BIOLOGICAL CONSEQUENCES OF OXYGEN DESATURATION AND RESPIRATORY EFFORT IN AN ACUTE ANIMAL MODEL OF OBSTRUTIVE SLEEP APNEA (OSA).

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ABSTRACT

Background. An animal model mimicking all the factors involved in obstructive sleep apnea (OSA) is useful for investigating mechanisms because the associated comorbididy usually present in such patients is an important limitation. **Aim:** To test the hypothesis that hypoxia/normoxia and the respiratory effort have different effects on the induction of the inflammatory response and endothelial dysfunction in an acute rat model of OSA.

Methods: Four groups of anesthetized rats were studied (n=8): 1) Sham; 2) Apnea: obstructions (15 s each, 60/h, for 3 h); 3) Apnea+O2: obstructions and breathing oxygen-enriched air to avoid hypoxia and 4) Intermittent hypoxia/normoxia. Inflammatory and endothelial mediators were measured as outcomes along with NF-kB in the lung and diaphragm..

Results: TNF-α and IL-1β significantly increased in all groups compared with Sham. NF-kB in the lung was increased in Apnea and Hypoxia/Normoxia groups, but not in Apnea+O2 group. In diaphragm tissue, NF-κB was only significant in Apnea compared to Sham. Significant differences were found in the ratio thromboxane-B2/6-keto-Prostaglandin-F1α between Apnea and Hypoxia/Normoxia compared to Sham but not in Apnea+O2.

Conclusions: Oxygen desaturations and respiratory efforts play a role in the induction of systemic inflammation but only hypoxia/normoxia induces endothelial dysfunction. These data suggest a potential role for oxygen therapy in patients with OSA.

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Key words: obstructive apnea, inflammation, endothelial dysfunction, respiratory effort, hypoxia, animal model.

INTRODUCTION

Obstructive sleep apnea (OSA) is the most common condition involving sleep-disordered breathing, affecting between 4-6% of the population (1). OSA is characterized by intermittent episodes of partial or complete obstruction of the upper airway during sleep and these disrupt normal ventilation and sleep architecture. OSA is typically associated with excessive daytime sleepiness, snoring, and witnessed apneas. This syndrome is also a cardiovascular risk factor (2), its association with hypertension (2-4) being one of the most clearly determined cardiovascular consequences.

The current evidence available shows that OSA is associated with systemic inflammation and endothelial dysfunction (2, 5-15), and this has been considered one of the potential mechanisms linking OSA with cardiovascular disease. In most cases, however, the data are partial and sometimes even contradictory. Associated comorbidity, such as obesity, may represent an important confounding factor, possibly leading to a distortion of the results obtained from patient studies. Animal models will therefore be useful for exploring the different mechanisms of OSA, as they not only avoid any associated comorbidity but will also allow us to analyse the roles of the various factors involved in the syndrome.

In our current research, we are testing the hypothesis that hypoxia/normoxia and respiratory effort have different effects on the induction of the inflammatory response and endothelial dysfunction present in OSA. To this end, we have used a previously described acute animal model of OSA (3 h, in anesthetized animals) that mimics the recurrent upper airway obstructions characteristic of this sleep breathing disorder (16). The model avoids the associated comorbidiity found in humans and allows us to individualize the various factors present in OSA. Of course, that this acute model can not be completely related to the chronic cardiovascular effects.

We analysed as outcomes of the different group experiments the circulating levels TNF-α and IL-1β, which served as markers of inflammatory response. Furthermore, in order to ascertain the source of these pro- inflammatory cytokines, we measured the transcription factor nuclear kappa B (NF-κB) in two target tissues involved in recurrent obstructive apneas: lung and diaphragm. Additionally, thromboxane-A_2 (TxA_2) and prostacyclin (PGI_2) were measured to evaluate the endothelial dysfunction. TxA_2 is a powerful starting agent of platelet aggregation and a potent vasoconstrictive, whereas, PGI_2 has vasodilatadory effects and acts against platelet aggregation. An imbalance of vasoconstrictor and vasodilator mediators has been implicated in vascular diseases.

A better knowledge of the mechanisms involved in the biological response of OSA might be very important, not only for understanding the disease but also for its potential relevance to treatment strategy. For instance, in the case that hypoxia/normoxia proved to be the major factor responsible for the systemic inflammatory/endothelial response, oxygen administration could also play a role in treatment, especially in patients with severe OSA who do not accept or tolerate CPAP.

2. MATERIALS AND METHODS

2.1 Animals

Pathogen-free male Sprague-Dawley rats, weighing 350-375 g, were obtained from Charles River Laboratories (Saint Germain sur L'arbresle, France). The rats were housed in a controlled environment and fed rodent chow (A04; Panlab, Barcelona, Spain) and tap water *ad libitum*. This study conformed to European Community (Directive 86/609/EEC) and Spanish guidelines for the use of experimental animals and was approved by the institutional committees of animal care and research.

2.2 Model of recurrent obstructive apneas

Recurrent obstructive apneas in rats were applied by means of a previously described computer-controlled collapsible segment based on a Starling resistor placed in the upper airway of the animal (16,17) (Figure 1). Briefly, the collapsible segment consisted of two identical cylindrical chambers separated by a circular flexible membrane. The base of one of the chambers had a tube connected to a computer-driven source of external pressure that produced controlled obstruction of the segment. A tube at the centre of the base of the other chamber connected the collapsible segment to the rat trachea. Another tube in this chamber wall was open to the atmosphere and acted as a pneumotachograph to measure breathing flow. A pressure port in the tube connecting the collapsible segment to the trachea enabled us to measure tracheal pressure. Arterial oxygen saturation in the rat leg was monitored by pulse oximetry (504 Inc Wauseda, WI, USA).

2.3 Experimental groups

Experimental animals were randomly divided into four groups (n=8 for each group): Sham, Apnea, Apnea+O2 and Hypoxia/Normoxia groups. The animals were anesthetized (Urethane 1.2 g/kg, intraperitoneal), a tracheotomy was performed, a cannula (2 mm ID) was inserted into the trachea, and the inlet of the cannula was connected to an upper airway collapsible segment (16). In the Sham group, no obstructions were applied to the upper airway collapsible segment (Figure 2). In the Apnea group, the animals were subjected to 60 obstructive apneas per hour lasting 15 s each for a period of 3 h. As shown in Figure 2, the respiratory efforts during obstructive apneas were three times that of the basal pressure. The Apnea+O2 group was treated in the same way as the Apnea group but a rich atmosphere of oxygen was applied: the oxygen concentration of the air was adjusted to maintain baseline oxygen saturation and avoid desaturations during apneas. The Hypoxia/Normoxia group was instrumented by a special device (Figure 1) that allows the induction of hypoxia/normoxia periods 60 times per hour. The cycles applied comprised a hypoxic phase (ambient O2

concentration 5 %) of 15 s followed by a rapid return to ambient O2 at 21 % for 45 s for a period of 3 h (Figure 2). The animals were sacrificed at the end of the experiment by exsanguination from the abdominal aorta.

2.4 Biochemical assays in plasma

The blood samples were put into microcentrifuge tubes containing ETDA, placed on ice and centrifuged at 3000 rpm for 15 min. The plasma was collected and frozen at −80°C until used. Tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) concentrations were measured by a commercial solid-phase sandwich enzymelinked immuno-sorbent assay (ELISA) from R&D Systems (Minneapolis, MN, U.S.A.). Circulating levels of Thromboxane-B2 (TxB2) and 6-keto-Prostaglandin-F1α (6kPGF1α) were measured with commercially available high sensitivity ELISA kits (Cayman Chemical, Tallinn, Estonia).

2.5 Nuclear protein extraction and determination of nuclear factor- B (NF-kB) binding activity.

Samples of lung and diaphragm tissue were obtained immediately after sacrifice, frozen in liquid nitrogen and kept at -80 ºC for further studies. For the measurement of NF-κB activation, nuclear fractions were prepared from lung and diaphragm tissues using a Nuclear Extract Kit (Active Motif, Rixensart, Belgium). Levels of nuclear p65 concentrations were determined by TransAMTM NF-κB p65 Chemi kit (Active Motiff, Rixensart, Belgium).

2.6 Statistics

Data are expressed as mean ± SEM. Statistical analysis was carried out by ANOVA. When differences were significant, appropriate post hoc tests, including the Newman-Keuls test (GraphPad Software Inc, San Diego, CA, USA), were performed. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Inflammatory biochemical markers

The inflammatory response was reflected by an increase in TNF-α and IL-1β. Both markers showed a significant increase in all the experimental groups, compared to the Sham group (Figure 3). There were no differences between groups. These data indicate that respiratory effort and recurrent hypoxia/normoxia induce systemic inflammation.

3.2 Nuclear factor-kB binding activity

NF-kB binding activity was increased in nuclear fractions from lungs in both the Apnea and the Hypoxia/Normoxia group compared to the Sham animals. This enhancement in NF-kB activity was not observed in the Apnea+O_2 group (Figure 4-top). Figure 4-bottom shows NF-κB activation in nuclear fractions from diaphragm tissue, indicating that, compared to Sham, there was a significant increase only in the Apnea group.

3.3 Vascular endothelial markers

As an indication of endothelial dysfunction, TxB_2 and $6kPGF_{1\alpha}$ were measured in plasma samples (Figure 5). The levels of TxB_2 showed a significant increase between the Apnea and Hypoxia/Normoxia group compared to the Sham group. However, the TxB_2 level in the Apnea+O_2 group did not show a significant increase compared to the Sham group. The levels of $6kPGF_{1\alpha}$ showed a significant decrease between the Apnea and Hypoxia/Normoxia group compared to the Sham group. This decrease of $6kPGF_{1\alpha}$ level was not found in the Apnea+O_2 compared to the Sham group (Figure 5). An increase in TxB_2 accompanied by a decrease in the $6kPGF_{1\alpha}$, which translates into an increase of the $TxB_2/6kPGF_{1\alpha}$ ratio, an index of a vasoconstrictor effect, was observed in the Apnea and Hypoxia/Normoxia

experimental groups compared to Sham animals. An oxygen-rich atmosphere reversed these changes.

4. DISCUSSION

There is a growing body of evidence in the literature to indicate that OSA is associated with increased levels of proinflammatory cytokines, and subsequent increased endothelial dysfunction. These could lead to cardiovascular disease (10-15). However, it is not clear to what extent hypoxia/normoxia and respiratory effort play a role as mechanisms that trigger the inflammatory and endothelial processes in OSA patients. Both stimuli could play an important role in the development of cardiovascular diseases. On the one hand, it has been reported in experimental models that hypoxia/normoxia without respiratory effort can trigger a vascular disease (18). On the other hand, the respiratory effort caused by the upper airway obstruction results in large negative swings in intrathoracic pressure that can also trigger systemic inflammation. (19).

In our study, we have discriminated between the different challenges that contribute to the pathology of OSA in order to identify the importance of these stimuli in the induction of systemic inflammation and endothelial dysfunction. The data from our study show that 3 hours of recurrent obstructive apneas were associated with an increase in inflammatory mediators, in line with various studies on OSA patients that have identified increased levels of circulating TNF-α and IL-1β. This increase occurs when either the hypoxia/normoxia or the respiratory effort appear on their own. We have also observed that apneas and hypoxia/normoxia episodes induce endothelial dysfunction, but this was not the case with recurrent respiratory efforts (obstructive apneas plus oxygen).

The experimental model allowed us to determine the importance of the different stimuli. It is important to take into account that in this experimental model the group of rats with only

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respiratory efforts maintained a normal oxygen, whereas experimental models of resistive breathing are always linked to tissue hypoxia. On the basis of our data, it seems reasonable to assume that the activation of inflammatory pathways is caused by the dual presence of hypoxia/normoxia and respiratory effort, as has also been suggested by Vassilakopoulos *et al.* (19).

It has been reported that subjects with OSA have a selective activation of NF-κB in monocytes compared with control subjects. Thus, the activation of NF-κB may be a molecular mechanism implicated in the development of OSA pathology,(7; 12; 20-22) as it is well- known that oxidative stress is considered the initial and the major stimulus for NF-κB activation, caused by resistive breathing and the hypoxia normoxia episodes (23,24). Our results therefore demonstrate an activation of NF-κB triggered by recurrent obstructive apneas, in the lung as well as in the diaphragm. Hypoxia/normoxia alone in lung tissue also contributes to NF-κB activation; respiratory effort alone does not activate the NF-κB in any of these tissues, however. These data suggest that oxygen desaturation plays the major role in the activation of NF-κB in the lung, whereas in the diaphragm the activation of NF-κB depends on both stimuli being present at the same time. Thus, the increased levels observed in TNF-α and IL-1β as a result of respiratory effort alone would have no relationship with NF-κB activation in the lung or diaphragm. In this case, TNF-α and IL-1β plasma levels do not correlate with NF-kB activity from either the lung or the diaphragm. These cytokines might be upregulated through a non-NF-kB mediated pathway in both tissues, or produced by circulating monocytes, as has been reported in OSA patients (7,20-22).

Furthermore, OSA patients present an increase in vascular endothelial dysfunction (12-14). Dysfunctional endothelium is characterized by an imbalance in the production of vasoactive hormones, increased adherence of inflammatory mediators to endothelial cells and

hypercoagulability, and it is a known risk factor for cardiovascular events (25). Another point that should be considered is the role of the CO2. We have not measured the CO2 levels and therefore unable to analyse its role. In fact, the results as regards CO2 are conflicting. On the one hand, Fletcher *et al.* (26) have suggested that exposure to hypercapnia during intermittent hypoxia may not be critical because the effect of intermittent hypoxia on diurnal blood pressure in rats does not vary according to any increase in the level of carbon dioxide. On the other hand, Tamisier *et al.* (27) have shown that hypercapnic hypoxia does lead to greater sympathetic activation than hypocapnic hypoxia.

$6kPGF_{1\alpha}$ has vasodilatadory effects, whereas TxB_2 results in vasoconstriction; more specifically, since they have markedly opposite effects on vascular tone, the ratio of $TxB_2/6kPGF_{1\alpha}$, is an index of vasoconstriction. In this way, it has been reported that patients with vascular disease and OSA, show a higher $TxB_2/6kPGF_{1\alpha}$ ratio, compared with controls, reflecting a predominance of vasoconstrictor activity. This could have cardiovascular consequences and suggests that OSA pathology could be associated with an abnormal release of prostanoids during sleep (28-29). The results obtained in this study show an increase in the $TxB_2/6kPGF_{1\alpha}$ ratio induced by recurrent obstructive apneas and hypoxia/normoxia. Therefore, these findings suggest a vasoconstrictor effect in apnea and hypoxia/normoxia conditions, and this could correlate with our previous studies in the chronic animal model, where the animals subjected to 5-s obstructions at a rate of 60 per hour, 6 h/day for 4 weeks showed higher $TxB_2/6kPGF_{1\alpha}$ ratio compared with controls (30).

In summary, in a rat model of sleep apnea we have shown that after only 3 hours any one of the tested stimuli – repetitive apneas, oxygen desaturations alone or respiratory efforts alone – plays a major role in the induction of an inflammatory process. However, hypoxia/normoxia episodes proved to be the main trigger for endothelial dysfunction. Even though this

information is derived from an acute animal model, it suggests a possible role for oxygen therapy in SAHS treatment, especially in patients where CPAP treatment is not possible.

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FIGURE LEGENDS

Figure 1: The system to induce realistic obstructive events in rats is depicted, with the general set-up on the top (A). A tracheostomized rat is surrounded by a transparent box. The tracheal tube is connected to an upper airway collapsible segment that is able to induce obstruction (see below). The box has two large holes. Air is flushed from one of these into the sham or apnea groups. A variable FiO2 was applied in the apnea + O2 group. In the case of the hypoxia/normoxia group, a number of cycles were applied to form a hypoxic phase (ambient O_2 concentration 5 %) of 15 s followed by a rapid return to ambient O_2 at 21 % for 45 s. Gas circulates through the box and reaches the other hole. On the bottom (B), the collapsible segment is shown. This consists of two chambers and a membrane (M) between them. One chamber is connected to a pressure (P) pump. If no pressure is applied, the membrane is in its central position (solid line) and there is no obstruction of the trachea. When an external pressure is applied, the membrane (dashed line) occludes the tracheal port. The other chamber is connected to the tracheostomy tube. Another tube in this clamber was open to the atmosphere and acted as pheumotachograph. The pump is controlled by a computer-driven source that is able to increase the pressure and move the membrane that occludes the thracheostomy. The obstruction can be performed at any frequency and for any duration and periodicity. Tracheal pressure and breathing flow can be measured by connecting pressure transducers to the corresponding ports in the valve

Figure 2: Examples of the signals recorded during the experiment. Figure 2A shows sham conditions; flow, pressure and the rat's arterial oxygen saturation (SaO_2) exhibited normal values. Figure 2B shows that during the apnea, flow was nil owing to valve closure, pressure swings in the trachea were markedly increased as a result of breathing effort and the SaO_2 exhibited a transient decrease. Figure 2C shows that during the apnea+O_2, flow was nil owing to valve closure, pressure swings in the trachea were markedly increased as a result of breathing effort but the SaO_2 showed no change. Figure 2D shows that during the

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hypoxia/normoxia, flow was nil owing to valve closure, there were no pressure swings in the trachea because of no breathing effort, while the SaO_2 exhibited a transient decrease.

Figure 3: Figure 3A shows a significant increase in plasma levels of Interleukin-1β (IL-1β) among the Apnea, Hypoxia/Normoxia and Apnea+O_2 groups compared to the Sham group. Figure 3B shows significant differences in plasma levels of Tumor necrosis factor-α (TNF-α) among the Apnea, Hypoxia/Normoxia and Apnea+O_2 groups compared to the Sham group. Data are means $\pm$ SEM of 8 animals per group (*p<0.05 *vs* Sham group).

Figure 4: Figure -upper shows a significant increase in the activation of NF-κB in the Apnea and the Hypoxia/Normoxia group, compared to the Sham group in nuclear fractions in lung tissue. Figure 4-bottom shows a significant in the activation of NF-κB in the Apnea group compared to the Sham group in nuclear fractions in diaphragm tissue. Data are means $\pm$ SEM of 8 animals per group (*p<0.05 *vs* Sham group).

Figure 5: Figure 5-upper shows a significant increase in plasma levels of Thromboxane-B_2 (TxB_2) between the Apnea and the Hypoxia/Normoxia groups compared to the Sham group. Figure 5-middle shows a significant decrease in plasma levels of 6-keto-Prostaglandin-$F_{1\alpha}$ (6kPGF$_{1\alpha}$) between the Apnea and the Hypoxia/Normoxia groups compared to the Sham group. Figure 5-bottom shows the ratio of TxB_2/6kPGF$_{1\alpha}$ between the Apnea and the Hypoxia/Normoxia groups compared to the Sham group. Data are means $\pm$ SEM of 8 animals per group (*p<0.05 *vs* Sham group).

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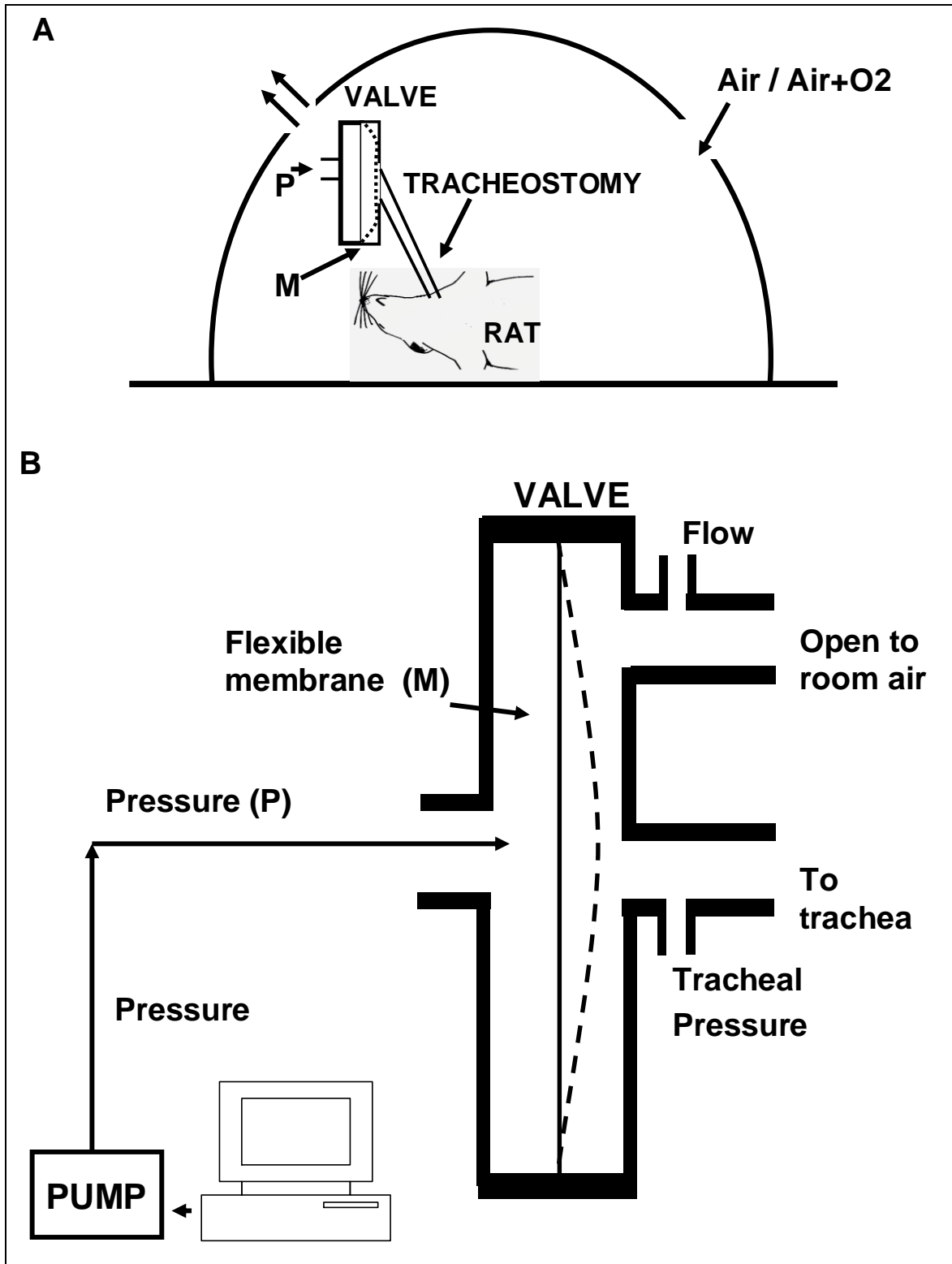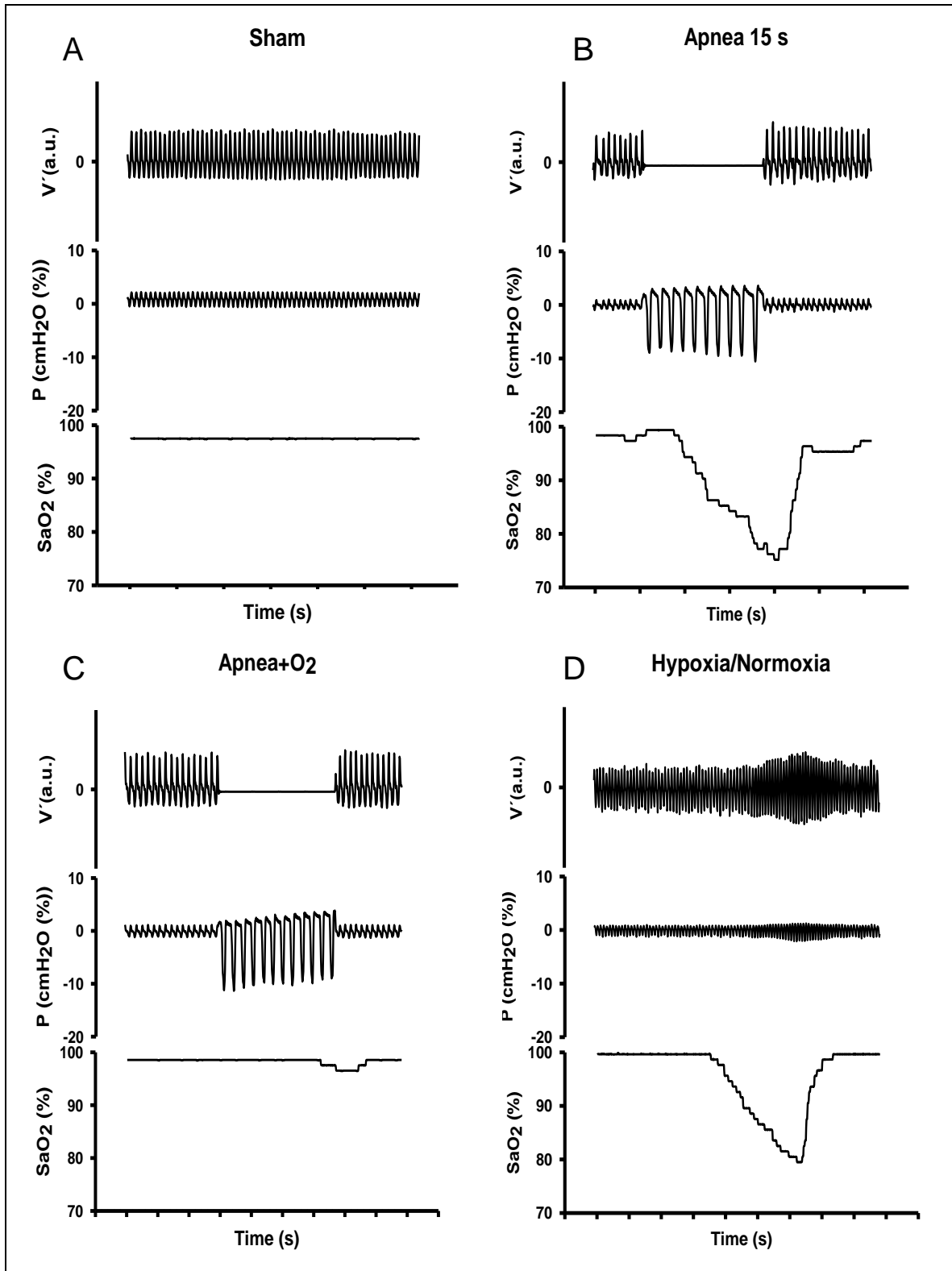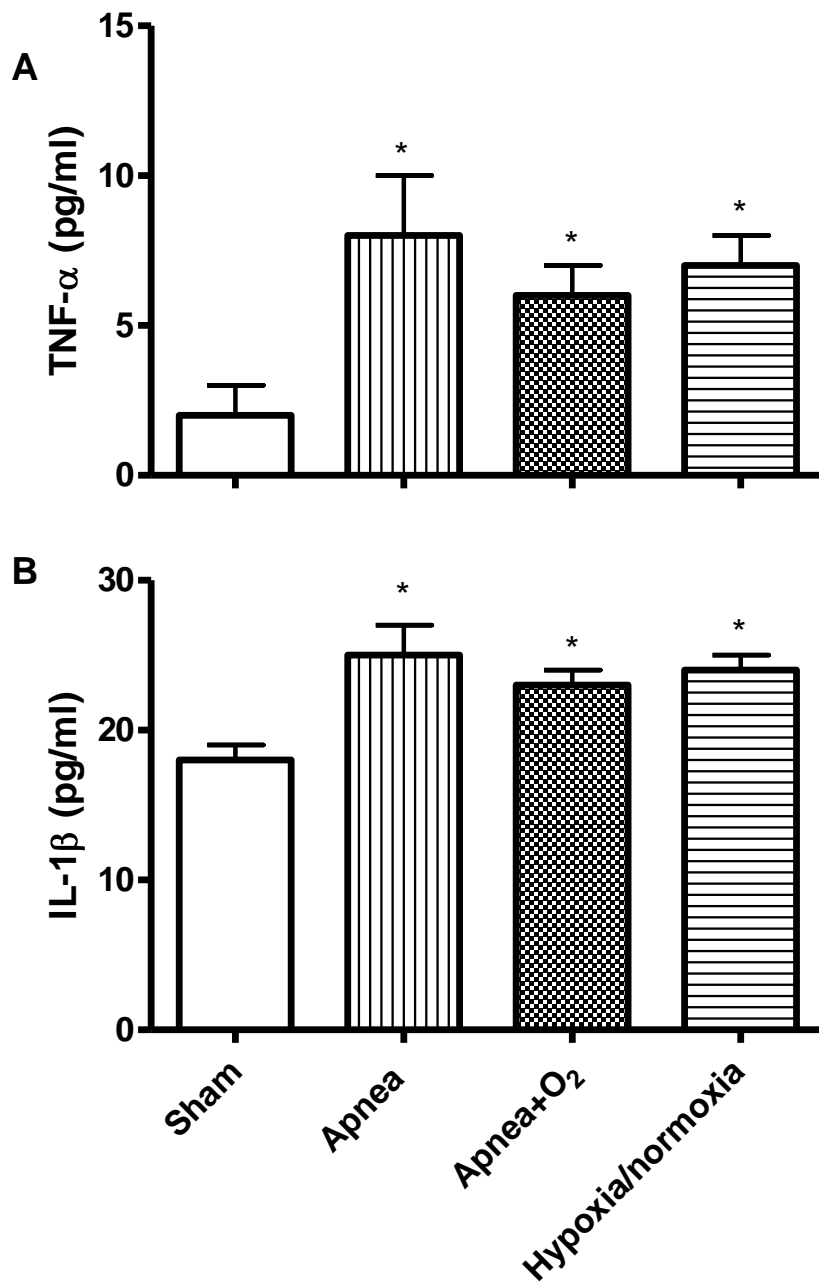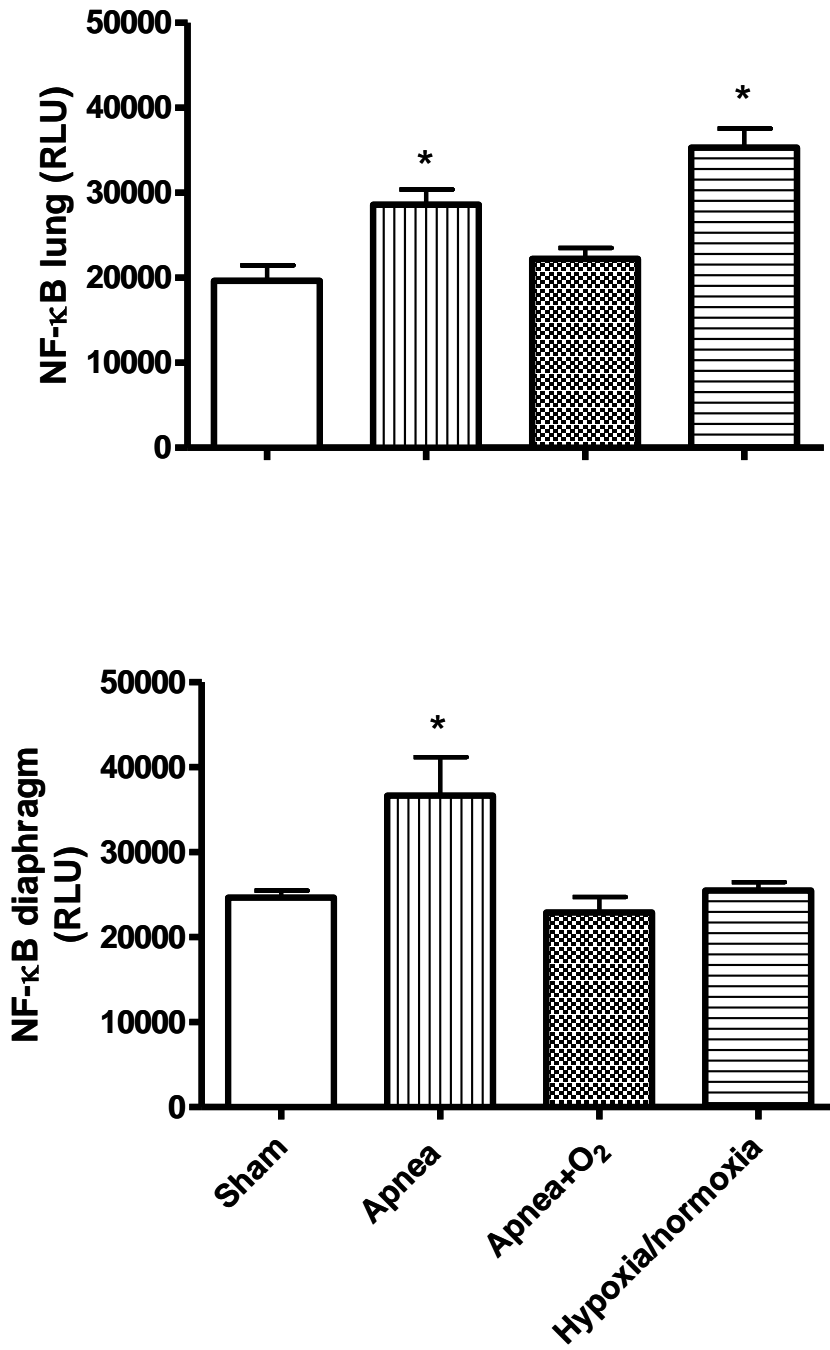
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