Major role of the extracellular matrix in airway smooth muscle phenotype plasticity
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

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Citation for published version (APA):

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Download date: 30-04-2019
Insulin-induced laminin expression promotes a hypercontractile airway smooth muscle phenotype

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Am J Respir Cell Mol Biol (2009) 41:494-504
Abstract

Airway smooth muscle (ASM) plays a key role in the development of airway hyperresponsiveness and remodeling in asthma, which may involve maturation of ASM cells to a hypercontractile phenotype. In vitro studies have indicated that long-term exposure of bovine tracheal smooth muscle (BTSM) to insulin induces a functional hypercontractile, hypoproliferative phenotype. Similarly, the extracellular matrix protein laminin has been found to be involved in both the induction and maintenance of a contractile ASM phenotype. Using BTSM, we now investigated the role of laminins in the insulin-induced hypercontractile, hypoproliferative ASM phenotype. The results demonstrate that insulin-induced hypercontractility after 8 days of tissue culture was fully prevented by combined treatment of BTSM-strips with the laminin competing peptides Tyr-Ile-Gly-Ser-Arg (YIGSR) and Arg-Gly-Asp-Ser (RGDS). YIGSR also prevented insulin-induced increases in α-smooth-muscle myosin expression and abrogated the suppressive effects of prolonged insulin treatment on PDGF-induced DNA-synthesis in cultured cells. In addition, insulin time-dependently increased laminin α2, β1 and γ1 chain protein, but not mRNA abundance in BTSM strips. Moreover, as previously found for contractile protein accumulation, signaling through PI3-kinase and Rho kinase dependent pathways was required for the insulin-induced increase in laminin abundance and contractility. Collectively, our results indicate a critical role for β1-containing laminins, likely laminin-211, in the induction of a hypercontractile, hypoproliferative ASM phenotype by prolonged insulin exposure. Increased laminin production by ASM could be involved in the increased ASM contractility and contractile protein expression in asthma. Moreover, the results may be of interest for the use of inhaled insulin administrations by diabetics.

Introduction

Remodeling of the airway wall is a feature of chronic airway inflammation and may importantly contribute to airway hyperresponsiveness and decline of lung function, as observed in patients with severe and persistent asthma (1-3). Airway wall remodeling in these patients is, amongst others characterized by increased airway smooth muscle (ASM) mass (4) and changes in extracellular matrix (ECM) (5, 6).

Increased ASM mass may result from both cellular hyperplasia and hypertrophy of individual ASM cells (4). In keeping with hyperplasia, ASM cells have been found to retain the ability to alter their phenotype in response to a broad range of environmental cues, varying from cell-cell contacts and mechanical strain to growth factors and ECM components (7). Modulation to a proliferative phenotype results from exposure of ASM cells to mitogenic stimuli leading to increased proliferative, synthetic and migratory capabilities and
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decreased contractile function (Chapter 3)(8-10). In asthmatic airways, accumulation of ASM cells with increased contractile properties and high expression levels of contractile proteins, but with low synthetic and proliferative capabilities has been described (11-14). In culture, induction of a (hyper)contractile ASM phenotype occurs as a consequence of growth arrest by serum deprivation, leading to augmented abundance of contractile proteins and contraction regulatory proteins (15, 16). Recently it has become apparent that insulin promotes ASM maturation to a greater extent than can be attained in its absence during serum deprivation. Long-term treatment (8 days) with insulin has been demonstrated to increase expression of specific contractile phenotype markers in bovine tracheal smooth muscle (BTSM) cells and strips (17), which is accompanied by decreased mitogenic responses and induction of a functionally hypercontractile phenotype (18). In addition, Rho kinase- and PI3-kinase-dependent signaling pathways were found to mediate insulin-induced ASM cell maturation (17).

Laminins are ECM proteins commonly found in basement membranes (19). Currently, five laminin α-, four β- and three γ-chains, forming at least 15 different laminin isoforms, have been identified in mammals (20). Laminin-111 (laminin-1) and laminin-211 (laminin-2) are produced in murine developing lung and found to be importantly involved in pulmonary branching and differentiation of naïve mesenchymal cells (20-22). Compared to healthy controls, increased expression of laminin α2 and β2 chains has been observed in the airways of asthmatics (23). Moreover, studies on the role of laminins in ASM function revealed that ASM cells, grown on an exogenously applied laminin-111 matrix, are retained in a hypoproliferative phenotype associated with increased contractile protein abundance (24). This inhibition of mitogenic responsiveness has been found of functional relevance, as recently demonstrated in this laboratory by the observation that the induction of a hypocontractile phenotype in BTSM strips during prolonged treatment with platelet-derived growth factor (PDGF) was prevented by combined treatment with exogenous laminin-111 (Chapter 3). A role for endogenously expressed laminin-211 in regulating ASM phenotype was recently suggested by Tran and colleagues, who found that increased expression of laminin α2, β1 and γ1 chains is required for maturation of human ASM cells under serum-deprived and insulin-supplemented conditions (25).

To elucidate whether laminins are functionally involved in the induction of a hypercontractile, hypoproliferative ASM phenotype, we now investigated the effects of insulin incubation in the absence and presence of the laminin competing peptides Tyr-Ile-Gly-Ser-Arg (YIGSR) and Arg-Gly-Asp-Ser (RGDS) on BTSM strip contractility. In addition, we assessed the effects of YIGSR on the insulin-induced α-sm-myosin accumulation in these strips and on the inhibitory effects of insulin stimulation on proliferative responses of isolated primary BTSM cells. Western analysis was carried out to study the effects of insulin stimulation on laminin α1, α2, β1 and γ1 chain abundance in BTSM strip homogenates.
Since PI3-kinase and Rho kinase have been associated with insulin-induced ASM maturation (17), the effects of the selective PI3-kinase inhibitor LY-294002 and the selective Rho kinase inhibitor Y-27632 on laminin-211 chain protein and mRNA abundance were investigated as well. Moreover, the roles of PI3-kinase and Rho kinase signaling in the induction of a functional hypercontractile phenotype by insulin were assessed. Our results indicate that the insulin-induced hypercontractile ASM phenotype is functionally dependent on signaling via α- and β1-containing laminins and that insulin increases laminin α2, β1 and γ1 chain protein abundance, via PI3-kinase- and Rho kinase-dependent signaling pathways. Accordingly, inhibition of both PI3-kinase and Rho kinase signaling normalized the insulin-induced hypercontractility. Increased laminin production by ASM could be involved in the increased ASM contractility and contractile protein expression as observed in asthma (13, 23). The results may also be of interest for the ongoing discussion on the treatment of diabetes mellitus type 1 and 2 by inhaled insulin administrations (26, 27).

**Materials and methods**

**Tissue preparation and organ-culture procedure.**

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in ice-cold Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, MgSO₄ 1.18, CaCl₂ 2.50, NaH₂PO₄ 1.28, NaHCO₃ 25.00 and glucose 5.50, pregassed with 5% CO₂ and 95% O₂; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips of macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in Medium Zero (sterile DMEM, supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1 :100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml), apo-transferrin (5 µg/ml, human) and ascorbic acid (0.1 mM)). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml Medium Zero was added per tissue strip. Strips were maintained in culture in an Innova 4000 incubator shaker (37°C, 55 rpm), under tightly controlled conditions, for 2, 4 or 8 days, refreshing the medium on day 4. When used, insulin (1 µM), Tyr-Ile-Gly-Ser-Arg (YIGSR, 100 µM), Arg-Gly-Asp-Ser (RGDS, 100 µM), Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 100 µM), Y-27632 (1 µM) or LY294002 (10 µM) were present during the entire incubation period. Effective concentrations of the antagonists were as per previous reports (Chapter 3)(17, 18). For determination of laminin-chain mRNA or protein expression some strips were flash frozen in liquid nitrogen directly after preparation.
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Isometric tension measurements. Tissue strips, collected from the suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5% CO₂ and 95% O₂, pH 7.4 at 37°C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37°C, continuously gassed with 5% CO₂ and 95% O₂, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two washouts, maximal relaxation was established by the addition of 0.1 μM (-)-isoproterenol. In > 95% of the experiments no basal myogenic tone was detected. Tension was readjusted to 3 g, immediately followed by two changes with fresh KH-buffer. After another equilibration period of 30 min, cumulative concentration response curves (CRCs) were constructed to stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM – 0.1 mM). When maximal tension was reached, the strips were washed several times and maximal relaxation was established using (-)-isoproterenol (10 μM).

Coating of culture plates with laminin. Mouse laminin, purified from Engelbreth-Holm-Swarm (EHS) sarcoma, consisting mainly of laminin-111 (laminin-1) (28), was diluted in PBS to a final concentration of 10 μg/ml. Diluted laminin (0.5 ml) was adsorbed to 24-well cluster plates overnight and air-dried at room temperature. Unoccupied protein-binding sites were blocked by a 30 min incubation with a sterile 0.1% bovine serum albumin (BSA) solution. Subsequently, plates were washed twice with unsupplemented DMEM and dried before further use.

Isolation of bovine tracheal smooth muscle (BTSM) cells. Bovine tracheal smooth muscle cells were isolated as described previously (29). In short, after the removal of mucosa and connective tissue, tracheal smooth muscle was chopped. Tissue particles were washed and enzymatic digestion was performed in Medium Plus (DMEM supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1 :100), gentamicin (45 μg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml) and FBS (0.5%), supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). After digestion, cells were filtered and washed three times in Medium Plus, supplemented with 10% FBS instead of 0.5% FBS.

[^3H]-Thymidine-incorporation. BTSM cells were plated on uncoated or laminin-coated 24-well culture plates at a density of 50,000 cells per well immediately after isolation and were allowed to attach overnight in Medium Plus, containing 10% FBS. Cells were washed twice with sterile PBS and made quiescent by incubation in Medium Zero,
supplemented with 0.1% FBS, apo-transferrin (5 μg/ml), ascorbic acid (0.1 mM), in the absence or presence of YIGSR (100 μM), for 7 days. When the effects of insulin were studied, 0.1% FBS was replaced by insulin (1 μM). After 7 days, cells were washed twice with PBS and incubated with or without PDGF (10 ng/ml) in Medium Zero for 28 h, the last 24 h in the presence of [methyl-\(^3\)H]-thymidine (0.25 μCi/ml). After incubation the cells were washed twice with 0.5 ml PBS at room temperature. Subsequently, the cells were treated with 0.5 ml ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [\(^3\)H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

**Western analysis.**

To obtain whole BTSM tissue homogenates, tissue strips were cultured as described above. Homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonification in homogenization buffer (composition in mM: Tris-HCl 50 mM, NaCl 150.0, EDTA 1.0, PMSF 1.0, Na₃VO₄ 1.0, NaF 1.0, pH 7.4, supplemented with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, Na-deoxycholate 0.25 % and 1% Igepal (NP-40)). Equal amounts of protein were subjected to electrophoresis and transferred onto nitrocellulose membranes. Membranes were subsequently blocked in blocking buffer (composition: Tris-HCl 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%, dried milk powder 5%) for 60 minutes at room temperature. Next, membranes were incubated overnight at 4 °C (anti-sm-myosin) or for 90 minutes at room temperature (anti-laminin-1 and anti-merosin (laminin α2)) with primary antibodies (anti-sm-myosin and anti-laminin-1 (α1, β1 and γ1), both diluted 1:200, anti-merosin and anti-β-actin, diluted 1:2000, all dilutions in blocking buffer). Specificity of the rabbit anti-laminin-1 (polyclonal) antibody for α1, β1 and γ1 chains was assessed using mouse EHS laminin (laminin-111) as a control (data not shown). After three washes with TBS-Tween 20 (0.1% TBST, containing 50.0 mM Tris-HCl, 150.0 mM NaCl and 0.1% Tween 20) of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary antibodies (dilution 1:2000 in blocking buffer) at room temperature for 90 min, followed by another three washes with 0.1% TBST. Antibodies were then visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (Totallab™). All bands were normalized to β-actin expression.

**Real-Time PCR**

To obtain BTSM mRNA, tissue strips were cultured as described above and total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. Total RNA (1 μg) was reverse transcribed using AMV transcriptase, incubated for 10 min at 25 °C followed by 45 min at 42 °C and 5 min at 99°C. cDNA was diluted 1:10 with RNase-free water. cDNA was then subjected to real time PCR, performed with an iQ5 Real-Time PCR Detection System (Biorad) and iQ5 SYBR Green supermix. Assay were performed in 25 μl
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... were: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s for 40 cycles. Real time PCR data were analyzed using the comparative cycle threshold (C\text{t} = \text{amplification cycle number}) method. The amount of target gene normalized to an endogenous reference (18s rRNA, designated as \Delta C\text{t}) and relative to a calibrator (day 8, control) is given by the equation 2^{-\Delta C\text{t}}.

Table 1: Primers for laminin chains and 18S rRNA used in real time PCR.

<table>
<thead>
<tr>
<th>Laminin chain</th>
<th>NCBI accession number</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin a2</td>
<td>XM_001787958.</td>
<td>Forward 5' GGA TCA ACC ACG CTG ATT TT 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversed 5' ATT GAT TTT GGT GGG GAT CA 3'</td>
</tr>
<tr>
<td>Laminin (\beta)1</td>
<td>XM_598260</td>
<td>Forward 5' ATG GTG GTT CGA GGA AAC TG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversed 5' TTG GTG TTA TGC CTG CAC AT 3'</td>
</tr>
<tr>
<td>Laminin (\gamma)1</td>
<td>BC105436</td>
<td>Forward 5' ATT GAG CCA TCC ACT GAA GG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversed 5' TAG CCG TGT CAG GTT TAC CC 3'</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>AF176811.</td>
<td>Forward 5' AAA CGG CTA CCA CAT CCA AG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversed 5' TCG CGG AAG GAT TTA AAG TG 3'</td>
</tr>
</tbody>
</table>

**Materials.**

Dulbecco’s modification of Eagle’s medium (DMEM), fetal bovine serum, sodium pyruvate solution (100 mM), non-essential amino acid mixture, gentamicin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml; 5000 µg/ml) and amphotericin B solution (250 µg/ml, Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Bovine serum albumin, mouse monoclonal anti-\(\beta\)-actin, apo-transferrin (human), soybean trypsin inhibitor, insulin (from bovine pancreas), leupeptin, aprotinin, pepstatin, Na-deoxycholate, Igepal (NP-40) and (-)-isoproterenol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse monoclonal anti-sm-myosin and polyclonal rabbit anti-laminin-1 were from Neomarkers (Fremont, CA, U.S.A.). Mouse monoclonal anti-merosin (laminin a2 chain) was from Chemicon (Temecula, CA, U.S.A.). [\methyl-\(3\)H]-Thymidine (specific activity 25 Ci/mmol) and Lumigen PS-3 detection reagent were from Amersham (Buckinghamshire, U.K.). Platelet derived growth factor (PDGF-AB) was from Bachem (Weil am Rhein, Germany). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Tyr-Ile-Gly-Ser-Arg (YIGSR), Arg-Gly-Asp-Ser (RGDS) and Gly-Arg-Ala-Asp-Ser-Pro (GRDSP) were obtained from Calbiochem (San Diego, CA, USA). Qiagen RNeasy Mini Kit was from Qiagen (Mississauga, ON, USA). AMV transcriptase was from Promega (Madison, WI, USA). iQ5 SYBR Green supermix was from Biorad (Hertfordshire, UK). PCR primers (Table 1) and EHS laminin were from
Invitrogen (Grand Island, NY, USA). Collagenase P and papain were from Boehringer (Mannheim, Germany). L(+)ascorbic acid was from Merck (Darmstadt, Germany). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) were from Tocris Bioscience (Ellisville, MO, USA). All other used chemical were of analytical grade.

Data analysis.
All data represent means ± SEM from n separate experiments. Statistical significance of differences was evaluated using one-way ANOVA for repeated measures, followed by a Bonferroni’s multiple comparisons test. Differences were considered to be statistically significant when $P<0.05$.

Results

Effect of the laminin competing peptides YIGSR and RGDS on insulin-induced hypercontractility.
To assess whether laminin $\alpha$ and $\beta 1$ chains were required for the induction of a functional hypercontractile phenotype, BTSM strips were incubated with insulin in the absence and presence of the laminin competing peptides Tyr-Ile-Gly-Ser-Arg (YIGSR, 100 $\mu$M), Arg-Gly-Asp-Ser (RGDS, 100 $\mu$M) or the negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 100 $\mu$M) for 8 days. In accordance with previous studies (18), we found that culturing of BTSM strips in the presence of insulin (1 $\mu$M) significantly increased maximal contractile force ($E_{\text{max}}$) to both methacholine and KCl ($P<0.05$, both) compared to vehicle treated controls (Figure 1, Table 2). Prevention of laminin binding by the laminin $\alpha$ chain competing peptide YIGSR normalized the insulin-induced increase in $E_{\text{max}}$ for both stimuli to control levels, whereas no apparent effects of pretreatment with YIGSR were observed under control (insulin-deficient) conditions. The laminin competing peptide RGDS, containing the RGD sequence found in the laminin $\alpha$ chains (30), but not its negative control GRADSP, also prevented the induction of a hypercontractile phenotype by insulin. Of note, we have reported previously that treatment with the RGDS peptide in the absence of other stimuli did not affect contractility (Chapter 3). The sensitivity ($pEC_{50}$) for either contractile stimulus was unaffected by all treatments. Collectively, these results indicate that endogenously expressed or synthesized laminin $\alpha$ and $\beta 1$ chains are required for the induction of a functional hypercontractile phenotype by insulin.
Figure 1: The induction of a hypercontractile phenotype by insulin is fully prevented by combined treatment with the laminin competing peptides YIGSR (A,B) or RGDS (C,D). Concentration-response curves of methacholine- (A,C) and KCl (B,D)-induced contractions of BTSM strips, pretreated with vehicle (control) or insulin (1 µM) in the absence or presence of the laminin β1 chain competing peptide YIGSR, the laminin α chain competing peptide RGDS or the negative control peptide GRADSP (100 µM) for 8 days. Data represent means ± SEM of 3-4 independent experiments each performed in triplicate.
Table 2: Contractile responses of BTSM strips to methacholine or KCl after 8 days of culturing in the absence or presence of insulin (1 μM), with or without the laminin blocking peptides YIGSR (100 μM), RGDS (100 μM) or the negative control GRADSP (100 μM).

<table>
<thead>
<tr>
<th></th>
<th>Methacholine</th>
<th>KCl</th>
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<tbody>
<tr>
<td></td>
<td>E&lt;sub&gt;max&lt;/sub&gt; (g)</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; (- log M)</td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>19.2 ± 2.6</td>
<td>6.81 ± 0.03</td>
</tr>
<tr>
<td>+ YIGSR</td>
<td>17.6 ± 2.4</td>
<td>6.84 ± 0.12</td>
</tr>
<tr>
<td>Insulin</td>
<td>24.4 ± 1.8**</td>
<td>6.71 ± 0.07</td>
</tr>
<tr>
<td>+ YIGSR</td>
<td>16.9 ± 1.8##</td>
<td>6.81 ± 0.13</td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>17.8 ± 2.3</td>
<td>6.68 ± 0.11</td>
</tr>
<tr>
<td>Insulin</td>
<td>24.1 ± 3.9*</td>
<td>6.79 ± 0.15</td>
</tr>
<tr>
<td>+ GRADSP</td>
<td>23.9 ± 3.0*</td>
<td>6.76 ± 0.11</td>
</tr>
<tr>
<td>+ RGDS</td>
<td>14.1 ± 1.3##</td>
<td>7.02 ± 0.14</td>
</tr>
</tbody>
</table>

Data represent means ± SEM of 3-4 independent experiments each performed in triplicate. Abbreviations: E<sub>max</sub>: maximal contraction; EC<sub>50</sub>: contraction of agonist eliciting half-maximal response; pEC<sub>50</sub>: negative logarithm of the EC<sub>50</sub> value. *P<0.05, **P<0.01 compared to vehicle-treated. #P<0.05, ##P<0.01, ###P<0.001 compared to insulin-treated.

Effects of YIGSR on insulin-induced sm-myosin accumulation.

The changes in E<sub>max</sub> in response to both receptor-dependent (methacholine) and receptor-independent (KCl) stimuli suggest post-receptor changes, which may occur at the level of the contractile apparatus. To assess these changes, we determined the expression of the sm-myosin, a specific marker for contractile ASM (16). In accordance with our previous studies (17), it was found that 8 days of insulin (1 μM) treatment increased sm-myosin protein expression in BTSM strips by approximately 2-fold as compared to controls (Figure 2). Combined treatment with YIGSR (100 μM) completely abrogated the insulin-induced increase in sm-myosin expression, in full agreement with the findings on contractility. No effects of the peptide were observed under vehicle-treated conditions. Interestingly, sm-myosin abundance in strips pretreated with both insulin and YIGSR appeared to be even lower compared to that in strips that were kept under insulin deficient conditions; however, this did not reach statistical significance. These results implicate that the insulin-induced increase in sm-myosin abundance is strongly dependent on the abundance of endogenous β1-containing laminins.
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Figure 2: YIGSR attenuates the insulin-induced increase in expression of sm-myosin in BTSM strips. Western analysis of sm-myosin protein expression in homogenates of BTSM strips pretreated with vehicle (control) or insulin (1 μM) in the absence or presence of YIGSR (100 μM) for 8 days. (A) Graph shows means ± SEM of 4 independent experiments after densitometric analysis. *P<0.05 compared to control. ††P<0.01 compared to insulin. (B) Representative immunoblots of sm-myosin (upper panel) and β-actin (lower panel).

Effects of YIGSR on inhibition of growth factor-induced proliferation by laminin.

As we previously demonstrated that laminin-111 inhibits BTSM cell proliferation in response to PDGF (Chapter 3), we were interested to learn whether this effect was dependent upon ligation of laminin with its receptors. Therefore, the effects of YIGSR on basal and growth factor-induced DNA-synthesis ([3H]-thymidine-incorporation) were examined, using freshly isolated BTSM cells cultured on plastic or on laminin matrices. As observed previously (Chapter 3)(24), culturing of cells on the exogenously-applied laminin matrix significantly reduced DNA-synthesis in response to PDGF, whereas no significant changes were observed on basal proliferative responses, both on plastic or laminin-coated surfaces (Figure 3). Similar effects were observed for cell number (data not shown). Treatment with YIGSR, by itself, had no effect on basal or growth factor-induced DNA-synthesis in cells grown on plastic. By contrast, the suppressive effects of laminin on the PDGF-induced DNA-synthesis were fully prevented in the presence of YIGSR, indicating that EHS laminin inhibits growth factor-induced DNA-synthesis through ligation with its receptors, which can be inhibited by the laminin β1 competing peptide YIGSR.
Effects of YIGSR on inhibition of growth factor-induced proliferation by insulin.

As pretreatment with insulin has been shown to decrease the mitogenic capacity of BTSM cells (18) and as our current study using YIGSR implies a role for β1-containing laminins in the induction of a hypercontractile phenotype by insulin (Figures 1 and 2), we next investigated the role of laminin signaling in the inhibitory effects of insulin pretreatment on growth factor-induced proliferation. To this aim, freshly isolated BTSM cells were cultured on plastic in the absence or presence of insulin, with or without YIGSR (100 μM) for 7 days. After pretreatment, the cells were exposed to either vehicle or PDGF (10 ng/ml). Consistent with our previous findings (18), we found that pretreatment with insulin for 7 days almost completely inhibited the mitogenic effects induced by PDGF (Figure 4). Importantly, this attenuation was fully prevented by combined YIGSR treatment, suggesting that signaling via β1-containing laminins is pivotal for the insulin-induced hypoproliferative BTSM phenotype. Of note, no effects of YIGSR or insulin pretreatment were found on basal DNA-synthesis or cell number (data not shown).
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Time-dependent effects of insulin on the accumulation of laminin protein expression in bovine tracheal smooth muscle strips.

As our results with the YIGSR peptide indicate that signaling via β1-containing laminins is required for the insulin-induced hypercontractile, hypoproliferative phenotype, we next investigated the effects of insulin on laminin protein accumulation. Previous studies have shown that mRNA and protein levels of laminin-211 chains, but not of laminin-111, are increased during ASM maturation (25). Therefore we investigated the effects of insulin on laminin α1, α2, β1 and γ1 chain protein accumulation in homogenates from BTSM strips cultured for up to 8 days. Insulin exposure led to a significant increase in the abundance of laminin α2, β1 and γ1 chains, whereas no effects were observed for α1 chain expression after 8 days of stimulation (Figure 5). Expression of laminin α2 chains increased rapidly and peaked at day 4 with an increase of approximately 2-fold compared to freshly isolated tissue, after which expression remained elevated. Expression of laminin β1 and γ1 chains was significantly increased by approximately 3-fold for both chains after 8 days of insulin exposure compared to freshly isolated tissue. No significant increases were observed under insulin-deficient conditions for all chains at any time point. These findings suggest that the accumulation of laminin α2, β1, and γ1 chains in BTSM tissue is increased after insulin stimulation.
Figure 5: Insulin time-dependently increases laminin α2, β1 and γ1 chain expression in BTSM strips. Western analysis of laminin α1, α2, β1 and γ1 chain expression in homogenates of BTSM strips pretreated with vehicle (control) or insulin (1 μM). (A) Representative immunoblots of laminin α1, α2, β1 and γ1 chains and corresponding β-actin. Graphs show means ± SEM of 4 independent experiments after densitometric analysis of (B) laminin α2, (C) laminin β1, and (D) laminin γ1 chains. *P<0.05, **P<0.01 compared to vehicle treatment (control). #P<0.05, ##P<0.01 compared to laminin chain expression in homogenates of freshly isolated BTSM strips.

Intracellular signaling associated with the insulin-induced laminin α2-, β1- and γ1-chain accumulation.
Both PI3-kinase- and Rho kinase-dependent signaling pathways have been shown to underlie the insulin-induced accumulation of the contractile phenotype marker proteins sm-myosin and calponin in ASM cells (17). To study the role of these enzymes in the accumulation of laminin α2, β1, and γ1 chains, we co-incubated BTSM strips with insulin and the selective pharmacological inhibitors of PI3-kinase (LY294002, 10 μM) and Rho kinase (Y27632, 1 μM) for 8 days. Inhibition of PI3-kinase and Rho kinase significantly reduced laminin α2-chain abundance and even fully prevented an increase in laminin β1 and γ1 chain expression in BTSM strip homogenates cultured in the presence of insulin (Figure 6). The inhibitors did not affect the expression of the laminin chains in
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strips cultured under insulin-deficient conditions. Collectively, these results suggest a key role for Rho kinase and PI3-kinase dependent signaling pathways in the excessive accumulation of laminin α2, β1 and γ1 chains by insulin.

Figure 6: PI3-kinase and Rho kinase signaling are required for the insulin-induced laminin chain accumulation. Western analysis of laminin α2, β1 and γ1 chain expression in homogenates of BTSM strips pretreated with vehicle (control) or insulin (1 μM) in the absence or presence of Y-27632 (1 μM) or LY-294002 (10 μM) for 8 days. Graphs show means ± SEM of 4 independent experiments after densitometric analysis of (A) laminin α2, (B) laminin β1 and (C) laminin γ1 chains. *P< 0.05, **P<0.01, ***P<0.001 compared to unstimulated controls. †P<0.05, ††P<0.01 compared to 8 days of insulin treatment. (D) Representative immunoblots of laminin α2 chains (left, upper panel), laminin β1 and γ1 chains (right, upper panel) and corresponding β-actin (lower panels).

Effects of insulin treatment on laminin mRNA expression.
As our studies indicated that abundance of laminin α2, β1, and γ1 chains in BTSM tissue is increased by insulin treatment and that these increases were dependent on signaling pathways associated with increased transcription (Rho kinase) (31) or translation (PI3-kinase) (32) in ASM cells, we next assessed whether the increased laminin chain expression was dependent on changes in mRNA abundance. Real time PCR analysis, performed on mRNA from BTSM strips cultured for 8 days in the absence and presence of insulin, revealed that insulin treatment did not affect mRNA levels for the α2, β1 or the γ1 laminin chain (Figure 7). Co-incubation of BTSM strips with LY294002 did not affect laminin chain mRNA abundance in the absence and presence of insulin either. Y27632 decreased laminin chain mRNA both under unstimulated (control) and
insulin-stimulated conditions. These findings suggest that increased expression of the laminin chains after insulin stimulation is independent of changes in gene transcription. Rho-kinase dependent signaling pathways, however, are important for the maintenance of laminin α2, β1, and γ1 chain mRNA abundance.

**Figure 7:** Effects of insulin treatment on accumulation of laminin chain mRNA in BTSM strips. Real time PCR analysis of laminin α2, β1, and γ1 chain mRNA in BTSM strips pretreated with vehicle (control) or insulin (1 μM), in the absence or presence of Y-27632 (1 μM) or LY-294002 (10 μM) for 8 days. Graphs show changes in (A) laminin α2, (B) laminin β1 and (C) laminin γ1 chain mRNA compared to vehicle (Control). Data represent means ± SEM of 5 independent experiments, each performed in duplicate. *P< 0.05, **P<0.01 compared to unstimulated controls.

**Effects of PI3-kinase and Rho kinase inhibition on insulin-induced hypercontractility.**

The requirement of endogenously expressed laminin in the induction of a functional hypercontractile phenotype and the role of PI3-kinase- and Rho kinase-dependent signaling pathways in the insulin-induced accumulation of laminin α2, β1, and γ1 chains, suggest that inhibition of these pathways would normalize the insulin-induced hypercontractile phenotype. Furthermore, we have previously shown that the effects of insulin on contractile protein accumulation were abrogated by combined pretreatment with LY294002 or Y27632 (17). To assess the role of these pathways in the induction of a functional hypercontractile BTSM phenotype, strips were incubated with insulin in the absence and presence of LY294002 or Y27632 for 8 days. Inhibition of PI3-kinase signaling by LY294002 fully normalized the insulin-induced increase in $E_{\text{max}}$ for both methacholine and KCl to control levels, whereas only a small effect of LY294002 was observed under control conditions (Figure 8). In agreement with previous findings (33), we found that treatment with Y27632 in the absence of insulin for 8 days decreased maximal contractility in response to methacholine, whereas for KCl-induced contractions a trend towards a decreased contractility
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was observed. Inhibition of Rho kinase by Y27632 also completely abrogated the insulin-induced increase in \( E_{\text{max}} \) for both agonists. The sensitivity \((\text{pEC}_{50})\) for either contractile stimulus was unaffected by all treatments (Table 3). Collectively, these findings indicate a key role for PI3-kinase and Rho kinase dependent signaling pathways in the induction of a hypercontractile phenotype by insulin.

Figure 8: Inhibition of the insulin-induced hypercontractile BTSM phenotype by combined treatment with the PI3-kinase inhibitor LY294002 (A,B) or the Rho kinase inhibitor Y27632 (C,D). Concentration-response curves of methacholine- (A,C) and KCl-induced (B,D) contractions of BTSM strips pretreated with vehicle (control) or insulin (1 \( \mu \text{M} \)), in the absence or presence of LY294002 (10 \( \mu \text{M} \)) or Y27632 (1 \( \mu \text{M} \)) for 8 days. Data represent means ± SEM of 4 independent experiments, each performed in duplicate.
Table 3: Contractile responses of BTSM strips to methacholine or KCl after 8 days of culturing in the absence or presence of insulin (1 μM) with or without the Rho kinase inhibitor Y-27632 (1 μM) or the PI3-kinase inhibitor LY-294002 (10 μM).

<table>
<thead>
<tr>
<th></th>
<th>Methacholine</th>
<th>KCl</th>
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<tbody>
<tr>
<td></td>
<td>E&lt;sub&gt;max&lt;/sub&gt; (g)</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; (- log M)</td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>20.6 ± 2.4</td>
<td>6.71 ± 0.15</td>
</tr>
<tr>
<td>+ LY294002</td>
<td>18.3 ± 3.0</td>
<td>6.80 ± 0.11</td>
</tr>
<tr>
<td>+ Y27632</td>
<td>15.1 ± 2.2*</td>
<td>6.70 ± 0.11</td>
</tr>
<tr>
<td>Insulin</td>
<td>25.8 ± 2.8*</td>
<td>6.92 ± 0.11</td>
</tr>
<tr>
<td>+ LY294002</td>
<td>16.3 ± 1.3**</td>
<td>6.81 ± 0.04</td>
</tr>
<tr>
<td>+ Y27632</td>
<td>16.2 ± 2.2**</td>
<td>6.57 ± 0.10</td>
</tr>
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</table>

Data represent means ± SEM of 4 independent experiments each performed in duplicate. Abbreviations: E<sub>max</sub>: maximal contraction; EC<sub>50</sub>: contraction of agonist eliciting half-maximal response; pEC<sub>50</sub>: negative logarithm of the EC<sub>50</sub> value. *P<0.05 compared to vehicle-treated. **P<0.01, ***P<0.001 compared to insulin-treated.

Discussion

In this study, we demonstrate for the first time that induction of a functional hypercontractile and hypoproliferative ASM phenotype by insulin is dependent on the interaction of laminin with its receptors. Moreover, 8 days of insulin exposure resulted in a time-dependent increase in the abundance of laminin α2, β1 and γ1 chains in isolated BTSM strip preparations, a process relying on PI3-kinase- and Rho kinase-mediated signaling. Inhibition of these pathways also fully inhibited the insulin-induced hypercontractile BTSM phenotype.

Accumulation of contractile ASM cells has been described in the airways of asthmatics (13, 14). In vitro, a functional hypercontractile phenotype can be induced by culturing isolated BTSM strips in the presence of insulin for 8 days. This hypercontractile phenotype is typically characterized by an increased maximal contractile response to both the muscarinic receptor agonist methacholine and the membrane-depolarizing agent KCl (18). The increased maximal contraction to both receptor-dependent and receptor-independent stimuli suggests post-receptor changes, which can occur at the level of the contractile apparatus. Accordingly, in the present study we have shown that increased contractility of BTSM strip preparations is associated with increased expression of the contractile phenotype marker protein sm-myosin. Moreover, we found that the insulin-induced hypercontractile BTSM phenotype and increased sm-myosin expression in BTSM strips was fully prevented by the YIGSR peptide, an antagonistic penta-peptide derived from the amino acid sequence of the β1-chain, which contains the receptor binding site in laminins (34). Similarly, treatment with the RGDS peptide, which contains the RGD
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binding motif present in several ECM proteins (35), including the laminin α chain (30), also fully normalized induction of a functional hypercontractile phenotype by insulin. These findings are consistent with the important role for laminin signaling in human ASM maturation (25), and show that interaction of laminin α and β1 chains with its receptors is pivotal for the induction of a functional hypercontractile ASM phenotype by insulin.

Changes in the maximal contractility of cultured BTSM strips induced by growth factors and ECM proteins have been shown to correlate inversely with the proliferative responses in isolated BTSM cells (Chapter 3)(8). In agreement with studies in cultured human (24) and bovine (Chapter 3) ASM cells, we found that culturing of cells on a laminin-111 matrix inhibited proliferation induced by the growth factor PDGF and maintained the cells in a hypoproliferative phenotype. This suppressive effect on PDGF-induced proliferation was fully prevented by YIGSR treatment, indicating that interaction of laminin-111 with its receptors is required for this inhibitory effect. The insulin-induced hypercontractile phenotype has previously been shown to be associated with a decreased mitogenic capacity as well (18). Similar to those findings, pretreatment of BTSM cells for 7 days with insulin did not affect basal [³H]-thymidine-incorporation or cell number, but did significantly attenuate the proliferative effects of PDGF-stimulation. We now show that co-incubation with the laminin β1 chain competing peptide YIGSR during insulin treatment normalized PDGF-induced DNA-synthesis to levels observed in cells grown in the absence of insulin, indicating that laminins containing the β1 chain, in addition to their important role in the induction of a hypercontractile phenotype, are also important for the induction of a hypoproliferative phenotype by insulin. The exact mechanism by which YIGSR inhibits laminin-111 signaling in PDGF-induced proliferation of BTSM cells is currently unknown. It may be envisaged that YIGSR interferes with existing interactions of immobilized laminin with its integrins. This interference would, however, occur without changes in cellular attachment, since we did not detect any changes in cell number or thymidine-incorporation on plastic or laminin in the presence of YIGSR. These observations are supported by previous findings (25), showing that YIGSR did not induce cellular detachment, changes in ASM morphology or cellular toxicity, but did inhibit laminin signaling in human ASM cells. Stimulation with PDGF could also possibly enhance expression of laminin-binding integrins, which would be blocked by YIGSR. In support of such a mechanism Nguyen et al found that culturing human ASM cells on plastic in the presence of PDGF specifically increased the expression of the laminin binding integrin α3 (36).

Increased expression of the contractile proteins desmin and calponin after serum deprivation in the presence of insulin has been shown to be associated with increased expression of laminin α2, β1 and γ1 chain mRNA and protein by human ASM cells (25). In different cell types, increased expression of several laminin chains has been shown to be dependent on insulin signaling as well (37-39). Further expanding those findings we found that prolonged insulin
exposure augments the protein abundance of laminin α2, β1 and γ1 chains, but not α1 chains, in a time-dependent fashion, implying the involvement of the laminin-211, but not the laminin-111 isoform in the induction of a hypercontractile, hypoproliferative ASM phenotype. Indeed, previous findings have shown that culture of BTSM strips in the presence of exogenously applied laminin-111 only induced a small increase in BTSM contractility and contractile protein expression (Chapter 3), suggesting that laminin-111, by itself, is not able to induce a hypercontractile BTSM phenotype.

Little is known on the signaling pathways involved in the biosynthesis of laminins. However, an essential role for the PI3-kinase-Akt1 pathway in insulin-regulated laminin β1-chain gene transcription (38) and/or translation (39) has been suggested. Insulin-induced β1-chain transcription in Chinese Hamster Ovary cells was inhibited by expression of dominant negative Akt/PKB (38), whereas in murine proximal tubular epithelial cells activation of the PI3-kinase-Akt-mTOR pathway was found to be important for the translation of laminin β1-chains. In these cells insulin increased phosphorylation of the translational protein 4E-BP1 (PHAS-1), which released eukaryotic initiation factor 4E (eIF4E) and subsequently increased laminin β1-chain translation (39). In ASM cells activation of PI3-kinase and downstream pathways was found to be required for protein synthesis, maturation and hypertrophy (32), also in response to ligands such as insulin (17). In particular, accumulation of SM22 and sm-MHC in canine ASM cells has been shown to be dependent on PI3-kinase-mediated signaling pathways, involving Akt1, mTOR and p70S6K (32); pathways which can be activated by insulin in BTSM cells as well (17). In full agreement with these findings, we now demonstrate that inhibition of PI3-kinase reduced the accumulation of laminin α2, β1 and γ1 chains in BTSM tissue and normalized insulin-induced hypercontractility. In addition, laminin α2, β1 and γ1 chain mRNA abundance in BTSM strips was not affected after insulin stimulation, both in the absence or presence of the PI3-kinase inhibitor LY294002, suggesting that the increased laminin chain protein expression was dependent on post-transcriptional mechanisms.

A signaling pathway shown to be essential for ASM maturation by regulating transcription of smooth muscle-specific genes is the Rho/Rho kinase pathway (3, 17, 31). Activation of this pathway promotes actin polymerization, which governs nuclear translocation and activation of serum response factor (SRF), ultimately resulting in the transcription of smooth muscle specific genes (31). Rho kinase-dependent signaling has recently been shown to be activated by insulin to promote accumulation of contractile proteins in BTSM cells (17). To our knowledge, no reports on the role of Rho kinase signaling in laminin chain expression have been published thus far. Interestingly, we found a significant reduction in the expression of α2, β1 and γ1 laminin chains after Rho kinase inhibition, indicating that pathways involved in the induction of contractile proteins and (hyper)contractility may similarly be involved in the expression of laminin chain-isoforms by ASM. In addition, we found that pharmacological
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Inhibition of Rho kinase decreased laminin α2, β1 and γ1 chain mRNA levels both under control and insulin stimulated conditions, indicating that although insulin is capable of activation Rho kinase dependent signaling pathways in BTSM (17), activation of this pathway by insulin is not directly required for the increase in laminin protein abundance. These findings further suggest that Rho kinase is necessary for the maintenance of laminin α2, β1 and γ1 chain mRNA abundance under basal conditions, which is conditional for increased protein translation after insulin stimulation. Clearly, further in depth investigation is warranted to more definitively determine the role of Rho kinase in regulating the expression of laminin chains.

No significant effects of inhibition of laminin signaling by YIGSR were observed on both contractile and mitogenic responsiveness under vehicle treated (control) conditions. Similarly, no changes in the expression of the contractile proteins desmin and calponin were found in human ASM after 7 days culture in the presence of YIGSR (25). Moreover, exogenously applied laminin-111, by itself, has no effects on maximal ASM contractility (Chapter 3) or contractile protein expression (25). Collectively, these findings imply that additional factors are likely to be required for the induction of a hypercontractile phenotype by insulin. One of these factors could be the laminin-binding integrin α7β1, which has recently been shown to be involved in ASM maturation (40).

Adverse effects of inhaled insulin administrations for treatment of diabetes type 1 and 2 have been reported, including decline in lung function in some of the patients (26). Acutely, insulin has been shown to have procontractile effects on ASM (41). Although the exposure of the ASM cells to insulin after inhaled administration is likely to differ from our experimental setup, insulin concentrations in the ASM compartment could reach high levels due to deposition in the bronchial tree. Bronchial deposition results in a low bioavailability (~10-20%) thus necessitating relatively high insulin doses for satisfactory glycemic control (42). Collectively, these findings may particularly be important for diabetic patients with asthma or COPD, in which lung function is already compromised and who, due to poor absorption, may require higher dosages (26).

In conclusion, the current study provides new insight in the development of a hypercontractile and hypoproliferative ASM phenotype induced by insulin. This phenotype modulation involves insulin-induced laminin α2, β1 and γ1 chain expression through PI3-kinase and Rho kinase dependent pathways, which may subsequently cause the induction of contractile proteins and inhibit ASM proliferation. Moreover, our findings indicate that increased laminin expression in the airways of asthmatic patients may contribute to the increased ASM contractility and contractile protein expression observed in these patients, on airway smooth muscle phenotype and function. In addition, the induction of laminin expression by insulin may limit the use of insulin as a substituent in serum-free media, as applied in cell culturing experiments in vitro.
Acknowledgements

The authors would like to thank Mariët van der Meer and Jeroen Lubbers for their expert technical assistance. Bart Dekkers is supported by a grant from the Netherlands Asthma Foundation (grant 3.2.03.36). Dedmer Schaafsma is the recipient of a CIHR/HSFC IMPACT Strategic Training Program Fellowship in Pulmonary and Cardiovascular Research. Thai Tran is supported by the Ministry of Education's Academic Research Fund – Tier 1 (T13-0702-P26).

References

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