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Original Research

Developmental regulation of molecular signalling in fetal and neonatal diaphragm protein metabolism

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Abstract

Structural and functional immaturity of the preterm diaphragm predisposes the preterm baby to respiratory muscle weakness and consequent impaired efficiency of spontaneous respiration, potentially necessitating mechanical respiratory support. The ontogeny of several proteolytic genes (calpain, caspase-3, MAFbx and MuRF-1) changes dynamically with gestational and early postnatal development. We aimed to define the molecular signal cascades and triggers responsible for the dynamic changes in the proteolytic pathways during *in utero* and early postnatal development. Costal diaphragm was obtained immediately following euthanasia of fetal and newborn lambs from 75 to 200 days postconceptional age (term = 150 days). Gene expression of insulin-like growth factor 1 (IGF-1), tumour necrosis factor α (TNF-α) and myostatin decreased steadily *in utero* from 75 to 145 days ($P < 0.05$) and the transcripts increased again after birth except of myostatin. Rapid activation of the fork-head transcriptional factors of the O class (FOXO1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways was observed at 24 h of postnatal age. Diaphragm reactive oxygen species (ROS) production increased over 29-fold at 24 h postnatal age, compared with the 145 days fetus ($P < 0.01$). Local (diaphragmatic) ROS accumulation occurred earlier and was more predominant than systemic (plasma) ROS. There were positive correlations between signalling transduction molecules (FOXO1 and NF-κB) and antioxidant gene expression (superoxide dismutase and glutathione peroxidase 1). We conclude that anabolic (IGF-1) and catabolic (TNF-α and myostatin) factors have a similar developmental pattern with a decreasing trend toward full term. This may reflect *in utero* integration of cellular events into low protein metabolism as the diaphragm matures in late gestation. On initiation of spontaneous breathing, ROS accumulated and potentially activated cascade of FOXO and NF-κB signal transduction. The finding provides new insights into developmental regulation of protein metabolism within development. The implication of these postnatal events for diaphragm adaptation to the *ex utero* environment needs further investigation.

Keywords: Development, ontogeny, diaphragm, infant, preterm, molecular signalling, protein synthesis, proteolysis

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Introduction

The diaphragm functions as the major respiratory pump for the establishment of spontaneous breathing. The phenotypic and functional integrity of this respiratory muscle largely depends on the finely tuned maintenance of muscle mass. Growth factors, hormones, cytokines, nutrients and mechanical loading are all environmental triggers capable of activating cellular signalling pathways which can push the balance in favour of protein synthesis or degradation leading to a gain or loss, respectively, of muscle mass.[1–3] Functional impairment of the immature diaphragm on commencement of spontaneous breathing after birth may contribute to the increased propensity of the preterm infant to development of neonatal respiratory distress syndrome. Understanding the ontogeny of diaphragm protein metabolism may provide insight into mechanisms that may contribute to postnatal respiratory muscle weakness. Further, elucidation of normal protein synthesis and proteolysis during *in utero* and early postnatal development is paramount to understanding how diaphragm development in the preterm subject may be additionally compromised by antenatal and early postnatal exposures such as impaired nutrition, steroids, infection and postnatal mechanical ventilation, and identify suitable targets for therapeutic interventions that promote improved respiratory muscle strength.

Loss of muscle protein (muscle atrophy) is a consistent feature of diaphragmatic dysfunction in human and

animal studies. The muscle loss is attributed to activated proteolytic systems leading to accelerated protein degradation. Among the proteolytic systems, the ubiquitin-proteasome pathway (UPP) is a principal regulator of catabolic processes for selective degradation of intracellular proteins. This pathway is constitutively active in muscle fibres and modulates both intracellular signalling events and normal protein metabolism.[4] Two muscle-specific ubiquitin ligases are required for muscle fibre atrophy: MAFbx (atrogin-1) and MuRF-1 (muscle ring finger-1),[5] as evidenced by the marked reduction of muscle wasting induced by denervation in *MAFbx* and *MuRF-1* knockout mice.[6]

The molecular signalling governing the process of protein degradation during catabolism is not fully understood. An intracellular signalling hierarchy of insulin-like growth factor 1 (IGF-1), phosphatidylinositol 3-kinases (PI3K), Akt and Fork-head transcriptional factors of the O class (FOXO) is established for muscle atrophy.[7–9] Following the atrophic signalling trigger, the IGF-1/Pl3K/Akt pathway is deactivated leading to dephosphorylated FOXO, which localizes to the nucleus to upregulate the mRNA expression of the UPP ligases (*MAFbx* and *MuRF-1*).[7,8] IGF-1/Pl3/Akt has been well characterized as a protein synthesis pathway with the downstream targets of mammalian target of rapamycin (mTOR) that activates 70-kDa ribosomal protein S6 kinase (S6K1) and 4 E-BP1 (eukaryotic initiation factor) and then controls translation and protein synthesis.[10,11] Thus, the IGF-1/Pl3K/Akt signalling pathway appears to function as a switch between protein synthesis and degradation.

A large body of research also highlights the role of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling in governing muscle wasting in catabolic states.[12,13] Activation of the NF-κB signalling cascade results in a complete degradation of IκB, a repressor of NF-κB, allowing the translocation of NF-κB to the nucleus, where it induces transcription of *MuRF-1* genes.[1] There is clear evidence that both FOXO and NF-κB activation alone are sufficient to cause muscle fibre atrophy.[8,13] However, they have a summative effect on atrophy signalling, as the inhibition of both pathways abolished disuse-induced muscle fibre atrophy to a greater extent than blocking either pathway alone.[14]

The activation of these catalytic signalling pathways is reported in a variety of disease states including fasting, systemic diseases, such as cancer and diabetes, and disuse, or denervation of specific muscle groups, although varied in the different atrophy conditions and regulated by unique triggering molecules in specific cases.[15,16] We hypothesized that FOXO and NF-κB pathways and protein turnover regulators (e.g. IGF-1, TNF-α and myostatin) also play critical roles in diaphragm muscle maturation during fetal and postnatal development, considering the significant implication of these signalling molecules in physiological and pathological conditions on adult. Recently, we established clearly that the ontogeny of the expression of several proteolytic and antioxidant genes changed dynamically with *in utero* and early postnatal development: calpain and caspase-3 pathways decreased progressively towards late gestation while UPP was activated in the early postnatal period and the antioxidant genes increased steadily throughout fetal life to early postnatal life.[17] We further anticipated that the selected molecular pathways and triggers accounted for the altered proteolytic signalling and antioxidant capacity. Thus, the objective of the study is to define ontogenic change of molecular signalling pathways from mid-fetal to early postnatal ovine diaphragm muscle, providing insights into molecular regulation of diaphragm protein metabolism within development.

Materials and methods

Animals and experimental design

Studies were approved by the Animal Ethics Committee of the University of Western Australia. A total of 38 fetal lambs were delivered from sedated (10 mg/kg ketamine, Parnell Labs; 0.02 mg/kg medetomidine, Pfizer Animal Health, NSW, Australia) date mated singleton naïve Merino ewes via caesarean section after spinal regional anaesthetic block (2% lignocaine, 3 mL, 60 mg) at 75 days ($n=3$), 100 days ($n=8$), 128 days ($n=12$) and 145 days ($n=15$) gestation (term = 150 days). The 15 lambs delivered at near full term (145 days) were allocated to either immediate study ($n=8$), or study after spontaneous breathing for 24 h ($n=7$). Ewes and their lambs were euthanized at delivery (sodium pentobarbitone, 100 mg/kg, Valabarb®, Pitman-Moore, Australia). All lambs were exsanguinated prior to postmortem. An additional group of seven singleton ewes delivered spontaneously, and their offspring ($n=7$) were studied at seven weeks postnatal age (~200 days postconceptional age). Blood and costal diaphragm were sampled immediately before and after lamb euthanasia, respectively.

Fractionation of cellular homogenates

Cytosolic and nuclear protein fractions were prepared from costal segments of the diaphragm using NE-PER® nuclear and cytoplasmic extraction reagents (CER) with inclusion of halt protease inhibitor cocktail (Thermo Scientific, MA, USA). Briefly, 20 mg muscle was washed with phosphate-buffered saline (PBS) and homogenized in CER I. After addition of CER II, the cytoplasmic extract was collected by centrifuging at $16{,}000 \times g$ for 5 min at 4°C. The remaining nuclear pellets were resuspended in nuclear extraction reagent, continually vortexed for 15 s every 10 min, for a total of 40 min at 4°C. The supernatants were obtained as cytosol-free nuclear protein extracts by centrifugation at $16{,}000 \times g$ for 10 min at 4°C and stored at −80°C for Western blotting.

Western blot analysis

Cytosolic and nuclear protein extracts were assayed using the Bradford method (Sigma, Sydney, Australia). One hundred microlitres of protein from each fraction were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (30 V for overnight at 4°C). After blocking with PBS containing 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Carlsbad, CA, USA) against Akt, phosphorylated Akt (Ser473), FOXO1, phosphorylated FOXO1

(Ser256), NF-κB p65 and α-Tubulin for 2 h at room temperature and used in 1 : 1000 dilutions. Bound antibodies were detected with anti-rabbit immunoglobulin conjugated with horseradish peroxidase (1 : 2000 dilution) for 1 h. After adding a chemiluminescent substrate (Thermo Scientific), immunoreactive protein signals were detected and quantified by computerized image analysis (ImageQuant™ 350, GE Healthcare). The values for each protein were standardized with the control sample and then normalized into α-tubulin abundance.

IGF-1 and TNF-α ELISA

Plasma insulin growth factor 1 (IGF-1) and TNF-α concentrations were measured using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits from Immunodiagnostic Systems Ltd (Fountain Hills, AZ, USA) and R&D Systems, Inc (Minneapolis, MN, USA), respectively.

RNA extraction and reverse transcription

Left diaphragm was homogenized and total RNA was purified using the RNeasy Mini Kit and DNase I digestion (Qiagen Pty Ltd., Doncaster, Australia). One microgram of total RNA was used to synthesize cDNA using QuantiTect® Reverse Transcription Kit (Qiagen Pty Ltd). Real-time amplification reactions were conducted using Rotor-Gene SYBR Green polymerase chain reaction (PCR) Kit (Qiagen Pty Ltd.) on Rotor-gene 3000 real-time PCR system (Corbett Life Science, Mortlake, Australia), under the following conditions: 5 min at 95°C, 40 cycles of 5 s at 95°C, 20 s at 60°C and 20 s at 72°C. The primers used were either from the published studies or designed in house on the specific mRNA sequences (Table 1). The fluorescence signal was analysed and normalized against 18 S RNA. Relative expression levels were obtained using $2^{-\Delta\Delta CT}$ method[18] and presented as fold increase relative to the near term group (145 days).

Hydrogen peroxide (H_2O_2) concentration and peroxidase activity

Serum and diaphragm H_2O_2 production and peroxidase activity were determined using Amplex Red (Molecular Probes®, Eugene, OR, USA). In the presence of peroxidase (10 U/mL), the Amplex Red reagent reacted with H_2O_2 in a 1 : 1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin formation was detected at an excitation and emission maxima of approximately 571 and 585 nm, respectively, using a multiwell plate reader fluorometer (BioTek, Winooski, VT, USA). The fluorescence reading value was interpolated into the H_2O_2 concentration with a standard curve. Conversely, peroxidase activity in the samples was determined using 20 mmol/L H_2O_2 and performed on the same procedure described above. The final value was presented as enzyme activity calculated from peroxidase standard curve.

Statistical analysis

Sigmaplot (version 11.0, Systat Software Inc, San Jose, CA, USA) was used for statistical analysis. Differences among multiple groups were assessed using one-way analysis of variance (ANOVA) with *post hoc* analysis. Non-parametric data were analysed using ANOVA on ranks. Pearson correlation index was calculated to determine the association among different variables using multiple regression analysis. $P < 0.05$ was considered statistically significant. Data are presented as mean (SEM) or median (range).

Table 1 Primer sequences designed in the study for real-time PCR.

Gene		Primer sequence (5'–3')	Accession No.	Product size (bp)	Designed by
IGF-1	F	TTGGTGGATGCTCTCCAGTTC	DQ152962	118	[41]
	R	AGCAGCACTCATCCACGATTC			
IGF-1 receptor	F	AAGAACCATGCCTGCAGAAGG	AY162434	105	[42]
	R	GGATTCTCAGGTTCTGGCCATT			
TNF-α	F	ACACCATGAGCACCAAAAGC	EU276079	103	[42]
	R	AGGCACAAGCAACTTCTGGA			
Myostatin	F	TTGCTGGCCCAGTGGATCTGA	NM_001009428	151	This study
	R	TCCAGGCGAAGCTTACTGAGG			
mTOR	F	AAGAGGAGTCTACTCGCTTCT	NM_001145455	151	This study
	R	AATCTTCCAATCCGAGTGGCA			
4 E-BP1	F	GTGCAGCTCCCGCCCGGGGA	NM_004095	123	This study
	R	CACAGGTGAGTTCCGACACTC			
S6K1	F	TGGCCACAGAAGTTGCTGC	DQ223557	132	This study
	R	GCAGGCGAACTCGGCCAT			
FOXO1	F	GCCCAACCAAAGCTTCCCACAC	XM_002691748	140	[43]
	R	TGGACTGCTTCTCTCAGTTCCTGCT			

F: forward; R: reverse.

Results

Systemic response

During *in utero* development, there was a peak increase in serum IGF-1 protein concentration at 100 days of gestational age (GA), with a 2.5-fold increase compared with 75 days of GA, followed by steady decrease in serum IGF-1 from 100 days until full term (145 days). IGF-1 level was significantly higher in the 100-day group than in any other gestational groups ($P < 0.05$). After birth, IGF-1 increased markedly: the IGF-1 level at 24 h postnatal age was significantly higher than that at 75, 125 and 145 days of GA ($P < 0.05$; Figure 1). Serum TNF-α protein was extremely low among the experimental groups, hence a reliable quantification in circulating TNF-α concentration was unobtainable.

Local catabolic and anabolic agents

For both IGF-1 and IGF-1 receptors, gene expression was up-regulated in the 100-day group ($P < 0.05$) approximately 60-and 8-fold change relative to 75 days of GA, respectively. Subsequently, there was gradual decrease of both IGF-1 and IGF-1 receptors with increasing maturation from mid to late gestation (Figure 2a and b). The developmental pattern remained consistent for both gene transcripts, and was similar to that of the systemic IGF-1 response. Both TNF-α and myostatin gene expression decreased steadily throughout development *in utero*: TNF-α and myostatin gene expression were significantly lower at 145 days compared with 100 days of gestation (Figure 2c and d). The overall decline was approximately 7-fold for TNF-α (Figure 2c) and 12-fold for myostatin (Figure 2d). However, at seven weeks of postnatal age, TNF-α mRNA level was markedly elevated (~13-fold) compared with the full-term group, while myostatin gene expression remained at a low level throughout the postnatal period examined.

Content of Akt signalling molecule

There was no difference observed in the protein content of Akt and phosphorylated Akt across all *in utero* and postnatal time points (Figure 3).

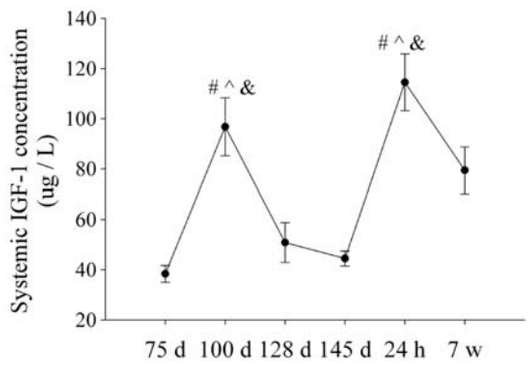

Figure 1 Systemic IGF-1 response: blood samples obtained from fetal lamb at 75, 100, 128 and 145 days (near term) gestation and postnatal ages (150 days of gestation) of 24 h and seven weeks. Values are mean (SEM). # ^ & indicate $P < 0.05$ compared with 75, 128 and 145-day groups, respectively

FOXO pathway

We observed a distinct effect of *in utero* and postnatal development on the cytosolic content of FOXO1 protein ($r^2 = 0.450$, $P < 0.001$). Total cytosolic FOXO1 protein increased with increasing maturation: compared with the 75 days of GA fetal diaphragm, muscle FOXO1 protein increased approximately 16-fold by 145 days of GA ($P < 0.001$) and was 22- and 40-fold higher in lambs after 24 h and seven weeks of the onset of spontaneous breathing, respectively (Figure 4a and d). Similarly, the phosphorylated FOXO1 (Ser 256) from the cytosolic fraction increased during fetal maturation and early postnatal development ($r^2 = 0.174$, $p = 0.007$), although there was no significant difference observed in any experimental groups (Figure 4a and c).

Consistent with its cytosolic counterpart, nuclear FOXO1 protein tended to be higher in late gestation and was significantly increased in the 24 h postnatal group compared with 128-day GA group ($P = 0.007$) with a trend towards lower levels at seven weeks after birth ($P = 0.186$; Figure 4b and e).

NF-κB pathway

Although the cytoplasmic NF-κB tended to increase with advancing gestation and decreased after birth (Figure 5a and c), the response was not significant due to variability of the data. In contrast, nuclear NF-κB content increased markedly at 24 h post-delivery compared with 100 days of GA ($P < 0.05$; Figure 5b and d).

Gene expression of signalling molecules

Gene transcripts of FOXO1, mTOR, 4 E-BP1 and S6K1 were unaltered in response to *in utero* and postnatal development ($P > 0.05$, data not shown).

Local and systemic reactive oxygen species (ROS) production and peroxidase activity

The diaphragm H_2O_2 level peaked at 100 days of GA then decreased throughout the remainder of *in utero* life with the difference becoming significant at 145 days of gestation ($P < 0.05$). H_2O_2 increased rapidly (29-fold) in the first 24 h of postnatal life (Figure 6a), accompanied by a similar increase (13-fold) in the diaphragm peroxidase activity (Figure 6b).

There was a clear time-dependent increase in plasma ROS throughout *in utero* and early postnatal life ($r^2 = 0.526$, $P < 0.00$[illegible]igure 6c). In contrast, while plasma peroxidase activity was high at 75 days (Figure 6d), there was time-related decrease over subsequent time points ($r^2 = 0.241$, $P = 0.006$). Notably, changes in plasma ROS and peroxidase activity were of a lower magnitude than those observed in the diaphragm.

Discussion

We showed previously that the *in utero* and postnatal developmental responses of the proteolytic pathways in the diaphragm included a progressive decrease of calpain and caspase-3 mRNA levels with increasing *in utero* maturation and a dramatic postnatal elevation of calpain, caspase-3,

MAFbx and MuRF-1 mRNA levels.[17] Our current study confirmed that FOXO1 and NF-κB pathways were activated after birth, consistent with our previous observation of elevated expression of atrophy genes. Moreover, ROS was dramatically accumulated in the fetal diaphragm immediately following birth at term, implying that this early postnatal regulatory mechanism was redox-sensitive.

FOXO pathway

FOXO1 is a primary mechanism of accelerated protein degradation by inducing atrophy-related ubiquitin ligase gene expression.[8] Furthermore, the distribution and activation of FOXO1 are likely regulated by insulin-stimulated Akt phosphorylation that sequesters phosphorylated FOXO in the cytoplasm and diminishes nuclear FOXO1 content.[16] We observed that total FOXO1 in the cytosolic fraction increased constantly throughout fetal and neonatal development, consistent with the observed increase in nuclear FOXO1 at 24 h after birth. These observations are consistent with our previous finding of increased proteolytic signalling after birth.[17]

In addition to its role in breakdown of muscle fibres, FOXO is implicated in muscle differentiation, cell cycle arrest, cell death and protection against oxidative stress.[19] Bois and Grosveld[20] reported that FOXO1 regulated the rate of myotube fusion during myogenic differentiation. In proliferating myoblasts, FOXO1 was localized to the cytoplasm, where it remained inactive. Cytosolic localization was presumed to occur through a non-Akt-dependent phosphorylation-mediated nuclear exclusion mechanism.[20] Increased total FOXO1 activity was unlikely to be regulated by the predominant Akt pathway given that the increased cytosolic FOXO1 throughout late fetal and early postnatal development was IGF-1/Akt independent and that Akt content and p-FOXO1 of Ser256 (Akt phosphorylation site) were unchanged over the same time. Such signal cascade was more probably initiated by the differentiation programme rather than catabolic triggers, particularly during *in utero* development. It is worth noting that *FOXO1* gene expression did not exhibit the corresponding response: therefore, the FOXO1 dynamics were not modulated through gene regulation. Post-translational modification such as dephosphorylation at different sites in an Akt-independent manner appeared to be the pivotal regulatory mechanism, resulting in the augmentation of cytosolic FOXO1 protein.

NF-κB pathway

To define whether the NF-κB pathway is involved in developmental regulation of the diaphragm, we investigated the cytosolic and nuclear NF-κB during maturation. Similar to the FOXO shuttling mechanism, NF-κB is normally sequestered into the cytoplasm of non-stimulated cells and consequently must be translocated into the nucleus to function.

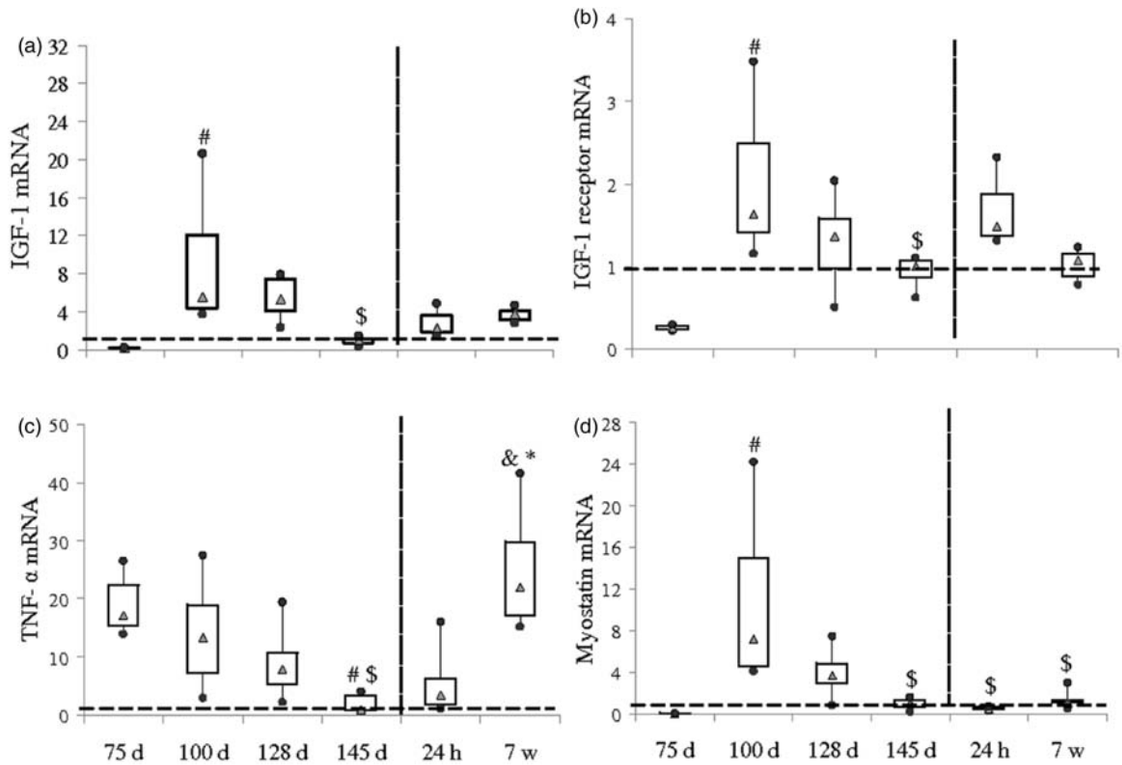

Figure 2 Expression of IGF-1, IGF-1 receptor, TNF-a and myostatin with gestational and postnatal development: graphs show IGF-1 (a), IGF-1 receptor (b), TNF-α (c) and myostatin (d) mRNA expression in diaphragm at 75, 100, 128 and 145 days (near term) gestation and postnatal ages of 24 h and seven weeks. Values are median (25th, 75th percentile). The error bars show 5th and 95th percentile. Vertical dotted line indicates change from fetal to postnatal life. # $ & * indicate $P < 0.05$ compared with 75-day, 100-day, 145-day and 24 h groups, respectively

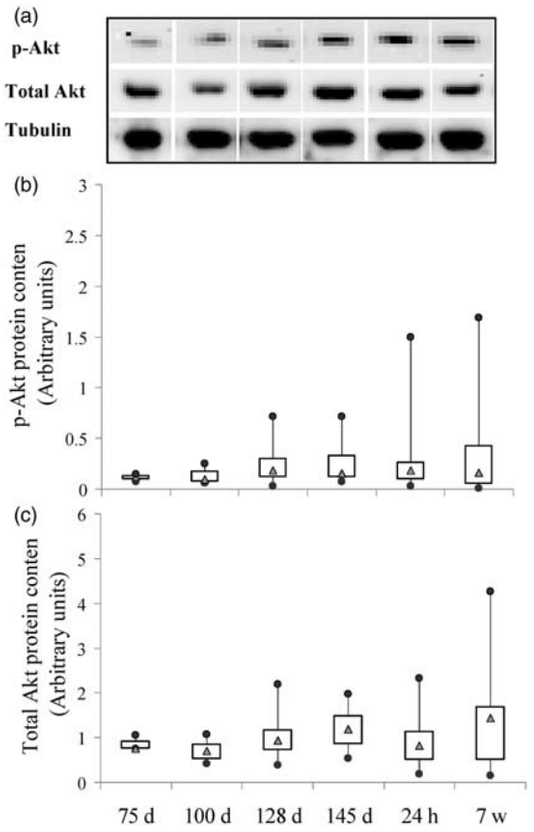

Figure 3 Cytosolic Akt protein expression: Western blots illustrate expression of phosphorylated (p−) Akt and total Akt using representative samples from each group (a). Graphs show absolute phosphorylated Akt (b), and total Akt (c) proteins in costal diaphragm at 75, 100, 128 and 145 days (near term) gestation and postnatal ages of 24 h and seven weeks. Values are median (25th, 75th percentile)

Our data indicated that nuclear NF-κB increased markedly at 24 h post-delivery, concomitant with a decreasing trend in cytoplasmic NF-κB. Therefore, we propose that NF-κB also participates in normal muscle growth and is a contributing factor in the elevated proteolytic pathways around the time of birth.

Regulatory factors in the control of diaphragmatic protein metabolism

To further explore how the signalling transduction was initiated, a number of anabolic and catabolic agents were analysed in concert including IGF-1, IGF-1 receptor, TNF-α, myostatin and ROS. These potential triggers play a causal role in atrophy or are involved in various aspects of cell homeostasis.[2,3,10]

The circulating IGF-1 (a major anabolic factor of protein metabolism) expression underwent three phases of dynamic changes, with a peak increase at 100 days of GA, falling to a low level at full term, before rising again after birth. The local (diaphragmatic muscle) IGF-1 and IGF-1 receptor mRNA expression also showed a similar profile. This pattern is consistent with a previous report that ovine IGF-I mRNA in the fetal muscle samples peak at 85–100 days of gestation,[21] consistent with accelerated growth at early gestation. Interestingly, both systemic and local IGF-1 signalling follow the same tendency as the proteolytic pathways, supporting our previous insight that the signalling associated with protein synthesis and degradation occurs in parallel to maintain protein homeokinesis across the different developmental stages.[17] However, we did not observe a corresponding response in the downstream Akt molecule, suggesting that IGF-1 may signal via alternative pathways such as the serum- and glucocorticoid-responsive kinase 1 (SGK1), a PI3K-dependent kinase with structural homology to Akt,[22] or the mitogen-activated protein kinase/extracellular signal-regulated receptor kinase (MAPK/ERK) pathway.[23] mTOR plays an important and central function in integrating a variety of growth signals, from simple nutritional stimulation to activation by protein growth factors, resulting in protein synthesis. Unfortunately, we were unable to determine the muscle contents of phosphorylated mTOR non-ambiguously due to non-specificity of the available antibodies.

In contrast to the anabolic function of IGF-1, TNF-α and myostatin are negative regulators of muscle mass[2,3] and important triggers of proteolysis during muscle wasting conditions.[24–27] The interactive inhibition between anabolic and catabolic factors was reported in *in vitro* studies.[28–33] Different from these observations, the expression patterns of catabolic genes within development were similar to that of IGF-1, and all had a positive correlation with calpain and/or caspase-3 gene expression (Table 2). This finding provides additional evidence that anabolic and catabolic signalling are integrated with maintenance of protein homeostasis during development. Furthermore, calpain and caspase-3 are the likely main downstream effectors of the complex regulation.

Redox regulation

Oxidative stress involves production of ROS that exceed the removal capacity of the antioxidant system and usually is defined as a pathological condition in which ROS is the damaging agent.[34] Nevertheless, emerging evidence suggests that ROS are major signals involved in muscle homeostasis, i.e. in maintaining normal skeletal muscle structure and function.[34,35] ROS generated during exercise activates mitogen-activated protein kinases, which in turn activate the NF-κB pathway and consequently the expression of antioxidant enzymes relevant to the adaptation of muscle cells to exercise.[36] Administration of H_2O_2 (one of the main ROS) was sufficient to stimulate protein degradation in C2C12 myotubes.[37] This protein degradation may involve both increased activity of FOXO and NF-κB pathways, since antioxidant supplementation that degrades H_2O_2 activity abolished immobilization-induced transactivation of both NF-κB and FOXO and attenuated the loss of muscle mass.[38] Thus, H_2O_2 may function as a signalling agent in initiating the molecular process of muscle atrophy.

The present study represents three different physiological phases: *in utero* maturation, transition to extrauterine life and early postnatal development (Figure S1). Therefore, the regulatory mechanisms should be viewed separately. Since antioxidant activity of the term newborn

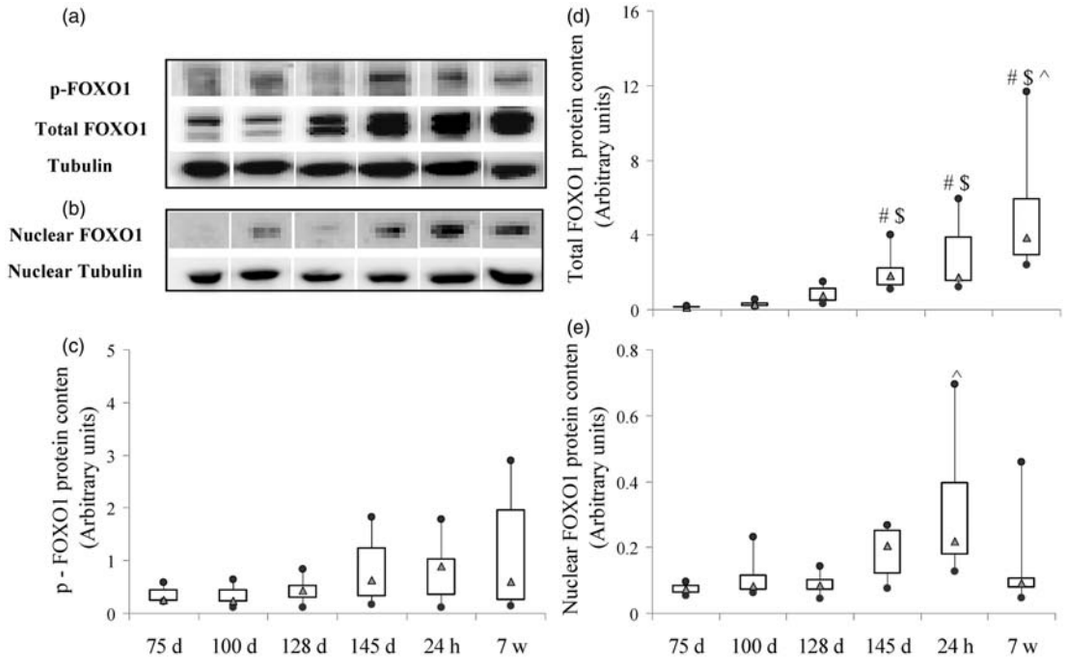

Figure 4 Cytosolic and nuclear FOXO1 protein expression: Western blots illustrate expression of phosphorylated (p–) FOXO1 and total FOXO1 using representative samples from each group (a and b). Graphs show phosphorylated FOXO1 (c) and total FOXO1 (d) proteins in cytosolic fraction and total FOXO1 in nuclear fraction (e) at 75, 100, 128 and 145 days (near term) gestation and postnatal ages of 24 h and seven weeks. Values are median (25th, 75th percentile). # $ ^ indicate $P < 0.05$ compared with 75, 100 and 128 days, respectively

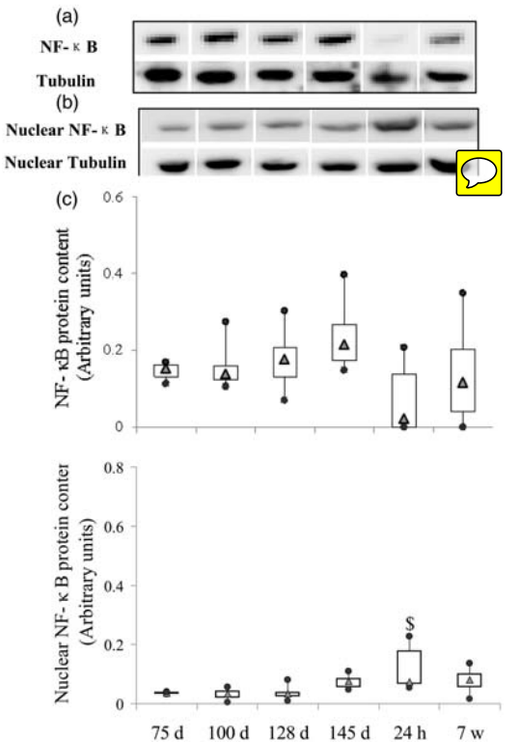

Figure 5 Cytosolic and nuclear NF-κB protein expression: Western blots illustrate cytosolic and nuclear expression of NF-κB (a and b) using representative samples from each group. Graphs show NF-κB (c) proteins in cytosolic fraction and NF-κB (d) in nuclear fraction at 75, 100, 128 and 145 days (near term) gestation and postnatal ages of 24 h and seven weeks. Values are median (25th, 75th percentile). $ indicates $P < 0.05$ compared with 100 days

is high compared with the preterm,[39] we hypothesized that the low oxidant-generating capacity of the full term diaphragm might be related to optimal *in utero* maturation of cellular antioxidant activity and hence defence systems.[40] Therefore, the decreased ROS at near term should be attributed to developed antioxidant capacity. On initiation of air breathing, a significant increase in ROS production was observed, likely a result of exposure to higher O_2 concentration than experienced in the hypoxic *in utero* environment. The transient increase in diaphragm H_2O_2 that occurred at 24 h postpartum is consistent with the elevated activity of FOXO and NF-κB. Therefore, ROS may promote FOXO and NF-κB activation and contribute to up-regulation of the atrophy genes at seven days of postnatal age, as evidenced by positive correlations (Table 2). Additional studies are needed to confirm the regulatory mechanisms.

Interestingly, the diaphragm was acutely sensitive to the higher O_2 of the postnatal environment than the plasma indicating that increased ROS in the diaphragm is more dominant and occurs earlier than systemic ROS. Additionally, concomitant increase of peroxidase in local tissue may reflect an immediate defensive response to resolve potential cytotoxic effects and maintain redox homeostasis, whereas an uncontrolled accumulation of oxidative stress might have pathological implications.[35]

FOXO and NF-κB are transcriptional factors that regulate a spectrum of downstream target genes. Both signalling molecules are implicated as mediators of redox signalling by positive feedback enhancement of antioxidant gene

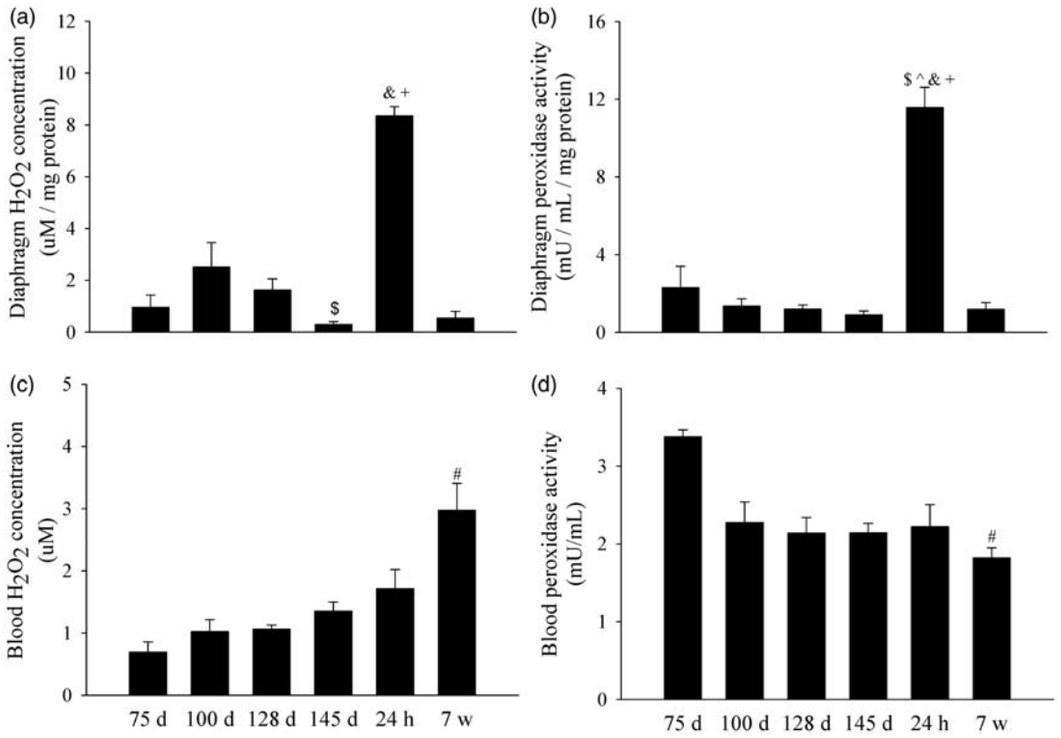

Figure 6 ROS production and peroxidase activity in diaphragm and blood: H_2O_2 concentration and peroxidase activity in costal diaphragm (a and b) and plasma samples (c and d) at 75, 100, 128 and 145 days (near term) gestation and postnatal ages of 24 h and seven weeks. Values are mean (SEM). # $ ^ & + indicate $P < 0.05$ compared with 75 days, 100 days, 128 days, 145 days and seven weeks, respectively

Table 2 Correlation between regulatory factors and proteolytic genes as well as signalling transduction molecules over the experimental period

	IGF-1	IGF-1 receptor	TNF-α	Myostatin	ROS
Calpain II	0.71**	0.62**	0.38*	0.64**	0.06
Caspase-3	0.83**	0.84**	0.22	0.74**	0.20
MuRF-1	0.01	−0.02	0.34*	−0.14	−0.05
MAFbx	0.06	−0.10	0.82**	0.02	−0.05
Cytosolic FOXO1	−0.22	−0.13	0.07	−0.34*	−0.00
Cytosolic p-FOXO1	−0.23	−0.17	−0.15	−0.30	−0.01
Nuclear FOXO1	−0.09	0.05	0.30	−0.07	0.39*
Cytosolic NF-κB	−0.10	−0.21	0.09	0.04	−0.28
Nuclear NF-κB	−0.34*	−0.13	0.06	−0.35*	0.43*

p-: phosphorylated.
Numbers indicate Pearson correlation.
***$P < 0.05$.**
****$P < 0.01$.**

expression. In our developmental model, we further confirmed the significant association of the regulatory molecules and superoxide dismutase (SOD) and glutathione peroxidase 1 (GPX1; Figure S2). Therefore, up-regulation of antioxidant genes expression should be regarded as a beneficial response for protecting cells from oxidant damage at birth. Of note, the key molecules of protein synthesis pathway such as mTOR, S6K1 and 4 E-BP1 were unaltered throughout the developmental period, excluding the possibility that they are transcriptional targets regulated by FOXO and NF-κB transcriptional factors.

Conclusions

Taken together, the data reported here indicate that the anabolic and catabolic factors exhibited a similar change during diaphragm development with a peak decrease at full term. The pattern may reflect that cellular events are integrated into low protein metabolism as the diaphragm matures *in utero*. On initiation of spontaneous breathing, ROS accumulated and activity of FOXO and NF-κB signalling pathways increased in the diaphragm. These changes are likely to promote expression of atrophy genes and up-regulate

transcripts of antioxidant genes e.g. SOD and GPX1 described previously,[17] leading to adaptation to the extra-uterine environment. Although further investigation is needed to establish the mechanistic link among different molecular and cellular pathways, the present results shed light on the underlying mechanism of susceptibility of the immature baby to diaphragm weakness. The compromised preterm oxidant defensive system may be unable to cope with the ROS produced after birth. Thus, an imbalanced redox system may be a major regulator for governing pathological process of diaphragmatic dysfunction.

Authors contribution

YS and JJP conceived and designed the study and prepared the paper. YS performed the laboratory work and data analysis. Both authors read and approved the final manuscript.

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