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UPPER AIRWAY COLLAPSE AND REOPENING INDUCE INFLAMMATION IN A SLEEP APNOEA MODEL

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Short title: Upper airway inflammation in sleep apnea

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ABSTRACT

The upper airway of obstructive sleep apnoea (OSA) patients is subjected to recurrent negative pressure swings promoting its collapse and reopening. The aim of the present work was to ascertain whether this mechanical stress induces upper airway inflammation in a rat model.

The upper airway of Sprague-Dawley rats was subjected to a periodic pattern of recurrent negative (-40 cmH_2O, 1s) and positive (4 cmH_2O, 2s) pressures inducing collapse and reopening for 5 h. Rats instrumented but not subjected to negative pressure swings were used as controls. The gene expression of the proinflammatory biomarkers macrophage inflammatory protein-2 (MIP-2), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and P-selectin in the soft palate and larynx tissues was assessed by real time PCR (n=8).

A marked over expression of MIP-2, TNF-α, IL-1β and P-selectin (~40, 24, 47 and 7-fold greater than controls, respectively) was observed in the larynx tissue. Similar results were found in the soft palate tissue: ~14, 7, 35 and 11-fold greater than controls, respectively.

Recurrent upper airway collapse and reopening mimicking those experienced by OSA patients triggered an early local inflammatory process. These results could explain the inflammation observed in the upper airway of OSA patients.

INTRODUCTION

The obstructive sleep apnoea (OSA) syndrome is a prevalent disorder characterised by recurrent upper airway obstructions with associated oxygen desaturations and disruption of sleep architecture. These recurrent obstructions and the associated breathing disturbances are caused by an increased collapsibility of the upper airway in OSA patients. Specifically, the negative pressure normally associated with the beginning of inspiration promotes a reduction in the lumen of the abnormally compliant upper airway with the consequent increase in airflow resistance. Sustaining inspiration through an airway with increased resistance requires a more negative inspiratory pressure which, in turn, contributes to a progressive obstruction of the upper airway and finally to the obstructive apnoea [1, 2]. The airway closure is resolved with the activation of the airway dilatory muscles caused by the patient's arousal.

As a consequence of the repetitive events of negative intraluminal pressure, collapse and reopening, the upper airway exhibits structural and functional alterations such as inflammation and oedema in the lamina propria of the uvula mucosa, in the soft palate and in the upper airway muscle [3-5]. This mechanical challenge also causes upper airway sensorial dysfunction to stimuli such as temperature, pressure and vibration [6-8]. Given that the upper airway plays a crucial role in the pathophysiology of OSA, a detailed understanding of its potential inflammatory mechanisms is of major interest. Indeed, the inflammation of the soft tissue structures in the pharynx could develop pharyngeal wall thickening, resulting in upper airway narrowing [3, 9] and also

could promote neuromuscular dysfunction, with the subsequent reduction in the upper airway dilator tone [5]. The loss of sensitivity in the pharyngeal wall could impair upper airway reflex to intraluminal inspiratory negative pressure, increasing its collapsibility, and, hence, exacerbating the apnoeic events.

Although it has been hypothesised that the events of negative pressure, collapse and reopening induce the inflammation resulting in upper airway dysfunction [3, 5], there is no evidence that this mechanical stress actually causes local inflammation. In fact, the well documented data obtained from patient studies [3-5] are inconclusive because different confounding proinflammatory stimuli (hypoxia, metabolic syndrome, obesity, etc) may contribute to the upper airway changes described in OSA patients. Accordingly, the aim of the present work was to setup a realistic and well controlled rat model to ascertain whether a collapse/reopening stimulus similar to that experienced by patients with OSA, is able to trigger *per se* an inflammatory response in the upper airway tissue.

METHODS

Animal preparation. The study, which was approved by the Ethics Committee for Animal Experimentation of the University of Barcelona, was carried out in 28 male Sprague-Dawley rats (250-300 g). The animals were anesthetised with urethane 10% (1 g/kg) and subjected to a double intubation at the tracheal level. One canula was inserted into the lower trachea and directed towards the lungs to allow the animal to breathe spontaneously. Another canula was inserted into the upper trachea and directed towards the upper airway to apply the recurrent negative pressure stimulus. A customized mask, open to the atmosphere through a solenoid electrovalve (L178B1, Sirai, Italy), was tightly adjusted to the rat nose (Figure 1).

Experimental setup. The experimental setting employed to apply recurrent negative pressure swings to the upper airway is shown in Figure 1. The ports of a three-way solenoid electrovalve (L377B03G, Sirai, Italy) were connected to the upper airway canula, to a -40 cmH_2O vacuum source, and to a 4 cmH_2O positive pressure source. By electronically controlling the valves, the upper airway was subjected to a periodic pattern of collapse (1 s) and reopening (2 s). Upper airway collapse was induced by simultaneously occluding the nasal mask valve and connecting the upper airway canula to the negative pressure source (Figure 1, top). Upper airway reopening was achieved by simultaneously opening the nasal mask to the atmosphere and connecting the upper airway canula to the positive pressure source (Figure 1, bottom). The temperature and humidity of the air in the positive pressure source were kept

under physiological conditions thereby avoiding upper airway mucosa drying. Pressures at the nasal mask and at the upper airway canula were continuously measured (Microswitch 176PC28HD2 Honeywell, ±70hPa; Scarborough, Ontario, Canada). Air flow at the trachea was measured with a pneumotachograph (Fleisch-000, Metabo, Epalinges, Switzerland) placed between the tracheal canula and the pressure source (not shown in Figure 1).

Figure 2 illustrates how the experimental setting (Figure 1) allowed to induce upper airway collapse and reopening. The magnitude of the negative tracheal pressure swings applied was progressively reduced (6 cycles) and then increased (4 cycles). When tracheal pressure was higher than P_{crit} (intraluminal pressure causing collapse) nasal pressure was the same as tracheal pressure, indicating that the upper airway remained open because the pressure applied to the trachea was transmitted to the nose. However, when tracheal pressure was lower than P_{crit}, nasal pressure was no longer reduced because the upper airway was collapsed and hence the negative pressure applied to the trachea could not be transmitted to the nose.

Experimental protocol. Fourteen rats were subjected to a recurrent pattern of collapse and reopening for 5 h (Figure 3). In 8 of these rats, the critical pressure (P_{crit}) of the upper airway was measured at the beginning and at the end of application of the recurrent negative pressure swings. At the end of the experiment the rats were sacrificed. In 8 of the rats, the soft palate and larynx tissues were excised [10], frozen and stored at -80ºC for further inflammatory gene expression analysis. In the other 6 rats, the upper airway was excised

completely [10] and embedded in a cutting temperature compound (OCT), frozen and stored at -80ºC for histopathological analysis. A group of 14 rats (control group) were subjected to the same instrumentation and protocol with the exception that the recurrent negative pressure swings were not applied (pressure sources not connected, Figure 1).

Gene expression analysis. The tissue samples were defrost and subsequently disrupted by means of a homogenizer (Polytron 2100, Kinematica, Luzern, Swizerland). The total RNA content of each sample was isolated using the system 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA) and the synthesis of cDNA from total RNA was performed by High Capacity cDNA Kit reagents (Applied Biosystems). The amplification of cDNA was carried out in triplicate by real-time PCR (7300 RT-PCR, Applied Biosystems). Specific gene expression assays for macrophage inflammatory protein-2 (MIP-2), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and P-selectin (Rn00586403_m1, Rn00562055_m1, Rn00580432_m1 and Rn00565416_m1, respectively) were used (Assays on DemandTM, Applied Biosystems). Gene expression of GAPD (Rn99999916_s1) was regarded as an endogenous reference for each rat. Finally, relative gene expression was quantified using the comparative method $2^{-\Delta\Delta Ct}$ [11]. To this end, the gen expression in the rats subjected to upper airway negative pressure was referenced to control rats.

Upper airway histopathology. A transversal section at the hypopharynx level was fixed in 4% formaldehyde for 48 h and paraffin-embedded. The paraffin blocks were cut in sections of 5 μm and stained with hematoxylin and eosin.

The degree of oedema and the presence of inflammatory cells in each sample were determined by two observers who did not know whether the preparation corresponded to rats subjected to the mechanical stimulus or to control rats.

Statistical analysis. Data are presented as mean±SE. Comparisons between different groups were carried out by the Student's t-test or the Mann-Whitney Rank Sum Test. The change in the P_{crit} of each rat induced by upper airway collapse/reopening was assessed by a paired t-test. Statistical significance was considered for $p<0.05$.

RESULTS

The 5 h pattern of recurrent collapse and reopening experienced by the upper airway induced an inflammatory process. A marked over expression of the proinflammatory biomarkers MIP-2, TNF-α, IL-1β and P-selectin (~40, 24, 47 and 7-fold greater than controls, respectively) was observed in the larynx tissue (Figure 4). Similar results were found in the soft palate tissue: ~14, 7, 35 and 11-fold greater than controls, respectively (Figure 5). By contrast, the histopathological analysis of the upper airway tissue showed no significant changes in oedema or in leukocyte infiltration when comparing the upper airway of rats subjected to collapse/reopening and controls (data not shown). On average, P_{crit} at the beginning of the experiment was -19.4 ± 1.6 cmH_2O. No significant ($p = 0.11$) change was observed after the 5 h of upper airway mechanical challenge (-15.7 ± 2.3 cmH_2O).

DISCUSSION

An experimental setting specifically designed to apply negative pressure swings to the rat upper airway enabled us to induce a recurrent collapse/reopening pattern similar to the one experienced by the upper airway in patients with OSA. The magnitude of the negative pressure applied to the upper airway (-40 cmH_2O) resembled the values of oesophageal pressures in OSA patients during obstructive events [2]. This mechanical stress was mild and short enough to avoid structural or functional effects in the upper airway tissue, as shown by the absence of histological differences between the rats subjected to the stimulus and the controls, and by the fact that P_{crit} did not change after the whole mechanical challenge. However, this realistic mechanical stimulus was able to trigger an early local inflammatory cascade. Indeed, a 5-h recurrent pattern of upper airway collapse and reopening caused by tracheal negative pressure swings resulted in a marked over expression of the proinflammatory genes studied in the larynx and soft palate tissues.

The *in vivo* isolated upper airway rat model was used in earlier studies to charactere the motor response to intraluminal pressure changes [12-14]. However, these studies did not mimic the dynamic process of recurrent upper airway closure and reopening characterizing OSA patients. Indeed, the upper airway was kept open and a progressive intratracheal negative pressure was applied to promote the reduction of the inspiratory flow (flow limitation) but without reaching full airway collapse [12, 13]. A similar approach has recently been used to study the intraluminal volume changes in response to negative

and positive pressures by means of magnetic resonance imaging [15]. Given that this technique demands the continuous application of positive or negative pressure over an extended period of time for image acquisition, the fast cycling dynamics characterising the upper airway events in OSA could not be mimicked. In another study, a quasi-static negative pressure was simultaneously applied to the inlet and the outlet of the upper airway with the result that airway closure could not be documented [14]. In contrast, the model designed in the present work was able to simulate the mechanical stress experienced in OSA since the sequence of mechanical events applied to the upper airway was induced by a dynamic pattern mimicking breathing. Another advantage of this experimental setting was that it allowed the direct measurement of P_{crit} (intraluminal pressure causing closure) of the upper airway instead of extrapolating data from partial collapse conditions using a Starling resistor model or by introducing catheters into the upper airway [12, 13, 15].

It has recently been reported that other mechanical stimuli indirectly associated with OSA can induce an early inflammatory response in the upper airway. The application of a vibration akin to snoring triggers a proinflammatory cascade in human airway epithelial cells [16] and in the rat soft palate [10]. Moreover, it has been shown that the mechanical compression associated with CPAP can promote inflammation in the nasal mucosa of rats [17]. However, there is no direct evidence that the recurrent mechanical stress consisting of negative pressure, collapse and reopening experienced by the upper airway induces local inflammation. The only available data on a similar mechanical stress refer

to bronchial and lung tissues. In particular, it was reported that collapse and reopening in the lower airways caused by mechanical ventilation in animal models [18] and in cultured foetal rat pulmonary epithelial cells [19] induced inflammation and cell damage, respectively. In addition, some earlier data supported the notion that negative pressure could be an inflammatory stimulus in the bronchial wall. Indeed, a recent study carried out in OSA patients showed a correlation between the levels of interleukin-8 in sputum and the apnoea/hypopnoea index [20]. The bronchial inflammation observed in these patients could be triggered by the increased negative pressure exerted on the bronchial tree during the recurrent inspirations with an increased upper airway resistance.

The present work provides evidence that the mechanical stress exerted by recurrent intraluminal negative pressure, collapse and reopening induces *per se* an early pro-inflammatory cascade in the upper airway. Specifically, a dramatic gene over expression of the inflammatory biomarkers MIP-2, TNF-α, IL-1 β and P-selectin has been observed in the larynx and soft palate tissues (Figures 4 and 5). The cytokine TNF-α causes endothelial dysfunction [21] and regulates the expression of reactive oxygen species and adhesion molecules [22], whereas the cytokine IL-1β can induce the expression of other inflammatory molecules such as MIP-2 which leads to leukocyte recruitment [23]. The adhesion molecule P-selectin, which is expressed in the endothelium, plays an important role in leukocyte extravasation through the endothelial barrier [24]. Given the complexity and redundancy of the signalling pathways of these inflammatory mediators, the interpretation of changes in the gene

expression for each individual marker should be treated with caution [25]. However, the considerable over expression observed in all the biomarkers analyzed (Figures 4 and 5) clearly shows that the acute stimulus applied triggered early local upregulation of the inflammatory cascade.

The inflammatory response induced by the mechanical stress in the upper airway may result in structural and functional consequences: thickening of the pharyngeal wall, local myopathy and/or neuropathy. Indeed, earlier studies have shown that the presence of cytokines such as TNF-α [26] and IL-1β [27] can cause weakness and contractile dysfunction of muscle fibres. Moreover, TNF-α could cause a loss of sensitivity in the upper airway by inducing neuropathy [28] and neurotoxicity [29]. In conclusion, this study shows that the mechanical stress associated with recurrent intraluminal pressure swings, collapse and reopening triggers an inflammatory process in the upper airway. Accordingly, it is expected that the chronic application of this mechanical challenge can give rise to inflammatory cellular infiltration and tissue changes causing the structural and functional upper airway injuries observed in patients, contributing to the “vicious circle” that characterises the progression of OSA [30].

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FIGURE LEGENDS

Figure 1. Diagram of the experimental setup to apply an intermittent pattern of upper airway collapse (1 s) and reopening (2 s) in anesthetised rats. EV_1 and EV_2 are electrovalves and PT are pressure transducers. *Top*: Upper airway collapse was achieved by occluding the nasal mask and connecting the upper airway canula to a -40 cmH_2O negative pressure source. *Bottom*: The upper airway was reopened by opening the nasal mask valve and connecting the upper airway canula to a 4 cmH_2O positive pressure source.

Figure 2. Example of the negative pressure applied intratracheally (grey thin line) and the pressure recorded at the nasal mask (black thick line). Both pressures were identical when tracheal pressure was higher than the critical pressure (P_{crit}) of the upper airway (≈ -15 cmH_2O in this rat). By contrast, when the negative pressure applied at the trachea was lower than P_{crit}, nasal pressure was no longer reduced because the upper airway was collapsed and hence the negative pressure applied to the trachea could not be transmitted to the nose.

Figure 3. Example of the signals recorded during the 5-h application of intermittent upper airway collapse and reopening (same rat as in Figure 2). *Top:* Negative pressure swings applied intratracheally (grey thin line) and pressure recorded at the nasal mask (black thick line). *Bottom*: Air flow recorded at the trachea. During application of negative tracheal pressure flow was nil indicating that the upper airway was collapsed. When positive pressure

was applied at the trachea the flow signal indicated that the upper aiway was open. The flow signal around the transition between positive and negative pressure were not plotted to avoid the high-frequency noise induced by valve closing/opening.

Figure 4. Relative gene expression of the pro-inflammatory biomarkers macrophage inflammatory protein-2 (MIP-2), tumour necrosis factor- α (TNF-α), interleukin-1β (IL-1β) and P-selectin in the larynx tissue (m±SE) of control rats and of rats subjected to recurrent negative pressure swings in the upper airway.

Figure 5. Relative gene expression of the pro-inflammatory biomarkers macrophage inflammatory protein-2 (MIP-2), tumour necrosis factor- α (TNF-α), interleukin-1β (IL-1β) and P-selectin in the pharynx tissue (m±SE) of control rats and of rats subjected to recurrent negative pressure swings in the upper airway.

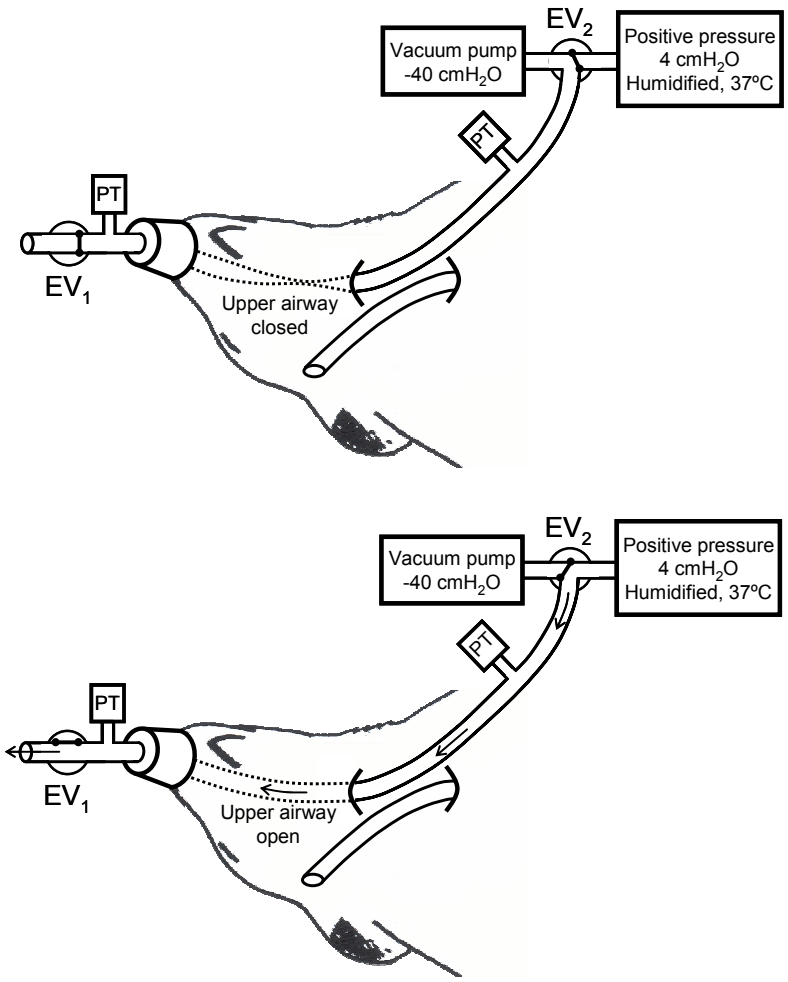
EV2
Vacuum pump
-40 cmH2O
Positive pressure
4 cmH2O
Humidified, 37ºC
PT
PT
EV1
Upper airway
closed
EV2
Vacuum pump
-40 cmH2O
Positive pressure
4 cmH2O
Humidified, 37ºC
PT
PT
EV1
Upper airway
open

Figure 1

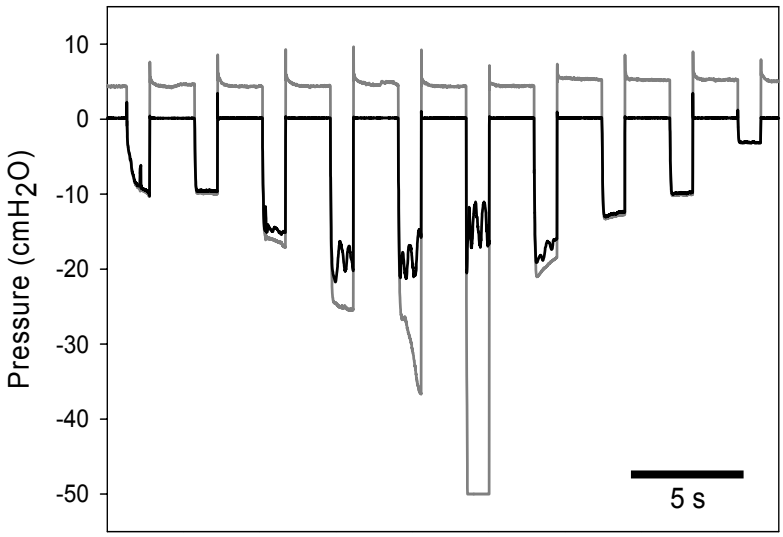

Figure 2

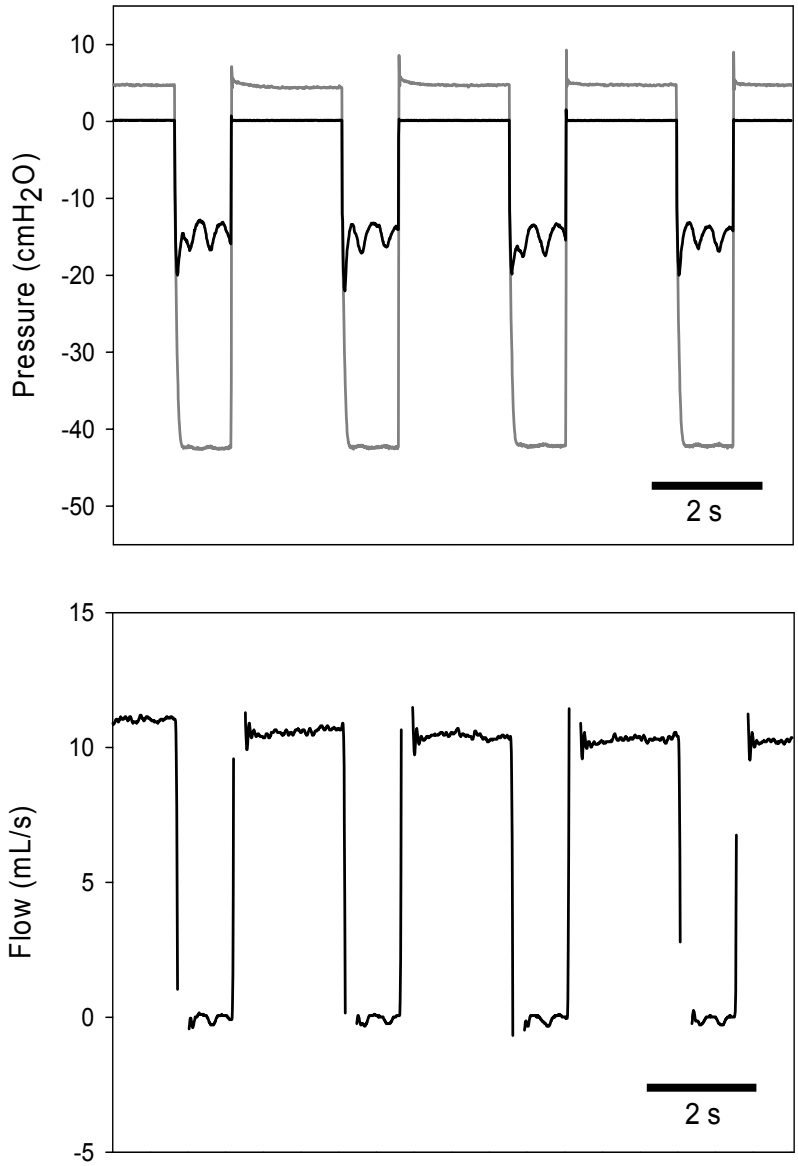

Figure 3

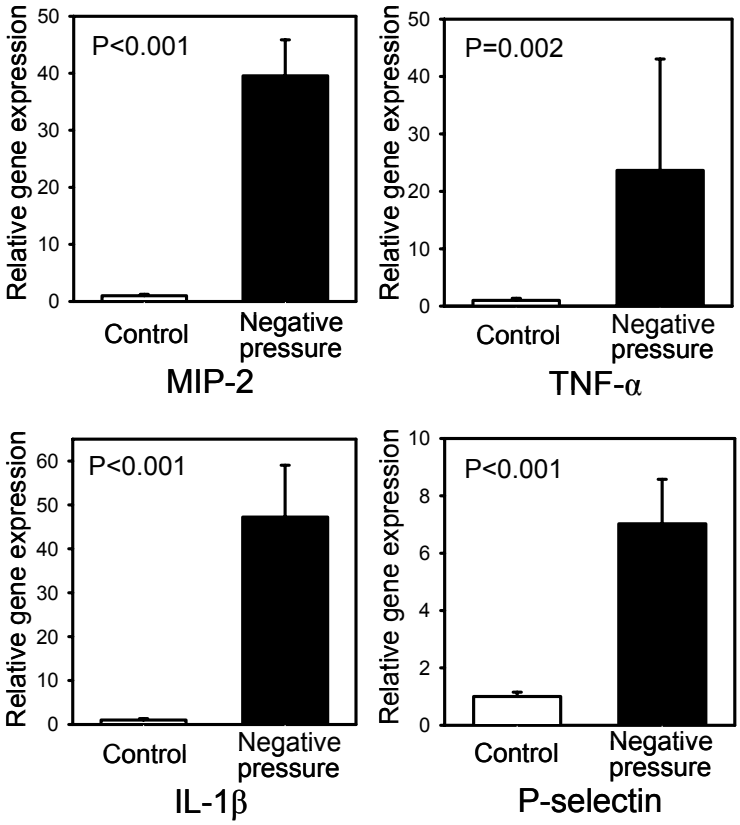
Relative gene expression
P<0.001
Control
Negative pressure
MIP-2
P=0.002
TNF-α
P<0.001
IL-1β
P<0.001
P-selectin

Figure 4

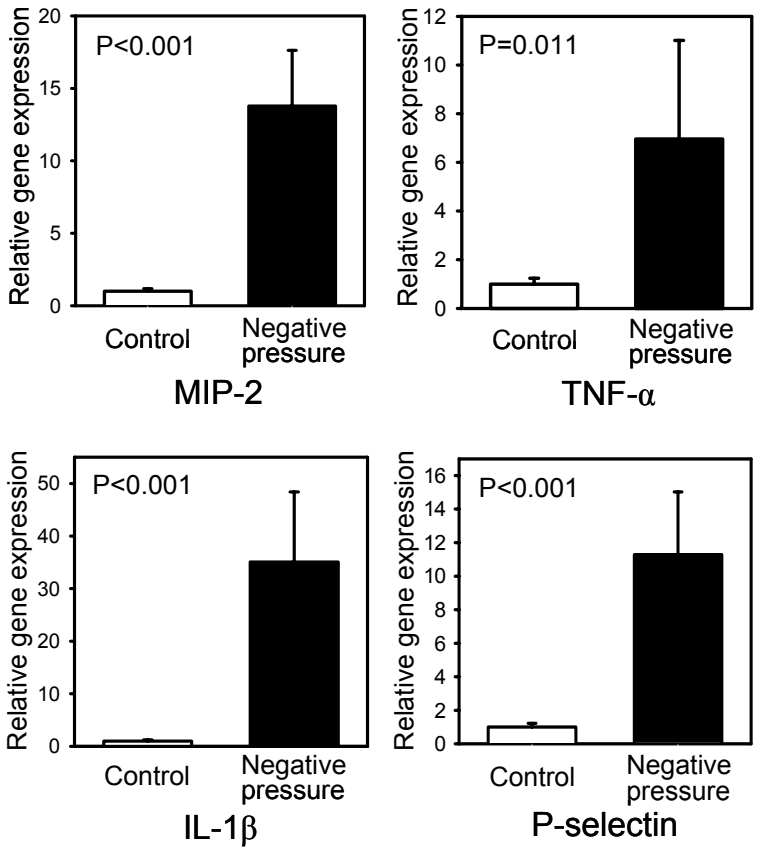
Relative gene expression
P<0.001
Control
Negative pressure
MIP-2
P=0.011
TNF-α
P<0.001
IL-1β
P<0.001
P-selectin

Figure 5