Molecular Response of the Human Diaphragm on Different Modes of Mechanical Ventilation

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Key Words

Angiogenesis • Vascular endothelial growth factor • Fibroblast growth factor-2 • Transforming growth factor beta 1 • Muscle relaxation • Controlled mechanical ventilation

Abstract

Background: The mechanical stress that the human diaphragm is exposed to during mechanical ventilation affects a variety of processes, including signal transduction, gene expression, and angiogenesis. **Objectives:** The study aim was to assess the change in the production of major angiogenic regulators [vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), and transforming growth factor beta 1 (TGFB1)] on the human diaphragm before and after contraction/relaxation cycles during mechanical ventilation. **Methods:** This observational study investigates the diaphragmatic mRNA expression of VEGF, FGF2, and TGFB1 in surgical patients receiving general anesthesia with controlled mechanical ventilation (CMV) with muscle relaxation (group A, n = 13), CMV without muscle relaxation (group B, n = 10), and pressure support of spontaneous breathing (group C, n = 9). Diaphragmatic samples were obtained from each patient at two time points: 30 min after the induction

of anesthesia (t₁) and 90 min after the first specimen collection (t₂). **Results:** No significant changes in the mRNA expression of VEGF, FGF2, and TGFB1 were documented in groups A and C between time points t₁ and t₂. In contrast, in group B, the mRNA levels of the above angiogenic factors were increased in time point t₂ compared to t₁, a finding which was statistically significant (p_{VEGF} = 0.003, p_{FGF2} = 0.028, p_{TGFB1} = 0.001). **Conclusions:** These findings suggest that the molecular response of the human diaphragm before and after application of diverse modes of mechanical ventilation is different. Angiogenesis via the expression of VEGF, FGF2, and TGFB1 was only promoted in CMV without muscle relaxation, and this may have important clinical implications.

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Introduction

It is well known that skeletal and cardiac muscle contractions create a powerful stimulus for the formation of new capillaries from existing ones, a process termed an-

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giogenesis. This is a physiologic adaptive response to increased muscle activity and exercise training in order to meet the metabolic demand [1–3].

Angiogenesis involves dissolution of the extracellular matrix underlying the endothelium, followed by endothelial cell migration and proliferation. Specific growth factors have been well documented to initiate and promote this extremely complex process. Vascular endothelial growth factor (VEGF) stimulates migration of endothelial cells as well as migration and proliferation of smooth muscle cells and nitric oxide (NO) production [4, 5]. Basic fibroblast growth factor-2 (FGF2) upregulates VEGF and NO production [6–8]. Transforming growth factor beta 1 (TGFB1), mainly produced by activated endothelial cells, promotes angiogenesis by recruiting pericytes to complete and stabilize the newly formed capillaries [9].

A vast amount of experimental evidence demonstrates that externally applied mechanical stresses (fluid shear stress, stretch, and pressure) regulate cytoskeletal organization, signal transduction, gene expression, and a wide variety of extracellular functions, including migration, proliferation, and extracellular matrix remodeling, which suggests a role of extrinsic stresses in angiogenesis [10–12].

The diaphragm, the main respiratory muscle that is continuously and rhythmically contracting, demonstrates characteristics of the skeletal muscles. Consequently, the mechanical stress that the human diaphragm is exposed to during mechanical ventilation affects a variety of processes, including signal transduction, gene expression, and angiogenesis [13–18].

The aim of the present study was to assess the molecular response of the major angiogenic regulators (VEGF, FGF2, and TGFB1) in the human diaphragm before and after the application of diverse modes of mechanical ventilation.

We hypothesized that active diaphragmatic contractions during spontaneous breathing in humans receiving general anesthesia would upregulate angiogenesis. Thus, this study investigated the immediate diaphragmatic angiogenic response as expressed by the mRNA levels of VEGF, FGF2, and TGFB1 in relation to: (a) active diaphragmatic contractions during spontaneous breathing with pressure support (PS) and (b) passive diaphragmatic movement during controlled mechanical ventilation (CMV), with and without muscle relaxation, in patients receiving general anesthesia, at two time points: 30 min after the induction of anesthesia (t_1) and 90 min after the first specimen collection (t_2).

Materials and Methods

The present study was approved by the Research Ethics Committee of the University Hospital of Heraklion and written informed consent was obtained from all participants.

Study Population

Women with ASA physical status I–II scheduled for elective abdominal hysterectomy (21 for uterine fibromas and 11 for uterine cancer) were enrolled into the study. Exclusion criteria were: (a) chronic obstructive pulmonary disease (COPD), asthma, interstitial and other pulmonary diseases; (b) severe scoliosis of thoracic spine with impaired respiratory function; (c) myasthenia and other neuromuscular diseases; (d) diabetes mellitus; (e) obesity (BMI >35), and (f) medication with corticosteroids or anabolics.

Anesthesia

Apart from the routine preoperative laboratory tests, all patients were assessed with spirometry (FEV₁, FEV₁/FVC) 24 h pre- and 48 h postoperatively; arterial blood gases were obtained at both time points. Plasma levels of pseudocholinesterase were measured in all patients. All patients received intraoperatively the same anesthetic technique with general and thoracic epidural anesthesia and were premedicated with midazolam 0.5–0.7 mg·kg⁻¹. Patients were randomized into three groups (A, B, and C) according to the mode of ventilation intraoperatively.

Group A: CMV with Muscle Relaxation

Patients received a nondepolarizing neuromuscular blocking agent of intermediate action (rocuronium 0.5 mg·kg⁻¹) for endotracheal intubation, and during anesthesia they were ventilated with the volume controlled mode (tidal volume 6–8 ml·kg⁻¹ of ideal body weight, respiratory rate 12–14 breaths per minute, PEEP 6–8 cm H₂O, and fresh gas flow 2 l·min⁻¹). Neuromuscular monitoring with train of four was recorded throughout surgery and targeted at zero [19].

Group B: CMV without Muscle Relaxation

Patients received a depolarizing neuromuscular blocking agent of short action (succinylcholine 1 mg·kg⁻¹) to facilitate endotracheal intubation. No other muscle relaxant was administered afterwards until emergence from anesthesia. Patients were ventilated with the volume-controlled mode with the same settings as in group A.

Group C: PS Ventilation

Patients received succinylcholine 1 mg·kg⁻¹ for endotracheal intubation and no further muscle relaxant. They were allowed to breathe spontaneously on PS mode. The ventilator triggering sensitivity was set at 1–1.5 l·min⁻¹ and the PS level was adjusted to ensure a tidal volume of 6–8 ml·kg⁻¹ of ideal body weight.

Preoperatively, all patients were volume preloaded with 1,000 ml Ringer's lactate and 500 ml colloid solution in order to avoid hypotension due to anesthesia induction and maintenance. Intraoperatively, patients were connected to standard noninvasive anesthetic monitoring [ECG, pulse oximetry (SpO₂), capnography, and esophageal temperature] and invasive monitoring of arterial blood pressure. Prior to induction of anesthesia, an epidural catheter was placed at the thoracic_{9–10} level. General anesthesia was

induced with fentanyl 2–3 $\mu\text{g}\cdot\text{kg}^{-1}$, propofol 2 $\text{mg}\cdot\text{kg}^{-1}$, and a muscle relaxant depending on the stratification group, and tracheal intubation was performed. Intraoperative analgesia was accomplished in all groups with ropivacaine 0.5% administered epidurally (12–15 ml bolus and 5–6 $\text{ml}\cdot\text{h}^{-1}$). General anesthesia was maintained in all patients with an inhaled anesthetic agent (sevo-flurane) titrated according to bispectral electroencephalography (BIS monitor; Aspect Medical Systems, Norwood, Mass., USA) [20]. In groups A and B, deep levels of anesthesia were maintained with BIS targeted at 40 ± 5 , while in group C a lighter level of anesthesia was maintained (BIS 60 ± 5) to avoid respiratory center depression. All patients breathed oxygen in air 35–50% to maintain a pulse oximetry (SpO_2) above 97%. If the systolic arterial pressure dropped below 20% from baseline for more than 10 min, a bolus dose of a vasoconstricting agent (ephedrine 5 mg) was administered.

Recordings

Hemodynamic (heart rate, systolic and mean blood pressure) and respiratory variables (respiratory rate, tidal volume, minute ventilation, peak and plateau inspiratory pressure, PEEP) were recorded every 15 min in each patient. At the same time arterial blood gases were obtained and PaO_2 , PaCO_2 , and SatO_2 were measured.

Sample Collection

At least three muscular samples (volume 1–1.5 cm^3) from the same region of the costal diaphragm of each patient were obtained at two different time points: the first at 30 min after induction of anesthesia (t_1) and the second 90 min later (t_2). All samples were taken by the same surgeon, immediately transferred to liquid nitrogen, and stored at -80°C until use.

RNA Extraction and cDNA Preparation

Tissue samples were homogenized in TRIzol[®] reagent (Invitrogen, Carlsbad, Calif., USA) using a power homogenizer and incubated at room temperature, followed by the addition of chloroform and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol, and resuspended in 20 μl of DEPC-treated water. The concentration and purity of RNA were calculated after measuring on a NanoDrop 1,000 spectrophotometer (NanoDrop Products; Wilmington, Del., USA) its 260 nm absorbance and 260/280 nm absorbance ratio, respectively.

cDNA was synthesized by reverse transcription (RT) with the RETROscript[®] Kit (Ambion, Austin, Tex., USA), using random decamers as amplification primers. In detail, 2.5 μg of total RNA and 5 μM of random decamers were heated at 80°C for 3 min in order to remove RNA secondary structures and placed on ice until the addition of cDNA synthesis mix, which contained 1 $\times$ RT buffer, 0.5 mM dNTPs mix, 10 units RNase inhibitor, and 100 units MMLV-RT reverse transcriptase. The final mix (volume 20 μl) was incubated at 55°C for 1 h. The reaction was terminated by heating at 92°C for 10 min and cDNA was stored at -20°C until use.

Quantitative Real-Time PCR

VEGF, TGF β 1, and FGF2 mRNA expression was measured using a real-time qPCR assay with SYBR[®] Green I dye. A house-keeping gene, beta-actin (β -Actin), was used as an internal control in order to normalize VEGF, TGF β 1, and FGF2 mRNA ex-

Table 1. Primer sequences used for quantitative real-time PCR

Primer pair	Sequence (5'–3')	Amplicon size, bp
VEGF	ATGACGAGGGCCTGGAGTGTG CCTATGTGCTGGCCTTGGTGAG	91
TGF β 1	AAGGACCTCGGCTGGAAGTG CCCGGGTTATGCTGGTTGTA	137
FGF2	CTGGCTATGAAGGAAGATGGA TGCCAGTTCGTTTCAGTG	149
β -Actin	CGGCATCGTCACCAACTG GGCACACGCAGCTCATTG	70

pression levels. The mRNA-specific primers, which were designed with Lasergene[®] 7.0 software (DNASTAR, Madison, Wisc., USA) and span at least one intron with an average length >800 bp, are listed in table 1. After initial experiments, in order to optimize the concentration and annealing temperature of the primers, 1 μl of cDNA from the different time point (t_1 and t_2) samples was amplified in a PCR reaction containing 2 $\times$ Maxima[™] SYBR Green qPCR Master Mix (Fermentas Inc., Glen Burnie, Md., USA) (containing 2.5 mM MgCl_2) and 300 nM of each primer in a final volume of 20 μl . To ensure the accuracy of the quantification measurements, a representative pool of all of the samples was diluted in a series of six 2 $\times$ dilutions and run on the same plate in order to construct a standard curve for the quantification process. After initial denaturation at 95°C for 10 min, samples were subjected to 40 cycles of amplification, comprised of denaturation at 95°C for 20 s, annealing at 60°C (VEGF, TGF β 1, β -Actin) or 55°C (FGF2) for 30 s, and elongation at 72°C for 30 s, followed by a melt curve analysis in which the temperature was increased from 60°C to 95°C at a linear rate of $0.2^\circ\text{C}/\text{s}$. Data collection was performed during both annealing and extension, with two measurements at each step, and at all times during the melt curve analysis. PCR experiments were conducted on an Mx3000P real-time PCR thermal cycler using software version 4.10, Build 389 Schema 85 (Stratagene, La Jolla, Calif., USA). Verification of the PCR products in terms of correct size and the absence of dimers was conducted using 2% (w/v) agarose gel electrophoresis. After amplification, standard curves were constructed from the samples used in the series of consecutive dilutions. Subsequently, using these standard curves and the Ct value of the samples, we calculated the mRNA expression of the genes studied. Samples with no amplification plots or with dissociation curves that exhibited signs of primer-dimer formation or by-products were excluded. To normalize the mRNA expression of each gene, its value was divided by the β -Actin mRNA value. In each PCR reaction two negative controls were included, one with no cDNA template and one with no RT treatment. All qPCR measurements were conducted in triplicate.

Statistical Analysis

All variables were first evaluated using the one-sample Kolmogorov-Smirnov goodness-of-fit test in order to determine whether they followed a normal distribution pattern or not. Sub-

Table 2. Patients' demographic data and respiratory and hemodynamic measurements

Variable	Group A (n = 13)	Group B (n = 10)	Group C (n = 9)	p _g value
Demographic data				
Age, years	50.3 ± 6.0	51.2 ± 7.7	53.0 ± 7.8	0.81
BMI	32.6 ± 3.7	30.1 ± 3.1	30.8 ± 5.5	0.45
Smoking, pack-years	12.7 ± 14.2	8.3 ± 13.3	2.0 ± 4.5	0.35
FEV ₁ , % predicted	112.6 ± 14.0	105.4 ± 10.4	94.4 ± 5.7	0.07
FEV ₁ /FVC, %	85.6 ± 8.6	81.4 ± 11.8	82.0 ± 2.3	0.77
Respiratory variables				
TV, ml				
t ₁	560.0 ± 83.2	528.3 ± 34.3	500.4 ± 63.0	0.35
t ₂	516.7 ± 51.7	518.3 ± 19.4	538.0 ± 41.5	0.42
p _t value	0.21	0.67	0.25	
RR, min ⁻¹				
t ₁	12.0 ± 1.0	11.8 ± 1.0	11.8 ± 1.9	0.66
t ₂	11.6 ± 1.4	11.7 ± 1.0	11.6 ± 1.5	0.39
p _t value	0.40	0.56	0.71	
PaO ₂ , mm Hg				
t ₁	143.1 ± 38.3	169.0 ± 61.0	140.0 ± 53.8	0.48
t ₂	139.8 ± 22.8	161.7 ± 40.0	132.0 ± 32.4	0.51
p _t value	0.72	0.87	0.92	
PaCO ₂ , mm Hg				
t ₁	35.2 ± 3.5	37.5 ± 5.3	40.3 ± 6.5	0.11
t ₂	37.7 ± 3.8	38.7 ± 2.7	37.1 ± 4.3	0.83
p _t value	0.14	0.46	0.30	
pH				
t ₁	7.46 ± 0.03	7.45 ± 0.04	7.42 ± 0.02	0.19
t ₂	7.44 ± 0.04	7.44 ± 0.02	7.43 ± 0.04	0.67
p _t value	0.62	0.68	0.34	
Hemodynamic variables				
SAP, mm Hg				
t ₁	105.9 ± 17.3	98.2 ± 9.4	106.4 ± 10.2	0.43
t ₂	97.6 ± 18.4	101.2 ± 7.1	105.2 ± 12.2	0.19
p _t value	0.29	0.52	0.92	
MAP, mm Hg				
t ₁	77.3 ± 13.4	74.0 ± 5.1	71.2 ± 7.3	0.73
t ₂	69.3 ± 16.2	74.5 ± 7.6	73.2 ± 10.5	0.26
p _t value	0.19	0.81	0.92	

p_g = p value between the three study groups (Kruskal-Wallis H test); p_t = p value within each group at time points t₁ (time of first diaphragmatic specimen) and t₂ (time of second specimen) (2-tailed Mann-Whitney U test); TV = tidal volume; RR = respiratory rate; SAP = systolic arterial pressure; MAP = mean arterial pressure.

sequently, the Kruskal-Wallis H test was used to examine the differences in patients' demographic data and respiratory and hemodynamic measurements within the three study groups, while a Mann-Whitney U test (2-tailed) was used to examine the differences in each group between time points t₁ and t₂. Normalized VEGF, FGF2, and TGFB1 mRNA levels were statistically compared in groups A, B, and C between time points t₁ and t₂ using Student's t test or the Mann-Whitney U test (2-tailed). p < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, Ill., USA).

Results

Thirty-two healthy women with normal FEV₁ and FEV₁/FVC values were included in the study: 13 in group A, 10 in group B, and 9 in group C. No statistically significant differences in patients' demographic characteristics and preoperative spirometric values between the three study groups were observed (table 2). Study subjects were ventilated with comparable tidal volumes, respiratory rates, and inspiratory pressures (P_{peak}, P_{plateau}, and

Table 3. VEGF, FGF2, and TGFBI mRNA expression data in the three study groups at time points t_1 and t_2

Patient number	VEGF		FGF2		TGFB1	
	t ₁	t ₂	t ₁	t ₂	t ₁	t ₂
Group A						
1	0.90	1.12	1.59	1.24	2.12	1.89
2	1.35	1.23	0.89	1.02	0.87	1.05
8	1.63	0.85	0.66	0.50	0.95	0.89
13	1.98	1.45	1.48	0.95	1.54	1.32
14	3.79	2.37	0.75	1.41	1.72	1.59
15	1.55	1.34	2.22	1.69	1.78	1.24
16	1.18	0.37	1.28	1.12	1.28	1.23
18	0.89	1.57	0.95	1.02	0.98	0.87
5	1.28	1.34	0.79	0.76	1.77	1.43
23	1.80	2.15	3.07	2.22	2.24	1.95
24	2.18	2.44	1.44	1.44	1.73	1.94
25	2.43	0.31	1.63	0.71	1.15	0.55
33	1.75	1.66	1.33	1.12	1.47	1.68
Average	1.75	1.40	1.39	1.17	1.51	1.36
SD	0.77	0.67	0.67	0.45	0.44	0.44
SEM	0.21	0.19	0.19	0.13	0.12	0.12
p value	0.29		0.44		0.44	
Group B						
3	1.10	5.37	2.01	6.57	0.32	1.12
4	0.29	0.73	0.32	1.17	0.18	0.75
7	0.78	5.06	2.44	4.78	0.58	1.45
11	0.16	0.65	1.78	2.65	0.09	0.49
12	1.23	3.45	0.97	2.17	0.52	0.78
26	0.85	1.33	1.16	1.54	0.84	0.91
27	0.93	1.28	0.82	1.11	0.27	0.77
28	0.54	3.12	1.97	3.68	0.38	1.02
29	0.37	2.44	1.58	4.42	0.44	0.88
30	0.67	1.98	2.21	3.87	0.62	0.84
Average	0.69	2.54	1.53	3.20	0.42	0.90
SD	0.35	1.69	0.68	1.78	0.22	0.26
SEM	0.11	0.53	0.22	0.56	0.07	0.08
p value	0.003		0.028		0.001	
Group C						
6	2.42	1.58	1.84	0.77	2.05	1.12
9	2.12	1.56	1.01	0.37	3.08	1.19
10	1.25	1.74	0.99	1.03	0.97	1.25
19	1.63	0.92	1.91	1.34	0.89	0.90
20	2.16	0.99	1.94	1.62	0.76	0.53
21	0.70	1.04	0.84	0.83	0.58	1.06
22	0.66	1.22	0.66	1.11	1.25	1.17
31	1.77	1.85	1.39	1.47	1.03	0.88
32	1.98	1.01	0.84	0.36	1.15	0.67
Average	1.63	1.32	1.27	0.99	1.31	0.97
SD	0.64	0.36	0.51	0.45	0.78	0.25
SEM	0.21	0.12	0.17	0.15	0.26	0.08
p value	0.17		0.31		0.60	

PEEP) between groups and within each group at time points t_1 and t_2 . All patients remained normoxic and hemodynamically stable (systolic arterial pressure and mean arterial pressure) throughout the procedure. The intraoperative blood loss range was approximately 200–300 ml (table 2), and no patient required ephedrine as a vasoconstricting agent.

Analysis of the expression data revealed that in group A all three angiogenic factors studied exhibited a small decrease in their expression from time point t_1 to t_2 , a finding which, however, was not statistically significant (VEGF: 1.75 ± 0.21 vs. 1.40 ± 0.19 , $p = 0.29$; FGF2: 1.39 ± 0.19 vs. 1.17 ± 0.13 , $p = 0.44$; TGFBI: 1.51 ± 0.12 vs. 1.36 ± 0.12 , $p = 0.44$). This non-statistically significant downregulation between time points t_1 and t_2 was also observed in group C (VEGF: 1.63 ± 0.21 vs. 1.32 ± 0.12 , $p = 0.17$; FGF2: 1.27 ± 0.17 vs. 0.99 ± 0.15 , $p = 0.31$; TGFBI: 1.31 ± 0.26 vs. 0.97 ± 0.08 , $p = 0.60$) (table 3; fig. 1).

On the contrary, in group B, VEGF was 3.7-fold overexpressed in time point t_2 when compared to time point t_1 (0.69 ± 0.11 vs. 2.54 ± 0.53), while both FGF2 and TGFBI were 2.1-fold overexpressed from time point t_1 to t_2 (FGF2: 1.53 ± 0.22 vs. 3.20 ± 0.56 ; TGFBI: 0.42 ± 0.07 vs. 0.90 ± 0.08). This finding was statistically significant for all three angiogenic factors studied ($p_{\text{VEGF}} = 0.003$; $p_{\text{FGF2}} = 0.028$; $p_{\text{TGFBI}} = 0.001$) (table 3; fig. 1).

Discussion

The present study showed increased diaphragmatic VEGF, FGF2, and TGFBI mRNA levels at time point t_2 when compared to t_1 during CMV without muscle relaxation in patients receiving general anesthesia for lower abdominal surgery. In contrast, the above factors did not exhibit significant changes either during CMV with muscle relaxation or during PS ventilation, disproving our original hypothesis.

So far, several animal and human studies have addressed the issue of upregulated VEGF after a short course of skeletal muscle exercise, demonstrating elevated mRNA levels of the growth factor [8, 21–23]. Since then, upregulation of VEGF protein expression has been documented during long-term skeletal muscle training over a period of days and weeks [24–26]. The present study investigated angiogenic activity in the human diaphragm by measuring the mRNA levels of three angiogenic factors by quantitative real-time PCR at two sequential time points after the application of diverse modes of ventilation.

Fig. 1. Box and whisker plots depicting the normalized expression of VEGF (a), FGF2 (b), and TGFB1 (c) in the three study groups (group A: CMV with muscle relaxation, group B: CMV without muscle relaxation, and group C: PS of spontaneous breathing) between time points t₁ and t₂. Only in group B were VEGF, FGF2,

and TGFB1 mRNA levels statistically significantly increased at time point t₂ compared to time point t₁ ($p_{\text{VEGF}} = 0.003$; $p_{\text{FGF2}} = 0.028$; $p_{\text{TGFB1}} = 0.001$; 2-tailed Mann-Whitney U test), whereas in groups A and C the differences in their expression among the two time points were not statistically significant.

Diaphragmatic angiogenesis in animals was first investigated by Siafakas et al. [13] who showed that the diaphragm exhibits the same angiogenic response to an increased workload as locomotive muscles do. His study showed that rats had increased diaphragmatic VEGF and FGF2 mRNA levels (3- and 1.5-fold, respectively) after 1 h of increased active ventilation, while mechanically ventilated rats with paralyzed diaphragms had no change in diaphragmatic VEGF and FGF-2 mRNA levels [13]. In a subsequent study by DeRuisseau et al. [27], VEGF mRNA expression was downregulated in diaphragmatic specimens of paralyzed rats after 6 and 18 h of CMV due to diaphragmatic unloading. In the first human study by Alexopoulou et al. [28], it was documented that VEGF mRNA levels were increased in COPD patients with a consequent increased respiratory load compared to normal subjects.

Angiogenesis in response to increased muscle activity has been attributed to mechanical forces: either hemodynamic acting on the luminal surface of the vessels, i.e. shear stress, wall tension, and capillary pressure, or extravascular acting on the abluminal surface as a result of muscular fiber stretch, contraction, and relaxation [29–31]. The latter mechanical forces associated with shortening and relaxation of the myocytes are imparted to the microvascular network via the extracellular matrix and connective tissue elements and initiate the angiogenic

process [32–35]. Although the impact of increased vessel wall stress and/or shear stress on muscle VEGF expression during exercise remains ambiguous, its regulation by extravascular stretch is undoubted [32, 36]. In a previous study by Roca et al. [37], no change in mRNA levels of VEGF was demonstrated in canine skeletal muscles after passive hyperperfusion without muscle contraction, whereas electrical stimulation of the skeletal muscle resulted in a 3-fold increase in VEGF mRNA abundance. The authors speculated that other factors including mechanical effects of contraction per se could be responsible for the increased angiogenic signal.

Our findings are in accordance with the above study as the upregulation of VEGF, FGF2, and TGFB1 mRNA levels in group B could be an immediate response to ventilator-induced diaphragmatic mechanical stretch due to lack of muscle relaxation. Presumably, the molecular and cellular changes implicated in the angiogenic process take place in a short time course, e.g. 90 min, and consequently any conclusion drawn regarding angiogenic activity in the diaphragm could be safely viewed at least at the gene level. It remains questionable as to what extent, and at what time the angiogenic stimuli are translated to actual angiogenic diaphragmatic activity. Future studies are needed to address these questions.

Our results should be attributed only to mechanical forces applied on the diaphragm since our patients did

not exhibit hemodynamic instability, hypoxemia, or acidosis, factors that might affect the process of angiogenesis [2, 3, 38]. Additionally, lower abdominal surgery was the ideal surgical procedure for the study since it has a minimum effect on diaphragmatic and respiratory function [39].

It is clear nowadays that neuromuscular blockade of the diaphragm during mechanical ventilation has been associated with prolonged mechanical ventilation, a longer stay in the ICU, and a higher incidence of ventilator-associated pneumonia [40]. Surgical patients with residual muscle paralysis had postoperatively prolonged intubation times and weaning periods from the ventilator [41]. Previous studies showed that the combination of mechanical ventilation with muscle-relaxing agents and/or high-dose steroids was associated with significant myopathy. Muscle weakness varied from mild to severe. Muscle biopsy showed generalized fiber atrophy, myofibril necrosis, and disorganization with loss of thick myosin filaments and fiber vacuolation [42, 43].

The results of the present study raise the question of whether an increased angiogenic response of a mechanically overloaded, non-paralyzed diaphragm may be a preventive measure against MV-induced diaphragmatic dysfunction. Further studies are needed to investigate any relation between increased diaphragmatic angiogenesis and improved functional performance during and

after discontinuation of mechanical ventilation. New therapeutic strategies may be developed under this consideration, focusing on intensive care unit patients with respiratory failure necessitating long-term mechanical ventilation.

A limitation of this study could be the small number of patients included. However, the recruitment of healthy women scheduled for lower abdominal surgery for the fairly invasive technique of taking diaphragmatic samples poses technical issues.

Regarding the observed variation in 'baseline' (t_1) gene expression (fig. 1), we should clarify that, even though samples at time point t_1 were obtained as early as possible, it was actually ~30 min after anesthesia had been applied to the patient (t_0). Therefore, an initial diaphragmatic response to the anesthetic agent, which could alter VEGF, FGF2, and TGF β 1 levels, especially in group B due to the lack of muscle relaxant, cannot be ruled out.

In conclusion, although our data did not confirm the initial hypothesis, we demonstrated that CMV without muscle relaxation constituted a rapid stimulus for increased diaphragmatic angiogenic activity in surgical patients during anesthesia. On the contrary, neither CMV with muscle relaxation nor spontaneous respiration with PS had any significant angiogenic effect on the diaphragm.

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