Toll-like Receptor 4 Signaling in Ventilator-induced Diaphragm Atrophy

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ABSTRACT

Background: Mechanical ventilation induces diaphragm muscle atrophy, which plays a key role in difficult weaning from mechanical ventilation. The signaling pathways involved in ventilator-induced diaphragm atrophy are poorly understood. The current study investigated the role of Toll-like receptor 4 signaling in the development of ventilator-induced diaphragm atrophy.

Methods: Unventilated animals were selected for control: wild-type (n = 6) and Toll-like receptor 4 deficient mice (n = 6). Mechanical ventilation (8 h): wild-type (n = 8) and Toll-like receptor 4 deficient (n = 7) mice.

Myosin heavy chain content, proinflammatory cytokines, proteolytic activity of the ubiquitin-proteasome pathway, caspase-3 activity, and autophagy were measured in the diaphragm.

Results: Mechanical ventilation reduced myosin content by approximately 50% in diaphragms of wild-type mice (*P* less than 0.05). In contrast, ventilation of Toll-like receptor 4 deficient mice did not significantly affect diaphragm myosin content. Likewise, mechanical ventilation significantly increased interleukin-6 and keratinocyte-derived chemokine in the diaphragm of wild-type mice, but not in ventilated Toll-like receptor 4 deficient mice. Mechanical ventilation increased diaphragmatic muscle atrophy factor box transcription in both wild-type and Toll-like receptor 4 deficient mice. Other components of the ubiquitin-proteasome pathway and caspase-3 activity were not affected by ventilation of either wild-type mice or Toll-like receptor 4 deficient mice. Mechanical ventilation induced autophagy in diaphragms of ventilated wild-type mice, but not Toll-like receptor 4 deficient mice.

Conclusion: Toll-like receptor 4 signaling plays an important role in the development of ventilator-induced dia-

What We Already Know about This Topic

- Mechanisms of ventilator-induced diaphragm dysfunction are poorly understood. Toll-like receptor 4 is involved in the inflammatory response after mechanical ventilation.

What This Article Tells Us That Is New

- Eight-hour mechanical ventilation in mice induced diaphragm atrophy in control mice but not in Toll-like receptor knockout mice.
- Toll-like receptor 4 signaling results in diaphragm atrophy most likely through increased expression of cytokines and activation of lysosomal autophagy.

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phragm atrophy, most likely through increased expression of cytokines and activation of lysosomal autophagy.

INVASIVE mechanical ventilation is a life-saving intervention in patients with acute respiratory failure. However, it is well known that mechanical ventilation is associated with important adverse events. For instance, studies in both rodents and humans have shown that controlled mechanical ventilation results in atrophy and weakness of the respiratory muscles.[1,2] This is an important clinical problem, as inspiratory muscle weakness plays a prominent role in patients difficult to wean from mechanical ventilation.[3]

The molecular pathways involved in the development of respiratory muscle atrophy during mechanical ventilation are incompletely understood. Increased muscle protein breakdown as well as reduced muscle protein synthesis have been associated with diaphragm atrophy induced by mechanical ventilation.[1,2,4,5] The upstream pathways that induce this imbalance in muscle protein turnover are currently unclear. Cytokines are well-known modulators of muscle protein turnover[6] and are involved in the development of respiratory muscle atrophy under inflammatory conditions.[7] During the past decade it has been established that mechanical ventilation is able to provoke a local and systemic inflammatory response.[8,9] Yet, whether mechanical ventilation induces an inflammatory response in the diaphragm has never been investigated. Toll-like receptors (TLR) are crucial receptors in the initiation of an inflammatory response. Different types of TLRs recognize specific ligands, which include microbial components, but also proteins released from damaged tissue ('alarmins').[10] Recent research demonstrated that the pulmonary inflammatory response to mechanical ventilation partly depends on TLR4 signaling.[11,12] TLR4 is also expressed on muscle tissue, including the diaphragm.[13] Accordingly, administration of the TLR4-specific ligand lipopolysaccharide to rodents elicits an up-regulation of proinflammatory genes in the diaphragm[14] and reduces diaphragm strength.[15] Whether TLR4 plays a role in ventilator-induced diaphragm atrophy is unknown, but may be of potential interest as TLR4 antagonists are available for use in humans.

Therefore, the first aim of the current study was to investigate whether mechanical ventilation-induced diaphragm atrophy is associated with an inflammatory response in the diaphragm. The second aim was to establish whether ventilator-induced diaphragm atrophy and inflammation depend on TLR4 signaling.

Materials and Methods

Animals

Experiments were carried out in male C57BL/6 mice (n = 14) aged 21 ± 0.7 weeks, body weight 27 ± 0.5 g (Charles River, Sulzfeld, Germany) and male TLR4 knockout (KO) mice (n = 13) aged 20 ± 0.6 weeks, weighing 31 ± 0.7 g (C57BL/6 background). All TLR4 KO mice were extensively backcrossed (at least 10 times) and were a gift from Professor Shizuo Akira, M.D., Ph.D. (Osaka University, Osaka, Japan). Animals were fed *ad libitum*.

To determine the role of TLR4 in ventilator-induced diaphragm atrophy, four groups of mice were studied: control wild-type (cWT; n = 6), mechanically ventilated wild-type (mvWT; n = 8), control TLR4 KO (cTLR4 KO; n = 6) and mechanically ventilated TLR4 KO (mvTLR KO; n = 7).

All experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care.

Controlled Mechanical Ventilation

Mice selected for ventilation were anesthetized and mechanically ventilated as described previously with minor modifications.[11] Briefly, mice were ventilated with a tidal volume of 8 ml/kg body weight, respiratory rate of 170/min, positive end-expiratory pressure of 1.5 cm H_2O, and inspired oxygen fraction of 0.45.

A sterile catheter was inserted in the carotid artery for continuous blood pressure monitoring. The cWT and cTLR4 KO mice were anesthetized and sacrificed without being mechanically ventilated as described previously.[9] Previous investigations from our laboratory have established that this experimental setting is free from contamination with lipopolysaccharide.[11]

Tissue Collection

After 8 h of mechanical ventilation (mechanical ventilation groups) or immediately after anesthesia (controls), mice were exsanguinated and a combined thoracotomy and laparotomy was performed. Left and right hemidiaphragm tissue was rinsed with the left part quickly frozen in liquid nitrogen and stored at −80°C for later biochemical analysis and the right hemidiaphragm submerged in cooled Krebs solution at pH 7.4 for single fiber isolation.

Methods of Measurement

Cytokines in Diaphragm and Plasma

Amounts of tumor necrosis factor-α, interleukin (IL)-1α, IL-1β, IL-6, and keratinocyte-derived chemokine (KC) in the diaphragm and IL-1β, IL-6, and KC in plasma were analyzed by enzyme-linked immunosorbent assay as published previously.[11] To determine cytokine concentration in the diaphragm, the muscle was homogenized in 100 volumes of ice-cold buffer, pH 7.2 (10 mM Tris/maleate, 3 mM EGTA, 275 mM sucrose, 0,1 mM dithiothreitol, 2 mg/ml leupeptine, 2 mg/ml aprotinin, 10 mg/l pepstatin A, 0.57 mM phenylmethylsulphonylfluoride), three cycles of freezing and thawing, and centrifuged at 17,000 G at 4°C for 30 min. Lower detection limits were 40 pg/ml for IL-1α and IL-1β; 32 pg/ml for tumor necrosis factor-α; 160 pg/ml for IL-6 and for KC.

Single Fiber Myosin Heavy Chain Content

As described previously, the content of myosin heavy chain was determined in isolated single fibers[16] with minor modifications. In short, after isolation, the length of a single fiber was measured by making a microscopic image on top of a metal raster. The single fiber length was analyzed using an image analysis system (ImageJ version 1.42, National Institutes of Health, Bethesda, MD). Subsequently, fibers were analyzed for myosin heavy-chain content by SDS–polyacrylamide gel electrophoresis.

Ubiquitinated Myosin Heavy Chain Content

Ubiquitinated myosin and total myosin were determined as described before.[17]

Diaphragm samples were homogenized in 100 volumes of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol, 0.5% SDS, and protease inhibitor cocktail (Sigma Chemical Company, Saint Louis, MO), boiled and centrifuged.

Soluble proteins were subjected to routine Western blotting. Antiubiquitin antibodies (PW8805, Biomol, Plymouth Meeting, PA) and antimyosine (my-32, Sigma Chemical Company) were used to stain ubiquitinated myosin and total myosin, *i.e.*, both ubiquitinated and not ubiquitinated myosin. Secondary goat antimouse-polyvalent immunoglobulin peroxidase conjugate (A0412, Sigma Chemical Company) and ECL kit (GE Healthcare, Buckinghamshire, United Kingdom) were applied for detection and analysis of ubiquitinated protein bands (optical densitometry software from Syngene, Cambridge, United Kingdom). Goat antimouse IRDye 800CW (LI-COR, Lincoln, NE) and subsequent Odyssey scan and Odyssey application software version 2.1 (LI-COR) were used for analysis of the myosin signal. For each lane the ratio of optical densities of ubiquitinated myosin per total myosin was calculated.

Ubiquitin-Proteasome Pathway and Caspase-3 Activity

To assess involvement of proteolysis we measured 20S proteasome proteolytic activity and caspase-3 activity as described previously.[17] The proteolytic activity of isolated 20S proteasomes was determined by measuring the generation of the fluorogenic cleavage product methylcoumarylamide from the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY) by spectrophotometry.

The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product methylcoumarylamide from the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by spectrophotometry. In addition, we measured the presence of 14 kD actin, a specific breakdown product of caspase-3[18] by Western blotting and antiactin antibody (A2066, Sigma Chemical Company).

To establish whether regulation of the ubiquitin-proteasome pathway was modulated, muscle RING-finger protein-1 (MuRF-1) protein content was assessed by Western blotting using anti-MuRF-1 antibodies (Ab77577, Abcam, Cambridge, United Kingdom) and messenger RNA concentrations of MuRF-1 and muscle atrophy factor box (MAFbx) were determined by quantitative polymerase chain reaction.[17] Concentrations of MAFbx and MuRF-1 messenger RNA were normalized to glyceraldehyde-3-phosphate dehydrogenase messenger RNA. Forward and reverse oligonucleotides used were as follows:

MAFbx, 5′-GACTGGACTTCTCGACTGCC-3′ and 5′-TCAGCCTCTGCATGATGTTC-3′, MuRF-1, 5′-CAACCTGTGCCGCAAGTG-3′ and 5′-CAACCTCGTGCCTACAAGATG-3′; Glyceraldehyde-3-phosphate dehydrogenase, 5′-TGATGGGTGTGAACCACGAG-3′ and 5′-GGGCCATCCACAGTCTTCTG-3′.

Induction of Autophagy

To study the role of lysosomal autophagy, the content of autophagy marker light chain 3B-II[19] (LC3B-II) was measured using standard Western blotting as described previously[20] using a specific antibody against LC3B 2775 (Cell Signaling Technology, Danvers, MA). Optical density of LC3B-II bands on blot were quantified using Odyssey scan and Odyssey application software version 2.1 (LI-COR).

Statistical Analysis

A two-sided unpaired Student *t* test was performed to evaluate the statistical significance of differences for myosin heavy chain and LC3B-II between cWT and mvWT animals, between cTLR4 KO and mvTLR4 KO animals.

Differences between the groups regarding cytokines, MAFbx, MuRF-1 messenger RNA, MuRF-1 and actin protein, caspase-3 activity were analyzed with one-way analysis of variance. Student-Newman-Keuls *post hoc* testing was used to test the probability level of differences between cWT and mvWT animals, between cTLR4 KO and mvTLR4 KO animals, between cTLR4 KO and cWT and between mvWT and mvTLR4 KO animals. For statistical analysis of cytokine measurements the value of the detection limit was used for samples that did not reach the detection limit. GraphPad Prism was used to conduct statistical analysis (GraphPad Software Inc., San Diego, CA). A probability level of P less than 0.05 was considered significant. All data, except plasma cytokines, are presented as mean ± SE. Plasma cytokines were presented as median (interquartile range, IQR) and mean.

Results

Although initially mean arterial blood pressure decreased in both groups, most likely resulting from anesthesia (fig. 1), hemodynamics stabilized thereafter. Blood gas analysis after 8 h of mechanical ventilation showed that Pa_{O_2}, Pa_{CO_2}, bicarbonate, and alveolar-arterial gradient were not significantly different between both ventilated groups (table 1). The mvWT mice were mildly acidotic after 8 h of mechanical ventilation.

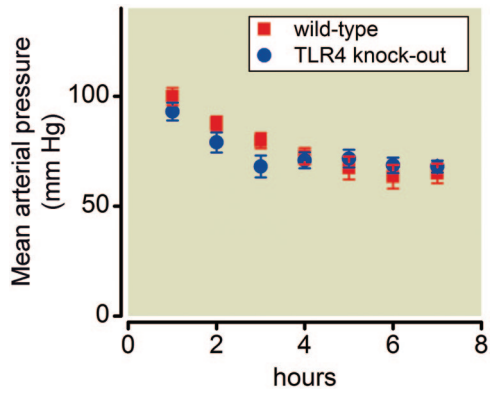

Fig. 1. Mean arterial pressure during mechanical ventilation. TLR4 = Toll-like receptor 4.

Cytokines in Diaphragm

Mechanical ventilation significantly increased concentrations of IL-6 (cWT 230 ± 26 pg/mg *vs.* mvWT 356 ± 37 pg/mg) by approximately 55% (fig. 2A, *P* less than 0.02) and KC (cWT 87 ± 28 pg/mg *vs.* mvWT 183 ± 22 pg/mg) by approximately 110% (fig. 2B, *P* less than 0.002) in the diaphragm of WT mice. Although concentrations of IL-1β (cWT 49 ± 11 pg/mg *vs.* mvWT 67 ± 10 pg/mg) were approximately 37% higher in the diaphragm of mechanically ventilated WT mice compared with unventilated WT mice, this difference did not reach statistical significance (fig. 2C). A similar trend regarding elevation of cytokine concentrations was observed in the diaphragm of mvTLR4 KO mice. Yet, KO of TLR4 clearly dampened the inflammatory response in the diaphragm upon mechanical ventilation, because none of the cytokine protein concentrations in the diaphragm were significantly different between ventilated and unventilated TLR4 KO mice. KC and IL-6 concentrations in the diaphragm were significantly lower in ventilated TLR4 KO than in ventilated WT mice (*P* less than 0.05). Diaphragmatic concentrations of IL-1α and tumor necrosis factor-α were not affected after 8 h of mechanical ventilation in either WT or TLR4 KO mice. Deficiency of TLR4 did not affect diaphragm muscle cytokine concentrations in unventilated animals.

Cytokines in Plasma

In ventilated WT mice, plasma concentrations of KC were significantly increased compared with those of unventilated mice (below detection limit for control WT and median 5060 [1440, 10910] pg/ml, mean 5952 pg/ml for ventilated WT; *P* less than 0.02). Although plasma concentrations of IL-1β (below detection limit for control WT and median 40 [40, 252] pg/ml, mean 125 pg/ml for ventilated WT) and IL-6 (below detection limit for control WT and median

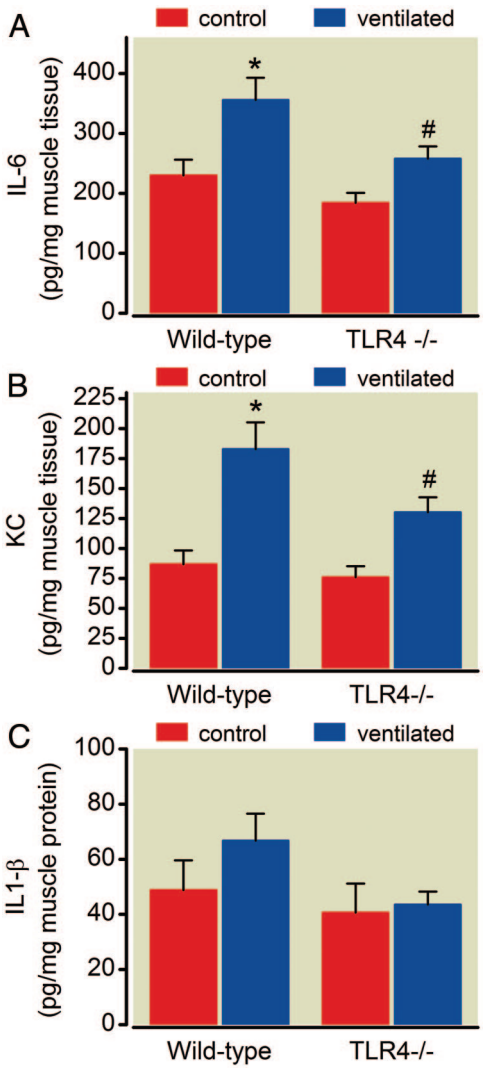

Fig. 2. (*A*) IL-6 cytokine concentration in diaphragm homogenates. **P* less than 0.02 *versus* control wild-type; #*P* less than 0.05 *versus* ventilated wild-type. (*B*) KC cytokine concentration in diaphragm homogenates. **P* less than 0.002 *versus* control WT; #*P* less than 0.05 *versus* ventilated WT. (*C*) IL-1β cytokine concentration in diaphragm homogenates. IL-6 = interleukin-6; IL-1β = interleukin-1β; KC = keratinocyte-derived chemokine; TLR4 = Toll-like receptor 4; WT = wild-type.

Table 1. Arterial Blood Gas and Alveolar-Arterial Gradient after 8 h of Mechanical Ventilation

	pH	Pa_{O_2} (mmHg)	Pa_{CO_2} (mmHg)	HCO_3 (mmol/l)	A-a Gradient (mmHg)	BE (mEq/l)
WT	7.31 ± 0.02*	203 ± 23	26 ± 3.6	16 ± 1	79 ± 21	−11.5 ± 1.4
TLR4 KO	7.40 ± 0.02	220 ± 20	24 ± 1.4	18 ± 1	64 ± 20	−8.0 ± 1.1

* $P < 0.05$ *vs.* ventilated TLR4 KO; values are mean ± SEM.

A-a gradient = Alveolar arterial gradient; BE = base excess; HCO_3 = bicarbonate; Pa_{CO_2} = arterial carbon dioxide tension; Pa_{O_2} = arterial oxygen tension; TLR4 KO = Toll-like receptor 4 knockout; WT = wild-type.

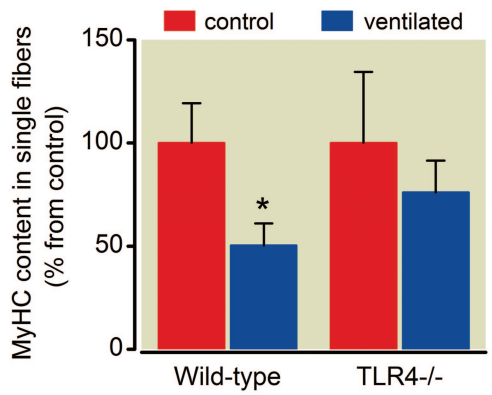

Fig. 3. Myosin heavy chain (MyHC) content in diaphragm single fibers from control wild-type (cWT) and Toll-like receptor 4 knockout (TLR4 KO), and ventilated WT and TLR4 KO mice. **P* less than 0.05 *versus* control WT. Data are represented as % from cWT for mechanically ventilated (mv) WT and as % from cTLR4 KO for mvTLR4 KO.

4130 [1295, 15575] pg/ml, mean 7574 pg/ml for ventilated WT) were higher in ventilated mice than in control mice, these differences did not reach statistical significance. Similarly, but also statistically not significant, mechanical ventilation of TLR4 KO mice led to increased plasma concentrations of KC (below detection limit for control TLR4 KO and median 2020 [1090, 2685] pg/ml, mean 1914 pg/ml for ventilated TLR4 KO) and IL-6 (below detection limit for control TLR4 KO and median 2810 [985, 3505] pg/ml, mean 2358 pg/ml for ventilated TLR4 KO). Plasma IL-1β was not different between control and ventilated TLR4 KO (both TLR4 KO groups were below detection limit). Plasma KC concentrations, but not IL-6 and IL-1β, were significantly higher in mvWT compared with those of mvTLR4 KO mice (*P* less than 0.05).

Myosin Heavy Chain Content

In 8 h ventilated WT mice myosin heavy chain content in diaphragm muscle single fibers was significantly reduced by approximately 50% (fig. 3; *P* less than 0.05). In contrast, myosin heavy chain content in diaphragm fibers from TLR4 KO mice was not significantly reduced by mechanical ventilation. Myosin heavy chain content was not different between unventilated WT and TLR4 KO mice, neither between ventilated WT and ventilated TLR4 KO mice.

Ubiquitin-Proteasome Pathway

Mechanical ventilation significantly enhanced transcription of MAFbx in both WT and TLR4 KO diaphragm (*P* less than 0.001; fig. 4A). MuRF-1 transcription levels and protein content in the diaphragm were not different after 8 h of mechanical ventilation of either WT or KO mice (fig. 4B, 4C, and 4D). TLR4-deficiency did not affect MAFbx nor MuRF-1 expression in unventilated animals (fig. 4A, 4B, and 4C).

The ratio of ubiquitinated myosin heavy chain over total myosin heavy chain was not significantly affected by mechanical ventilation nor by TLR4 KO, and levels between cWT and cTLR4 KO were not different (fig. 5).

Proteasome activity in the diaphragm was not affected by mechanical ventilation nor by TLR4 deficiency (fig. 6).

Caspase-3 Activity

No differences in diaphragm caspase-3 activity were observed between groups (fig. 7A). To support this observation we determined amounts of 14 kD actin fragments, a product of caspase-3 activation. Indeed, no 14 kD fragment was found in diaphragm homogenates of any group, supporting the absence of caspase-3 activation (fig. 7B).

Autophagy

Content of the autophagy marker LC3B-II was significantly increased by approximately 31% in diaphragm muscle of 8 h mechanical ventilated WT mice compared with control WT mice (fig. 8). In contrast, diaphragmatic LC3B-II content in ventilated TLR4 KO mice, was not different from that in unventilated TLR4 KO. LC3B-II was not different between unventilated WT and TLR4 KO mice, neither between ventilated WT and TLR4 KO mice.

Discussion

The current study investigated the signaling pathways of ventilator-induced diaphragm muscle atrophy in particular related to TLR4. The main new findings are that (1) mechanical ventilation-induced diaphragm muscle atrophy is associated with increased expression of cytokines in the diaphragm and (2) TLR4 signaling is involved in myosin loss, the inflammatory response and lysosomal autophagy in the diaphragm during controlled mechanical ventilation. These findings are of potential clinical interest, as diaphragm atrophy plays a prominent role in weaning from mechanical ventilation.

Inflammatory Response in Diaphragm upon Mechanical Ventilation

This is the first study to examine the effects of mechanical ventilation on inflammatory responses in the diaphragm. We found that mechanical ventilation increases diaphragm concentrations of IL-6, KC, and to a lesser extent IL-1β. It has previously been proposed that increased expression of cytokines induces skeletal muscle atrophy. For example, overexpression of IL-6 in transgenic mice engenders profound skeletal muscle atrophy, which can be completely blocked by administration of an IL-6 antagonist.[21] Subcutaneous administration of IL-6 also results in skeletal muscle atrophy in rats.[22] Noticeably, dose-response experiments showed diaphragm weight loss at concentrations where no peripheral muscle weight loss was detected, suggesting that the diaphragm is more sensitive to the atrophic effects of IL-6 than peripheral muscles. Furthermore, local infusion of IL-6 into the tibialis anterior in rats causes a preferential loss of myofibrillar proteins, such as myosin.[23] A recent study from our laboratory showed that IL-6 in plasma of patients with sepsis

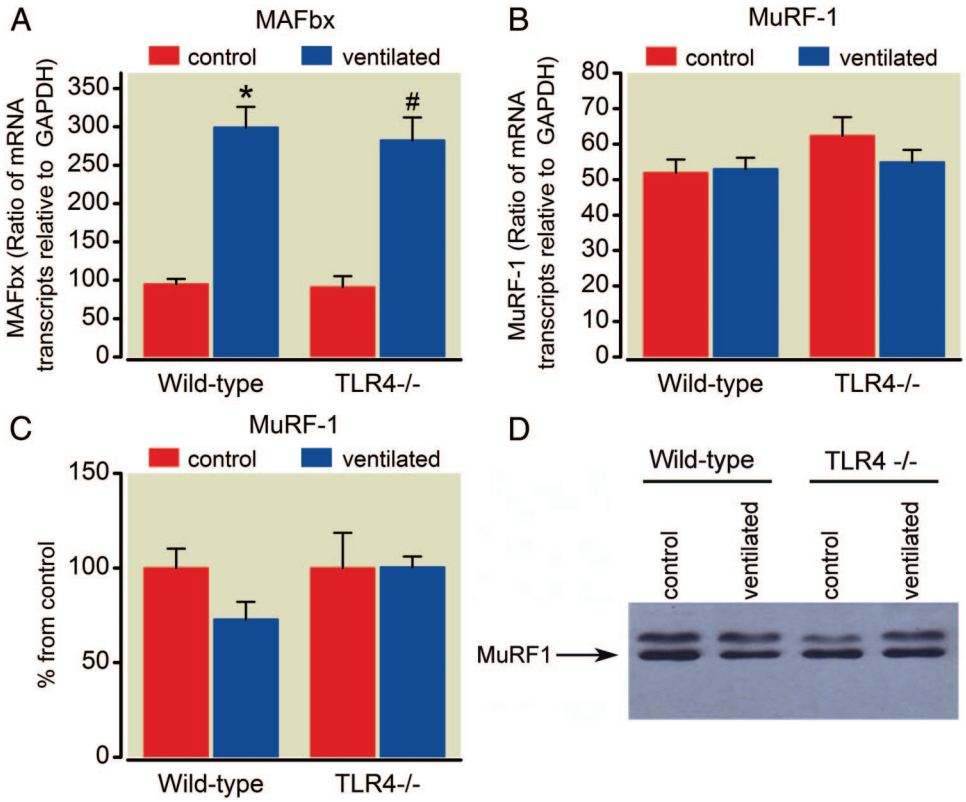

Fig. 4. E3-ligase expression concentrations in the diaphragm of control WT and TLR4 KO and ventilated WT and TLR4 KO mice (ratio of mRNA transcripts relative to GAPDH). (*A*) MAFbx messenger RNA (mRNA) concentratinos. **P* less than 0.001 *versus* control WT; #*P* less than 0.001 *versus* control TLR4 KO. (*B*) MuRF-1 mRNA. (*C*) MuRF-1 protein concentrations. Data are represented as % from control wild-type (cWT) for mechanically ventilated wild-type (mvWT) and as % from cTLR4 KO for mvTLR4 KO. (*D*) Representative Western blot stained against MuRF-1. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; KO = knockout; MAFbx = muscle atrophy factor box; MuRF-1 = muscle RING-finger protein-1; TLR4 = Toll-like Receptor 4; WT = wild-type.

plays a prominent role in inducing muscle atrophy.[24] In addition to IL-6, we found increased concentrations of KC and IL-1β in the diaphragms of mechanically ventilated mice. As far as we know, no previous studies have investigated the role of KC and IL-1β on muscle atrophy *in vivo*. Nevertheless, high circulating concentrations of IL-1β have been associated with skeletal muscle wasting.[25] Some evidence for a causative role for IL-1β in skeletal muscle wasting comes from *in vitro* studies. For example, exposure of skeletal

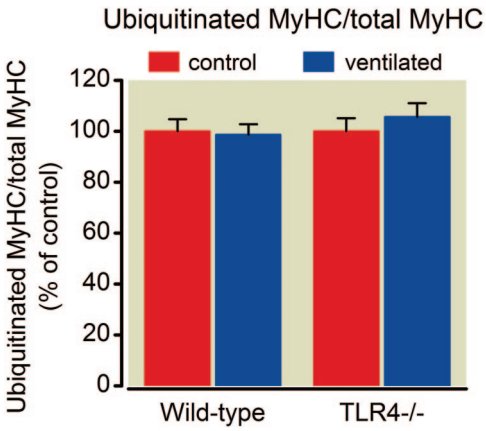

Fig. 5. Ubiquitinated myosin concentrations in diaphragm from control wild-type (cWT) and Toll-like receptor 4 knockout (TLR4 KO) and ventilated WT and TLR4 KO mice. Data are represented as % from cWT for mechanically ventilated (mv) WT and as % from cTLR4 KO for mvTLR4 KO. MyHC = myosin heavy chain; TLR4 = Toll-like receptor 4.

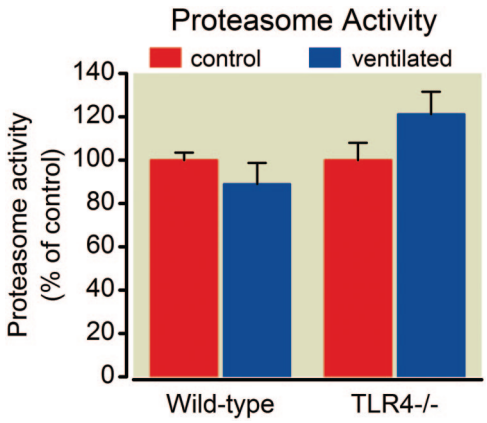

Fig. 6. Proteasome activity in the diaphragm of control wild-type (cWT) and Toll-like receptor 4 knockout (TLR4 KO), and ventilated WT and TLR4 KO mice. Data are represented as % from cWT for mechanically ventilated (mv) WT and as % from cTLR4 KO for mvTLR4 KO.

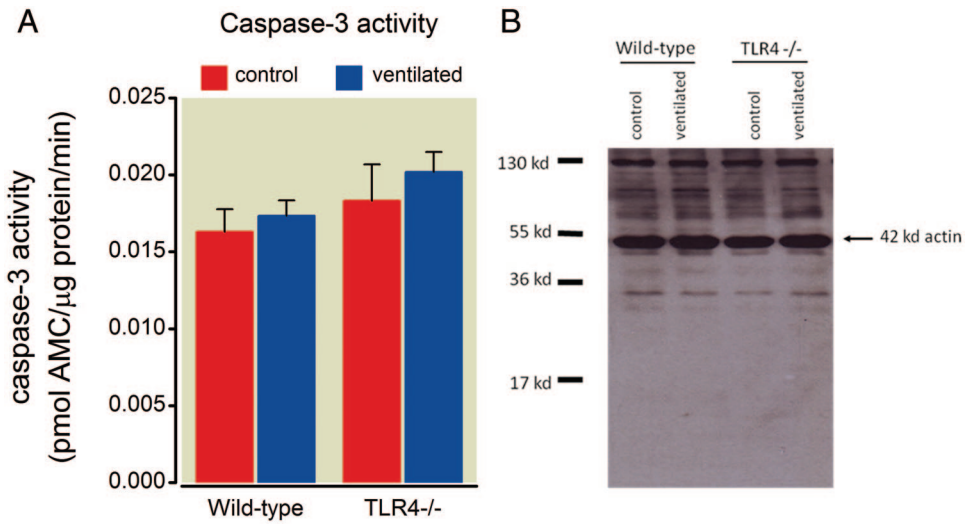

Fig. 7. (*A*) Caspase-3 activity in the diaphragm of control wild-type (WT) and Toll-like receptor 4 knockout (TLR4 KO), and ventilated WT and TLR4 KO mice. (*B*) Representative Western blot stained against actin. AMC = amido-4-methylcoumarin.

myotubes to IL-1β during 48 h results in muscle atrophy.[26] In line with these studies, results from the current study show a relationship between increased expression of cytokines and myosin loss in the diaphragm, *i.e.*, in contrast to WT mice, mechanical ventilation of TLR4-deficient mice did not result in enhanced cytokine expression nor in reduced myosin content. Although the current data support the concept that the increased concentrations of cytokines elicited by mechanical ventilation are associated with myosin loss in the diaphragm, we did not investigate a causal relationship. Future studies should examine whether administration of IL-6 antagonists can prevent the induction of diaphragm atrophy during mechanical ventilation.

Role for TLR4 in Ventilator-induced Diaphragm Atrophy

The current study shows that knocking out the TLR4 gene prevented increased expression of cytokines and loss of myosin in the diaphragm upon mechanical ventilation. This implicates a role for TLR4 signaling in ventilator-induced diaphragm dysfunction. TLRs are wellknown for their role in innate immunity. Each TLR homolog senses a specific set of conserved microbial molecules. The postreceptor signaling of TLRs is very complex,[27] but noteworthy is that activation of TLRs eventually results in the release of inflammatory cytokines, necessary to combat infection. Recent discoveries show that TLRs, in particular TLR4, are expressed in skeletal muscle.[13,28,29] Moreover, several studies have demonstrated that stimulation of TLR4 by lipopolysaccharide increases the expression of cytokines such as IL-6, KC, IL-1β and tumor necrosis factor-α, in skeletal muscles.[13,28,30] The inflammatory response to lipopolysaccharide administration also occurs in the diaphragm, where it is even more vigorous than in limb muscle.[14] In line with these data, the current study shows that TLR4-deficiency attenuated the up-regulation of cytokines in the diaphragm upon mechanical ventilation. Moreover, we found that knocking out the TLR4 gene partially prevented myosin loss in the diaphragm of mechanically ventilated mice. Because myosin plays a central role in muscle contraction, these data indicate that TLR4 signaling is involved in mechanical ventilation-induced diaphragm dysfunction.

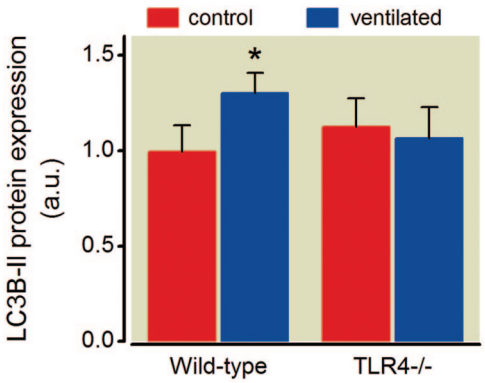

Fig. 8. Light chain 3B-II (LC3B-II) concentration in the diaphragm of control wild-type (WT) and Toll-like receptor 4 knockout (TLR4 KO), and ventilated WT and TLR4 KO mice, data are presented as arbitrary units (a.u.) from Western blot analysis. Mechanical ventilation increases amounts of LC3B-II in diaphragm of WT mice (* *vs.* control WT; $P = 0.05$) and not in TLR4 KO mice.

Although it was not a main objective of this study, our data provide some additional insight into the downstream mechanisms by which TLR4 signaling induces loss of myosin. For instance, considering the well-established effect of increased expression of cytokines on myosin content as described previously, it seems likely that myosin loss in the diaphragm upon mechanically ventilation is caused by TLR4-mediated up-regulation of inflammatory cytokines.

A recent publication by Doyle *et al.* suggested that TLR4 activation might also directly induce myosin loss by the p38 mitogen-activated protein kinase pathway, *i.e.*, independent from actions of cytokines.[20] Myosin loss in that study was

provoked by coordinate downstream activation of the ubiquitin-proteasome and autophagy-lysosome pathways. Interestingly, our data indicate that mechanical ventilation activates autophagy in the diaphragm already after 8 h. This is in line with a recent study in humans, which showed activation of autophagy after prolonged mechanical ventilation.[31] More importantly, our data show that TLR4 plays a prominent role in inducing autophagy during mechanical ventilation, because TLR4 KO mice did not show up-regulation of LC3B-II after 8 h of mechanical ventilation. Remarkably, our data do not support an important role for the ubiquitin-proteasome pathway in ventilator-induced diaphragm atrophy. First, 8 h of mechanical ventilation did not enhance proteasome activity. Second, reduced total myosin content was not accompanied by increased ubiquitination of myosin. In accordance, expression of the E3-ligase MuRF-1, which is known to ubiquitinate myosin,[32] was unaffected by mechanical ventilation. Third, although mechanical ventilation increased MAFbx expression, the protective effects of TLR4 deficiency on myosin content were independent from MAFbx activation. In contrast, some previous studies showed that diaphragm atrophy was associated with activation of the ubiquitin-proteasome pathway in the diaphragm of mechanically ventilated rats[5,33,34] and brain-dead humans.[35] Yet, those studies do not provide unambiguous evidence that this pathway is responsible for the loss of myosin. In contrast with the current study, ubiquitination of myosin was not specifically studied. Our data indicate that 8 h of mechanical ventilation does not increase ubiquitination of myosin. Furthermore, attenuation of mechanical ventilation-induced diaphragm atrophy by antioxidants occurs independent from increased MuRF-1 and MAFbx expression.[36] Caspase-3 is known to cleave the contractile protein actin, which may induce release of myosin from the sarcomere.[18] However, data from the current study do not support a role for caspase-3, as its cleaving activity in the diaphragm was not affected by mechanical ventilation and actin fragments could not be detected. In apparent contrast with our study, previous studies have shown enhanced content of activated caspase-3 in the diaphragm of mechanically ventilated animals[37,38] and humans.[1] However, in those studies cleavage activity of caspase-3 itself was not measured nor were concentrations of contractile proteins determined. With respect to those studies, therefore we do not exclude that the ubiquitin-proteasome pathway and caspase-3 may be activated, in particular after long periods of mechanical ventilation, but in our opinion there is currently no solid evidence that activation of this pathway is responsible for loss of myosin. Our data suggest that increased proteolysis through lysosomal autophagy is involved after 8 h of mechanical ventilation. More importantly, our results show that activation of lysosomal autophagy depends on TLR4 signaling. This is in line with previous data of Doyle *et al.*, who showed that the TLR4 agonist lipopolysaccharide induces lysosomal autophagy in cultured muscle cells.[20]

After 8 h of mechanical ventilation, WT mice exhibited mild metabolic acidosis, despite insignificant difference in Pa_{CO_2} and HCO_3^-. Unfortunately, this acidosis could not be prevented because sequential blood gas analysis is not feasible in mice due to low circulating volume. However, it is unlikely that this mild acidosis explains the differences in myosin and cytokine analysis between groups. Previous studies have demonstrated myosin loss after mechanical ventilation in nonacidotic animals.[37,39]

Endogenous Ligands for TLR4 during Mechanical Ventilation

Questions remain about the nature and origin of the ligands that activate TLR4 during mechanical ventilation. Evidence is accumulating that TLR4 can be activated by nonmicrobial molecules such as endogenous ligands[40] including hyaluronan and heat shock protein 70[41,42] These ligands are released from the lung upon mechanical ventilation with high tidal volumes.[43,44] In the current study we chose to ventilate with relatively low tidal volumes because we wanted to resemble the clinical setting. Nevertheless, a recent study from our laboratory showed that even lowtidal volume mechanical ventilation results in the appearance of TLR4 ligands in the bronchoalveolar lavage fluid.[11] An attractive hypothesis is therefore that TLR4s in the diaphragm are activated by ligands released from the mechanically ventilated lung.

Clinical Implications

Respiratory muscle weakness plays an important role in difficult weaning from mechanical ventilation. Recently, the development of respiratory muscle atrophy in mechanically ventilated humans has been demonstrated.[1] Currently, no proven strategies are available to prevent or reverse ventilator-induced respiratory muscle atrophy. The current study provides a rationale to test the effects of TLR4 antagonists on ventilator-induced muscle atrophy. Interestingly, a phase 2 trial with the TLR4 antagonist eritoran tetrasodium (Eisai Research Institute of Boston, Andover, MA), showed a trend toward lower mortality rate in severe septic patients.[45] However, this article did not study the effects of eritoran on duration of mechanical ventilation or respiratory muscle function. Ideally, TLR4 should be selectively blocked in the diaphragm muscle without compromising the innate immune system.

In conclusion, controlled mechanical ventilation induces loss of myosin, autophagy and an increased expression of cytokines in the diaphragm muscle. The current study demonstrates that TLR4 signaling is involved in eliciting this response. These findings may prove helpful in the development of strategies to attenuate ventilator-induced diaphragm dysfunction.

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