Diaphragmatic angiogenic growth factor mRNA responses to increased ventilation caused by hypoxia and hypercapnia

N.M. Siafakas*,#, M. Jordan*, H. Wagner*, E.C. Breen*, H. Benoit*, P.D. Wagner*

Diaphragmatic angiogenic growth factor mRNA responses to increased ventilation caused by hypoxia and hypercapnia. N.M. Siafakas, M. Jordan, H. Wagner, E.C. Breen, H. Benoit, P.D. Wagner. ©ERS Journals Ltd 2001.
ABSTRACT: This study investigates the effect of increased ventilation on the expression of messenger ribonucleic acid (mRNA) levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β_1 (TGF-β_1) in the diaphragm of intact, awake, spontaneously breathing rats, compared with responses in paralysed, mechanically-ventilated animals at similar blood gas and ventilatory levels.

Four groups of intact, rats were studied in a body box, each group breathing one of four gases: room air, 12% oxygen (O_2), 5% carbon dioxide (CO_2), or 12% O_2+5% CO_2 for 1 h. Another 4 groups of paralysed, mechanically-ventilated animals were matched for arterial blood gas and ventilatory level.

The results showed that VEGF mRNA abundance was increased three-fold and that of bFGF 1.5-fold when 12% O_2+5% CO_2 were breathed, but TGF-β_1 did not change. A significant linear relationship of VEGF and bFGF mRNA to minute ventilation was observed in awake animals (r=0.98, p<0.02 and r=0.87, p<0.03, respectively). The paralysed, mechanically-ventilated animals showed no mRNA increases for any probe. Systemic hypoxia had no additional effect on VEGF or bFGF levels in the diaphragm.

It was concluded that messenger ribonucleic acid for vascular endothelial growth factor and basic fibroblast growth factor in the diaphragm rises significantly as a result of active ventilation and not due to blood gas/pH changes or to passive muscle shortening *per se*.
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*Dept of Medicine, University of California, San Diego La Jolla, CA, USA. #Dept of Thoracic Medicine, University of Crete, Heraklion, Greece.

Correspondence: N.M. Siafakas, Dept of Thoracic Medicine, Medical School, University of Crete, Heraklion, Greece. Fax: 30 81542650

It is well known that a major adaptation of skeletal muscles to repeated exercise (endurance training) is the formation of new capillaries (angiogenesis) [1–3]. Angiogenesis is an extremely complex process involving, among other steps dissolution of extracellular matrix underlying the endothelium, cell migration and endothelial cell proliferation [4, 5]. In malignant tumour growth or in development, it has been shown that factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β_1 (TGF-β_1) are involved in the formation of new capillaries [6–8].

It has been shown that a single, 1-h bout of exercise of normal skeletal muscles increases the messenger ribonucleic acid (mRNA) levels of VEGF, bFGF and TGF-β_1 [9] (more so in hypoxia for VEGF, in particular). In addition, electrical stimulation increases VEGF levels [10]. Recently, it was demonstrated that passively-induced hyperperfusion does not increase muscle VEGF mRNA, and it was speculated that either intracellular hypoxia or mechanical effects of contraction *per se* (such as fibre stretch) may be responsible for this increase [11].

The respiratory muscles are skeletal muscles and respond to endurance training by increasing their capillary density, as do the locomotive muscles. However, the diaphragm differs from locomotive skeletal muscles in that it continuously and rhythmically contracts, and thus it is subjected to life long "endurance training". These findings have accordingly focused the authors attention on the capillary growth of the respiratory muscles in response to contraction, and particularly that of the diaphragm. Since it is well known that respiratory muscle failure is a life-threatening condition, it is of importance to understand the regulation of capillary growth of the respiratory muscles. In addition, patients in intensive care units (ICUs) are usually mechanically ventilated for some time. It is thus possible that a lack of active respiratory muscle effort under these conditions could reduce respiratory muscle capillarity, which could contribute to difficulty in respiratory weaning.

The aim of this study was to investigate the response of VEGF, bFGF and TGF-β_1 mRNA in the diaphragm to increased ventilation stimulated by hypoxia and/or hypercapnia. These responses were compared with those of paralysed, mechanically-ventilated animals at similar blood gas and ventilatory levels, to determine whether active contraction, passive movement or altered partial pressure of oxygen (P_{O_2}) is primarily responsible for changes in mRNA.

It was demonstrated that mRNA levels of VEGF and bFGF were increased after 1 h of exposure to these stimuli and that this rise was proportional to minute

ventilation. Paralysed, mechanically-ventilated animals showed no mRNA change, for any angiogenic factor, suggesting that neither passive shortening nor hypoxia were involved. The possible clinical implications of these findings are discussed.

Methods

Adult female Wistar rats were used throughout the study. Forty-eight animals were studied (mean±SD age 9.2±1.1 weeks and weight 272±13 g). The study was approved by the Animal Subjects Committee of the University of California, San Diego, USA.

Protocol I: intact animals

Four groups of six animals each were studied. An arterial line (PE-50) was placed in the femoral artery under short duration halothane anaesthesia, using sterile techniques. The catheter was positioned under the skin and exited at the back of the neck of the animal. The catheter was heparinized, capped and the animal was allowed to recover from anaesthesia for at least 24 h. The next day, the intact animal was comfortably positioned into a body box (30 cm × 18 cm). The arterial line was connected to an airtight opening of the box in order to withdraw arterial blood samples during the experiments. The animals spontaneously breathed room air for 10–20 min (baseline conditions), and measurements of ventilator parameters (tidal volume (V_T) and respiratory frequency (f_R)) were made according to the method of BARTLETT and TENNEY [12]. Minute ventilation V'_E was calculated using the equation $V'_E = V_T \times f_R$. In addition, an arterial blood sample (1 mL) was taken for the measurement of blood gases (Instrumentation Laboratories, Model 1306). Thereafter, the animal spontaneously breathed either room air (A=control group) or 12% O_2+88% N_2 (B=hypoxic group), or 5% CO_2,+21% O_2+74% N_2 (C=hypercapnic group) or 12% O_2+5% CO_2 +83% N_2 (D=hypoxic+hypercapnic group) for 1 h. The concentrations of the inspired gases in the body box were continuously measured by a mass spectrometer (Perkin Elmer MGA 1100; Perkin Elmer, Saira Louis, MO, USA).

Measurements of V_T, f_R, and blood gases were made after 30, 45 and 60 min of breathing the various mixtures. After 1 h of exposure to the designated gas, the animals were euthanized by administrating 1 mL of pentobarbital sodium *via* the arterial line. The diaphragm was immediately removed and quickly frozen in liquid nitrogen for isolation of total cellular mRNA.

Protocol II: Anaesthetized, paralysed, mechanically-ventilated animals.

Another four groups of animals (six each) were anaesthetized with intraperitoneal pentobarbital sodium (40 mg·kg^{-1}) and paralysed with pancuronium promide *i.v.* (2 mg·kg^{-1}) The animals were tracheostomized and mechanically-ventilated by a rodent ventilator (Har-

vard # 683; Harvard, Boston, MA, USA). The dead space of the connecting tubing was <1 mL. In addition, an arterial catheter was placed into the femoral artery. The ventilator was set to produce a minute ventilation equivalent to that of the intact animals of protocol I during quiet room air breathing in order to match their blood gases. After 15 min baseline measurements, the animals breathed room air (control) or 12% O_2+88% N_2 (hypoxic), or 5% CO_2+21% O_2+74% N_2 (hypercapnic), or 12% O_2+5% CO_2+83% N_2 (hypoxic+hypercapnic), gas mixtures. Again, the ventilator was set (f_R and V_T) to match the ventilatory levels of the intact animals and their blood gases at each corresponding experimental condition of Protocol I. After 1 h of mechanical ventilation, the animals were euthanized, and the diaphragm was removed and processed as in the intact, spontaneously breathing animals (Protocol I).

Ribonucleic acid isolation and Northern blot analysis

Total cellular ribonucleic acid (RNA) was isolated from each muscle sample by the method of CHOMCZYNSKI and SACCHI [13]. RNA preparations were quantitated by absorbance at 260 nm, and intactness was assessed by ethidium bromide staining. Ten micrograms of total cellular RNA were separated by electrophoresis in 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to a Zeta probe membrane (Bio-Rad, Hercules, CA, USA). RNA was cross-linked to the membrane by ultraviolet irradiation by using an ultraviolet crosslinker (model FD-UVXL 1000; Fisher Scientific, Hampton, NH, USA) and stored at 4°C. The blots were then probed with oligolabelled [a-^{32}Phosphorus] deoxycytidine triphosphate complementary deoxyribonucleic acid (cDNA) probes, which had a specific activity of $\geqslant 1 \times 10^9$ disintegrations·min^{-1}·µg DNA^{-1} [14]. The human VEGF probe is a 0.93-k cDNA fragment isolated from the *EcoR* I site of pUC-derived plasmid [6]. The human TGF-β_1 cDNA probe is a 0.985-kb *Hind* III.Xba I insert cloned into pBlue-script II KS$^+$ vector [15]. The bFGF probe is a 1-kb *Xho* I fragment of human bFGF cDNA [5]. Prehybridization and hybridizations were performed in 50% formamide, 5X SSC (20X SSC is 0.3 M sodium chloride and 0.3 M sodium citrate), 10X Denhardt's solution (100X Denhardt's solution is 2% Ficoll and 2% polyvinyl pyrrolidone), 50 mM sodium phosphate (pH 6.5), 1% SDS, and 250 µg·mL^{-1} of sonicated salmon sperm DNA at 37 or 42°C. Blots were washed with 2X SSC and 0.1% SDS at room temperature, with O.1X SSC and 0.1% SDS at 55°C for the VEGF mRNA, and with 1X SSC and 0.1% SDS at 60°C for TGF-β_1 and bFGF mRNAs. Blots were exposed to XAR-5 radiograph film (Eastman Kodak, New Haven, CT, USA) by use of a Cronex Lightning Plus screen at -70°C. In all analyses, quantitative densitometry of the autoradiographs was used to measure the mRNA levels for all three growth factors. Each blot was subsequently reprobed (after prior complexes were stripped) with a cDNA specific for 18S ribosomal RNA, and this signal was used to normalize the mRNA signal for minor variation in the lane loading.

Statistical analysis

In all statistical treatments, quantitative densitometry of measured mRNA levels for all three growth factors was used. Lane loading variability was controlled by the normalization of the signals with the ribosomal 18S RNA as the control band. A one-way analysis of variance was used to determine changes in mRNA levels with increased ventilation. Linear relationships were tested with the least square method, and the Mann-Whitney test was used to determine differences between awake intact (I) and paralysed ventilated (P) animals. Statistical significance was accepted if $p \leqslant 0.05$.

Results

The results of the ventilatory parameters (V_T, f_R, V'_E) and of the blood gases (pH, oxygen tension in arterial blood (P_{a,O_2}), carbon dioxide tension in arterial blood (P_{a,CO_2}) in the awake, intact (I) spontaneously breathing and in the paralysed (P), mechanically-ventilated animals are summarized in table 1. No statistically significant differences were found in the mean values of the variables discussed between the intact (I) and paralysed (P) animals breathing the same gas mixture. Although, the mean pH value in paralysed animals was lower (7.28) than the one in intact (I) (7.38) during hypoxic+hypercapnic runs, suggesting a mild metabolic acidosis, those differences were not significant. Therefore, the experimental requirements to test the intact and paralysed animals under similar ventilatory levels, blood gases and acid-base conditions were met. In addition, table 1 shows that minute ventilation (V'_E) increased significantly due to hypoxia and/or hypercapnia in the spontaneously breathing animals.

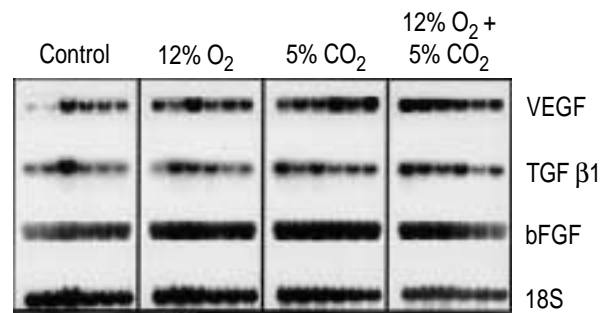Figure 1 shows the set of Northern blots of VEGF, bFGF and TGF-β_1 mRNA levels in the diaphragm

Fig. 1. – Northern blots of vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), basic fibroplast growth factor (bFGF) normalized to 18S ribosomal RNA (Rn 18S) in awake, spontaneously breathing animal: control (room air), hypoxic (12% O_2), hypercapnic (5% CO_2) and hypoxic and hypercapnic (12% O_2+5% CO_2) conditions.

from protocol I in intact spontaneously breathing animals after 1 h of increased ventilation caused by hypoxia and/or hypercapnia. It is apparent that with hypoxia and/or hypercapnia VEGF and bFGF mRNA abundance was increased.

The Northern blots of the same angiogenic factors from protocol II where the animals were anaesthetized, paralysed and mechanically-ventilated are shown in figure 2. It can be seen that no apparent change of VEGF occurred during those experimental conditions.

The normalized values of mRNA for VEGF, bFGF and TGF-β_1 in the diaphragm of the intact animals (protocol I) and the paralysed, mechanically-ventilated animals (protocol II) are shown in figure 3. VEGF mRNA increased more than 2.5-fold from the control condition (breathing room air) in the intact spontaneously breathing animals after 1 h of increased ventilation (5% CO_2 plus 12% O_2). Similarly, bFGF increased approximately 1.5-fold, and both increases were statistically significant ($p<0.0017$ and $p<0.001$

Table 1. – Ventilatory parameters and blood gases (mean±SD) in intact (I) awake spontaneously breathing and in paralysed (P) mechanically-ventilated animals

Ventilatory parameter	Inspired mixtures			
	Control (room air)	Hypoxia (12% O_2)	Hypercapnia (5% CO_2)	Hypoxia+hypercapnia (12% O_2 + 5% CO_2)
V_T mL				
I	0.526±0.81	0.797±0.23	0.941±0.21	1.160±0.18
P	0.510±0.11	0.800±0.26	0.910±0.26	1.100±0.2
f beats·min^{-1}				
I	82±12	99±15	128±12	146±23
P	85±5	98±14	125±6	134±6
V'_E mL·min·kg^{-1}				
I	163±30	278±87	480±19	610±141
P	164±41	280±35	387±77	503±54
pH				
I	7.46±0.04	7.53±0.02	7.36±0.02	7.38±0.03
P	7.44±0.02	7.47±0.08	7.33±0.02	7.28±0.05
P_{a,O_2} mmHg				
I	95.0±8.4	44.8±1.8	118.0±2.8	68.0±4.2
P	82.0±2.3	42.7±5.5	117.0±8.6	55.0±5.3
P_{a,CO_2} mmHg				
I	34±3.8	29±0.8	44±2.1	42±1.2
P	32±4.2	25±3.3	45±1.5	43±1.9

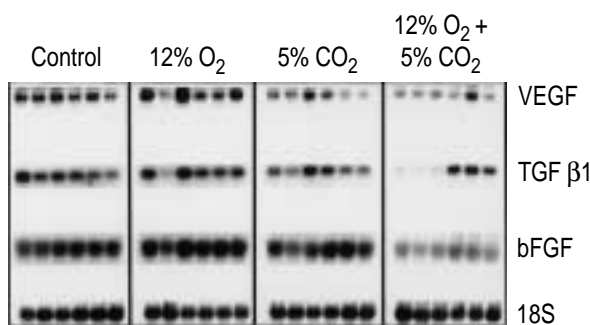No significant difference between I and P was observed in any of the above parameters.

Fig. 2. – Northern blots of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-β1) and normalized to RN18S. In anaesthetized, paralysed and mechanically-ventilated animals.

respectively), with increased ventilation. In contrast, TGF-β_1 mRNA levels did not change after 1 h breathing under the four experimental conditions in the intact animals (fig. 3).

No significant change in any of the three growth factor mRNA levels was observed in the paralysed animals when they were mechanically-ventilated at high ventilatory levels (similar to those in intact animals) and with similar pH and blood gases. A trend towards an increase in mRNA levels of the three factors was seen in the paralysed animals during hypoxia (fig. 3, hatched bars), but this did not reach statistical significance. The mRNA level of bFGF during hypoxia and hypercapnia (fig. 3, hatched bar) actually decreased.

Figure 4a shows the relationship between minute ventilation (V'E) and the mean normalized mRNA levels of VEGF, bFGF and TGF-β_1, in the diaphragm. A statistically significant linear relationship was found between V'E and VEGF (r=0.98, p<0.02) and between V'E and bFGF (r=0.87, p<0.03) but not between V'E and TGF-β_1.
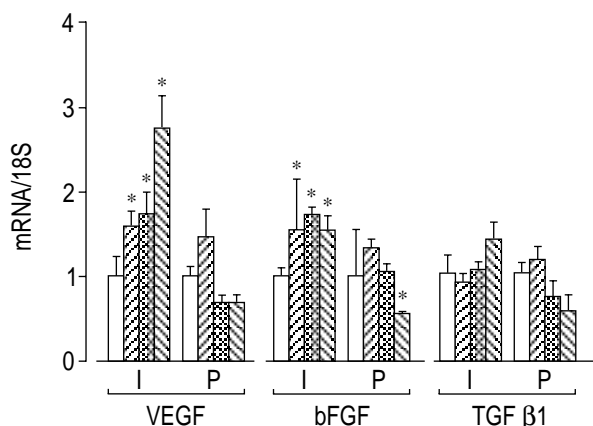

Fig. 3. – Mean±SEM values of normalized messenger ribonucleic acid (mRNA) of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-β1) in the diaphragm of intact, awake, spontaneously breathing (I) and anaesthetized paralysed (P), mechanically-ventilated animal breathing. □ : room air; ▨ : 12% O₂; ▩ : 5% CO₂ and ▧ : 12% O₂+5% CO₂. *: significantly different from room air.
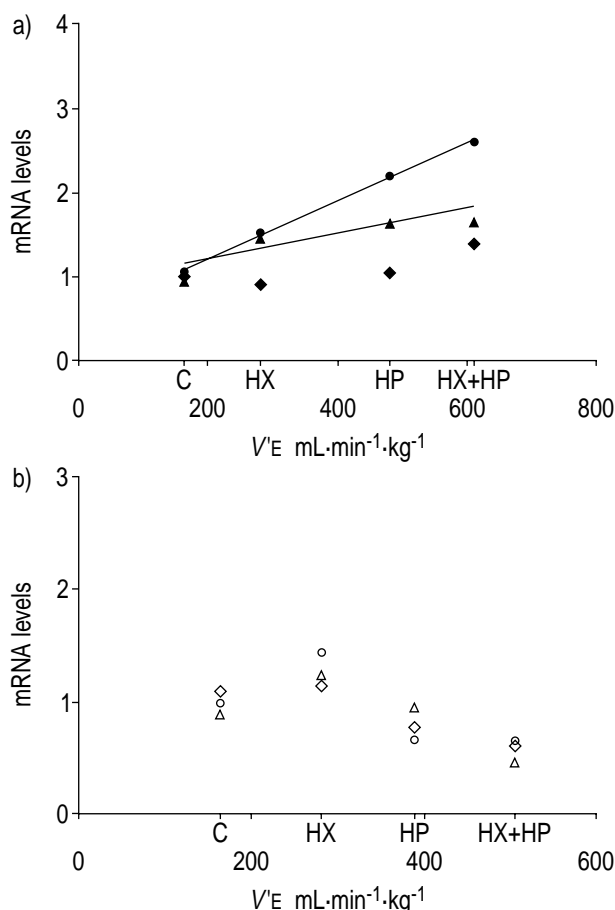

Fig. 4. – Relationship between minute ventilation (V'E) and mean messenger ribonucleic acid (mRNA) levels of vascular endothelial growth factor, basic fibroblast growth factor and transforming growth factor-β1 in the diaphragm under control, hypoxic and/or hypercapnic conditions. a) in awake, intact, spontaneously breathing animals; b) in paralysed mechanically-ventilated animals. ● : VEGF intact (r=0.980, p<0.002); ▲ : bFGF intact (r=0.870, p<0.03); ◆ : TGF-β1 intact (nonsignificant; NS); ○ : VEGF paralysed (NS); △ : bFGF paralysed (NS); ◇ : TGF-β1 (NS).

No significant relationship was observed between VE and mRNA abundance of the three growth factors in the paralysed ventilated animals (fig. 4b).

Finally, no significant relationship was found between mRNA levels and Pa,O$_2$, Pa,CO$_2$, (fig. 5) or arterial pH in the intact, awake, spontaneously breathing or paralysed animals.

Discussion

The main findings of this study were that mRNA levels for VEGF and bFGF in the diaphragm increase after 1 h of actively increased ventilation, and do so in proportion to ventilation, but are unrelated to Pa,O$_2$ and Pa,CO$_2$. Those for TGF-β_1 did not change under the same experimental conditions.

The design of this study has allowed for the assessment of the separate role of: a) active contraction; b) systemic hypoxaemia; and c) passive muscle shortening during mechanical ventilation on the growth

The findings of AMEREDES *et al.* [20] support the present results demonstrating that the diaphragm is relatively insensitive to short (24 min) changes in O_2 supply during moderate hypoxia (inspiratory oxygen fraction (FI,O_2)=0.13). They speculated that this could reflect a difference in either the metabolic or blood flow characteristics of shortening contractions of the diaphragm. This could be visualized better if the geometry of the diaphragm is considered, which is like a dome (curved) in contrast to the more linear nature of the skeletal muscles. Diaphragmatic blood flow is enhanced at shorter muscle lengths [21], and active muscle shortening may optimize capillary configuration [22]. Thus, arterial hypoxia could not accurately reflect the partial pressure of O_2 in the diaphragmatic muscle fibres. Another explanation for the different diaphragmatic response to systemic hypoxaemia than that seen in the gastrocnemius could be that VEGF in the diaphragm is regulated by another mechanism namely, the adenosine pathway [23].

The lack of change in TGF-β_1 after active ventilation was not unexpected. The study of BREEN *et al.* [9] showed a higher signal for VEGF and bFGF than TGF-β_1 after exercise. In addition, the study of ROCA *et al.* [11] showed an increase in the mRNA of VEGF after electrical stimulation only and not in mRNA levels of bFGF and TGF-β_1. Although those results were obtained in another species (canine) and under different experimental conditions, it is apparent that the major angiogenic factor gene response for the skeletal and respiratory muscles is VEGF. The short half-life of VEGF mRNA levels in vascular smooth muscles [24] could explain the acute increase in VEGF as an early response to increased metabolic needs.

Basic FGF, although a direct angiogenic factor, has a broader cell-type specificity. It has been speculated that bFGF may represent a longer-term response to muscle stimulation [8]. The present experiments only lasted 1 h, and may be insufficient to test this hypothesis. However, the smaller increase seen in bFGF than in VEGF is in agreement with this suggestion.

It is well known that VEGF expression is regulated at the transcriptional [25], post-transcriptional [26–28] and translational [29] levels. VEGF upregulation by hypoxia has been demonstrated both *in vitro* and *in vivo* and induces neovascularization [30]. Furthermore, there have been some studies reporting that stretch upregulates VEGF expression in the heart [31] and in cultured cardiac myocytes [32]. This stretch induction of VEGF appears to be mediated in part by TGF-β. However, this network interaction between the various angiogenic factors is not fully understood and the results of this study provide a rationale for further studies of the regulatory regions in the genes of angiogenic growth factor. In particular, further experiments are needed to investigate the relationship between mRNA levels and the protein levels of these angiogenic factors in the diaphragm.

Overall, angiogenic growth factors serve an important physiological role of mediating the dynamic vascularization of tissues to meet metabolic, nutritional and oxygen supply demands. The results of this study suggest that upregulation of VEGF mRNA levels is an early response to increased mechanical tension in
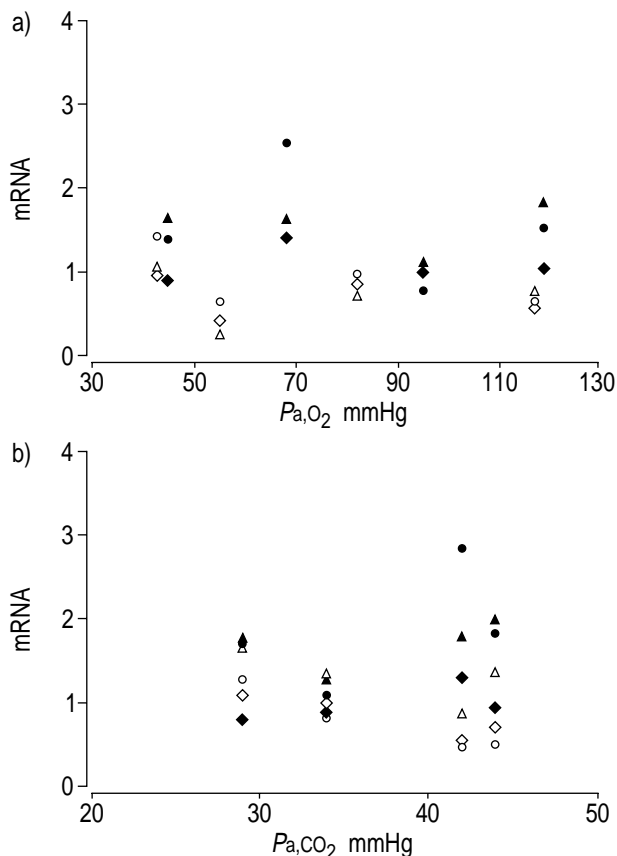

Fig. 5. – Plots of messenger ribonucleic acid (mRNA) levels of vascular endothelial growth factor (VEGF; ●, ○), basic fibroblast growth factor (bFGF; ▲, △) and transforming growth factor (TGF-β1; ◆, ◇) *versus* arterial oxygen tension (Pa,O_2) in intact (closed) and paralysed (open symbols) animals under control, hypoxic and/or hypercapnic conditions. b) Similar plots of mRNA levels of VEGF, bFGF and TGF-β1 *versus* arterial carbon dioxide tension (Pa,CO_2) in intact and paralysed animals. None of the above relationships were statistically significant.

factor gene response. Based on the authors prior work in locomotor skeletal muscle [9, 16–19], showing an enhanced mRNA response of VEGF to hypoxia, studying paralysed, ventilated animals as controls with similar blood gas values as the actively breathing animals provided the means of distinguishing a) from b). Unlike locomotor skeletal muscle, systemic hypoxia alone did not stimulate mRNA levels of the diaphragm during the paralysed experiments. Accordingly, the data suggest that for the diaphragm, active contraction is required for increased gene expression and that hypoxia *per se* is not contributory. The other conclusion that can be made from the paralysed control animals was that passive hyperventilation and thus fibre shortening can also be ruled out as contributing to the gene response. Why the diaphragm appears unresponsive to systemic hypoxia at a level that increases VEGF mRNA in even the resting gastrocnemius of the same species is not clear, but without the ability to measure intracellular Pa,O_2 in either case, it is not possible to provide a clear answer. Another weakness of the present protocol is that a limb muscle to clarify the effect of systemic hypoxia was not included.

the diaphragm resulting from increased ventilation. Furthermore, these data show that angiogenic growth factor gene expression in the diaphragm is sensitive to diaphragmatic load, and this raises the future possibility that therapeutic enhancement of diaphragmatic vascular growth in patients with respiratory failure could contribute to their recovery (weaning from a ventilator). While further work would have to be done to evaluate this hypothesis, studies of the myocardium [33, 34] and of ischaemic vascular disease [35] have shown that targeted growth factor administration improves local blood supply [36–40].

In summary, the results of this study showed that active increases of ventilation by hypoxia and/or hypercapnia upregulate vascular endothelial growth factor and basic fibroblast growth factor messenger ribonucleic acid levels in the diaphragm, and this increase is proportional to minute ventilation. In paralysed mechanically-ventilated animals at similar arterial blood gas and ventilatory levels, no change in messenger ribonucleic acid levels of any of the three angiogenic factors studied was observed. Thus, moderate hypoxia and passive shortening of the muscle had no additional effect. To conclude, angiogenic gene responses of the diaphragm depend on active contraction of the diaphragm.

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