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Effects of genetic obesity on rat upper airway muscle and diaphragm contractile properties

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ABSTRACT: The contractile properties of pharyngeal respiratory muscle are altered in sleep apnoea and in conditions associated with sleep apnoea, such as ageing. We hypothesized that the contractile properties of the pharyngeal musculature are also altered by obesity, another factor associated with sleep apnoea.

Studies compared a pharyngeal muscle, the sternohyoid, with the diaphragm. These were chosen as representative muscles whose contraction has opposing effects on upper airway patency. Both muscles were removed from nine lean and nine obese male Zucker rats (a genetic model of obesity), and isometric contractile properties were studied *in vitro* at 37°C.

For the sternohyoid muscle, in obese compared to lean animals there were no significant differences in isometric contraction time (15.2±0.3 *vs* 14.2±0.6 ms, respectively), half-relaxation time (13.6±0.5 *vs* 12.6±0.9 ms, respectively), twitch-to-tetanic tension ratio (0.22±0.02 *vs* 0.24±0.02, respectively), force-frequency relationship, fatigue resistance (2 min fatigue index 0.20±0.03 *vs* 0.18±0.02, respectively), or maximal degree of force potentiation during repetitive stimulation (52±11 *vs* 74±20% increase, respectively). For the diaphragm, the only significant effect of obesity was a lowering of the twitch-to-tetanic tension ratio (0.25±0.01 *vs* 0.29±0.02, respectively). In obese, as in lean animals, the sternohyoid had faster isometric twitch kinetics, a larger degree of force potentiation, and lower resistance to fatigue, than the diaphragm. In lean, but not obese, animals the sternohyoid twitch-to-tetanic tension ratio was lower than and the force frequency relationship was located to the right of that of the diaphragm.

In this study, genetic obesity in rats was not associated with any significant alterations in the contractile properties of the pharyngeal muscle, and only small changes in the relationship between the contractile properties of the sternohyoid and diaphragm muscle.

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Obstructive sleep apnoea is a disorder characterized by intermittent collapse of the pharyngeal upper airway with consequential cessation of ventilation during sleep [1, 2]. There is a close association between obesity and sleep apnoea, in that there is a high prevalence of obesity among adults with obstructive sleep apnoea and, conversely, a high prevalence of sleep apnoea among obese adults [1–4]. Obesity reduces chest wall compliance [5], and in a rat model has recently been found to alter the structural properties and to a smaller extent the contractile properties of the diaphragm [6]. The effects of obesity on the upper airway musculature are not known.

Maintenance of pharyngeal patency during breathing is dependent on sufficient activation and contraction of the skeletal muscles which dilate the pharyngeal upper airway. Alterations in structural and/or contractile properties of the pharyngeal muscle have recently been noted in humans and dogs with sleep apnoea [7, 8], in humans who snore [9], and in conditions associated with obstructive sleep apnoea, including Down's syndrome [10], hypothyroidism [11], development [12, 13] and ageing [14]. Conversely, subjects with primary muscle disease (*e.g.* muscular dystrophy) have an abnormally high prevalence of obstructive sleep apnoea [15, 16]. These data suggest a role for altered intrinsic properties of the pharyngeal musculature in the pathophysiology of obstructive sleep apnoea.

The present study tested the hypothesis that obesity alters the contractile properties of the pharyngeal dilator musculature. To test this hypothesis, isometric contraction and relaxation rates, force-frequency relationships, fatigue resistance and degree of force potentiation of the sternohyoid and diaphragm muscle were examined in an animal model of genetic obesity, the Zucker rat [17, 18]. Comparisons were made between representatives from muscle groups whose contraction has opposing effects on upper airway patency and which are involved in the pathogenesis and relief of obstructive apnoeas [1, 2, 7, 8]: the sternohyoid muscle, which dilates the airway, thereby promoting upper airway patency, and the diaphragm whose contraction generates subatmospheric pressures in the upper airway thereby promoting closure of the upper airway. The diaphragm was examined directly in the present study to avoid possible effects of inter-animal and methodological variability, which may have affected comparisons had we relied exclusively on previously published data for the diaphragm [6].

Methods

Muscle contractile properties were assessed *in vitro*. Muscle from nine obese male Zucker rats (mean weight 612±3 g) was compared with that from nine lean male Zucker rats (mean weight 389±12 g) of similar age (3–4 months). The animals were anaesthetized with intraperitoneal urethane (1–1.5 $g \cdot kg^{-1}$). The sternohyoid muscle was removed *via* a midline cervical incision, and the diaphragm muscle was removed *via* thoracic and abdominal incisions. Both muscles were removed sequentially in rapid succession from each animal and placed in oxygenated physiological solution (see composition below). The muscles were cut into small rectangular strips (width 1–1.5 mm), maintaining the integrity of the bony and/or tendinous origins and insertions. One diaphragm strip and one sternohyoid strip were studied from each animal, except that for one of the obese animals the sternohyoid muscle was damaged during removal and not studied.

The muscle strips were mounted vertically in a double-jacketed bath, whose temperature was maintained at 37°C. The composition of the preparation and bathing solutions were as follows (in mM): 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 15 $NaHCO_3$, and 11 glucose. The solution was aerated with 5% CO_2-95% O_2, and had its pH adjusted to 7.3–7.4. After a 5 min equilibration period, the muscle strips were stimulated electrically (supermaximal voltage, pulse width 1 ms for both muscles) *via* platinum field electrodes, and muscle length was adjusted to that at which twitch tension was maximal. A high sensitivity isometric transducer (Kent Scientific Corporation-Radnotti Glass Technology, Monrovia, CA, USA) was used to measure isometric twitch force. The addition of curare (0.025 mM) to the bath did not alter twitch force in preliminary studies, indicating direct activation of the muscles. For all data reported, sample sizes are nine muscle strips for lean sternohyoid, lean diaphragm and obese diaphragm, and eight muscle strips for obese sternohyoid.

To determine isometric twitch kinetics, muscle strips were stimulated at a frequency of 0.1 Hz. Thereafter, they underwent testing of the force-frequency relationship by stimulation at frequencies of 1, 5, 10, 15, 20, 30, 40, 50, 60, 80 and 100 Hz. Following a brief recovery period, a standard stimulation protocol was used to assess muscle fatigue: 40 Hz pulses lasting 0.33 s were delivered every 1 s for a total of 5 min [8, 13, 14, 19, 20]. Force was quantified by measuring the peak value at any time during the sequence of pulses. Fatigue indices were defined as the ratio of peak force at the end of 2 and 5 min of repetitive stimulation to initial force. A high fatigue index, therefore, indicates a greater degree of resistance to fatigue. To assess force potentiation during repetitive stimulation, during the first 40 s of the fatigue protocol each sequence was analysed to determine the presence and extent of augmentation of force relative to that induced during the initial sequence. Force potentiation at 10 s was defined as force after 10 s of stimulation relative to initial force (a negative value indicates force decline rather than force potentiation). The maximum degree of force potentiation was defined as the highest force during any sequence; and expressed relative to force during the initial sequence. In a previous study of the sternohyoid and diaphragm [20], close agreement was found between degree of force potentiation assessed in this manner and force potentiation assessed with staircase and post-tetanic potentiation protocols.

The output from the force transducer was fed *via* an analogue-to-digital converter to the hard drive of a computer using a standard data acquisition program (Axotape; Axon Instruments, Foster City, CA, USA). Force measurements were made on screen with the use of manually-controlled cursors. Isometric twitch kinetics were quantified by the contraction time (time required to attain maximal twitch force) and the half relaxation time (time required for maximal force to decay by 50%). During the repetitive stimulation protocols, force was normalized to that produced during the first simulation sequence, and expressed as percentage of the initial value. Mean values±SEM were calculated for data from each muscle. Statistical comparisons of data for each muscle between rat strains, and between muscles for a given rat strain, were performed using the unpaired t-test. Statistical comparisons of force-frequency relationships were performed with two-way repeated measures analysis of variance (ANOVA), followed by the Newman-Kuels test when the ANOVA indicated a significant difference. A p-value of less than 0.05 (two-tailed) was considered to indicate statistical significance.

Results

Mean values for the isometric contraction and half relaxation times of the sternohyoid and diaphragm of lean and obese Zucker rats are depicted in figure 1. There were no significant differences between obese and lean animals for sternohyoid contraction time (15.2±0.3 *vs* 14.2±0.6 ms, respectively) or half-relaxation time (13.6±0.5 *vs* 12.6±0.9 ms, respectively), nor did obesity significantly affect diaphragm twitch kinetics. In obese animals, the sternohyoid had faster contraction ($p<0.001$) and half relaxation times ($p<0.001$) than the diaphragm, as was the case for lean animals ($p<0.001$ for both).

The twitch-to-tetanic tension ratio was not different for obese compared to lean animals for the sternohyoid

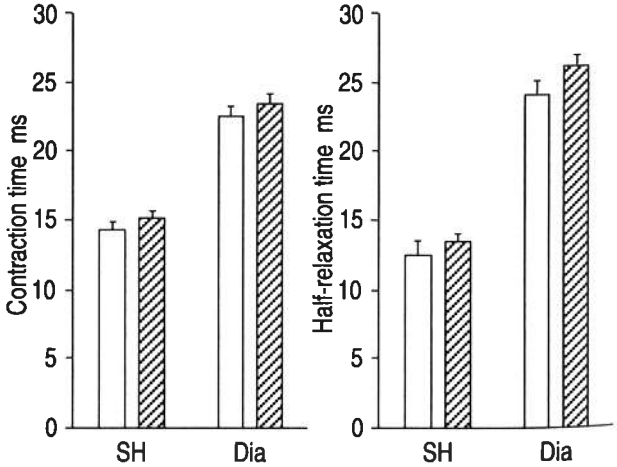

Fig. 1. – Isometric contraction and half relaxation times of the sternohyoid (SH) and diaphragm (Dia) muscles of lean and obese Zucker rats. Values are presented as mean±SEM. ▭: lean rats; ▨: obese rats.

muscle (0.22±0.02 *vs* 0.24±0.04, respectively), but was significantly lower for obese than lean animals for the diaphragm muscle (0.25±0.01 *vs* 0.29±0.02, respectively) ($p<0.05$). In obese animals, there was no significant difference between the two muscles in twitch-to-tetanic tension ratios, in contrast to lean animals in which differences were found ($p<0.05$). Obesity did not significantly alter the force-frequency relationship either for the sternohyoid or diaphragm muscle (figure 2). In obese animals, there was no significant difference between the two muscles in the force-frequency relationship, whereas in lean animals the force-frequency relationship of the sternohyoid was located significantly to the right of that of the diaphragm ($p<0.02$).

Values for the 2 and 5 min fatigue indices during repetitive 40 Hz stimulation are shown in figure 3. For the sternohyoid muscle, there were no significant differences between obese and lean rats in the degree to which force declined following 2 and 5 min of repetitive stimulation (2 min fatigue index 0.20±0.03 *vs* 0.18±0.02, respectively; 5 min fatigue index 0.13±0.02 *vs* 0.11±0.01, respectively). Furthermore, obesity did not affect diaphragm fatigue resistance significantly. In obese animals, the diaphragm had significantly higher 2 and 5 min fatigue indices than the sternohyoid muscle ($p<0.02$ and $p<0.01$, respectively), as was the case for lean animals ($p<0.001$ and $p<0.001$, respectively).

During repetitive stimulation, force of the sternohyoid muscle initially increased prior to a rapid rate of decline (fig. 4a). There were no significant differences between obese and lean animals in the degree to which sternohyoid muscle force changed at specific time intervals (fig. 4a) or in the maximal force at any point in time (52±11 *vs* 74±20% increase, respectively) (fig. 4b). The diaphragm

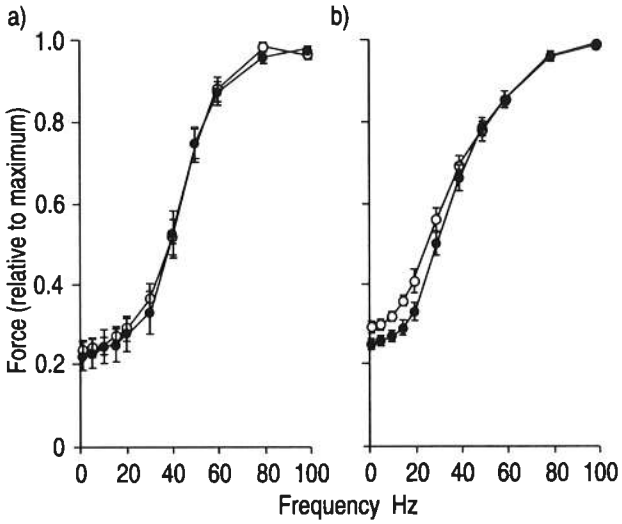

Fig. 2. – Force-frequency relationships of: a) the sternohyoid; and b) the diaphragm muscles of lean and obese rats Zucker rats. Values are presented as mean±SEM. ○ : lean rats; ● : obese rats.

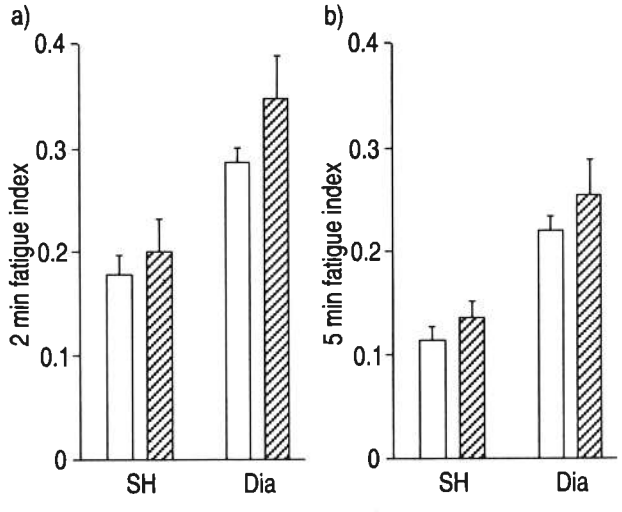

Fig. 3. – a) 2 min; and b) 5 min fatigue indices of the sternohyoid (SH) and diaphragm (Dia) muscles of lean and obese Zucker rats. Values are presented as mean±SEM. ▭ : lean rats; ▨ : obese rats.

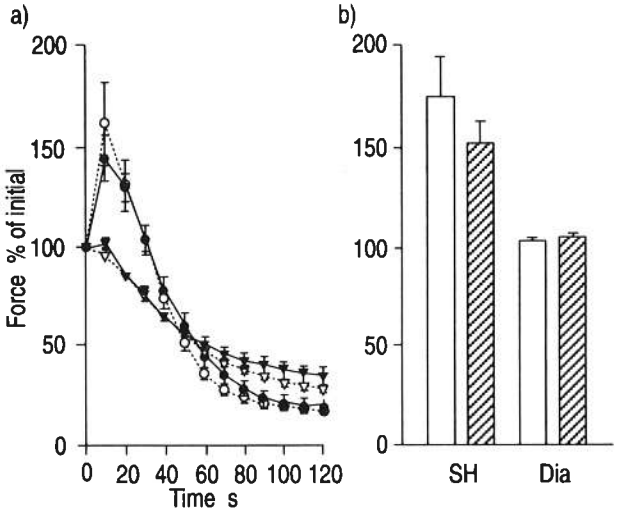

Fig. 4. – Changes in force of the sternohyoid (SH) and diaphragm (Dia) muscles during repetitive stimulation in lean and obese Zucker rats. Force was expressed as a percentage of that during the initial stimulus sequence for each muscle. Values are presented as mean±SEM. a) changes in force over time; ···○··· : lean SH; —●— : obese SH; ···▽···: lean Dia; —▼— : obese Dia. b) maximal force at any time during repetitive stimulation. ▭ : lean rats; ▨ : obese rats.

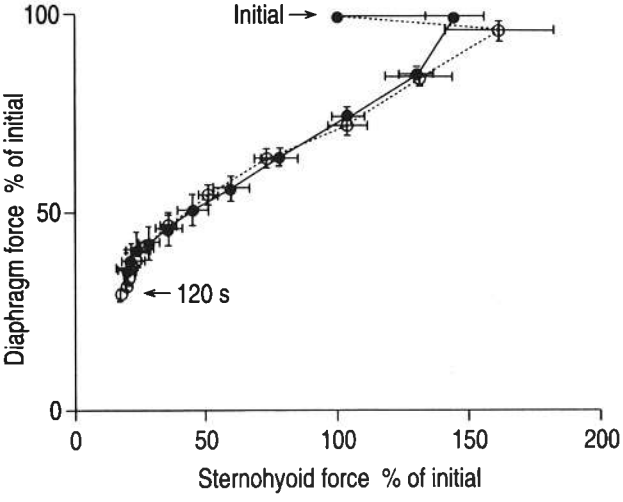

Fig. 5. – Relationship between changes in sternohyoid and diaphragm force of lean and obese Zucker rats during the first 2 min of repetitive contraction. Force was expressed as a percentage of that during the initial stimulus sequence for each muscle. Values are presented as mean±SEM. ○ : lean rats; ● : obese rats.

exhibited a much smaller and shorter-lived force increase, which was also not affected by obesity. In obese animals, the maximal force was significantly greater for the sternohyoid than the diaphragm ($p<0.001$), as was the case for lean animals ($p<0.005$) (fig. 4b).

Although obesity did not significantly affect force potentiation or fatigue of the sternohyoid or diaphragm, there were some trends which if in opposite directions for the two muscles could potentially affect the relationship between force output of these two muscles over time. Figure 5 examines this relationship directly, and indicates that obesity produced only a small change in the relationship between sternohyoid and diaphragm force, which was limited to the early portion of repetitive stimulation.

Discussion

The sternohyoid muscle is one of many pharyngeal muscles which dilates the upper airway [21, 22]. Considerable information is available about its structural and contractile properties in various species [7, 11, 13, 14, 19, 20, 23]. Alterations have been found in this muscle (but not in the geniohyoid muscle) in a dog model of sleep apnoea: a higher proportion of fast fibres; an increased proportion of morphologically abnormal fibres, consistent with previous or ongoing injury; and a greater connective tissue content, consistent with fibrosis [7]. The sternohyoid muscle was chosen for the current study based on the above considerations, as well as the fact that it is easier to distinguish from surrounding tissues and, hence, easier to dissect than other muscles, such as the geniohyoid and especially the genioglossus muscles, minimizing the chance of tissue damage. Previous studies have examined the contractile properties of the sternohyoid muscle in the adult rat, the sternohyoid, sternothyroid, geniohyoid, and genioglossus muscles in the adult cat, and the musculus uvulae in adult humans [8, 14, 19, 20, 24, 25]. These muscles generally have fast isometric contraction and relaxation rates, low twitch-to-tetanic tension ratios, a more rightward force-frequency relation than the diaphragm, a high degree of force potentiation, and variable resistance to fatigue (which differs among muscles and among species). The present data for the sternohyoid muscle in lean Zucker rats are consistent with previous studies in normal Sprague-Dawley and Fischer 344 rats [14, 20].

The present study found that genetic obesity in rats had no significant effects on the following physiological properties of the sternohyoid muscle: isometric twitc kinetics, twitch-to-tetanic tension ratio, force-frequency relationship, force potentiation and fatigue resistance. Furthermore, the relationship between the contractile properties of the sternohyoid and diaphragm was not altered by obesity, with the following exceptions: in lean animals the sternohyoid twitch-to-tetanic force ratio was lower than and the force-frequency relationship was shifted to the right of that of the diaphragm, whereas in obese animals these two properties did not differ between the sternohyoid and the diaphragm. The differences in these relationships between obese and lean animals will impact the transduction of motoneuronal firing rates into muscle force, specifically making the transduction between the two muscles more similar in obese than lean animals. Obese Zucker rats have blunted ventilatory responses to hypercapnia, but no alterations in resting breathing or ventilatory responses to hypoxia, compared to lean animals [5]. Whether obese animals differ from lean animals in phrenic and/or hypoglossal motoneuronal firing rates during breathing is not known, so the impact of altered relationships between sternohyoid and diaphragm force-frequency relationships in the intact animal is difficult to predict.

The paucity of changes in contractile properties of the sternohyoid muscle with obesity contrasts with the effects of development and ageing, both of which significantly affect sternohyoid fatigue resistance [13, 14]. The effects of ageing were examined in Fischer 344 rats: compared to young muscle (3–4 months), old muscle (20–21 months) had impaired fatigue resistance (the 2 min fatigue index decreased from 0.18 to 0.10, and the 5 min fatigue index decreased from 0.10 to 0.03) despite no change in isometric twitch kinetics [14]. In that study, the old animals weighed more than the young animals (~470 *vs* ~300 g, respectively). The present data indicating no effects of obesity on sternohyoid muscle fatigue resistance suggest that the altered fatigue resistance noted with ageing in the previous study was not a consequence of the higher body weight of the older animals in that study. Developmental effects on sternohyoid muscle function have been examined in piglets: 14–20 day old animals had reduced fatigue resistance (the 2 min fatigue index decreased from 0.52 to 0.36 with development) but no differences in isometric twitch kinetics compared to 1–7 day old animals [13]. SERIES and co-workers [8] compared the musculus uvulae (a palatal muscle) of humans with sleep apnoea with that of snorers with similar body mass index, and found no differences in contraction and half-relaxation times, fatigue resistance, or tetanic tensions normalized for cross-sectional area, but an increase in absolute tetanic tension in subjects with sleep apnoea. No comparative data on subjects who neither snored nor had sleep apnoea were reported. More recently, SERIES and co-workers [24] found that the contractile properties of musculus uvulae correlated with upper airway collapsibility among subjects with snoring and sleep apnoea, suggesting a greater modulation of pharyngeal muscle properties with disease than had been indicated by their previous study [8].

The findings of the present study concerning the diaphragm are in general agreement with those described previously by FARKAS *et al.* [6] for the diaphragm of 8–10 month old female Zucker rats. In both studies, obesity decreased the twitch-to-tetanic force ratio, but did not alter the half-relaxation time, the normalized force-frequency relationship, the rate of muscle fatigue, or (based on inspection of figure 6 of [6]) the degree of force potentiation during the early part of the fatiguing stimulation. The only discrepancy between the two studies with regard to the effects of obesity is that FARKAS *et al.* [6] found that obesity significantly prolonged diaphragm contraction time (from 21.2 to 23.1 ms), whereas in the present study there was a more modest prolongation of contraction time (from 22.7 to 23.6 ms), which was not statistically significant. The reason for the small difference between studies is not entirely clear. The methodology for determining contraction time is highly standardized, so that differences in technique between studies are an

unlikely explanation. On the other hand, FARKAS *et al.* [6] used 8–10 month old female rats, whereas 3–4 month old male rats were used in the present study, so differences in age or gender between studies are a more likely explanation.

In the present study, it was found that both for obese and lean animals the sternohyoid muscle demonstrated considerable force potentiation (maximum increases of 74 and 52% in lean and obese animals, respectively) whereas the diaphragm had relatively little force potentiation (maximum increases of 2 and 5% in lean and obese animals, respectively) during the early portion of the fatigue run. The recent study comparing the contractile properties of the diaphragm in lean and obese rats did not specifically address force potentiation, but inspection of the fatigue data indicates force potentiation of ~10% for lean animals and ~3% for obese animals at a stimulation frequency of 35 Hz (figure 6 of [6]). In contrast, in a previous study by KUEI *et al.* [26] no apparent force potentiation can be discerned for Sprague-Dawley rat diaphragm at stimulation frequencies of 20, 40 or 75 Hz (figure 6 of [26]). The present data are consistent with our previous study in Sprague-Dawley rats, in which substantially greater force potentiation was noted for the sternohyoid muscle than the diaphragm (maximum force increases of 33 and 3%, respectively, during 20 Hz stimulation; and 40 and 1%, respectively during 40 Hz stimulation). Thus, the degree of force potentiation noted for the diaphragm in the present study is within the range reported previously.

Upper airway patency during sleep is determined by the balance of forces which, on the one hand, dilate or stiffen the airway (determined by contraction of pharyngeal dilator muscles) and, on the other hand, produce subatmospheric intraluminal pressures (determined among other factors by the vigour of thoracic muscle contraction during inspiration) [1, 2]. Therefore, changes in the efferent neural output to and the contractile properties of the upper airway muscles are best interpreted in light of changes in efferent output to and contractile properties of the thoracic respiratory muscles. In the present study, we found a nonsignificant trend for obesity to improve the fatigue resistance of the sternohyoid muscle, which was in the same direction as the nonsignificant trend for the diaphragm muscle. On the other hand, there were nondivergent trends in the effect of obesity on force potentiation, which resulted in a transient alteration in the relationship between sternohyoid and diaphragm force during repetitive stimulation. During obstructive sleep apnoeas, upper airway muscle activity rises and falls considerably, so that it is possible that the force potentiating properties of the upper airway musculature could impact on upper airway patency. However, whether this occurs has not been examined specifically in either human or canine sleep apnoea.

Previous studies have found that the structural and metabolic properties of limb muscles are altered by obesity. These changes (which are not uniform among limb muscles) include: increased levels of the oxidative enzymes cytochrome oxidase, citrate synthase, and β-hyproxyacetyl-coenzyme A (CoA) dehydrogenase; decreased proportions of Type I fibres, and reduced capillary density [27–29]. The diaphragm is also altered structurally by obesity, as follows: increased proportions of Type I fibres with reduced proportions of Type IIa and IIb fibres; increased size of Type I and IIa fibres with reduced size of Type IIb fibres; increased contribution to total cross-sectional area of Type I fibres and decreased contribution to total cross-sectional area of Type IIb and IIx fibres; and decreased succinate dehydrogenase activity of Type I fibres [6]. These structural and metabolic changes in the diaphragm with obesity were not necessarily associated with functional changes (*e.g.* fatigue resistance did not change with obesity) [6]. Therefore, the absence of significant functional changes in the sternohyoid with obesity does not imply an absence of structural or metabolic changes with obesity.

The extent to which abnormalities in pharyngeal muscle structural and contractile properties are the cause *versus* the consequence of obstructive sleep apnoea and snoring is not clear. That a relationship between the two does exist is supported by data, both from humans and dogs, reporting changes in pharyngeal muscle properties with sleep apnoea and snoring [7–9]. Recent preliminary data further support such a relationship, in that absolute twitch and tetanic tensions of the musculus uvulae (a palatal muscle) were found to correlate positively, and the fatigue index tended to correlate negatively, with upper airway critical pressure in humans with sleep apnoea and/or snoring [24]. Postulated abnormalities which may contribute to obstructive sleep apnoea in obesity include changes in upper airway structure, *e.g.* fat deposition [30], alterations in the neural regulation of pharyngeal muscles, and alterations in pharyngeal muscle contractile properties.

The present data argue against alterations in the contractile properties of the pharyngeal muscle playing a major role in the pathogenesis of obstructive apnoea in obesity. However, it is not clear whether data in genetically obese rats can be generalized to humans or dogs with sleep apnoea. Firstly, effects of genetic obesity may differ from acquired obesity. Secondly, effects of obesity on muscles in rats may differ from their effects in humans. Thirdly, sleep, and especially the propensity to develop sleep apnoea, may differ in humans and rats; specifically, there have been no reports of naturally occurring obstructive sleep apnoea in rats. Thus, it is possible that in humans obesity may lead to sleep apnoea which secondarily alters pharyngeal muscle properties, whereas in rats obesity does not lead to obstructive apnoeas, so that the pharyngeal muscles do not deteriorate. It is also possible that obesity has a primary effect on pharyngeal muscles in humans which leads to sleep apnoea, whereas in rats obesity has no physiological effects on pharyngeal muscles, so that the animals do not develop sleep apnoea. Studies of pharyngeal muscles in obese and nonobese humans with and without sleep apnoea are needed to better address these issues.

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