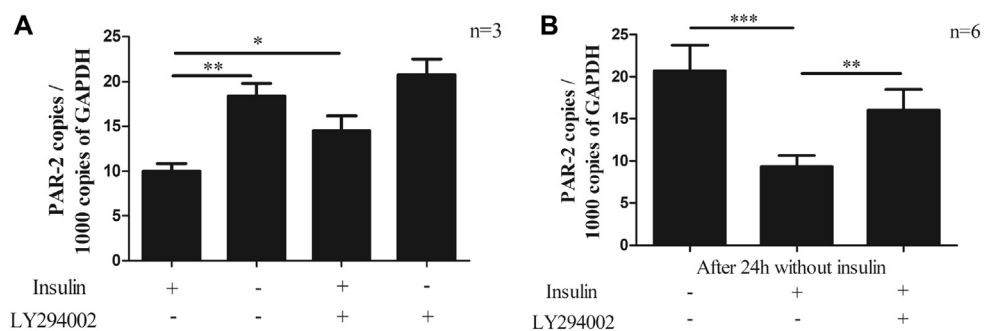


FIG E5. Insulin regulates PAR-2 mRNA expression in AECs through PI3K activation. **A**, Cells were cultured in the presence or absence of insulin with or without the PI3K inhibitor LY294002. PI3K inhibition resulted in increased PAR-2 expression in the presence of insulin. **B**, Cells were first cultured in the absence of insulin for 24 hours to upregulate PAR-2 expression. Cells were then further cultured for 24 hours in the absence or presence of insulin or in the presence of insulin with PI3K inhibitor. PI3K inhibition prevented insulin-mediated downregulation of PAR-2 expression. Data are presented as means $\pm$ SEMs. *ns*, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$ between the groups indicated by horizontal lines.