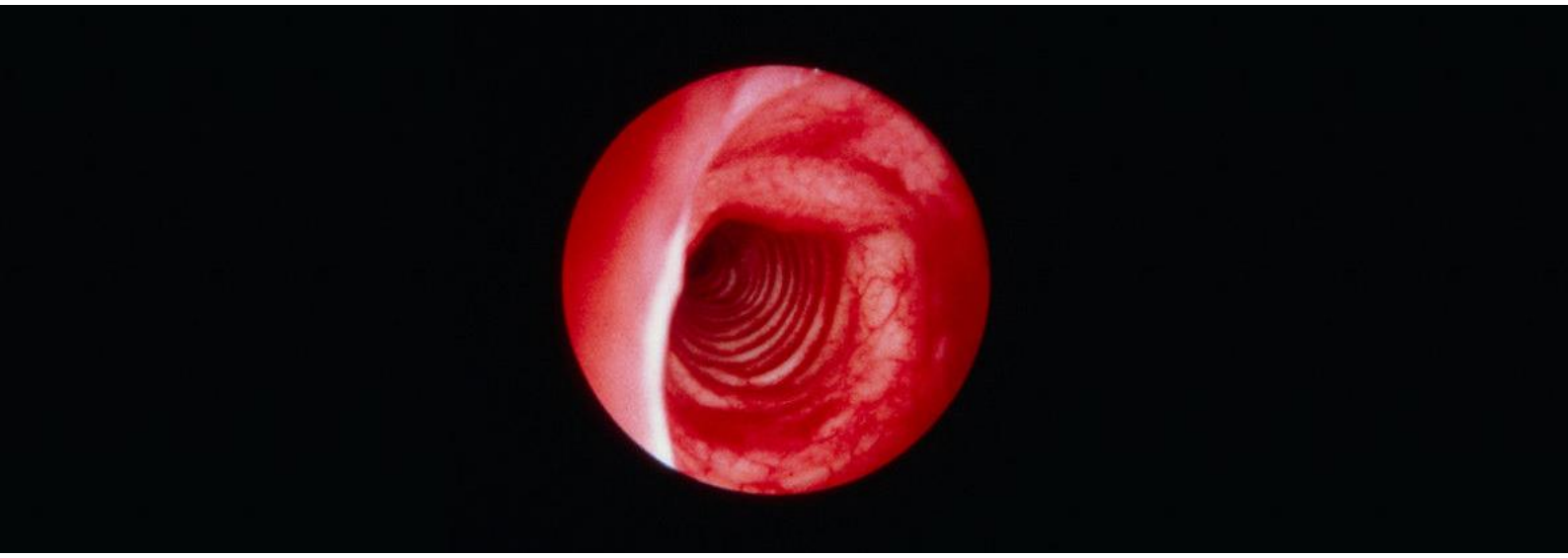
Mechanisms involved in the induction of airway remodeling due to bronchoconstriction



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Summary

Chronic airway diseases, like asthma or COPD, are characterized by hyperresponsiveness resulting in inflammation and bronchoconstriction. During these diseases, also remodeling of the airway tissue occurs. Smooth muscle hypertrophy, epithelial goblet-cell hyperplasia and collagen deposition are examples of airway remodeling. Recent findings suggest a role for muscarinic receptor stimulation regulating remodeling in chronic airway diseases. Besides, other recently published data suggest that mechanical stress caused by bronchoconstriction may cause remodeling independent of inflammation. In this study, methacholine was used to induce bronchoconstriction, but methacholine is also a muscarinic agonist. Mainly due to this last article, the question arose which mechanisms are involved in the induction of airway remodeling by bronchoconstriction. That is why we designed a model to examine if the effect of methacholine is attributable to cell signaling or to the mechanical forces caused by bronchoconstriction. We used isometric and isotonic contraction experiments to induce mechanical strain in bovine airway smooth muscle strips and added methacholine to induce both bronchoconstriction and cell signaling via muscarinic receptors. The strips were tested on several protein levels with western blot. Contractile protein levels in chronic stimulated strips did not show strain-dependent increases. The acute stimulated strips with isometric settings did show a significant increased phosphorylated ERK 1/2 and GSK-3 level at 10 grams of tension. In case of phosphorylated ERK 1/2, this increase is probably strain-dependent. Methacholine was able to further increase the levels, but not on a strain-dependent manner. Besides, we tested if the phosphorylation of ERK 1/2 was attributable to Rho kinase-induced contraction, but this was not demonstrated. However, we did see an inhibitory effect on the contractility of methacholine induced strips. With regard to this last point, more experiments are recommended to investigate this properly. The main aim of this study to design a model to investigate whether the increasing effect of methacholine on airway remodeling markers was due to cell signaling or to the mechanical forces caused by constriction of the trachea has not yet been achieved.

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Introduction

Chronic airway diseases

Patients with a chronic airway disease, like asthma or COPD, have difficulty breathing attributable to their tendency toward reversible bronchoconstriction. They are hyperresponsive to everyday environmental factors. Asthma and COPD are characterized by airway inflammation and airway remodeling. Mucus gland and smooth muscle hypertrophy, fibrosis, collagen deposition and epithelial goblet-cell (mucin-secreting cells) hyperplasia are the main features of remodeling in asthma and COPD (Grainge et al., 2011; Tschumperlin et al., 2011; Kistemaker et al., 2012). Besides, remodeling may contribute to early-onset and persistent airway dysfunction and evidence indicate that remodeling can be detected early in life (Tschumperlin et al., 2011). Because of these findings, it is interesting to determine the mechanisms by which remodeling occurs.

Airway remodeling and the role of acetylcholine

Muscarinic receptor stimulation

Acetylcholine (ACh) is the main parasympathic neurotransmitter in the airways, acting on muscarinic receptors (Kistemaker et al., 2012; Gosens et al., 2006, Oenema et al., 2010a). It is a key player in the induction of airway smooth muscle (ASM) contraction and mucus secretion. Muscarinic receptor regulation of ASM tone is increased in airway diseases by two major mechanisms: increased expression and enhanced function of essential signaling molecules for contraction, and exaggerated release of neuronal ACh due to inflammation associated neuronal mechanisms (Gosens et al., 2006). Recent findings suggest a role for muscarinic receptor stimulation regulating additional functions in the airways, like remodeling, in obstructive airway diseases (Gosens et al., 2006). The effects on the different remodeling characteristics mentioned above are described below.

Mucus gland, epithelial goblet-cell hyperplasia and subepithelial fibrosis

Different *in vitro* and *in vivo* studies showed that acetylcholine plays an important role in mucus gland hypertrophy and mucus production and that this effect can be enhanced by epidermal growth factor (EGF) (Kistemaker et al., 2012). In addition, anticholinergic tiotropium reduces mucus gland hypertrophy and epithelial goblet cell numbers (Pera et al., 2011).

Subepithelial fibrosis is one of the characteristics of asthma patients; fibrosis in COPD is mostly peribronchial (Hogg et al., 2004). Proliferative and pro-fibrotic responses of airway epithelial cells may be induced by acetylcholine stimulation (Grainge et al., 2011). Grainge et al. found an increased expression level of the pro-fibrotic cytokine epithelial-TGF-β in mild asthmatic patients with methacholine (the synthetic form of acetylcholine) induced bronchoconstriction. A bronchodilator,

albuterol, reduced this effect and for this reason it can be stated that it was related to the constrictive effect of acetylcholine. No anticholinergic was tested, but in another study it was shown that tiotropium can inhibit fibrosis progression (Cui et al., 2010). So there is an important role for muscarinic receptor stimulation in mucus gland hypertrophy, mucus production and subepithelial fibrosis.

Smooth muscle hypertrophy

Smooth muscle hypertrophy, which is mainly a characteristic of asthma, is induced by growth factors like EGF and PDGF and this effect can be enhanced by acetylcholine (Gosens et al., 2007). Additionally, it was described that TGF-β-induced contractile protein expression in human ASM cells is enhanced by acetylcholine (Oenema et al., 2010b). Gosens et al. showed that inhibition of GSK-3 plays a key role in ASM proliferation induced by stimulation of muscarinic receptors and PDGF receptors (Gosens et al., 2007). Later, it was showed that TGF-β stimulated ASM can inhibit GSK-3, by which β-catenin can be released and activate transcription (Baarsma et al., 2011). This is consistent with the findings of Oenema et al. and Gosens et al. described above. Together, these findings suggest an important role for muscarinic receptor stimulation and a key role for GSK-3 inhibition.

Collagen deposition

Fibroblasts are mesenchymal cells which produce extracellular matrix and collagen. It is demonstrated in *in vitro* studies that stimulation of muscarinic receptors induces cell proliferation and collagen synthesis of lung fibroblasts via activation of the mitogen-activated protein kinase pathway (MAPK) (Haag et al., 2008). The acetylcholine-induced cell proliferation is increased in human lung fibroblasts from COPD patients compared with those from healthy non-smokers and healthy smokers without COPD. This was associated with elevated levels of phosphorylated ERK 1/2, a MAP kinase, and phosphorylated NF-κB, a transcription factor (Profita et al., 2009). Grainge et al. also showed that methacholine induced bronchoconstriction can increase the thickness of the collagen band (Grainge et al., 2011). Collectively, these findings demonstrate an important role for muscarinic receptor stimulation and MAPK in collagen deposition.

Airway remodeling and the role of mechanical stress

Patients with chronic airway diseases experience some forms of mechanical loading during lifetime, including bronchoconstriction and acute hyperinflation (Kumar et al., 2003a). Cells exposed to these kinds of mechanical forces are stimulated in growth and modulation of the extracellular matrix occurs (Tschumperlin and Drazen, 2001). Therefore it is plausible that mechanical forces associated with bronchoconstriction may play a role in the thickening and remodeling of the airway wall in patients with a chronic airway disease. The structural changes in the airways have been mainly attributed to inflammation, but *in vitro* studies indicate, however, that bronchoconstriction

accompanied by compressive mechanical forces may induce remodeling independently of inflammation (Grainge et al., 2011). Therefore Grainge et al. performed a clinical study with mild asthma patients who were divided into four challenge groups: allergen, methacholine, methacholine after albuterol and saline (Grainge et al., 2011). The allergen induced subjects would develop eosinophilic inflammation together with bronchoconstriction, while in the methacholine induced subjects bronchoconstriction alone would occur. Albuterol is a bronchodilator, therefore the group of methacholine after albuterol would be a control for the non-bronchoconstrictor, receptor-mediated response of methacholine. The study showed that the methacholine challenged group could induce an increase in several airway remodeling markers as well as the allergen group. This effect was reduced in the methacholine after albuterol challenge group. Collectively, these results show that bronchoconstriction alone can induce airway remodeling independently of inflammation.

The mechanical environment of the airway wall is dependent on a few things, i.e. the lung volume, surface tension in the airway, mechanical properties of the airway wall and state of activation of airway smooth muscle (Tschumperlin and Drazen, 2006). They are all connected and can change in case of airway diseases. In an open-chest mechanical ventilation experiment, four hours of increased lung volume induced up-regulation of mRNA for procollagen, fibronectin and TGF-β (Berg et al., 1997). Changing lung volumes by direct mechanical stretch of isolated mouse lobes led to increased activation of NF-κB and MAP kinases (Kumar et al., 2003a).

When stretching airway smooth muscle, there is an elevated level of α-actin expression only in inactive samples, not in histamine- or carbachol-contracted samples. This indicates antagonistic interactions between mechanical and biochemical signaling (Wahl et al., 2004). Wahl et al. also demonstrated that in stretched ASM proliferation is enhanced and contractile protein expression and activity are increased. Besides, he showed the contractions are increased in magnitude and velocity and stretch led to stiffening of smooth muscle cells as well. Last but not least, increased activity of ERK 1/2, JNK1 and p38 which are all MAP kinases was observed which i.e. enhanced IL-8 inflammatory cytokine synthesis (Kumar et al., 2003b).

When looking at the effect on epithelium, mechanical stress leads to higher levels of calcium, IP3, PKC and ATP/UTP signaling pathways (Tschumperlin and Drazen, 2006). These pathways are involved in coordination of ciliary beating activity. In epithelium the levels of IL-8 are elevated via MAPK pathways as well (Tschumperlin and Drazen, 2006). It is also shown that ERK 1/2 phosphorylation can change gene expression and signaling in epithelium. ERK 1/2 plays a key role in downstream biological effects and is dependent of the EGF receptor autocrine activity (Tschumperlin et al., 2002).

Kumar et al. likewise showed elevated levels of ERK 1/2 and p38 MAP kinases after both constant static stretch and methacholine induced lung parenchyma (Kumar et al., 2003a). These elevated levels contribute to increased activity of AP-1 and NF-κB transcription factors.

To conclude, cells in the airways feel and react to the mechanical environment and changes in this environment represent both normal physiology and the response to disease. Besides, MAP kinase activation may link the effects of mechanical stretch to biological responses and gene expression.

Aim of this study

In the study of Grainge et al. methacholine was used to induce bronchoconstriction and it was concluded that bronchoconstriction can induce airway remodeling independent of inflammation. Methacholine is a muscarinic receptor agonist and may induce bronchoconstriction and remodeling both via cell signaling. But remodeling may be attributable to the mechanical forces caused by bronchoconstriction as well. Therefore, we our aim was to investigate which mechanisms are involved in the induction of remodeling due to bronchoconstriction independent of inflammation.

To this aim, a model was designed to investigate whether the increasing effect of methacholine on airway remodeling markers was due to cell signaling or to the mechanical forces caused by constriction of the trachea. Therefore, we used contraction experiments to induce mechanical strain in airway smooth muscle strips and added methacholine to induce bronchoconstriction and activation of the cell signaling pathways through the muscarinic receptors. When using inhibitors of strain and muscarinic receptors, we could also check of there is a cross-talk between them. For the chronic contraction studies, the levels of proteins which play an important role in contraction were tested: calponin and actin, which are filament proteins; myosin, which is a motor protein; and GAPDH, which is important in the generation of energy. For the acute contraction studies, we chose ERK 1/2 because it is a key player in intracellular signaling. ERK activation plays a major role in cell proliferation, differentiation and survival. In addition, as mentioned above, it is associated with remodeling characteristics like collagen deposition, gene expression and signaling of epithelium. (Tschumperlin and Drazen, 2006; Kumar et al., 2003a) Kumar et al. showed that mechanical stretch can lead to elevated levels of ERK 1/2 in lung parenchyma as well (Kumar et al., 2003a). Besides, G protein coupled muscarinic receptors can activate signaling pathways which lead to the activation of ERK 1/2 MAP kinase, Rho kinase and phoshatidyl-inositol-3-kinase (PI3K) (Gosens et al., 2006). Therefore, we found ERK 1/2 suitable for this study in which the difference between mechanical stress and muscarinic receptor stimulation was studied. Also phosphorylated GSK-3α/β was chosen for acute contraction studies because it was shown within our research group that it has an important role in ASM proliferation (Gosens et al., 2007). Besides, asthma and COPD patients have

elevated levels of TGF- β and TGF-β can induce GSK-3 phosphorylation, which in turn can be induced by acetylcholine (Oenema et al., 2010b; Baarsma et al., 2011). Our hypothesis was that stimulated airway smooth muscle would result in increased TGF-β release by which GSK-3 gets phosphorylated and transcription, and therefore remodeling, is enhanced.

During our study, the acute protocol with isometric settings became the main focus. That's why we made an overview of this model which is showed below (figure 1).

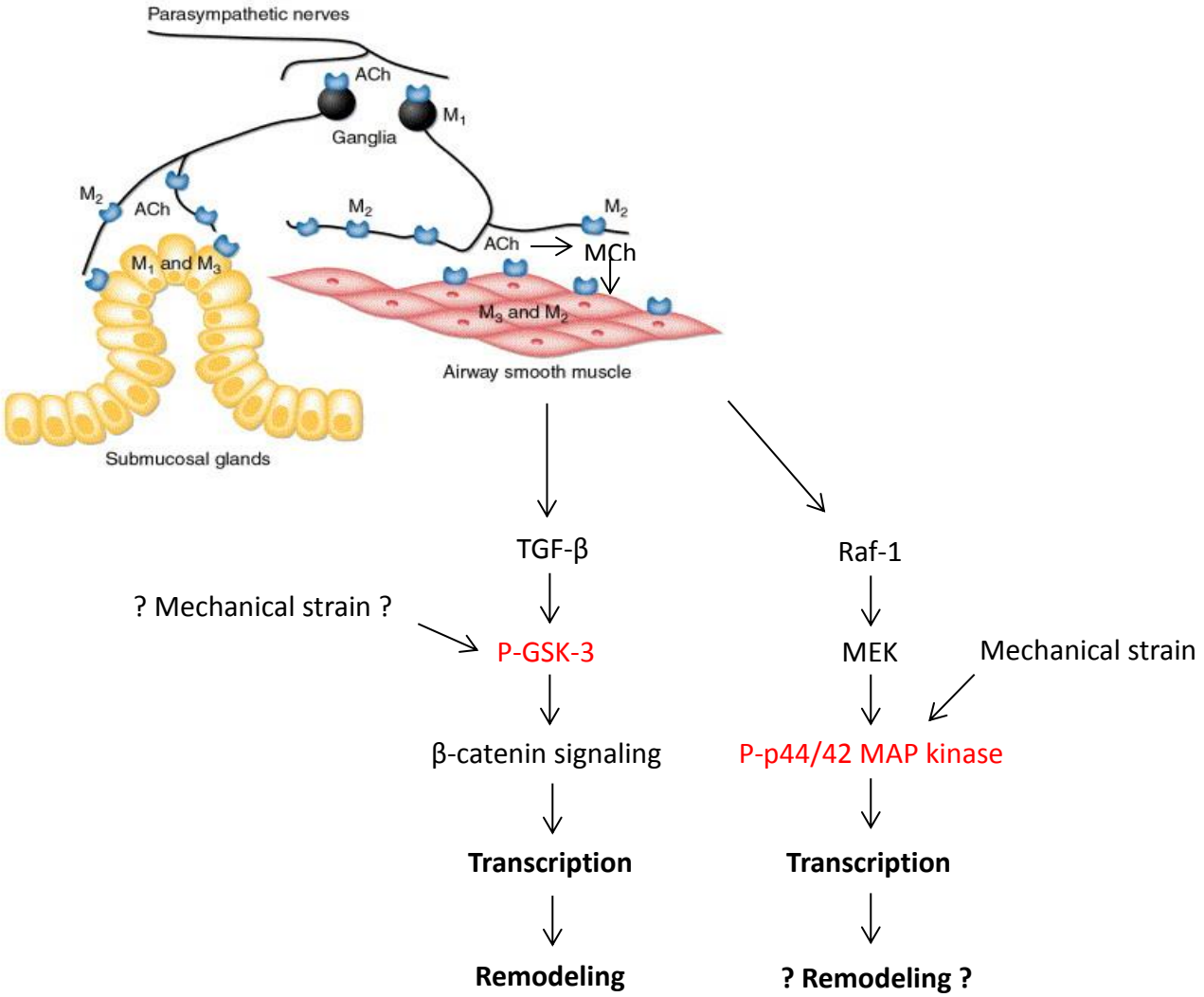

Figure 1: Overview of the induction of remodeling by methacholine stimulated airway smooth muscle. With this model it was our intention to study the effect of cell signaling, via muscarinic receptor stimulation with methacholine, and mechanical strain on remodeling. We therefore tested phosphorylated p44/42 (ERK 1/2) MAP kinase levels, because ERK 1/2 plays an important role in cell signaling, and GSK-3 for its role in airway smooth muscle proliferation. We hypothesized that mechanical strain may lead to phosphorylation of GSK-3 and that phosphorylated p44/42 could lead to remodeling.

MATERIAL & METHODS

Obtained airway smooth muscle tissue

The airway smooth muscle tissue was obtained either from guinea pigs from the UMCG central service laboratory animals (Universitair Medisch Centrum Groningen, NL) or from bovine from the slaughterhouse (Kroon vlees, Groningen, NL). For contraction experiments with guinea pigs, cartilage rings were isolated from the trachea and cut open on the opposite side of the muscle. This tissue was used for contraction studies immediately after isolation, hence no preservation was needed. For contraction studies with bovine tissue, the muscle was gently isolated from a part of the trachea. Epithelia and connective tissue were carefully stripped of and equal sized strips were cut. All of the above was carried out in a preparation bath with Krebs buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, pH 7.4) with a constant carbogen gas flow. Bovine muscle strips for acute stimulation were preserved in primary human tracheal smooth muscle cells (HTSM) S0 medium (500 ml DMEM: 3.3 ml amphothericin B, 11 ml penicillin/streptomycin, 5.5 ml sodium pyruvate, 5.5 non-essential amino acids, 3.1 ml gentamycin) in a shaking incubator at 37°C until contraction studies. For chronic stimulation (± three days), one end was tied to a weight: one strip with 1.7 grams, one with 5 grams and one with 8.5 grams. They were hung in an upright culture bottle with HTSM S0 medium. The control (0 gram) floated loose in the same bottle.

Contraction studies

Preparation

The muscle strips or cartilage rings for acute stimulation contraction experiments were tied at both ends with surgical thread in a preparation bath with Krebs buffer with a constant carbogen gas flow and, for preserved bovine tissue, at 37°C. For the chronic stimulated bovine tissue, the weights were cut off and directly put into nitrogen for western blot analysis. For acute stimulation, the ends of each muscle strip were tied with surgical thread. On one end of each strip a small eye was tied to attach the strip to a hook that was connected to the arm of a lever. The other end was tied with a longer threat to secure it at a clamp connected to a length manipulator. To control and record force during the experiment, a computerized lever system was used.

Isotonic

Determination of the appropriate methacholine concentration and relaxant agent

For this experiment, guinea pig tissue was used. The cartilage rings were hung in the incubation baths which contained 20 mL Krebs buffer with 60 µl indomethacin and a constant carbogen gas flow. They were tied to isotonic force transducers which were all in pairs connected to a recorder. Each transducer had a counterbalance of 0.5 grams. First the muscles were stabilized for 30 minutes in the baths. Five minutes after changing the Krebs buffer and adding new indomethacin, a series of 10^{-7} M, 10^{-6} M and 10^{-5} M methacholine (MCh, acetyl-β-methylcholine chloride) was added in succession. Methacholine was purchased from Sigma-Aldrich (St. Louis, MO, USA). After the series, the muscles were washed two times fast and an additional two times ten minutes. The concentration which caused 50% of the total contraction was then again added and the relaxant effect of three different substances in four different concentrations was studied. The relaxant agents used for this study were SB431542, ML-7 hydrochloride and Latrunculin A, all purchased at Tocris Bioscience (Abingdon, UK). Afterwards, the muscles were washed until baseline was reached.

Isotonic

Acute stimulation of bovine strips

Twelve strips were hung in the incubation baths which contained 20 mL Krebs buffer at 37°C and a constant carbogen gas flow. They were tied to the isotonic force transducers which were all in pairs connected to a recorder. Each transducer had a counterbalance of 0.5 grams. First the muscles were stabilized for 30 minutes in the baths. After stabilization, the length of the strips was changed by adding weight to the transducer. We split the strips into three series with four strips per series: a control (0.5 grams), 2 grams weight, 5 grams weight and 10 grams weight. After half an hour, they were washed until they were stable again. Then two series were additionally stimulated with either 10^{-6} M or 10^{-5} M methacholine. As soon as the line on the recorder again ran stable, the strips were cut between the nodes and put into nitrogen. They were stored at -80°C.

Isometric

Acute stimulation of bovine strips

First the system was calibrated by making a calibration line from 1 to 10 grams. The bovine strips were hung half an hour at 0.5 grams (control strips) or 1 gram (other strips) tension in the incubation baths with 20 mL Krebs buffer at 37°C and a constant carbogen gas flow. The strips, except controls, were pre-stressed with 2 (maximum of the 2 grams strips) and 3 grams tension in succession by using the isometric force transducer for a half hour, followed by 15 minute of washing with clean KREBS. *

Subsequently, the muscles were stimulated with 0.5 grams (controls), 2 grams, 5 grams or 10 grams tension. Then two series were additionally stimulated with either 10^{-6} M or 10^{-5} M methacholine. As soon as the line on the recorder again ran stable, the strips were cut between the nodes and put into nitrogen. They were stored at -80°C.

* For a few experiments, we tested the effect of the Rho-associated protein kinase (ROCK) inhibitor Y-27632 dihydrochloride (Tocris Bioscience, Abingdon, UK). Therefore, we added 10 µM Y-27632 to four strips (instead of the MCh 10^{-6} M series) after pre-stress and incubated for half an hour. Two strips were then stimulated with 0.5 grams and two with 10 grams tension. Then, one 0.5 grams and one 10 grams tension stimulated strip received additional stimulation with 10^{-5} M methacholine.

Protein purification

Strips were crushed in a mortar with nitrogen. They were dissolved in 300 µl SDS lysis buffer (composition (10mL): 10 µl Aproteinine (1 mg/ml), 10 µl pepstatin A (1 mg/ml), 10 µl leupeptin (1 mg/ml), 50 µl ortho-vanadate (NO_3VO_4), 50 µl Na-fluoride and 10,8 mg β-glycerophoshpate) and then sonificated to be further used for western blot analysis.

Protein assay

After protein purification, the amount of protein in each sample was determined by using the Thermo Scientific Pierce BCA Protein Assay Kit (Rockford, USA) following the manufacturer instruction. In short, the bovine samples and the calibration curve samples are mixed with working reagent in a 96-wells plate. The plate was 30 minutes incubated in a shaking incubator at 37°C. The protein concentration was measured with a plate reader at 595nm.

Western blot analysis

According to the protein assay results, the samples were diluted with UP and loading buffer to an equal amounts of protein. The diluted samples were electrophoresed on polyacrylamide gels and transferred to nitrocellulose membranes. After transfer, the membranes were blocked with 5% milk powder in 0.1% TBST. Then specific primary antibodies were used for protein analysis. For the acutely stimulated samples the main proteins of interest were P-p44/42 MAPK (phosphorylated ERK 1/2) and p44/42 (total ERK 1/2), both obtained from Cell Signaling Technology (Boston, MA, USA). Beside, P-GSK-3-alpha/beta (Cell Signaling Technology, Boston, MA, USA) and GSK-3α/β (Santa Cruz Biotechnology, St. Louis, MO, USA) were of interest too. For the chronically stimulated samples,

monoclonal Anti-Actin (α-smooth muscle) and monoclonal Anti-Calponin, purchased from Sigma-Aldrich (St. Louis, MO, USA), GAPDH (Santa Cruz Biotechnology, St.Louis, MO, USA), and smooth muscle Myosin (Neomarkers Immunologics, Duiven, The Netherlands) were of interest. The membranes were overnight incubated at 4°C with the primary antibodies. Next day they were washed three times 10 minutes with 0.1% TBST. Afterwards, the membranes were incubated for one hour with secondary antibody. The secondary antibodies were horseradish peroxidase (HRP)-conjugated, purchased from Sigma-Aldrich (St. Louis, MO, USA), followed by washing. The protein bands on the membranes were highlighted after addition of chemiluminescence reagents and then visualized using the G:BOX connected to a computer with GeneSnap image acquisition software.

Results

Determination of the appropriate methacholine concentration and relaxant agent

The appropriate concentration of methachonline to accomplish 50% contraction appeared to be $3x10^{-7}$. After addition of this concentration, the relaxing effect at different concentrations of some relaxant agents could be tested. ML-7 hydrochloride appeared to give a 100% relaxation at 30 µM, Latrunculine A at 1 µM and SB431542 was tested up to 10 µM but could only achieve a maximum relaxation of about 70%.

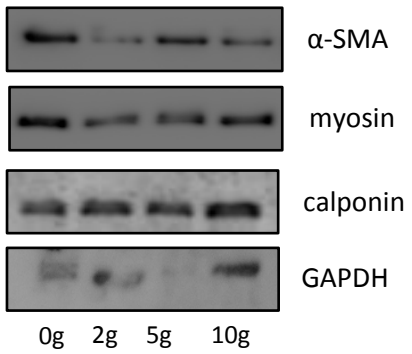

Figure 2: Contractile protein levels of chronic stimulated strips. Here you can see the levels of α-smooth muscle actin, myosin, calponin (n=3) and GAPDH (n=1) after chronic stimulation of bovine smooth muscle strips with either 0g, 2g, 5g or 10g of tension

Chronic stimulated bovine strips

The levels of α-smooth muscle actin (α-SMA), myosin, calponin and GAPDH contractile proteins of the chronic stimulated bovine strips are shown in figure 2. There was no increase in those levels after increase of tension. GAPDH was only shown once. Because these results from the chronic experiments (n=3) were not suitable to achieve our aim, we continued with only the acute protocol.

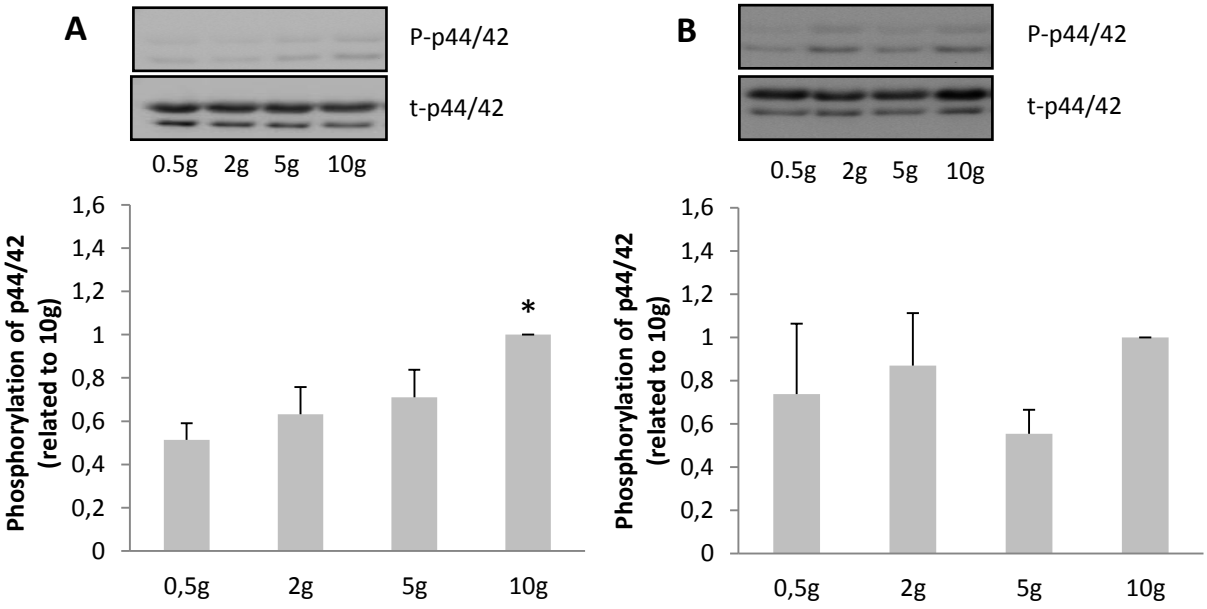

Figure 3: Phosphorylation of p44/42 of acute stimulated bovine smooth muscle strips. A: Isometric settings (n=9). At 10 grams of tension, there is a significant difference with 0.5 grams ($P<0.05$). B: Isotonic settings (n=5). On top, the western blot results of the effect of strain on phosphorylated ERK 1/2 are shown. The intensity of the western blot bands are represented in the graphs below.

Acute stimulated bovine strips

In each figure the results of both isometric and isotonic settings are placed next to each other so a comparison can be made between the results of both settings. In figure 3, the results of the effects of 0.5 grams, 2 grams, 5 grams and 10 grams of strain on the phosphorylation of p44/42 are shown. There is a significant increase of phosphorylated p44/42 after 10 grams of tension ($P<0.05$) with isometric settings. The results of the isotonic experiment are not significant and inconsistent.

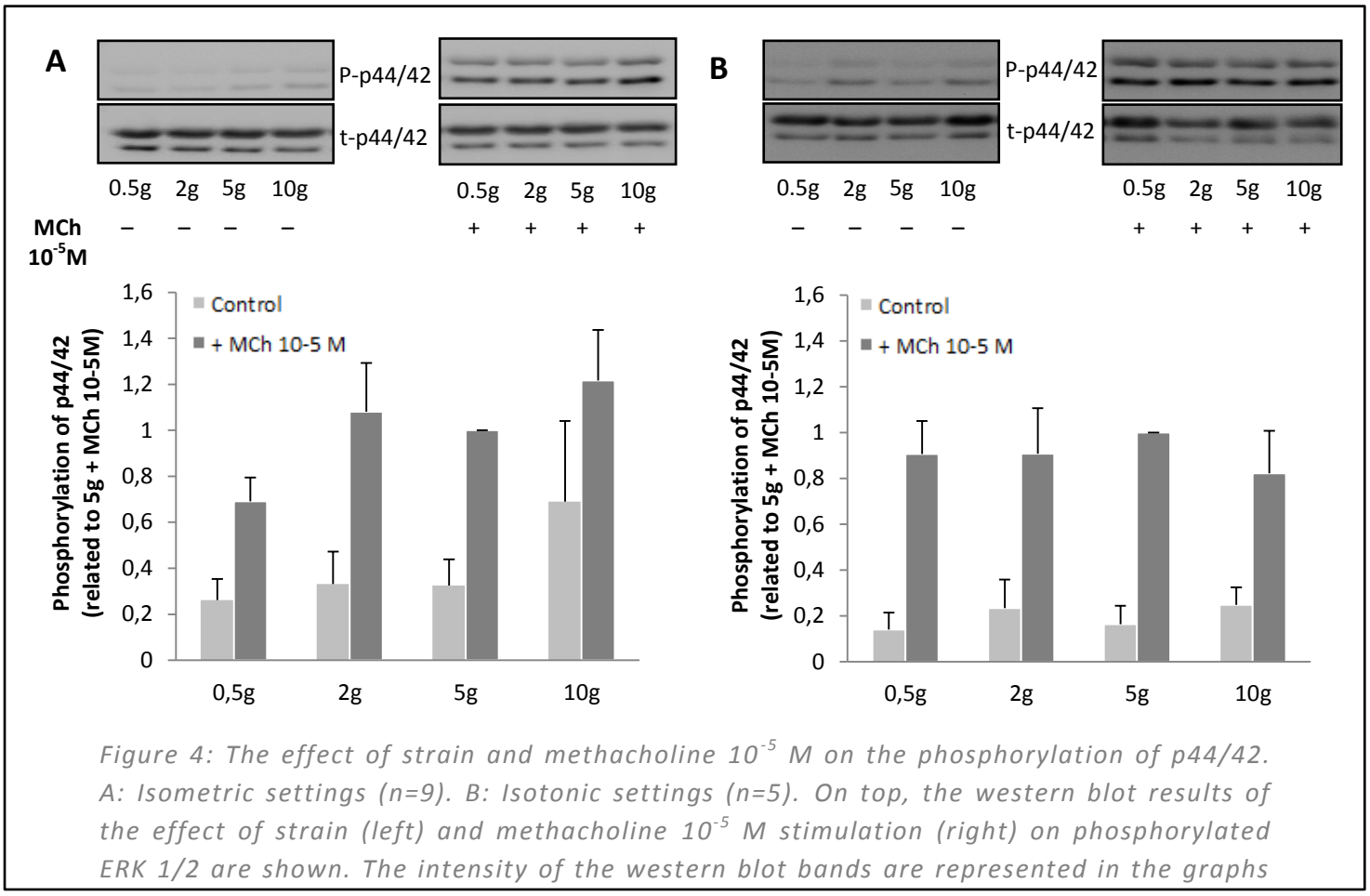

Figure 4: The effect of strain and methacholine 10^{-5} M on the phosphorylation of p44/42. A: Isometric settings (n=9). B: Isotonic settings (n=5). On top, the western blot results of the effect of strain (left) and methacholine 10^{-5} M stimulation (right) on phosphorylated ERK 1/2 are shown. The intensity of the western blot bands are represented in the graphs

For investigation of the effect of cell signaling induced by muscarinic receptor stimulation, methacholine (MCh) was added. We used 10^{-5} M MCh and 10^{-6} M MCh. Because the strips stimulated with 10^{-5} M MCh gave more consistent results than the ones with 10^{-6} M MCh, these results are shown in a separate graph (see figure 4). In figure 5, the results of both 10^{-5} M MCh and 10^{-6} M MCh are shown. Methacholine could increase the phosphorylation levels with both settings and concentrations, but not on a strain-dependent manner and 10^{-6} M MCh shows a lot of variety.

To test whether these effects on the phosphorylation of p44/42 were attributable to Rho kinase-induced contraction, strips were pre-incubated with 10µM Rho kinase-inhibitor Y-27632. This could not reduce the effects of strain or methacholine 10^{-5} M on the phosphorylation of ERK 1/2 (figure 6).

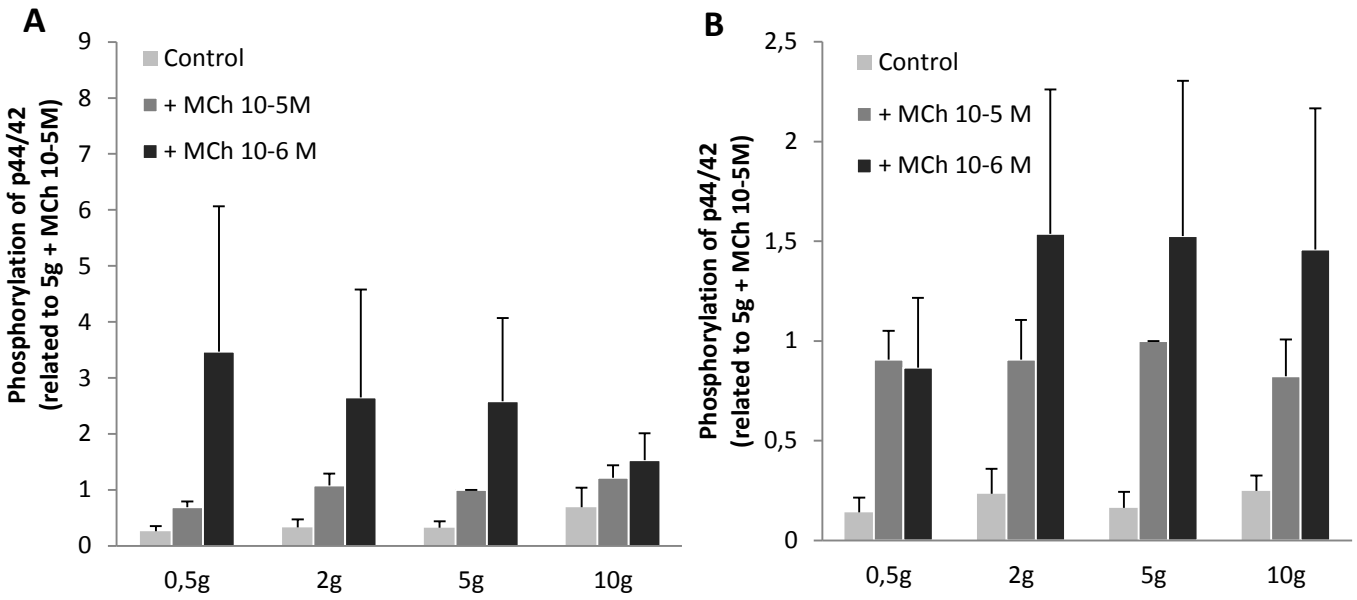

Figure 5: The effect of strain, methacholine 10^{-5} M and 10^{-6} M on the phosphorylation of p44/42. A: isometric settings (control and MCh 10^{-5} M: n=9 and MCh 10^{-6} M: n=5). B: isotonic settings (n=5). The intensity of the western blot bands are represented in the graphs.

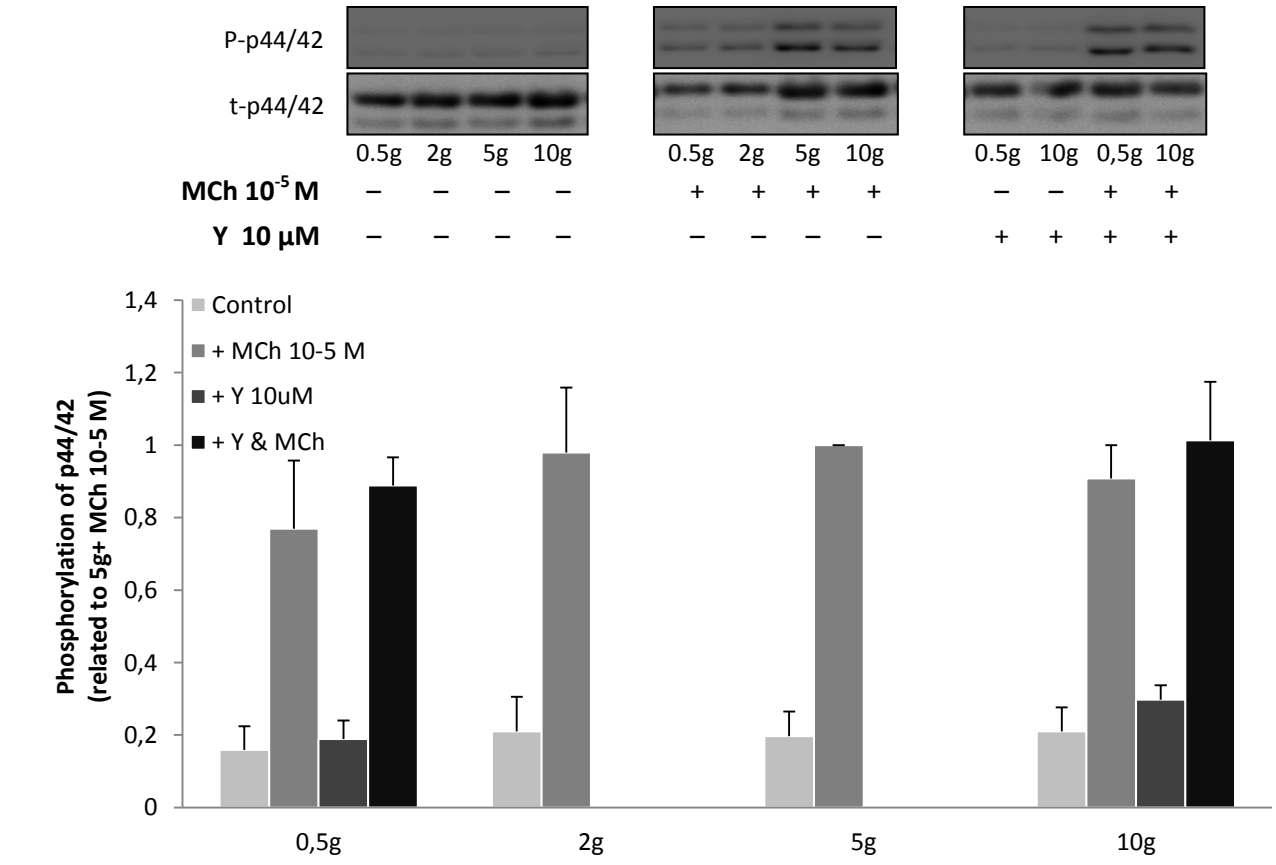

Figure 6: The effects of Rho kinase inhibitor Y-27632 on the phosphorylation of p44/42. Isometric settings (n=4). On top, the western blot results of the effects of strain, methacholine 10^{-5}M and Y-27632 10µM on phosphorylated ERK 1/2 are shown. The intensity of the bands is represented in the graphs below. Y-27632 was only tested on a control (0.5g) and 10 grams of tension without and in combination with methacholine 10^{-5}M stimulation.

Although the Rho-inhibition had no effect on the levels of phosphorylated ERK 1/2, we observed a decrease in the contractile effect of methacholine during the contraction experiments.

Besides the effects of strain and cell signaling on the phosphorylation of p44/42, the effect on phosphorylation of GSK-3 was investigated likewise with both isotonic and isometric settings. In figure 7, the effects of strain are showed.

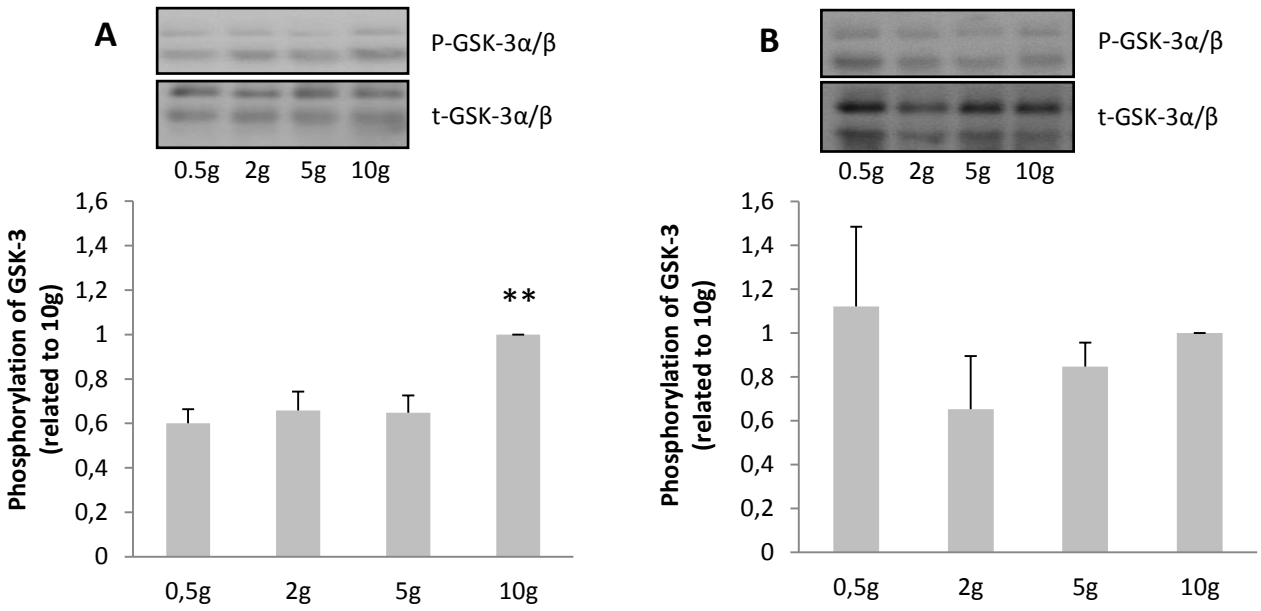

Figure 7: Phosphorylation of GSK-3α/β of acute stimulated bovine smooth muscle strips. A: isometric settings (n=5). There is a significant up-regulation of phosphorylation of GSK-3 at 10 grams of tension (P<0.01). B: isotonic settings (n=4). The western blot results of the effect of strain on phosphorylated GSK-3 are shown on top and the intensity of the bands are represented in the grapsh below.

The effects of cell signaling on the phosphorylation of GSK-3 were also tested by addition of methacholine. Again both MCh 10^{-5} M and MCh 10^{-6} M were added and the total graph with both concentrations is depicted in figure 8. With isotonic settings, no constant up-regulation after methacholine stimulation is observed. With isometric settings there is a constant up-regulation of phosphorylated GSK-3, but it is not significant.

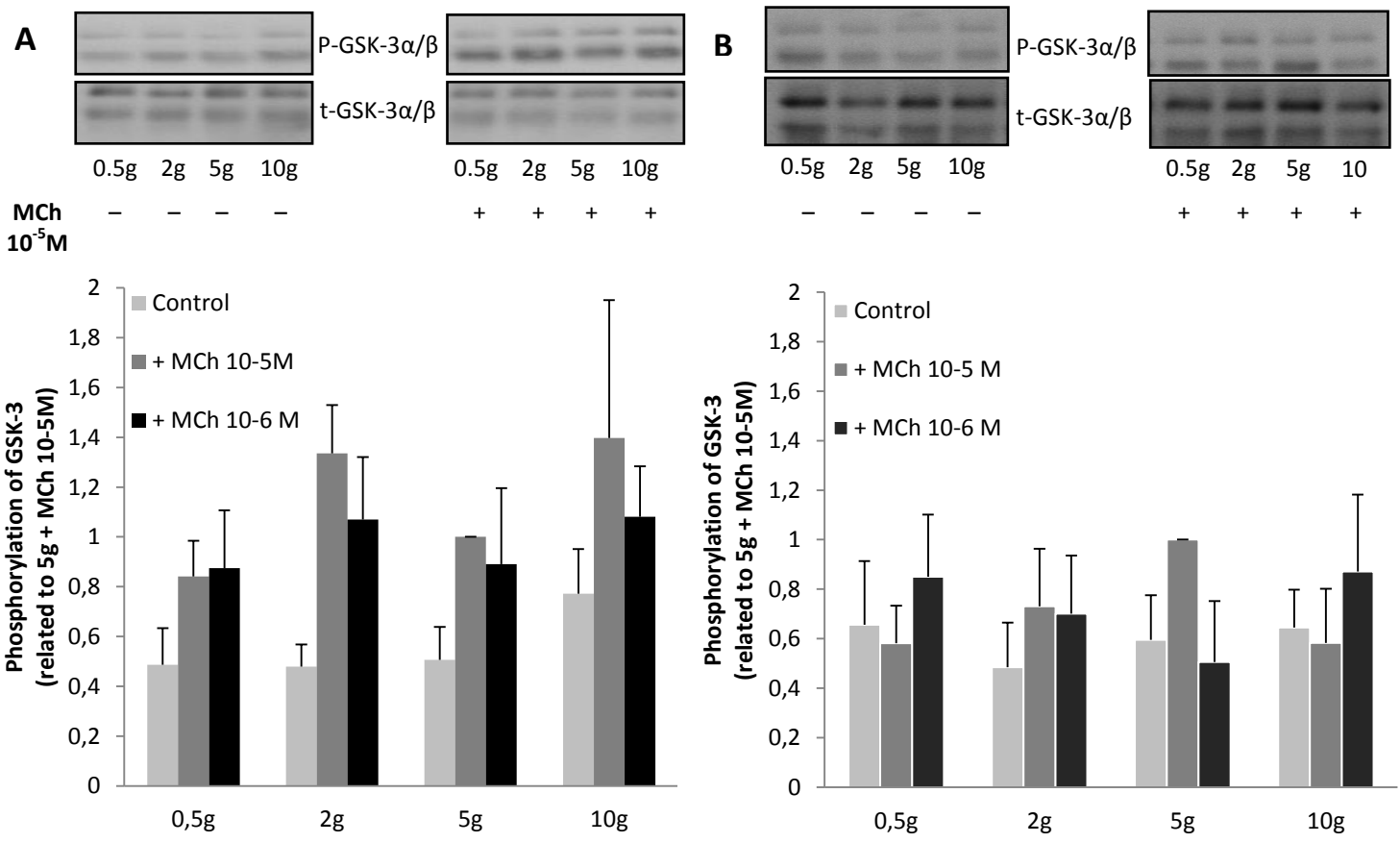

Figure 8: The effect of strain and methacholine 10^{-5} M and 10^{-6} M on the phosphorylation of GSK-3α/β. A: isometric (n=5, MCh 10^{-5} M 10g n=3). B: isotonic (n=4, control 0,5g n=3). The blots of the western blot on top of the figure show the effect of strain and methacholine 10^{-5} M on the phosphorylation of GSK-3. Below, the intensity of the bands of strain, methacholine 10^{-5} M and 10^{-6} M are represented.

Discussion

This study shows that, when using isometric settings, phosphorylation levels of ERK 1/2 and GSK-3 in airway smooth muscle can be significantly increased after 10 grams of tension. Although not significant, the phosphorylation of ERK 1/2 is probably strain-dependent with isometric settings. This is not apparent from the observations of GSK-3. Our hypothesis that mechanical strain can lead to GSK-3 phosphorylation is therefore not yet completely confirmed. Stimulation with methacholine could increase the levels of phosphorylated ERK 1/2, but at a methacholine concentration of 10^{-6} M the effects were very variable. Rho-kinase inhibition is probably able to decrease the constrictive effect of muscarinic receptor stimulation, however no effect on the phosphorylation of ERK 1/2 is observed. The effect of methacholine on phosphorylated GSK-3 was only constantly increasing with isometric settings. The hypothesis that phosphorylation of ERK 1/2 is able to induce remodeling was not tested with this model. Because ERK 1/2 proteins are widely expressed and involved in general processes like cell proliferation, differentiation and survival, we are not able to conclude that the up-regulation of ERK 1/2 due to increasing strain is an indication for the induction of remodeling. The main aim of this study was to design a model to investigate whether the increasing effect of methacholine on airway remodeling markers was due to cell signaling or to the mechanical forces caused by constriction of the trachea. This aim has not yet been achieved.

We started this study with both chronic and acute stretched bovine airway smooth muscle strips. The chronic stretched strips were tested for some contractile protein levels. Levels of myosin, α-actin and calponin contractile proteins did not show a strain-dependent increase. We would expect this to happen since Wahl et al. showed in their study that in stretched airway smooth muscle contractile protein expression and activity are increased (Wahl et al., 2004). Maybe the chronic stretch-period was too long and the contractile protein levels were already decreased. In addition, it is possible that the weights are too heavy and non-physiological by which the normal contraction-related responses did not occur. GAPDH was only detected once; therefore no conclusions can be drawn from this result.

After three bovine tracheas with both chronic and acute stimulation, we proceeded with only the acute protocol. The levels of GSK-3 and ERK 1/2 were significantly up-regulated after 10 grams of tension. As 10 grams of tension is unlikely to occur during *in vivo* bronchoconstriction and ERK i.e. plays a role in cell survival, we expect elevated phosphorylation of ERK 1/2 is due to the healing process the muscle needs during such a large tension. It is already shown that ERK 1/2 controls the endothelial repair process (Ranzato et al., 2010). Furthermore, GSK-3 phosphorylation levels could be increased to protect the muscle as well. It is known that phosphorylated GSK-3 plays an inhibitory

role in apoptosis and necrosis of cardiac muscle (Miura and Miki, 2009). Whether this is also applicable for smooth muscle has to be studied first.

Except for some phosphorylated GSK-3 levels with isotonic settings, methacholine could always increase the effect of strain on the phosphorylation levels of both ERK 1/2 and GSK-3. But there was no cross-talk observed because the effect was not strain-dependently increased. Other studies already showed that increased ERK 1/2 phosphorylation is attributable to muscarinic receptor stimulation (Kumar et al., 2003a; Gosens et al., 2006). The increased levels of phosphorylated GSK-3 in presence of methacholine are possibly induced by TGF-β released during bronchoconstriction (Baarsma et al., 2011). To confirm this assumption, the levels of TGF-β must be checked.

When adding a Rho-kinase inhibitor, by which Rho-induced contraction would be downregulated, we expected to see a decrease in the mechanical strain induced phosphorylated ERK 1/2 levels because reduction in ERK 1/2 phosphorylation after Rho-inhibition was already shown in osteoblasts, glioblastoma cells and epithelial cells (Khatiwala et al., 2009; Zohrabian et al., 2009; Nakabayashi and Shimizu, 2011; Chaturvedi et al., 2011). During the contraction experiment, it was seen that the pre-incubation with the Rho-inhibitor reduced the contractile effect of methacholine. But Rho-inhibition did not affect the phosphorylated ERK levels.

These results and the consequential assumptions suggest that this is not the right model to investigate the mechanisms which are involved in the induction of airway remodeling attributable to bronchoconstriction. Despite that, it would be interesting to complement this study with some experiments and/or to make some adjustments. In the first place, it would be nice to check the TGF-β levels with western blot. And if these levels are increased with increasing strain and with methacholine, the strips should be pre-incubated with specific TGF-β receptor blocker SB-431542 and tested for GSK-3 phosphorylation levels. In this way it can be determined whether the increased levels of phosphorylated GSK-3 are attributable to high TGF-β levels. On the other hand, the results for phosphorylated GSK-3 were variable and do not appear to be a good read-out, hence it would be better to look for a substitute. The experiments with Rho-inhibitor should be repeated and it might be good to test with higher concentrations. For example, Zohrabian et al. used 30 µM with glioblastoma cells (Zohrabian et al., 2009). On the other hand, for osteoblasts and epithelial cells 10 µM was used (Khatiwala et al., 2009; Chaturvedi et al., 2011). But there possibly is a difference between different cell types and between cells and tissue to react on the inhibitor. Besides, another strain inhibitor has to be tested, for example Latrunculin A. This inhibitor prevents constriction by binding the actin monomers by which they cannot polymerize. Because we started with isotonic settings and the counterbalance was 0.5 gram, the control had to be 0.5 grams. Consequently, when

we started to use isometric settings, the control was again set on 0.5 grams of tension. But for future experiments, when only isometric settings are used, the control has to be as near as possible to 0 gram. Because when you look at the strips during the application of 0.5 grams of tension, it is a large stretch. As for the rest of the weights, mainly 5 grams and 10 grams, they are probably not physiological. Maybe it would be wiser to test with tensions up to, for example, 3 grams. And when the chronic experiment would be repeated, we would suggest to test the strips for contractile proteins after about 24 hours.

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