行政院國家科學委員會專題研究計畫 成果報告

兒茶酚胺能調控缺氧所誘發在人類單核球 TNF- alpha 及 MMP-9 的表現

研究成果報告(精簡版)

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中 華 民 國 96 年 11 月 01 日

行政院國家科學委員會補助專題研究計畫 ■ **成 果 報 告**
□**期中進度報告**

Catecholamine can modulate hypoxia induced TNF-alpha and MMP-9 expression in human monocytes

計畫類別：▓ 個別型計畫 □ 整合型計畫
計畫編號：NSC 95 － 2314 － B － 002 － 090 －
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成果報告類型(依經費核定清單規定繳交)：▓精簡報告 □完整報告

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□出席國際學術會議心得報告及發表之論文各一份
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執行單位：臺大醫院內科部

中 華 民 國 96 年 10 月 26 日

目錄

中文計畫成果摘要

阻塞性睡眠呼吸中止症候群(OSA) 是一個普遍的疾病，其特徵為在睡眠中上呼吸道重複性的塌陷，造成間歇性缺氧及交感神經活化，因而引起心血管疾病。OSA 相關的心血管疾病包括動脈粥狀硬化、高血壓、冠狀動脈疾病、心律不整及心衰竭。在 OSA 患者身上可發現某些發炎媒介的上升，包括發炎指數(CRP)、粘接因子(adhesion molecule)、血管生長因子(VEGF)，細胞激素(cytokine)及基質金屬蛋白酵素 (MMP)等，這些物質的生成則為心血管疾病形成的重要機制。在我們前期的實驗可發現，OSA 患者血中細胞激素 IL-6, TNFα及 CRP 的濃度明顯比正常人高，且 IL-6 與 CRP 濃度皆與血中最低含氧量成高度反比。由此證實在 OSA，間歇性缺氧是誘發發炎反應的決定性因子。 在這些發炎物質中，TNFα是與動脈粥狀硬化形成有關，且為冠狀動脈疾病患者預後的預測因子。

慢性間歇性缺氧已被證實可以引起交感神經的過度活化，臨床上我們可發現 OSA 患者血中兒茶酚胺濃度明顯比正常人高，且濃度與缺氧時間成正相關。在 OSA 患者血中 TNFα表現亦增加，而且與兒茶酚胺濃度相關。在體外實驗已證實腎上腺素及正腎上腺素分別經由β及α2 受體，來加強人類巨噬細胞中脂多糖（LPS）所誘發 TNFα 表現。然而，兒茶酚胺對缺氧所誘發 TNFα表現的影響仍未被研究過。

本次實驗利用人類單核球細胞株 U937，分別在正常含氧量及缺氧情況下，在不同時間點處理以 LPS，cobalt chloride 以及不同濃度的兒茶酚胺。結果顯示兒茶酚胺可降低 LPS，cobalt chloride 以及缺氧狀況下引起的 TNFα表現，但對於正常氧氣下 TNFα表現則無影響。對於缺氧引起的 TNFα表現抑制在腎上腺素可達 60%, 而在正腎上腺素可達 70%。對 TNFα表現作用時間則是從兒茶酚胺處理後一小時開始，可持續十二小時。本實驗此結果對於臨床上處理 OSA 病患合併心臟血管疾病的藥物選擇上有重要的影響。

英文計畫成果摘要: (約二百字)

Introduction: Obstructive sleep apnea can result in intermittent hypoxia and sympathetic hyperactivity. Several cytokines, especially TNF-α, were reported to increase in the in OSA patients. In vivo studies showed catecholamine could attenuate lipopolysaccharide-induced expression of TNF-α. Therefore, the objectives of this study were to prove our hypothesis that catecholamine could potentiate hypoxia- induced TNF-α expression and to explore the potential therapeutic effects of α or β agonist/antagonists.

Methods and Materials: We used the human monocyte cell line U937 as target cells. In hypoxic condition, we used 0.1% O_2 and 5% CO_2 at a controlled incubator and maintained PO_2 of the medium lower than 40 mmHg the. In nomoxic condition, we used 21% O_2 and 5% CO_2 and PO_2 in the medium was above 150 mmHg. Cell line U937 was treated with catecholamine (epinephrine, norepinephrine), lipopolysaccharide (LPS) and cobalt chloride ($CoCl_2$) for 12 hours at both normoxic and hypoxic conditions. The supernatant and cells were harvested at 0, 0.5, 1, 3, 6 and 12hr after drug treat. The supernatant was harvested for ELISA and cells were harvested for PCR to assess the TNF-α expression. The TNF-α expression in hypoxic condition was compared to normoxic condition.

Results: Both epinephrine and norepinephrine could attenuate the TNFα expressions in LPS, $CoCl_2$ and hypoxia induced TNF-α expression. In normoxia, neither epinephrine nor norepinephrine had effect on TNFα expressions. In hypoxia, the epinephrine worked best at concentrations of 10^{-7} M to attenuate TNF-α expression and norepinephrine worked best at 10^{-5}M, which is 100 and 1000 times of plasma level in OSA patients respectively. The epinephrine could reduce TNFα expression up to 60% and norepinephrine could reduce TNFα expression up to 70%. The effect of catecholamine on TNFα expression started from one hour after drug treat and lasted for 12 hours.

Conclusion: Catecholamines could attenuate the LPS, $CoCl_2$ and hypoxia induced TNF-α expression but not at normoxia. The result would help us with treating OSA patients with cardiovascular disease.

研究計畫成果報告

簡介（Introduction）

Obstructive sleep apnea syndrome (OSAS) is a major public health problem affecting at least 4% of middle-aged men and 2% women (50). OSAS is characterized with recurrent collapse of upper airway during sleep which results in intermittent hypoxia and sleep fragmentation (3, 31, 51) The repeated episodes of hypoxia and bursts of sympathetic activity provoke surges in blood pressure and heart rate (32, 58, 59) , which results in hypertension, atherosclerosis, coronary artery disease, heart failure and stroke (3, 16, 33, 51-57).

The hypoxia in OSA is characterized as chronic and intermittent. As we know, many transcriptional factors and critical signaling pathways were involved in hypoxia induced transcription of specific genes, which included hypoxia inducible factor, P53, NF-κB, activator protein-1 and critical signaling pathways (74-78). Several inflammatory mediators have been reported to increase in patients with OSA, which include C-reactive protein (CRP), oxidative stress, adhesion molecules, vascular endothelial growth factor, proinflammatory cytokines, adhesion molecules like intracellular adhesion molecule-1 and vascular cell adhesion molecule-1. (24, 66, 68-71, 93, 94) Elevations of serum levels of these inflammatory mediators may lead to endothelial injury and adverse cardiovascular function in patients with OSA. Our data showed the levels of TNFα were higher in OSA patients than control subjects and the levels were highly correlated with the lowest pulse oxygen saturation (SpO_2) (r=0.38, $p<0.01$) which could decrease after one-month CPAP treatment (Fig. 1). Therefore, TNFα is a good biomarker for studying OSA associated cardiovascular complications.

Sympathetic hyperactivity in OSA is resulted from hypoxia and repeated arousal. Both sustained and Intermittent hypoxia can alter chemoreflex control of sympathetic tone and induce prolonged sympathetic hyperactivity in human.(95-98) Rats exposed to intermittent hypoxa could have higher dopamine and norepinephrine content in carotid body than sustained hypoxia.(35) This finding supported that chronic intermittent hypoxia had stronger influence on sympathetic activity than sustained hypoxia. The presentations of sympathetic hyperactivity in OSA include hypercatecholaminemia and elevated sympathetic tone of peripheral nerve. Our study demonstrated plasma levels of norepinephrine highly correlated with the proportion of time with pulse oxymetry <90% (r=0.36, $p=0.01$), which would go down after one-month CPAP treatment (Fig. 2). Hypersympathetic tone is known for attributing to the developments of cardiovascular diseases. Our data showed OSA patients had a surge of blood pressure in the early morning, which disappeared after one-month CPAP treatment (Figure 3). Our data also showed the plasma levels of norepinephrine highly correlated with serum levels of TNFα (r=0.63, p<0.01).

Catecholamine was known to be able to modulate the production of pro/ anti-inflammatory cytokines in the condition of sepsis and autoimmune disease. (93, 99-104) In vivo studies showed norepinephrine could potentiate LPS-induced expression of TNFα through α2 adrenergic receptor, which could be blocked by α2 adrenergic antagonist.(104) Epinephrine and other β adrenergic agonists (isoproterenol) could reduce TNFα expression when exposing the cell to adrenaline and LPS at the same time. But incubating cells with isoproterenol for 24 h before LPS stimulation would increase TNFα expression. Both regulations of norepinephrine and epinephrine on LPS induced TNFα expression are

mediated by changes in intracellular cAMP concentrations, which are exerted at a posttranscriptional level. (102) However, the effect of catecholamine on TNFα expression in the hypoxic microenvironment has never been studied.

To test if catecholamine could affect TNFα expression in the hypoxic environment, we chose monocyte cell line U937 as the study model and treated the U937 with various concentrations of catecholamine in both normoxic and hypoxic condition. The preliminary results showed epinephrine had no effect on TNF expression in normoxic condition but could attenuate the TNF expression in hypoxic condition (Figure 4). Therefore, we hypothesize that catecholamines can modulate intermittent hypoxia induced TNFα and further affect the developments of cardiovascular complications in OSA. In this project, we'll use the human peripheral blood monocyte from healthy subjects and OSA patients as the target cells, which were serially treated with catecholamine and α or β agonists/antagonists in both normoxic and hypoxic microenvironment, to achieve the following 3 objectives:

(1). To examine the effect of catecholamines on the modulation of intermittent hypoxia induced TNF-α in human monocytes from both healthy subjects and OSA patients

(2). To map the signaling pathway of catecholamines regulating the intermittent hypoxia induced TNF-α expression.

(3). To explore the potential therapeutic effects of α or β agonists/antagonists on intermittent hypoxia induced TNF-α expression

Figure 1. Serum of TNF before and after one-month CPAP treatment

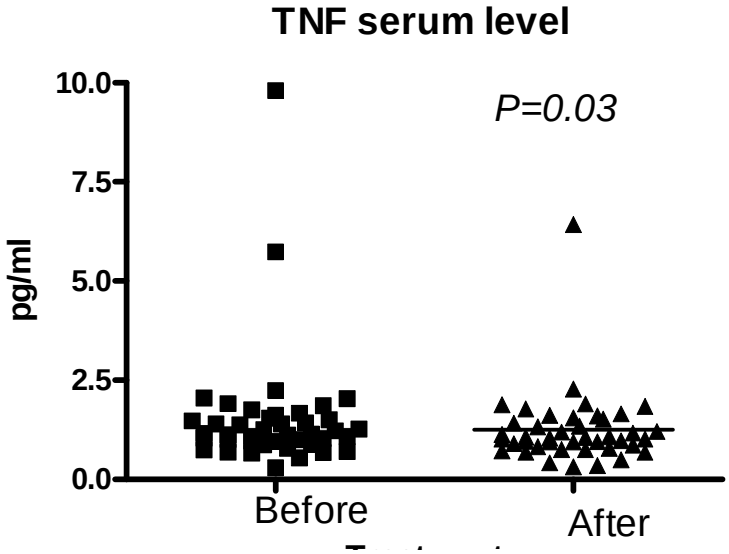
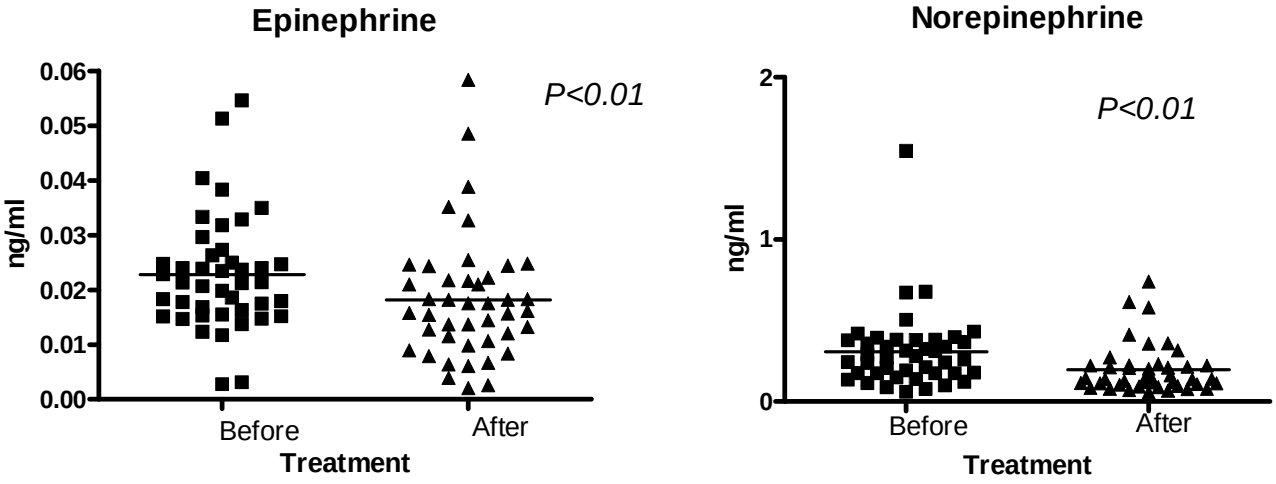


Figure 2. Seru

PAP treatment



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Diastolic BP

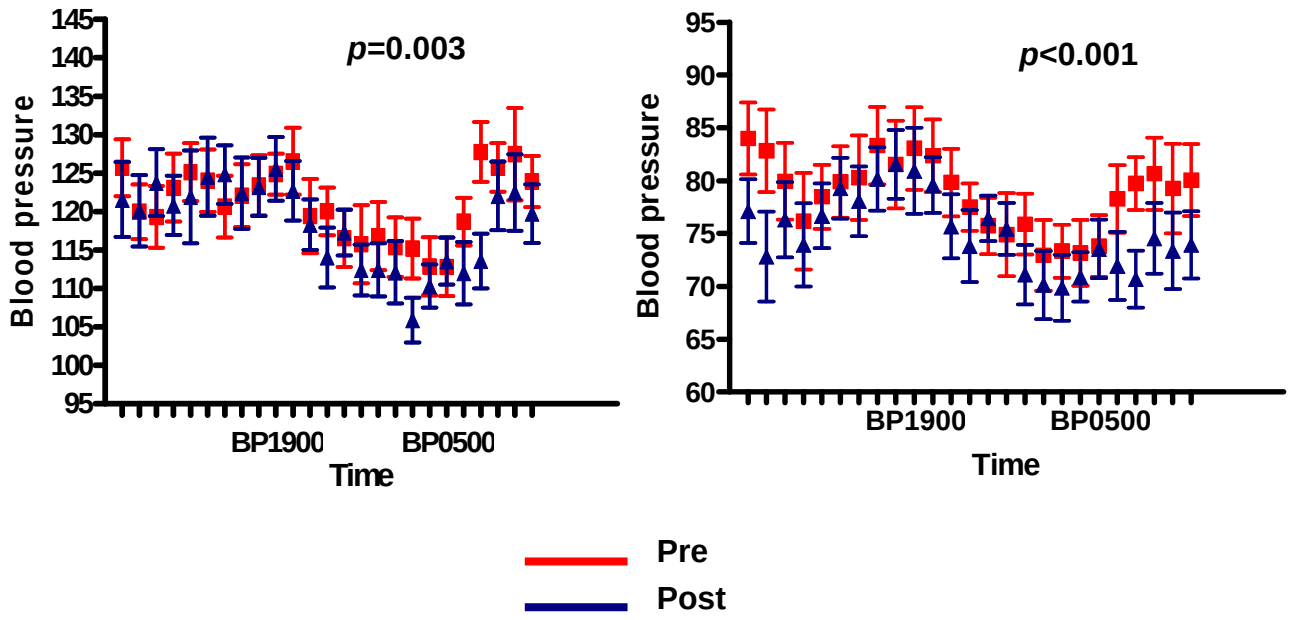
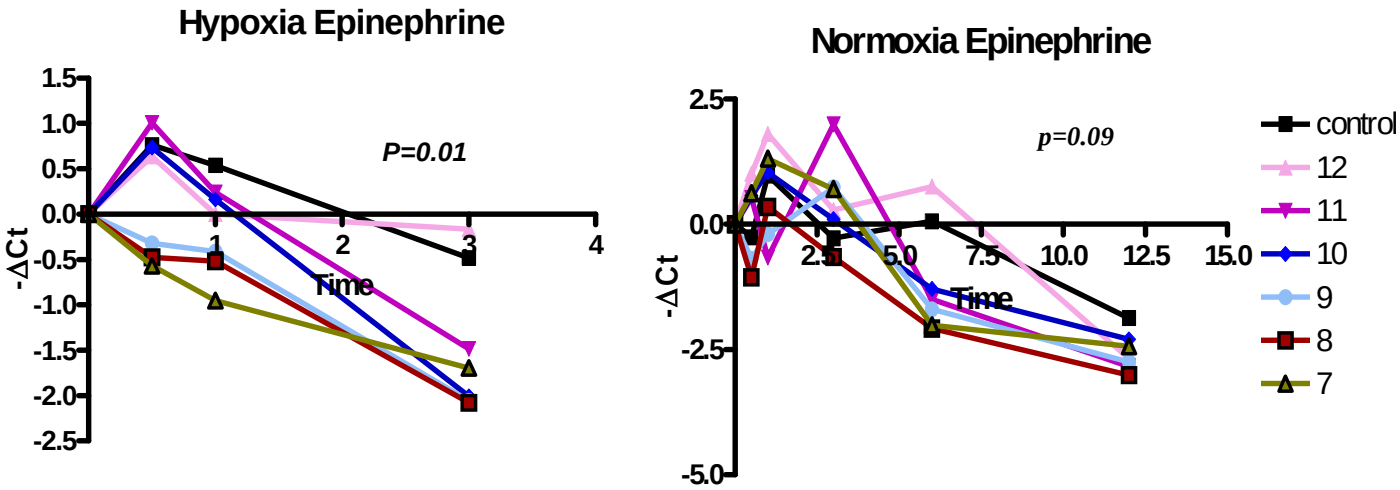


Figure 4. The effects of epinephrine on TNF-α expression in U937 in both normoxia and hypoxia



研究目的 (Specific Aim)

(1). To examine the effect of catecholamines on the modulation of intermittent hypoxia induced TNF-α in human monocytes from both healthy subjects and OSA patients

(2). To map the signaling pathway of catecholamines regulating the intermittent hypoxia induced TNF-α expression.

(3). To explore the potential therapeutic effects of α or β agonists/antagonists on intermittent hypoxia induced TNF-α expression

材料及方法 (Subjects and Methods)

Study Design:

We use both human monocyte from healthy subjects and OSA patients as the target cells. Isolated monocyts were seriously treated with catecholamine and α or β agonists/antagonists in both normoxic and hypoxic microenvironment. The effects of catecholamine on TNF-α expressions were measured with TNF-α concentration in supernatant and level of mRNA expression. The protocol of the experiment is shown in Fig 5.

Material and Methods

Cell culture experiments

Human monocyte isolation: Peripheral blood mononuclear cells (PBMCs) from healthy subjects and OSA patients were isolated with a Ficoll-Hypaque gradient. CD14+ monocytes were isolated by positive selection with magnetic beads (MACS microBeads, Miltenyi Biotec). To separate T cells, natural killer cells, B cells, dendritic cells, and basophils from PBMCs, they were indirectly magnetically labeled with a cocktail of hapten-conjugated CD3, CD7, CD 19, CD 45RA, CD 56, and anti-Ig E antibodies and MACS MicroBeads coupled to an anti-hapten monoclonal antibody. The magnetically labeled cells were removed by retaining them on a MACS column in the magnetic field of the VarioMACS (Miltenyi Biotec). Purity of these preparations of monocytes was determined by FACS-analysis employing CD 14 antibodies. The supernatant and cells were harvested at 0', 30min, 1hr, 3hr, 6hr, 12hr and 24hr after cell stimulation with catecholamineα or β agonists/antagonists.

Cell culture and harvest: Isolated monocytes were resuspended with medium containing RPMI-1640, 10% FCS (Northumbria Biologicals Ltd., Cramlington Northumberland, UK), penicilline (100 U/ml), streptomycin (100 μg/ml), 1% glucose, 1% HEPES, 1% L-glutamin and 1% sodium pyruvate. Cells were maintained for 24 hr at 37°C in humidified atmosphere of 5% CO2:95% air before experiment. One- milliliter cell suspension was seeded per well into 24-well plates at a cell density of $3x10^5$ in 24-well plate and treated with catecholamine, α or β agonists/antagonists for up to 24 hr. Supernatant and cell were harvest at 0, 30min, 1hr, 3hr, 6hr, 12hr after treat.

Assay for Cell viability and aptosis: Cell viability as determined by trypan blue exclusion immediately before cell seeding and after respective cell harvest. Cell aptosis were assayed with annexin-V.(105, 106)

Cell stimulation

Epinephrine (bitartrate salt), norepinephrine (bitartrate salt), propranolol (hydrochloride salt), phenoxybenzamine (hydrochloride salt), metoprolol, butoxamine, phenoxybenzamine, doxazosine, yohimbine were all obtained from Sigma Chemical Co. (Poole, Dorset, UK). They were dissolved and diluted in culture medium. Dilutions were made in phosphate buffer saline. The concentrations of epinephrine were 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} μM. The concentrations of norepinephrine were 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}μM.

Cytotoxicity Assay: Possible toxic effects of catecholamine, α and β receptor antagonists used were excluded by a lactate dehydrogenase (LDH)-based cytotoxivcity assay kit (Sigma) and Trypan blue

staining.

Normoxia and Hypoxia

The hypoxic condition was achieved with oxygen concentration of 0.1%, which was maintained using a controlled incubator with CO_2/O_2 monitoring and CO_2/N_2 sources (Edwards Instrument Co., Wilmington, MA). The normoxic condition was achieved with oxygen concentration of 21%. CO_2 was maintained at 5% in both hypoxic and normoxic conditions. The culture medium was preequlibrated for 24 hr before cell exposure and maintained at a PH of 7.3. The PO_2 of the medium was measured with arterial blood gas machine just before seeding cell, which was kept about 40 mmHg in hypoxic condition and about 150 mmHg in normoxic condition.

Measurement of TNF-α expression

Expression of TNF-α was assessed with measuring level of TNF-α in supernatant and level of mRNA expression. The TNF-α in supernatant was assayed with high-sensitivity ELISA kit (R&D, Minneapolis, USA) and mRNA expression was assessed with real-time PCR.

RNA preparation: Total RNA was isolated using Trizol reagent as recommended by the manufacturer's instructions from peripheral blood monocytes isolated. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide and the 18S and 28S RNA bands were visualized under U.V. light.

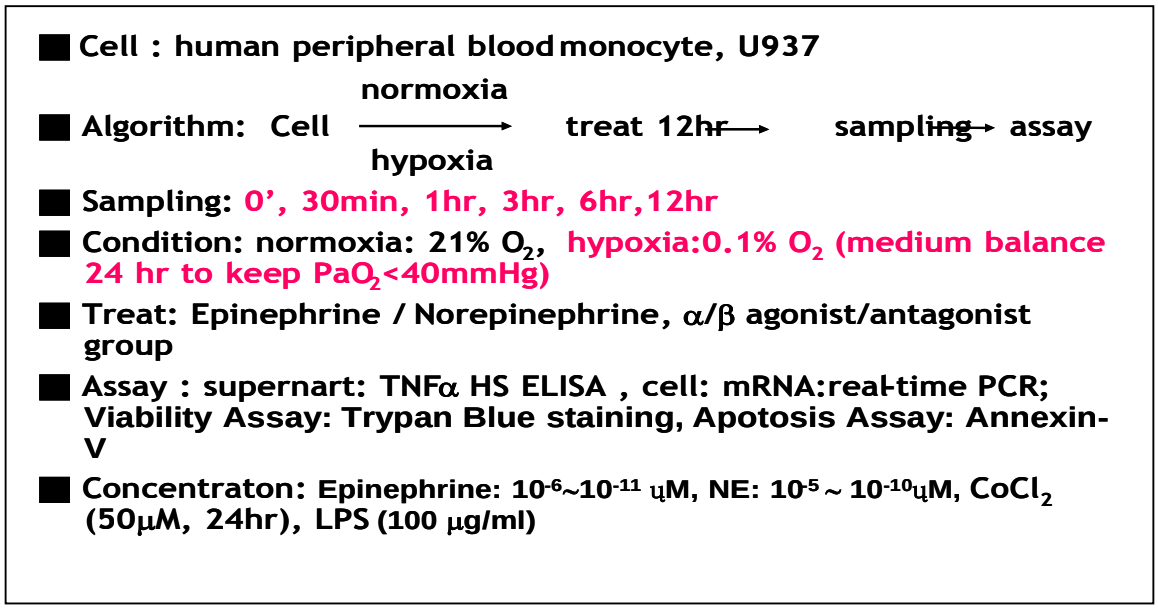
cDNA probe preparation: The reverse transcriptase reaction mixture was made up to 100μl with 1xTaq polymerase buffer containing 0.5μg of 5'primer and 2U of Taq polymerase and subjected to 30 cycles amplification at 94°C for 1min, 60°C for 2min, and 72°C for 3 min.

Real-time PCR: The real-time quantitative PCR of TNF-α mRNA was performed using TaqMan dye (Applied Biosystems, Foster City, CA, U.S.A.) with standard protocol. The primer and probe of TNF-α and TBP for real-time PCR was selected with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, U.S.A). The standard curve samples used for real-time quantitative PCR were prepared by serial dilution of a specific RNA sample to cover the range of 62.5 ng, 125 ng, 250 ng and 500 ng. The serially diluted samples were aliquotted and stored at -80 °C until use. Each assay included a standard curve, a no-template control, and duplicate total RNA samples. The fluorescence emitted by the reporter dye was detected on-line in real-time using the ABI prism 7500 sequence detection system (PE Applied Biosystem, Foster City, California).

Mediation of catecholamine-modulated TNF-α gene expression

cAMP analysis: After drug stimulation, the culture medium was aspirated after the indicated time and the cells were treated as reported in the literature. Briefly, 0.8 cc 75%ethanol with 1mM EDTA was added to each well, and after 10 min, cell was harvested by scraping. Ethanol was removed by SpeedVac centrifugation and pellets were suspended in 0.5ml of 4xTE buffer and sonicated 5sec. After centrifugation, a 25ų supernatant aliquot from each sample was used for determination of cAMP levels with the cyclic AMP assay system (Amersham Pharmacia Biotec) according to manufacturer's instructions.

Figure 5. Flow chart of study design



結果 (Results)

Summary of experimental algorithm

Treat / Drug / TNF	$CoCl_2$*	Hypoxia	Normoxia	LPS**
Control	A	C	E	G
Catecholamine	B	D	F	H

*$CoCl_2$ treat 24 hr in normoxia before catecholamine treat
** LPS concomitant with catecholamine

- Prove A>C>E=F; (A-B)>(C-D)>(E-F)
- Statistics: compare TNFα expression between control and catecholamine group by GEE repeated measurement

2. Experimental condition:

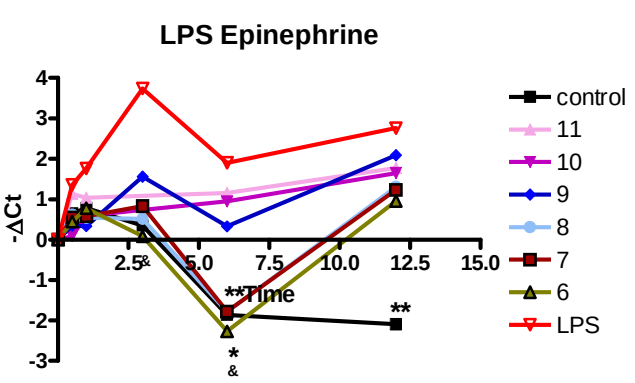
	LPS (100μg/ml)		$CoCl_2$(μM)		Normoxia		Hypoxia	
	E	NE	E	NE	E	NE	E	NE
Cell number	$3x10^5$	$4x10^5$	$3x10^5$	$3x10^5$	$3x10^5$	$3x10^5$	$2.6x10^5$	$3.58x10^5$
Concentration of catecholamine								
Max	-11	-10	-11	-10	-11	-10	-11	-10
Mini	-6	-5	-6	-5	-6	-5	-6	-5
Survival at 12th hr (%)	-	-	92	92	100	100	99.7	96.2
Medium gas								
PH	-	-	-	-	7.45	7.45	6.79	6.88
PCO_2	-	-	-	-	25.6	25.6	165.65	166.2
PO_2	-	-	-	-	204.7	204.7	35.65	44.7

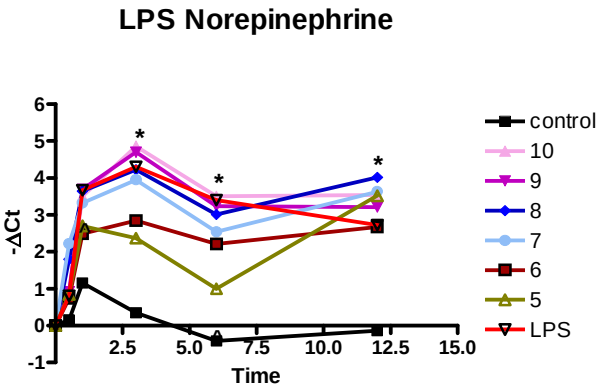
E: epinephrine, NE: norepinephrine

3. Gene expression of TNF:

A. LPS:

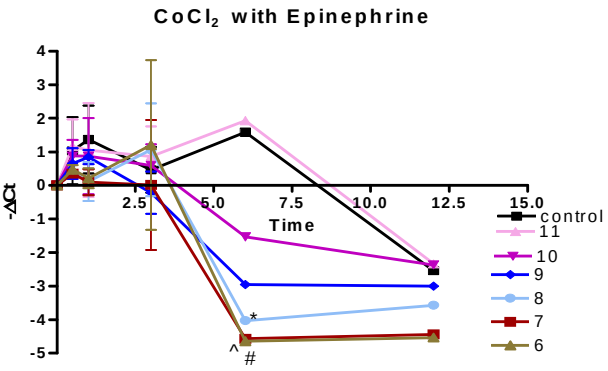
- Both epinephrine and norepinephrine could attenuate the LPS induced TNFα expression
- Epinephrine could suppress LPS induced TNFα expression most at concentration of 10^{-6}M

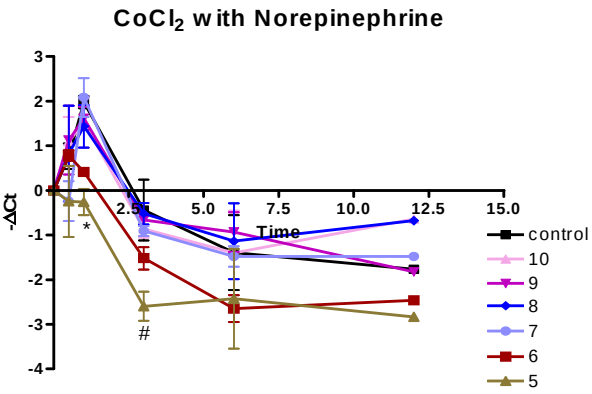


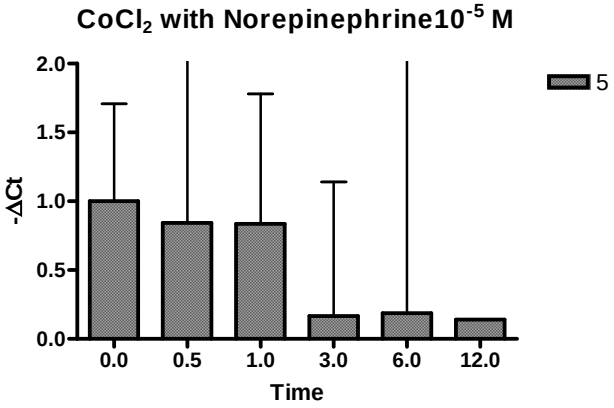


B. $CoCl_2$:

- Both epinephrine and norepinephrine attenuate the LPS induced TNFα expression
- Epinephrine could suppress $CoCl_2$ induced TNFα expression most at concentration of 10^{-6}M
- Norepinephrine could suppress $CoCl_2$ induced TNFα expression most at concentration of 10^{-5}M



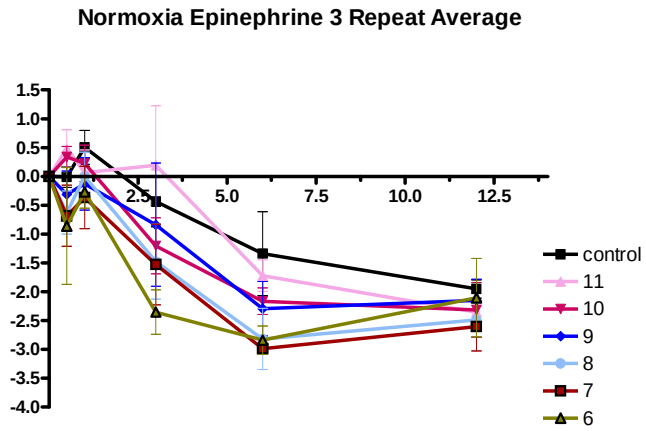


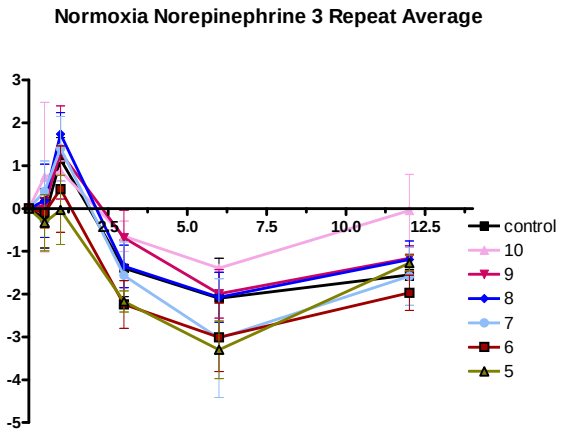


C. Catecholamine:

a. Normoxia

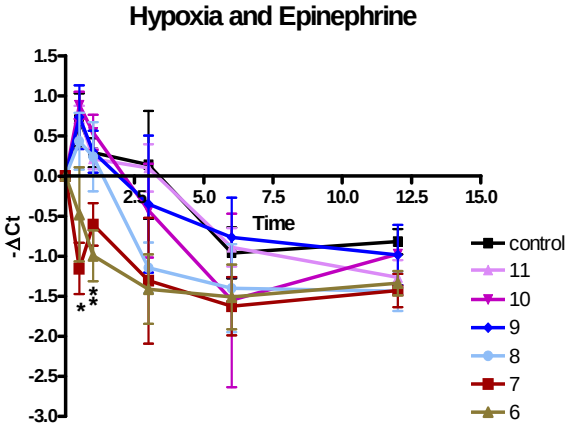
- There's no difference of TNFα expression between control and catecholamine treated

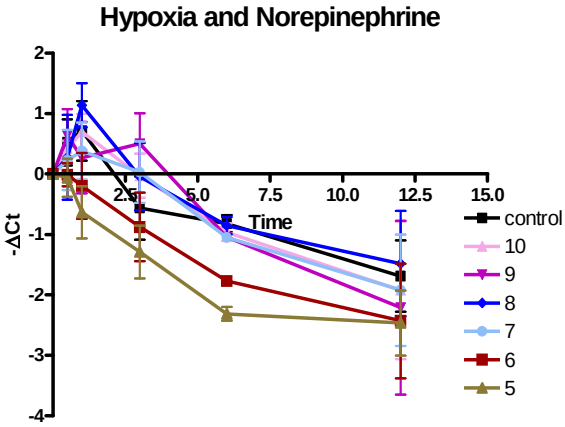


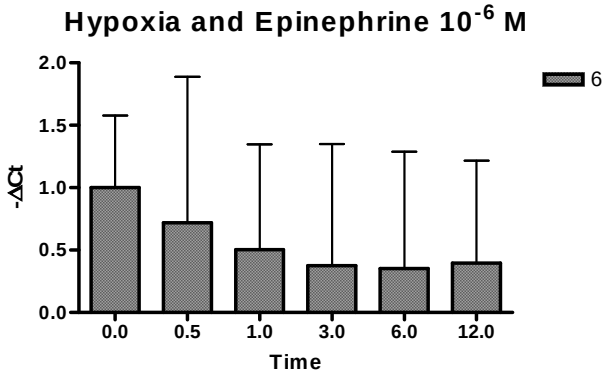


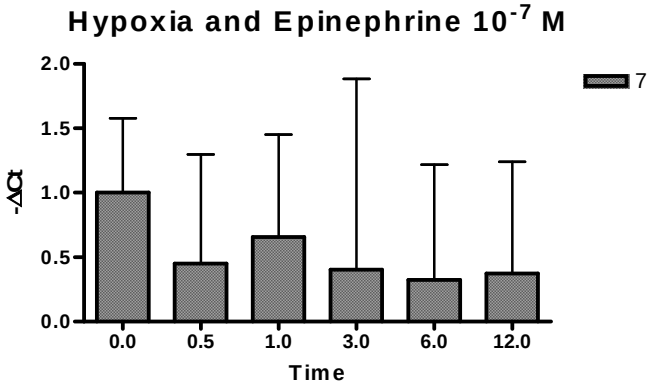
b. Hypoxia

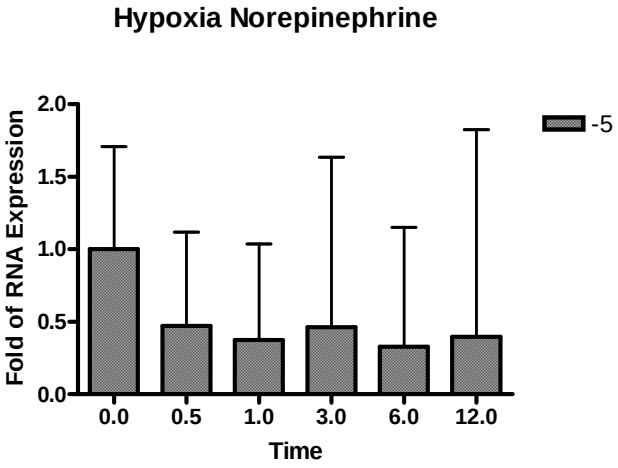
- Both epinephrine and norepinephrine could attenuate the TNFα expression in hypoxia induced TNF-α expression.
- In hypoxia, the effective concentrations of catecholamine were 10^{-7} for epinephrine and 10^{-5} for norepinephrine, which is 100 and 1000 times of plasma level in OSA patients.
- The epinephrine could reduce TNFα expression up to 60 % and norepinephrine could reduce TNFα expression up to 70%.
- The effect of catecholamine on TNFα expression started from one hour after drug treat and lasted for 12 hours






Hypoxia and Epinephrine 10-6 M
-ΔCt
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
3.0
6.0
12.0
Time
6


Hypoxia and Epinephrine 10-7 M
-ΔCt
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
3.0
6.0
12.0
Time
7


Hypoxia Norepinephrine
Fold of RNA Expression
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
3.0
6.0
12.0
Time
-5

討論 (Discussion)

1. Limitation of the study

The limitations of our study included the followings

- No continuous infusion of catecholamine: the level of catecholamine in culture medium: The catecholamine is likely to degrade during 12-hour incubation, which would reduce the concentration catecholamine in culture medium
- The effective concentration of catecholamine is 100 and 1000 times of plasma level in OSA patients, which limited the clinical application. Further work on medications with high-affinity to α, β receptors would lower drug dose needed to suppress TNFα expression.

2. **Future work:**

- Test the effects of α, β agonists/antagonists on TNFα with the specific concentration of catecholamine. The specific concentrations for epinephrine are 10^{-6} and 10^{-7}M. The optimal concentrations for norepinephrine are 10^{-5}M.
- Measure the concentration of catecholamine in the culture medium of different time series

結論 (Conclusion)

Catecholamines could attenuate the LPS, $CoCl_2$ and hypoxia induced TNF-α expression but not at normoxia. The result would help us with treating OSA patients with cardiovascular disease

文献 (References)

1. . Sleep-related breathing disorders in adults: recommendations for syndrome definition and measurement techniques in clinical research. The Report of an American Academy of Sleep Medicine Task Force. *Sleep* 1999; 22:667-689.
2. Drazen JM. Sleep apnea syndrome. *N Engl J Med* 2002; 346:390.
3. Malhotra A, White DP. Obstructive sleep apnoea. *Lancet* 2002; 360:237-245.
4. Marin JM, Carrizo SJ, Vicente E, Agusti AG. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. *Lancet* 2005; 365:1046-1053.
5. Goncalves MA, Paiva T, Ramos E, Guilleminault C. Obstructive sleep apnea syndrome, sleepiness, and quality of life. *Chest* 2004; 125:2091-2096.
6. Schneider C, Fulda S, Schulz H. Daytime variation in performance and tiredness/sleepiness ratings in patients with insomnia, narcolepsy, sleep apnea and normal controls. *J Sleep Res* 2004; 13:373-383.
7. Chervin RD. Sleepiness, fatigue, tiredness, and lack of energy in obstructive sleep apnea. *Chest* 2000; 118:372-379.
8. Black J. Sleepiness and residual sleepiness in adults with obstructive sleep apnea. *Respir Physiol Neurobiol* 2003; 136:211-220.
9. Vgontzas AN, Papanicolaou DA, Bixler EO, Hopper K, Lotsikas A, Lin HM, Kales A, Chrousos GP. Sleep apnea and daytime sleepiness and fatigue: relation to visceral obesity, insulin resistance, and hypercytokinemia. *J Clin Endocrinol Metab* 2000; 85:1151-1158.
10. Black JE, Hirshkowitz M. Modafinil for treatment of residual excessive sleepiness in nasal continuous positive airway pressure-treated obstructive sleep apnea/hypopnea syndrome. *Sleep* 2005; 28:464-471.
11. Lloberes P, Marti S, Sampol G, Roca A, Sagales T, Munoz X, Ferrer M. Predictive factors of quality-of-life improvement and continuous positive airway pressure use in patients with sleep apnea-hypopnea syndrome: study at 1 year. *Chest* 2004; 126:1241-1247.
12. Harsch IA, Schahin SP, Radespiel-Troger M, Weintz O, Jahreiss H, Fuchs FS, Wiest GH, Hahn EG, Lohmann T, Konturek PC, Ficker JH. Continuous positive airway pressure treatment rapidly improves insulin sensitivity in patients with obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 2004; 169:156-162.
13. Choi S, Bennett LS, Mullins R, Davies RJ, Stradling JR. Which derivative from overnight oximetry best predicts symptomatic response to nasal continuous positive airway pressure in patients with obstructive sleep apnoea? *Respir Med* 2000; 94:895-899.
14. Bennett LS, Langford BA, Stradling JR, Davies RJ. Sleep fragmentation indices as predictors of daytime sleepiness and nCPAP response in obstructive sleep apnea. *Am J Respir Crit Care Med* 1998; 158:778-786.
15. Barbe F, Mayoralas LR, Duran J, Masa JF, Maimo A, Montserrat JM, Monasterio C, Bosch M, Ladaria A, Rubio M, Rubio R, Medinas M, Hernandez L, Vidal S, Douglas NJ, Agusti AG. Treatment

with continuous positive airway pressure is not effective in patients with sleep apnea but no daytime sleepiness. a randomized, controlled trial. *Ann Intern Med* 2001; 134:1015-1023.
16. Pepperell JC, Ramdassingh-Dow S, Crosthwaite N, Mullins R, Jenkinson C, Stradling JR, Davies RJ. Ambulatory blood pressure after therapeutic and subtherapeutic nasal continuous positive airway pressure for obstructive sleep apnoea: a randomised parallel trial. *Lancet* 2002; 359:204-210.
17. Kingshott RN, Vennelle M, Hoy CJ, Engleman HM, Deary IJ, Douglas NJ. Predictors of improvements in daytime function outcomes with CPAP therapy. *Am J Respir Crit Care Med* 2000; 161:866-871.
18. Patel SR, White DP, Malhotra A, Stanchina ML, Ayas NT. Continuous positive airway pressure therapy for treating sleepiness in a diverse population with obstructive sleep apnea: results of a meta-analysis. *Arch Intern Med* 2003; 163:565-571.
19. Robinson GV, Smith DM, Langford BA, Davies RJ, Stradling JR. Continuous positive airway pressure does not reduce blood pressure in nonsleepy hypertensive OSA patients. *Eur Respir J* 2006; 27:1229-1235.
20. Rechtschaffen A KA. 1968. A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. UCLA brain information service/brain research institute, Los Angles.
21. Johns MW. A new method for measuring daytime sleepiness: the Epworth sleepiness scale. *Sleep* 1991; 14:540-545.
22. Krupp LB, LaRocca NG, Muir-Nash J, Steinberg AD. The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus. *Arch Neurol* 1989; 46:1121-1123.
23. Taylor RR, Jason LA, Torres A. Fatigue rating scales: an empirical comparison. *Psychol Med* 2000; 30:849-856.
24. Yokoe T, Minoguchi K, Matsuo H, Oda N, Minoguchi H, Yoshino G, Hirano T, Adachi M. Elevated levels of C-reactive protein and interleukin-6 in patients with obstructive sleep apnea syndrome are decreased by nasal continuous positive airway pressure. *Circulation* 2003; 107:1129-1134.
25. Ryan S, Taylor CT, McNicholas WT. Predictors of elevated nuclear factor-kappaB-dependent genes in obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 2006; 174:824-830.
26. Riha RL, Brander P, Vennelle M, McArdle N, Kerr SM, Anderson NH, Douglas NJ. Tumour necrosis factor-alpha (-308) gene polymorphism in obstructive sleep apnoea-hypopnoea syndrome. *Eur Respir J* 2005; 26:673-678.
27. Vgontzas AN, Zoumakis E, Lin HM, Bixler EO, Trakada G, Chrousos GP. Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor-alpha antagonist. *J Clin Endocrinol Metab* 2004; 89:4409-4413.
28. Ip MS, Lam KS, Ho C, Tsang KW, Lam W. Serum leptin and vascular risk factors in obstructive sleep apnea. *Chest* 2000; 118:580-586.
29. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28:412-419.
30. Hanley JA, Negassa A, Edwardes MD, Forrester JE. Statistical analysis of correlated data using

generalized estimating equations: an orientation. *Am J Epidemiol* 2003; 157:364-375.
31. Brooks D, Horner RL, Kozar LF, Render-Teixeira CL, Phillipson EA. Obstructive sleep apnea as a cause of systemic hypertension. Evidence from a canine model. *J Clin Invest* 1997; 99:106-109.
32. Leung RS, Bradley TD. Sleep apnea and cardiovascular disease. *Am J Respir Crit Care Med* 2001; 164:2147-2165.
33. Shahar E, Whitney CW, Redline S, Lee ET, Newman AB, Javier Nieto F, O'Connor GT, Boland LL, Schwartz JE, Samet JM. Sleep-disordered breathing and cardiovascular disease: cross-sectional results of the Sleep Heart Health Study. *Am J Respir Crit Care Med* 2001; 163:19-25.
34. Punjabi NM, Sorkin JD, Katzel LI, Goldberg AP, Schwartz AR, Smith PL. Sleep-disordered breathing and insulin resistance in middle-aged and overweight men. *Am J Respir Crit Care Med* 2002; 165:677-682.
35. Hsu AA, Lo C. Continuous positive airway pressure therapy in sleep apnoea. *Respirology* 2003; 8:447-454.
36. Sullivan CE, Issa FG, Berthon-Jones M, Eves L. Reversal of obstructive sleep apnoea by continuous positive airway pressure applied through the nares. *Lancet* 1981; 1:862-865.
37. Naegele B, Pepin JL, Levy P, Bonnet C, Pellat J, Feuerstein C. Cognitive executive dysfunction in patients with obstructive sleep apnea syndrome (OSAS) after CPAP treatment. *Sleep* 1998; 21:392-397.
38. Farre R, Hernandez L, Montserrat JM, Rotger M, Ballester E, Navajas D. Sham continuous positive airway pressure for placebo-controlled studies in sleep apnoea. *Lancet* 1999; 353:1154.
39. Montserrat JM, Ferrer M, Hernandez L, Farre R, Vilagut G, Navajas D, Badia JR, Carrasco E, De Pablo J, Ballester E. Effectiveness of CPAP treatment in daytime function in sleep apnea syndrome: a randomized controlled study with an optimized placebo. *Am J Respir Crit Care Med* 2001; 164:608-613.
40. Kapur VK, Baldwin CM, Resnick HE, Gottlieb DJ, Nieto FJ. Sleepiness in patients with moderate to severe sleep-disordered breathing. *Sleep* 2005; 28:472-477.
41. Aguillard RN, Riedel BW, Lichstein KL, Grieve FG, Johnson CT, Noe SL. Daytime functioning in obstructive sleep apnea patients: exercise tolerance, subjective fatigue, and sleepiness. *Appl Psychophysiol Biofeedback* 1998; 23:207-217.
42. Hossain JL, Ahmad P, Reinish LW, Kayumov L, Hossain NK, Shapiro CM. Subjective fatigue and subjective sleepiness: two independent consequences of sleep disorders? *J Sleep Res* 2005; 14:245-253.
43. Becker HF, Jerrentrup A, Ploch T, Grote L, Penzel T, Sullivan CE, Peter JH. Effect of nasal continuous positive airway pressure treatment on blood pressure in patients with obstructive sleep apnea. *Circulation* 2003; 107:68-73.
44. Wallin BG, Sundlof G, Eriksson BM, Dominiak P, Grobecker H, Lindblad LE. Plasma noradrenaline correlates to sympathetic muscle nerve activity in normotensive man. *Acta Physiol Scand* 1981; 111:69-73.
45. Hedner J, Darpo B, Ejnell H, Carlson J, Caidahl K. Reduction in sympathetic activity after long-term CPAP treatment in sleep apnoea: cardiovascular implications. *Eur Respir J* 1995; 8:222-229.
46. Can M, Acikgoz S, Mungan G, Bayraktaroglu T, Kocak E, Guven B, Demirtas S. Serum cardiovascular risk factors in obstructive sleep apnea. *Chest* 2006; 129:233-237.

47. Sharma SK, Kumpawat S, Goel A, Banga A, Ramakrishnan L, Chaturvedi P. Obesity, and not obstructive sleep apnea, is responsible for metabolic abnormalities in a cohort with sleep-disordered breathing. *Sleep Med* 2007; 8:12-17.
48. Makino S, Handa H, Suzukawa K, Fujiwara M, Nakamura M, Muraoka S, Takasago I, Tanaka Y, Hashimoto K, Sugimoto T. Obstructive sleep apnoea syndrome, plasma adiponectin levels, and insulin resistance. *Clin Endocrinol (Oxf)* 2006; 64:12-19.
49. O'Brien E, Petrie J, Littler W, de Swiet M, Padfield PL, O'Malley K, Jamieson M, Altman D, Bland M, Atkins N. The British Hypertension Society protocol for the evaluation of automated and semi-automated blood pressure measuring devices with special reference to ambulatory systems. *J Hypertens* 1990; 8:607-619.
50. Young T, Palta M, Dempsey J, Skatrud J, Weber S, Badr S. The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* 1993; 328:1230-1235.
51. Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP. Intermittent hypoxia increases insulin resistance in genetically obese mice. *J Physiol* 2003; 552:253-264.
52. Faccenda JF, Mackay TW, Boon NA, Douglas NJ. Randomized placebo-controlled trial of continuous positive airway pressure on blood pressure in the sleep apnea-hypopnea syndrome. *Am J Respir Crit Care Med* 2001; 163:344-348.
53. Javaheri S, Parker TJ, Liming JD, Corbett WS, Nishiyama H, Wexler L, Roselle GA. Sleep apnea in 81 ambulatory male patients with stable heart failure. Types and their prevalences, consequences, and presentations. *Circulation* 1998; 97:2154-2159.
54. Newman AB, Nieto FJ, Guidry U, Lind BK, Redline S, Pickering TG, Quan SF. Relation of sleep-disordered breathing to cardiovascular disease risk factors: the Sleep Heart Health Study. *Am J Epidemiol* 2001; 154:50-59.
55. Peppard PE, Young T, Palta M, Skatrud J. Prospective study of the association between sleep-disordered breathing and hypertension. *N Engl J Med* 2000; 342:1378-1384.
56. Sin DD, Fitzgerald F, Parker JD, Newton G, Floras JS, Bradley TD. Risk factors for central and obstructive sleep apnea in 450 men and women with congestive heart failure. *Am J Respir Crit Care Med* 1999; 160:1101-1106.
57. Suzuki M, Guilleminault C, Otsuka K, Shiomi T. Blood pressure "dipping" and "non-dipping" in obstructive sleep apnea syndrome patients. *Sleep* 1996; 19:382-387.
58. Parker JD, Brooks D, Kozar LF, Render-Teixeira CL, Horner RL, Douglas Bradley T, Phillipson EA. Acute and chronic effects of airway obstruction on canine left ventricular performance. *Am J Respir Crit Care Med* 1999; 160:1888-1896.
59. Tkacova R, Rankin F, Fitzgerald FS, Floras JS, Bradley TD. Effects of continuous positive airway pressure on obstructive sleep apnea and left ventricular afterload in patients with heart failure. *Circulation* 1998; 98:2269-2275.
60. Chin K, Nakamura T, Shimizu K, Mishima M, Miyasaka M, Ohi M. Effects of nasal continuous positive airway pressure on soluble cell adhesion molecules in patients with obstructive sleep apnea syndrome. *Am J Med* 2000; 109:562-567.

61. Schulz R, Mahmoudi S, Hattar K, Sibelius U, Olschewski H, Mayer K, Seeger W, Grimminger F. Enhanced release of superoxide from polymorphonuclear neutrophils in obstructive sleep apnea. Impact of continuous positive airway pressure therapy. *Am J Respir Crit Care Med* 2000; 162:566-570.
62. Dyugovskaya L, Lavie P, Lavie L. Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* 2002; 165:934-939.
63. Schulz R, Hummel C, Heinemann S, Seeger W, Grimminger F. Serum levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea and severe nighttime hypoxia. *Am J Respir Crit Care Med* 2002; 165:67-70.
64. Alberti A, Sarchielli P, Gallinella E, Floridi A, Mazzotta G, Gallai V. Plasma cytokine levels in patients with obstructive sleep apnea syndrome: a preliminary study. *J Sleep Res* 2003; 12:305-311.
65. Vgontzas AN, Papanicolaou DA, Bixler EO, Kales A, Tyson K, Chrousos GP. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. *J Clin Endocrinol Metab* 1997; 82:1313-1316.
66. Imagawa S, Yamaguchi Y, Higuchi M, Neichi T, Hasegawa Y, Mukai HY, Suzuki N, Yamamoto M, Nagasawa T. Levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea--hypopnea syndrome. *Blood* 2001; 98:1255-1257.
67. Ohga E, Tomita T, Wada H, Yamamoto H, Nagase T, Ouchi Y. Effects of obstructive sleep apnea on circulating ICAM-1, IL-8, and MCP-1. *J Appl Physiol* 2003; 94:179-184.
68. Carpagnano GE, Kharitonov SA, Resta O, Foschino-Barbaro MP, Gramiccioni E, Barnes PJ. Increased 8-isoprostane and interleukin-6 in breath condensate of obstructive sleep apnea patients. *Chest* 2002; 122:1162-1167.
69. Guillemin K, Krasnow MA. The hypoxic response: huffing and HIFing. *Cell* 1997; 89:9-12.
70. Jung Y, Isaacs JS, Lee S, Trepel J, Liu ZG, Neckers L. Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. *Biochem J* 2003; 370:1011-1017.
71. Lukiw WJ, Ottlecz A, Lambrou G, Grueninger M, Finley J, Thompson HW, Bazan NG. Coordinate activation of HIF-1 and NF-kappaB DNA binding and COX-2 and VEGF expression in retinal cells by hypoxia. *Invest Ophthalmol Vis Sci* 2003; 44:4163-4170.
72. Entzian P, Linnemann K, Schlaak M, Zabel P. Obstructive sleep apnea syndrome and circadian rhythms of hormones and cytokines. *Am J Respir Crit Care Med* 1996; 153:1080-1086.
73. Minoguchi K, Tazaki T, Yokoe T, Minoguchi H, Watanabe Y, Yamamoto M, Adachi M. Elevated production of tumor necrosis factor-alpha by monocytes in patients with obstructive sleep apnea syndrome. *Chest* 2004; 126:1473-1479.
74. Seta KA, Millhorn DE. Functional genomics approach to hypoxia signaling. *J Appl Physiol* 2004; 96:765-773.
75. Semenza GL. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 2001; 107:1-3.
76. Koong AC, Chen EY, Giaccia AJ. Hypoxia causes the activation of nuclear factor kappa B through

the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Res* 1994; 54:1425-1430.
77. Royds JA, Dower SK, Qwarnstrom EE, Lewis CE. Response of tumour cells to hypoxia: role of p53 and NFkB. *Mol Pathol* 1998; 51:55-61.
78. Singh S, Darnay BG, Aggarwal BB. Site-specific tyrosine phosphorylation of IkappaBalpha negatively regulates its inducible phosphorylation and degradation. *J Biol Chem* 1996; 271:31049-31054.
79. Beitner-Johnson D, Leibold J, Millhorn DE. Hypoxia regulates the cAMP- and Ca2+/calmodulin signaling systems in PC12 cells. *Biochem Biophys Res Commun* 1998; 242:61-66.
80. Beitner-Johnson D, Rust RT, Hsieh TC, Millhorn DE. Hypoxia activates Akt and induces phosphorylation of GSK-3 in PC12 cells. *Cell Signal* 2001; 13:23-27.
81. Conrad PW, Freeman TL, Beitner-Johnson D, Millhorn DE. EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. *J Biol Chem* 1999; 274:33709-33713.
82. Conrad PW, Rust RT, Han J, Millhorn DE, Beitner-Johnson D. Selective activation of p38alpha and p38gamma by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J Biol Chem* 1999; 274:23570-23576.
83. Wright J, Johns R, Watt I, Melville A, Sheldon T. Health effects of obstructive sleep apnoea and the effectiveness of continuous positive airways pressure: a systematic review of the research evidence. *Bmj* 1997; 314:851-860.
84. Davies RJ, Stradling JR. The efficacy of nasal continuous positive airway pressure in the treatment of obstructive sleep apnea syndrome is proven. *Am J Respir Crit Care Med* 2000; 161:1775-1776.
85. Henke KG, Grady JJ, Kuna ST. Effect of nasal continuous positive airway pressure on neuropsychological function in sleep apnea-hypopnea syndrome. A randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 2001; 163:911-917.
86. Malhotra A, Ayas NT, Epstein LJ. The art and science of continuous positive airway pressure therapy in obstructive sleep apnea. *Curr Opin Pulm Med* 2000; 6:490-495.
87. Loube DI, Gay PC, Strohl KP, Pack AI, White DP, Collop NA. Indications for positive airway pressure treatment of adult obstructive sleep apnea patients: a consensus statement. *Chest* 1999; 115:863-866.
88. Rosenthal L, Gerhardstein R, Lumley A, Guido P, Day R, Syron ML, Roth T. CPAP therapy in patients with mild OSA: implementation and treatment outcome. 2000; 1:215-220.
89. Liu CC, Lin CC, Chen WS, Chen HY, Chang PC, Chen JJ, Yang PC. CRSD: a comprehensive web server for composite regulatory signature discovery. *Nucleic Acids Res* 2006; 34:W571-577.
90. Tseng HM LJ, Tsai YJ. Assessment of Health-Related Quality of Life (II): Norming and Validation of SF-36 Taiwan Version. *Taiwan Journal of Public Health* 2003; 22:512-518.
91. Lu JR TH, Tsai YJ. Assessment of Health-Related Quality of Life in Taiwan (I): Development and Psychometric Testing of SF-36 Taiwan Version. *Taiwan Journal of Public Health* 2003; 22:501-511.
92. Kazumi T, Kawaguchi A, Sakai K, Hirano T, Yoshino G. Young men with high-normal blood pressure have lower serum adiponectin, smaller LDL size, and higher elevated heart rate than those with optimal blood pressure. *Diabetes Care* 2002; 25:971-976.
93. Speidl WS, Toller WG, Kaun C, Weiss TW, Pfaffenberger S, Kastl SP, Furnkranz A, Maurer G, Huber

K, Metzler H, Wojta J. Catecholamines potentiate LPS-induced expression of MMP-1 and MMP-9 in human monocytes and in the human monocytic cell line U937: possible implications for peri-operative plaque instability. *Faseb J* 2004; 18:603-605.
94. Tazaki T, Minoguchi K, Yokoe T, Samson KT, Minoguchi H, Tanaka A, Watanabe Y, Adachi M. Increased levels and activity of matrix metalloproteinase-9 in obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 2004; 170:1354-1359.
95. Cutler MJ, Swift NM, Keller DM, Wasmund WL, Burk JR, Smith ML. Periods of intermittent hypoxic apnea can alter chemoreflex control of sympathetic nerve activity in humans. *Am J Physiol Heart Circ Physiol* 2004; 287:H2054-2060.
96. Somers VK, Mark AL, Zavala DC, Abboud FM. Contrasting effects of hypoxia and hypercapnia on ventilation and sympathetic activity in humans. *J Appl Physiol* 1989; 67:2101-2106.
97. Hausberg M, Hoffman RP, Somers VK, Sinkey CA, Mark AL, Anderson EA. Contrasting autonomic and hemodynamic effects of insulin in healthy elderly versus young subjects. *Hypertension* 1997; 29:700-705.
98. Somers VK, Dyken ME, Clary MP, Abboud FM. Sympathetic neural mechanisms in obstructive sleep apnea. *J Clin Invest* 1995; 96:1897-1904.
99. Black PH, Garbutt LD. Stress, inflammation and cardiovascular disease. *J Psychosom Res* 2002; 52:1-23.
100. Elenkov IJ, Chrousos GP. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann N Y Acad Sci* 2002; 966:290-303.
101. Annane D, Bellissant E, Cavaillon JM. Septic shock. *Lancet* 2005; 365:63-78.
102. Severn A, Rapson NT, Hunter CA, Liew FY. Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists. *J Immunol* 1992; 148:3441-3445.
103. Torres KC, Antonelli LR, Souza AL, Teixeira MM, Dutra WO, Gollob KJ. Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. *J Neuroimmunol* 2005; 166:144-157.
104. Spengler RN, Allen RM, Remick DG, Strieter RM, Kunkel SL. Stimulation of alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. *J Immunol* 1990; 145:1430-1434.
105. Guo M, Song LP, Jiang Y, Liu W, Yu Y, Chen GQ. Hypoxia-mimetic agents desferrioxamine and cobalt chloride induce leukemic cell apoptosis through different hypoxia-inducible factor-1alpha independent mechanisms. *Apoptosis* 2006; 11:67-77.
106. Huang Y, Du KM, Xue ZH, Yan H, Li D, Liu W, Chen Z, Zhao Q, Tong JH, Zhu YS, Chen GQ. Cobalt chloride and low oxygen tension trigger differentiation of acute myeloid leukemic cells: possible mediation of hypoxia-inducible factor-1alpha. *Leukemia* 2003; 17:2065-2073.