

## Hyperoxia Influences mRNA Expression of Cytokines in Cultured Human Umbilical Vein Endothelial Cells

Min Soo Park<sup>1</sup> and Heather M. Wallace<sup>2</sup>

*High concentrations of oxygen, indispensable for the treatment of severe hypoxemia from neonatal as well as adult respiratory distress syndrome, increase the risk of oxygen toxicity. Biochemical mechanisms are lipid peroxidation, protein sulfhydryl oxidation, enzyme inactivation, and DNA damage. Recent reports suggest that cytokines might be involved in free radical injury as well as in adaptive response to hyperoxic injury. However, actual signal transduction pathways involving cytokines have not yet been clarified. In this study we exposed cultured human umbilical vein endothelial cells (HUVECs) to either ambient air or 100% oxygen, and compared for the rate of DNA synthesis (<sup>3</sup>H)thymidine uptake) at different time points up to 72 h. After exposing the cells to each treatment condition, we extracted RNA, constructed complementary DNA using reverse transcriptase, amplified the specific DNA segments of cytokines by polymerase chain reaction (PCR), and used the PCR products for gel electrophoresis to examine the bands which signified mRNA levels of corresponding cytokines. There was a significant decrease in the rate of DNA synthesis as early as 24 h. The mRNA expression of IL-1 $\beta$  and TNF $\alpha$  seemed less influenced by hyperoxia, while IL-8 and TGF $\beta$  showed marked increase in mRNA levels at 6 h of 100% oxygen exposure.*

**Key Words:** Hyperoxia, reactive oxygen species, HUVEC, cytokine, RT-PCR

Although oxygen is a life-saving as well as life-sustaining gas in aerobic organisms, including humans, it is not as completely harmless as one wishes it would be (Stogner and Payne, 1992). Toxic effects are reported after exposure to varying degrees of high concentrations of oxygen in clinical and experimental settings and range from mild pulmonary problems, such as airway inflammation and edema

formation, to blindness, convulsion, respiratory failure and death (Deneke and Fanburg, 1980).

Depending on the type of cells in different tissues *in vivo*, the oxygen tension at which cells are normally exposed differs. For example, the oxygen concentration of alveolar air is 14% (or PO<sub>2</sub> of 100 mmHg); that of arterial blood 13% (95 mmHg); umbilical vein blood ~4% (27 mmHg); and adult cerebral cortex <1% (2~5 mmHg). Therefore, any oxygen concentrations above these physiological levels for respective tissues would augment the oxidative stress to cells that are usually under the constant threat of reactive oxygen species which are produced but effectively neutralized by defence mechanisms in physiological conditions. Usually, if oxygen concentration is above 40~60% (PO<sub>2</sub> of 300~500 mmHg), excessive production of highly-reactive, partially-reduced oxygen intermediates, so-

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<sup>1</sup>Department of Pediatrics, Yonsei University College of Medicine, Seoul, Korea and <sup>2</sup>Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK

Address reprint request to Dr. M.S. Park, Department of Pediatrics, Yongdong Severance Hospital, Yonsei University College of Medicine, Yongdong P.O. Box 1217, Seoul 135-270, Korea, e-mail: minsark@yumc.yonsei.ac.kr

called reactive oxygen species (ROS), overwhelms the capacity of cells to detoxify these toxic intermediates (Deneke and Fanburg, 1980; Small, 1984; Youngman, 1984; Jamieson *et al.* 1986).

A morphological study using different oxygen concentrations on human endothelial cells showed that during the early stages the exposure to very high PO<sub>2</sub> tensions first (after 24 h) stimulated the cells to enter the S phase of the cell cycle; thereafter (after 48 h) there was a conspicuous and substantial decrease of S phase cells, resulting in an almost complete halt in cell proliferation (Bjerkvig *et al.* 1992).

In humans short-term exposure to hyperoxia causes tracheitis, damage to airway epithelium, impaired mucociliary clearance, atelectasis, and alveolar-capillary leakage. Long-term exposure produces pulmonary oedema and impaired gas exchange, the characteristic features of ARDS and BPD (Bancalari, 1992).

Even though prolonged exposure to hyperoxia is fatal to experimental animals of various ages, pretreatment with TNF and IL-1 is known to induce tolerance to subsequent hyperoxia (White *et al.* 1989). In addition, other types of oxidant stress, such as UV and ionizing radiation, cause an increase in mRNA levels of TNF and IL-1, which induces MnSOD (Kupper *et al.* 1987; Hallahan *et al.* 1989; Janssen *et al.* 1993). As to TGF $\beta$ , there are conflicting findings in its role as a prooxidant or as an inducer of antioxidant enzymes (Das and Fanburg, 1991; Thannickal *et al.* 1993).

Antioxidant enzymes, either genetically engineered to overexpress (White *et al.* 1991) or exogenously administered (Turrens *et al.* 1984; Beckman *et al.* 1988; Walther *et al.* 1990), showed protective effects against hyperoxic injury. Interestingly, studies demonstrated that after exposure to hyperoxia, cells exhibited an increase in gene expression, production, and activity of these antioxidant enzymes (Housset and Junod, 1982; Deneke *et al.* 1987; Kong *et al.* 1993; Rusakow *et al.* 1993). However, the complex regulatory mechanisms are still to be clarified and there are discrepancies in the findings of studies using different cell types. In human endothelial cells, an increase in CuZnSOD, as well as an increase in glutathione peroxidase (GP), were observed after 3 or 5 days of exposure to hyperoxia;

while little change was found in fibroblast cultures (Kong and Fanburg, 1992).

Although there have been many reports of adaptive responses to hyperoxia, such as an increase in antioxidant enzymes (Janssen *et al.* 1993), which could also be induced by certain cytokines, there are in fact only a few papers which studied the direct effect of hyperoxia on the expression of cytokines in the cells exposed to hyperoxia. Among these, a number of reports showed that the expression or production of certain cytokines such as IL-8 was increased by hyperoxia in various cell types such as monocytes, lung fibroblasts, type 2 alveolar epithelial cells, and alveolar macrophages (Metinko *et al.* 1992; DeForge *et al.* 1993; Stancombe *et al.* 1993; Deaton *et al.* 1994).

This study was carried out to determine the effect of hyperoxia in cultured human umbilical vein endothelial cells (HUVEC) on the rate of DNA synthesis, as well as the changes in the expression of mRNAs of cytokines (IL-1b, IL-8, TNF $\alpha$ , and TGF $\beta$ ) in cultured HUVEC on exposure to hyperoxia.

## MATERIALS AND METHODS

### Isolation and culture of HUVEC

HUVEC was prepared by modified method of Jaffe *et al.* (1973). Briefly, umbilical cords of 20~30 cm in length were collected immediately after delivery, and after removing the blood from the vein by flushing with PBS A, 0.05% collagenase H (Boehringer Mannheim Corp., Mannheim, Germany) was infused into the vein. The cord was incubated at 37°C in the incubator for 10 min, and collagenase H solution containing cells was withdrawn using syringes. About 10 to 20 ml of Medium 199 (M199) (Life Technology, Paisley, U.K.) was injected into the vein and withdrawn to recover the rest of the cells. The solution containing the cells was centrifuged for 3 min at 1,000  $\times$ g. The cell pellet was resuspended and dispersed with 5 ml of MS20 [M199+10% foetal calf serum (Life Technology, Paisley, U.K.)+10% horse serum (Life Technology, Paisley, U.K.)+2 mM L-glutamine (Sigma Chemical Co. Ltd., Poole, U.K.)]. Cell suspension was placed into a flask and incubated in the Gallenkamp

**CO<sub>2</sub> Incubator.**

When the primary HUVEC reached confluence, it was subcultured onto 75 cm<sup>2</sup> flasks. Beginning with the first passage, subsequent passage cells were given endothelial cell growth supplement (ECGS)/Heparin (Sigma Chemical Co. Ltd., Poole, U.K.) in order to optimize the cell proliferation. Cells were allowed to grow to confluence at the second passage and were seeded for the experiment in their third passage. The cell number and viability were determined using trypan blue.

**Measurement of protein content**

Protein content was measured by modified Lowry method. 50 µl aliquots of samples and standard were dispensed in triplicate into 96-well plates. 100 µl of Lowry reagent [2%(w/v) solution of Na<sub>2</sub>CO<sub>3</sub> containing copper tartrate solution (50:1)] was added to each well and left for 10 min at room temperature. 100 µl Folin's reagent [1 part Folin-Ciocalteu's phenol reagent (BDH Chemicals Co. Ltd., Poole, U.K.); 14 parts dH<sub>2</sub>O] was then added to each well and left for 15 min at room temperature to develop. Absorbance was read at 690 nm using a Titrek microplate reader linked to an Atari computer. Protein content was automatically calculated in µg/ml using log-log regression analysis of the standard data.

**Determination of DNA synthesis rate by [<sup>3</sup>H]thymidine uptake**

At each time point plates were taken out and appropriate amounts of 50 µCi/ml [<sup>3</sup>H]thymidine solution (Amersham International, Amersham, Bucks, U.K.) was added to give a final concentration of 0.5 µCi/ml.

At the end of one hour of [<sup>3</sup>H]thymidine pulse, the medium was poured off and the cell layer was washed twice with cold PBS. 0.2 M HClO<sub>4</sub> was added and incubated on ice or in the refrigerator for at least 15 min. Cells were scraped off with a rubber policeman and transferred into an eppendorf tube. Plates were washed with a further 0.2 ml of 0.2 M HClO<sub>4</sub>, and the solution was added to the eppendorf. Tubes were centrifuged at 11,600 g<sub>av</sub> in MSE for 3 min. 1 ml of 0.3 M NaOH was added to the remain-

ing insoluble fraction (pellet), which was incubated at 37°C overnight to dissolve it and stored at 4°C for protein content and [<sup>3</sup>H]thymidine uptake determination.

One tenth of the volume of the sample dissolved in 0.3 M NaOH was taken (made up to 100 µl with 0.3 M NaOH, if less) and placed in a scintillation vial. Added to it were 100 µl 3 M HCl, 100 µl dH<sub>2</sub>O and 4 ml of Ultima Gold scintillation fluid (Cambera Packard, Pangbourne, U.K.).

**Determination of mRNA expression of cytokines**

**RNA extraction:** RNA was extracted using the modified acid guanidinium thiocyanate-phenol-chloroform mixture (AGPC) method (Chomczynski and Sacchi, 1987). Briefly, after washing with and removing complete PBS, 1 ml RNAzol (Cinna Biotech, Biogenesis, U.K.) was added to each plate of cell monolayer. Each plate was evenly scraped and mixed. The solution was transferred to a sterile, screw-cap 2 ml microfuge tube on ice, and 1/10th volume chloroform was added. The tubes were shaken vigorously for 15 sec then put on ice for 5 min. They were spun at 11,500 rpm for 15 min at 0°C. The upper phase was carefully aspirated into a fresh tube, without disturbing the interface and the bottom phase. An equal volume of cold isopropanol was added to the upper phase, shaken well and kept on ice for 15 min. The tubes were centrifuged at 11,500 rpm at 0°C for 15 min. Isopropanol was carefully aspirated from the pellet, and washed with 1 ml cold 75% ethanol and centrifuged at 11,500 rpm for 5 min. Then ethanol was aspirated until virtually dry. The RNA pellet was dissolved in 52 µl diethyl pyrocarbonate (DEPC) treated water. 2 µl of this solution was taken and added into 998 µl sterile water (1 in 500 dilution) in a separate eppendorf tube for spectrophotometric determination of RNA content. RNA concentration was calculated by OD at 260 nm × 0.04 × dilution factor; total RNA content was calculated by multiplying the total volume left; and then the volume of RNA solution which would yield 5 µg of RNA was calculated.

**Reverse transcription:** Thus obtained, RNA was reverse-transcribed using a superscript reverse transcriptase kit (Life Technologies, Paisley, U.K.). 1 µl oligo dT primer (0.5 µg/µl) (Pharmacia Biotech,

Hertfordshire, U.K.) was added to RNA and mixed well. Tubes were heated at 70°C for 10 min in thermocycler. They were cooled on ice for 2~3 min and the reaction was pelleted by pulse spin. The remaining reagents were added to the reaction in the following order: 4  $\mu$ l of 5 $\times$  reaction buffer, 1  $\mu$ l of 10 mM dNTP mix (Promega Corp., Southampton, U.K.), 2  $\mu$ l of 0.1 M DTT, and 1  $\mu$ l of reverse transcriptase; and the reaction was mixed gently by tapping the side of the tube and pulse-spun to collect the reaction at the bottom of the tube. The reaction was left for 10 min at room temperature on the bench, and placed in a thermocycler at 42°C for 50 min and heated to 95°C for 5 min. The reaction volume was brought to 100  $\mu$ l by adding 80  $\mu$ l DEPC water. The cDNA was stored frozen at -20°C.

**Polymerase chain reaction (PCR):** 2.5  $\mu$ l of primer mix (IL-1 $\beta$ , IL-8, TNF $\alpha$ , or TGF $\beta$ ) (Oswell DNA Service, Edinburgh, U.K.) was added to each tube. Master mix was prepared by mixing all the components [water 15.375  $\mu$ l, dNTP 2  $\mu$ l, buffer 2.5  $\mu$ l and Taq polymerase (Promega Corp., Southampton, U.K.) 0.125  $\mu$ l per tube]. They were mixed well by pipetting and 20  $\mu$ l was dispensed to each tube. First 2.5  $\mu$ l cDNA solution and then 25  $\mu$ l of mineral oil were added to each tube. Tubes were mixed by tapping the side. Reaction was collected in the bottom of the tube by pulse spin and tubes were placed in a Hybaid OmniGene thermocycler. The PCR Program used was as follows: one cycle of stage 1: 94°C for 4 min; 55°C for 1 min; 72°C for 1 min 30 sec --- 33 cycles of stage 2: 94°C for 50 sec; 55°C for 1 min; 72°C for 1 min 30 sec --- and 1 cycle of stage 3: 94°C for 50 sec; 55°C for 1 min; 72°C for 5 min.

**Agarose gel electrophoresis:** 1.5% agarose gel was prepared by melting 1.2 g of agarose in 80 ml of TBE buffer [Tris, boric acid, EDTA (Na<sub>2</sub>)] in a microwave oven. After cooling for a few minutes, 3  $\mu$ l of ethidium bromide was added to the solution which was then poured onto the gel maker with the combs in place. When the gel was hardened, buffer was poured onto the electrophoresis kit. 8  $\mu$ l of PCR product of each sample was added to each well after mixing with 2  $\mu$ l of DNA loading dye.  $\phi$ X174RF DNA/Hae III Fragments were used as a DNA size marker. Voltage was set at 100 volts. Electrophoresis was performed for one hour. Gel was examined

under UV light and an image was produced using a computerized system.

**Scoring system of band intensity for comparison:** An arbitrary scoring system was employed to assign the degree of band intensity relative to the band intensity of 0 h control. The band intensity of 0 h control was set to 0. The score ranged from -4 ~ +4 based on the darkness and the size of bands.

### Experiments for the effects of 100% oxygen exposure on HUVEC

**Oxygen exposure environment:** In order to create an oxygen exposure environment comparable to an air-control environment, Modular Incubator Chambers (ICN Flow, Thame, UK) were used. The chamber used for oxygen exposure was filled with 100% oxygen. O<sub>2</sub> concentration inside the chamber was tested using a Servomex Oxymeter and found to be 100% at 0 h, 99.5% at 6 h and 98% at 24 h. The control chamber was clamped after exposing the chamber to ambient air. After each time point when the chambers had to be opened, the original conditions were restored as quickly as possible.

**Determination of rate of DNA synthesis:** When cells were confluent at Passage 2 in 75 cm<sup>2</sup> flasks, they were seeded onto culture plates at the density of 2 $\times$ 10<sup>4</sup>/cm<sup>2</sup> and grown in MSHE20 (M199 with 25 mM Hepes+10% foetal calf serum+10% horse serum+2 mM L-glutamine+2% ECGS/Heparin) at 37°C in 5% CO<sub>2</sub> Gallenkamp Incubator. Plates were divided into air-control and 100% oxygen-exposure groups. At confluence, plates were placed in Modular Incubator Chambers with either air or 100% oxygen. The chambers were placed in the cell-culture incubator. Plates were taken out of the exposure environment at designated time points: 0, 6, 12, and 24 h for short-term effects and 0, 24, 48, and 72 h for long-term effects.

**Determination of changes in mRNA expression of cytokines:** Cells were seeded at Passage 3 onto 10 cm plates at the density of 2 $\times$ 10<sup>4</sup>/cm<sup>2</sup> and were exposed under each treatment condition at confluence using Modular Incubator Chambers.

**Treatment condition:**

Air	O <sub>2</sub>
Air + NAC* 20 mM	O <sub>2</sub> + NAC 20 mM
Air + NAC 20 μM	O <sub>2</sub> + NAC 20 μM
Air + Dexa† 10 μM	O <sub>2</sub> + Dexa 10 μM

\*: NAC = N-acetylcysteine; pH adjusted to 7.0

†: Dexa = dexamethasone

Cells were taken out of the chambers at 0, 6 and 24 h of oxygen exposure for extraction of RNA, reverse transcription, and PCR.

**RESULTS**

**Effects of 100% oxygen on the rate of DNA synthesis**

A significant decrease in the rate of DNA synthesis expressed as [<sup>3</sup>H]thymidine uptake in scintillation count (dpm) per mg protein was observed as early as 6 h of 100% oxygen exposure compared to the air control. There was a continuing decrease at

12, 24, 48 and 72 h of 100% oxygen exposure compared to the corresponding air controls. The rate of DNA synthesis dropped to a negligible level after 24 h (Fig. 1). However, it was noticeable that there was a minimal increase in [<sup>3</sup>H]thymidine uptake at 6 h compared to 0, 12 and 24 h values (Fig. 1).

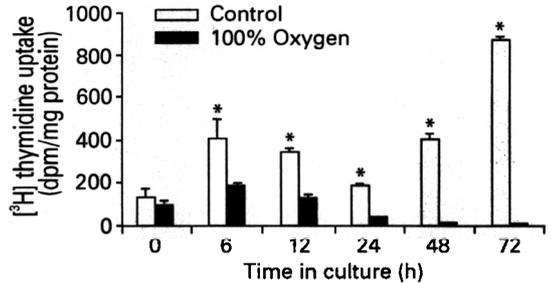


Fig. 1. [<sup>3</sup>H] thymidine uptake was compared at 0, 6, 12, 24, 48 and 72 h in culture in air- and 100% oxygen-exposed groups. \*: *p* < 0.001 compared both to air control of the same time point and to 0 h values.

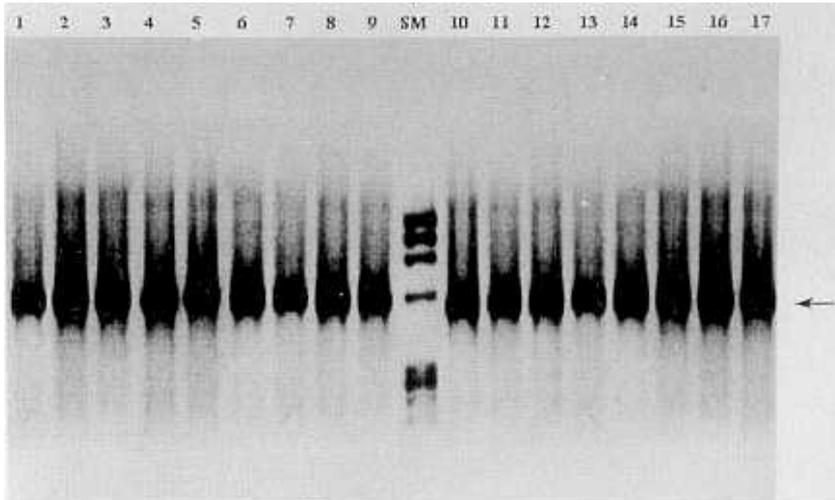
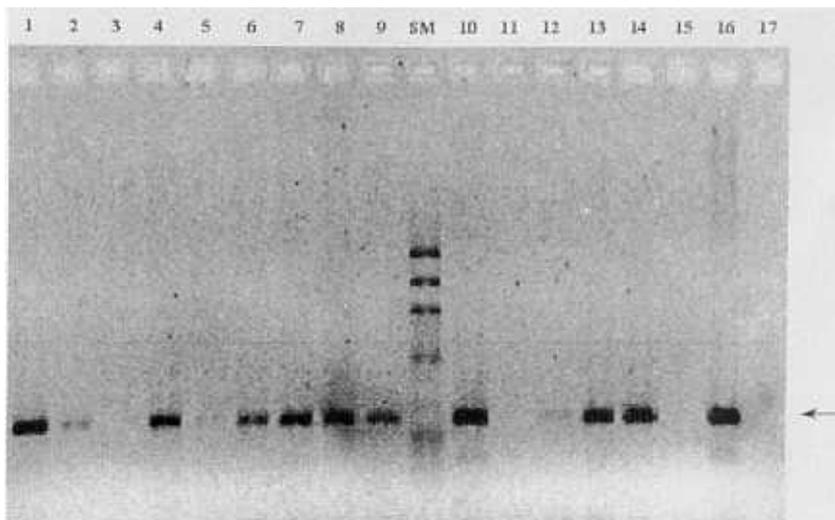


Fig. 2. The result of agarose gel electrophoresis of PCR products produced by using β-Actin (arrow: band at 543 bp) primers and templates (cDNAs) which had been reverse transcribed from mRNAs extracted from HUVECs under different treatment conditions. Lane 1: 0 h control; Lane 2: 6 h air control; Lane 3: 6h air +NAC 20mM; Lane 4: 6h air +NAC20 μM; Lane 8: 6 h 100%O<sub>2</sub>+NAC 20 μM; Lane 9: 6 h 100% O<sub>2</sub> +Dexa 10 μM; SM: size marker φX174RF DNA/Hae III Fragments; Lane 10: 24 h air control; Lane 11: 24 h air+NAC 20 mM; Lane 12:24 h air+NAC 20 μM; Lane 13: 24 h air+Dexa 10 μM; Lane 14: 24 h 100%, alone; Lane 15: 24 h 100% O<sub>2</sub>+NAC20 mM; Lane 16: 24 h 100% O<sub>2</sub>+NAC 20 μM; Lane 17: 24 h 100% O<sub>2</sub>+Dexa 10 μM.



**Fig. 3.** The result of agarose gel electrophoresis of PCR products produced by using *IL-1 $\beta$*  (arrow: band at 376 bp) primers and templates (cDNAs) which had been reverse transcribed from mRNAs extracted from HUVECs under different treatment conditions. Lane 1: 0 h control; Lane 2: 6 h air control; Lane 3: 6 h air + NAC 20 mM; Lane 4: 6 h air + NAC 20  $\mu$ M; Lane 5: 6 h air + Dexa 10  $\mu$ M; Lane 6: 6 h 100% O<sub>2</sub> alone; Lane 7: 6 h 100% O<sub>2</sub> + NAC 20 mM; Lane 8: 6 h 100% O<sub>2</sub> + NAC 20  $\mu$ M; Lane 9: 6 h 100% O<sub>2</sub> + Dexa 10  $\mu$ M; SM: size marker  $\phi$ X174RF DNA/Hae III Fragments; Lane 10: 24 h air control; Lane 11: 24 h air + Nac 20 mM; Lane 12: 24 h air + NAC 20  $\mu$ M; Lane 13: 24 h air + Dexa 10  $\mu$ M; Lane 14: 24 h 100% O<sub>2</sub> + NAC 20 mM; Lane 16: 24 h 100% O<sub>2</sub> + NAC 20  $\mu$ M; Lane 17: 24 h 100% O<sub>2</sub> + Dexa 10  $\mu$ M.

### Effects of 100% oxygen on cytokine mRNA expression

**Expression of  $\beta$ -actin mRNA as internal control (Fig. 2):** For the normalization of the RT-PCR data,  $\beta$ -actin was used as an internal control. The gel electrophoresis definitely showed the consistent expression of  $\beta$ -actin in each lane.

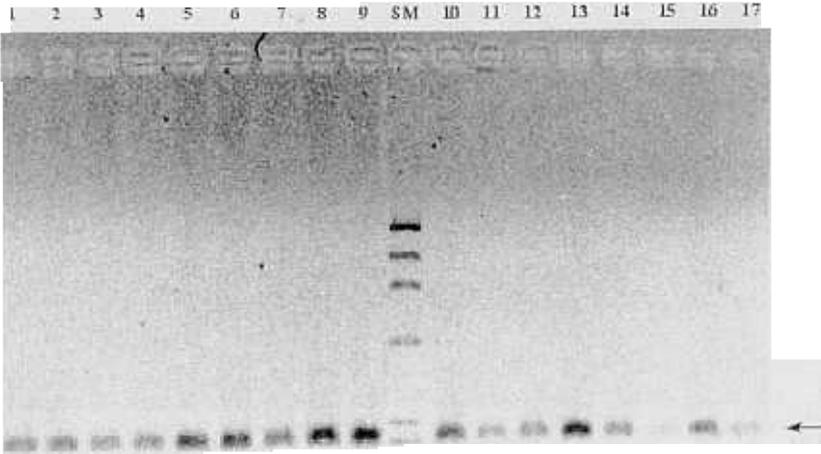
**Expression of *IL-1 $\beta$*  mRNA (Fig. 3):** At 6 h the band intensities of air control, air with 20 mM NAC group (Lane 3) and air with 10  $\mu$ M dexamethasone group (Lane 5) were conspicuously weaker than 0 h control, while that of air with 20  $\mu$ M NAC was only slightly weaker. The group exposed to 100% oxygen alone (Lane 6) showed the band with weaker intensity ( $-2$ ) than 0 h control. The band intensities of cells exposed to 100% oxygen with any of the drug treatments (Lanes 7, 8, 9) were also weaker than 0 h control, but slightly stronger compared to that of oxygen alone.

At 24 h the intensity of air control group (Lane 10) was rather prominent and slightly stronger than 0 h control and definitely stronger than 6 h control.

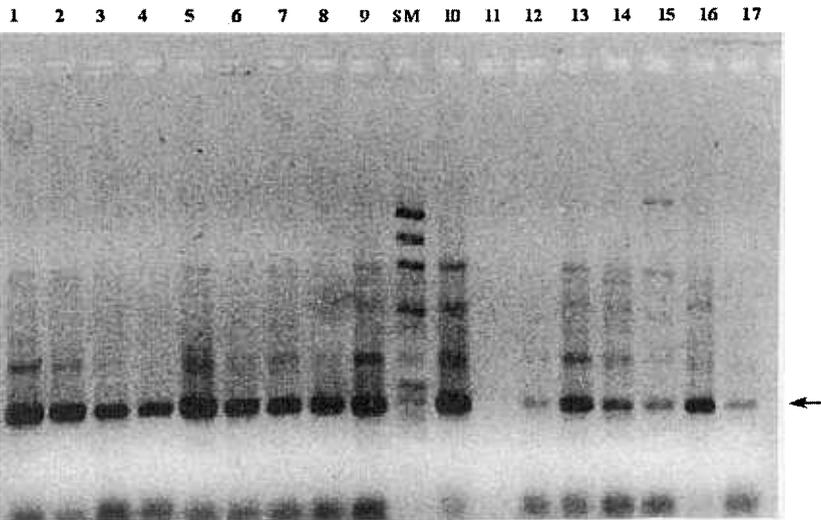
The intensity of air group with 20 mM NAC (Lane 11) remained almost undetectable as at 6 h, while that with 20  $\mu$ M NAC (Lane 12) decreased further from the 6 h level to an undetectable state. The intensity of air group with Dexa (Lane 13) was comparable to 0 h, but was much stronger compared to the 6 h equivalent. The groups with oxygen alone (Lane 14) and with 20  $\mu$ M NAC (Lane 16) showed increased intensity compared to the 6 h equivalents (Lanes 6 & 8, respectively), while those of 20 mM NAC (Lane 15) and Dexa (Lane 17) dropped markedly from 6 h levels to almost undetectable levels.

**Expression of *IL-8* mRNA (Fig. 4):** At 6 h air control (Lane 2) and groups in air with either concentration of NAC (Lanes 3 & 4) showed band intensities comparable to 0 h control, while the air group with Dexa (Lane 5) had a band with slightly stronger intensity. All oxygen-exposed groups (Lanes 6~9) had stronger bands than 0 h or 6 h air controls. However, the band of oxygen group with 20 mM NAC (Lane 7) was slightly weaker than that of oxygen alone (Lane 6), while those with 20  $\mu$ M NAC and 10  $\mu$ M Dexa (Lane 8 & 9) were slightly

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**Fig. 4.** The result of agarose gel electrophoresis of PCR products produced by using IL-8 (arrow: band at 287 bp) primers and templates (cDNAs) which had been reverse transcribed from mRNAs extracted from HUVECs under different treatment conditions. Lane 1: 0 h control; Lane 2: 6 h air control; Lane 3: 6 h air+NAC 20 mM; Lane 4: 6 h air+NAC 20  $\mu$ M; Lane 5: 6 h air+Dexa 10  $\mu$ M; Lane 6: 6 h 100% O<sub>2</sub> alone; Lane 7: 6h 100% O<sub>2</sub>+NAC 20 mM; Lane 8: 6 h 100% O<sub>2</sub>+NAC 20  $\mu$ M; Lane 9: 6 h 100% O<sub>2</sub>+Dexa 10  $\mu$ M; SM: size marker  $\phi$ X174RF DNA/Hae III Fragments; Lane 10: 24 h air control; Lane 11: 24 h air+NAC 20 mM; Lane 12: 24 h air+NAC 20  $\mu$ M; Lane 13: 24 h air+Dexa 10  $\mu$ M; Lane 14: 24 h 100% O<sub>2</sub> alone; Lane 15: 24 h 100% O<sub>2</sub>+NAC 20 mM; Lane 16: 24 h 100% O<sub>2</sub>+NAC 20  $\mu$ M; Lane 17: 24 h 100% O<sub>2</sub>+Dexa 10  $\mu$ M.



**Fig. 5.** The result of agarose gel electrophoresis of PCR products produced by using TNF  $\alpha$  (arrow: band at 439 bp) primers and templates (cDNAs) which had been reverse transcribed from mRNAs extracted from HUVECs under different treatment conditions. Lane 1: 0 h control; Lane 2: 6 h air control; Lane 3: 6 h air+NAC 20 mM; Lane 4: 6 h air+NAC 20  $\mu$ M; Lane 5: 6 h air+Dexa 10  $\mu$ M; Lane 6: 6 h 100% O<sub>2</sub> alone; Lane 7: 6h 100% O<sub>2</sub>+NAC 20 mM; Lane 8: 6 h 100% O<sub>2</sub>+NAC 20  $\mu$ M; Lane 9: 6 h 100% O<sub>2</sub>+NAC 20  $\mu$ M; Lane 9: 6 h 100% O<sub>2</sub>+Dexa 10  $\mu$ M; SM: size marker  $\phi$ X174RF DNA/Hae III Fragments; Lane 10: 24 h air control; Lane 11: 24 h air+NAC 20 mM; Lane 12: 24 h air+NAC 20  $\mu$ M; Lane 13: 24 h air+Dexa 10  $\mu$ M Lane 16: 24 h 100% O<sub>2</sub>+NAC 20  $\mu$ M; Lane 17: 24 h 100% O<sub>2</sub>+Dexa 10  $\mu$ M.

stronger.

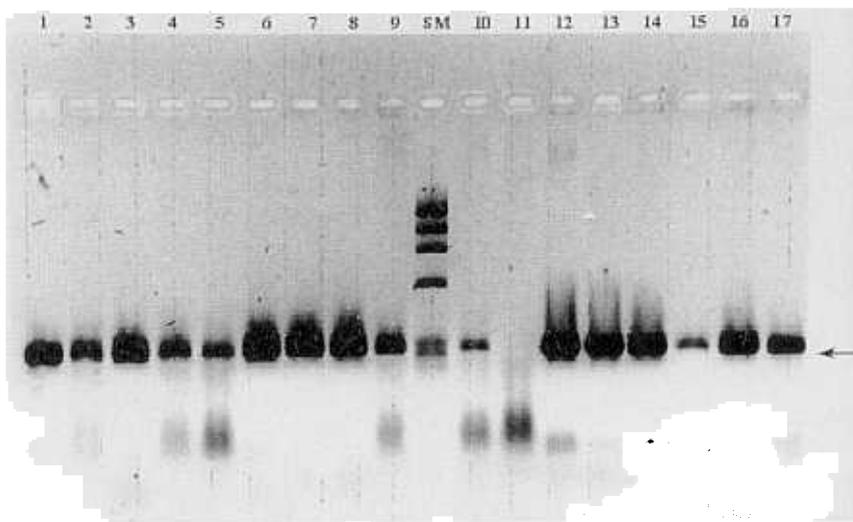
The band intensity of air control at 24 h (Lane 10) grew stronger compared to 6 h one. Air with either concentration of NAC (Lane 11 & 12) remained the same, while that with Dexta (Lane 13) increased further. On the other hand, the band intensities of all oxygen-exposed groups (Lanes 14 to 17) decreased markedly compared to 6 h. The expression of IL-8 was effectively blocked by 20 mM NAC and Dexta (Lane 15 & 17).

**Expression of TNF $\alpha$  mRNA (Fig. 5):** At 6 h air control group (Lane 2) and air groups with both concentrations of NAC (Lanes 3 & 4) had bands with intensity slightly weaker than the 0 h control, but the air group with Dexta (Lane 5) had a slightly stronger band compared to air control. Oxygen exposed groups had bands with intensity slightly weaker (Lanes 6, 7, 8) than or comparable (Lane 9) to 0 h air control, but they were similar in intensity to the air equivalents. NAC in both air and oxygen-exposed groups did not affect the expression of TNF $\alpha$  mRNA. When the band intensities of air with Dexta

and of oxygen with Dexta were compared with air control or oxygen control (oxygen alone), respectively, they were slightly stronger in both cases.

At 24 h band intensities of air groups with NAC decreased further and markedly (Lanes 11 & 12) compared with 6 h equivalents as well as with 24 h air control. The air group with Dexta (Lane 13) had a band with weaker intensity, but not as markedly as NAC groups compared with 24 h air control. The band intensity of the group exposed to oxygen alone (Lane 14) was markedly weaker compared with 0 and 6 h air controls and 6 h oxygen alone as well as with 24 h air control. 20 mM NAC (Lane 15) and 10  $\mu$ M Dexta (Lane 17) did not have any effect on the oxygen-exposed group at 24 h, while 20  $\mu$ M NAC group (Lane 16) had a band intensity stronger than the oxygen-alone group.

**Expression of TGF $\beta$  mRNA (Fig. 6):** Air control group, air groups with 20  $\mu$ M NAC and with 10  $\mu$ M Dexta at 6 h (Lanes 2, 4, 5) had slightly weaker bands, while 20 mM NAC group (Lane 3) had a band slightly stronger than 0 h control. The



**Fig. 6.** The result of agarose gel electrophoresis of PCR products produced by using TGF  $\beta$  (arrow: band at 299 bp) primers and templates (cDNAs) which had been reverse transcribed from mRNAs extracted from HUVECs under different treatment conditions. Lane 1: 0 h control; Lane 2: 6 h air control; Lane 3: 6 h air + NAC 20 mM; Lane 4: 6 h air + NAC 20  $\mu$ M; Lane 5: 6 h air + Dexta 10  $\mu$ M; Lane 6: 6 h 100% O<sub>2</sub> alone; Lane 7: 6 h 100% O<sub>2</sub> + NAC 20 mM; Lane 8: 6 h 100% O<sub>2</sub> + NAC 20  $\mu$ M; Lane 9: 6 h 100% O<sub>2</sub> + Dexta 10  $\mu$ M; SM: size marker  $\phi$ X174RF DNA/Hae III Fragments; Lane 10: 24 h air control; Lane 11: 24 h air + NAC 20 mM; Lane 12: 24 h air + NAC 20  $\mu$ M; Lane 13: 24 h air + Dexta 10  $\mu$ M; Lane 14: 24 h 100% O<sub>2</sub> alone; Lane 15: 24 h 100% O<sub>2</sub> + NAC 20 mM; Lane 16: 24 h 100% O<sub>2</sub> + NAC 20  $\mu$ M; Lane 17: 24 h 100% O<sub>2</sub> + Dexta 10  $\mu$ M.

group exposed to 100% oxygen-alone and oxygen groups with 20 mM and 20  $\mu$ M NAC (Lanes 6, 7, 8) showed conspicuously stronger bands, while that with Dexa (Lane 9) had a band with intensity much weaker compared to the group with oxygen alone.

At 24 h the band of air control (Lane 10) was much weaker compared to 6 h as was that of 20 mM NAC (Lane 11). Air groups with 20  $\mu$ M NAC and Dexa (Lanes 12 & 13) had bands of much stronger intensity compared with air control at 24 h. The band of the oxygen-alone group at 24 h (Lane 14) was much stronger than air control but was slightly weaker than the 6 h equivalent. Oxygen with 20 mM NAC group (Lane 15) showed a band much weaker compared with that of oxygen alone, while those of 20  $\mu$ M NAC and 10  $\mu$ M Dexa (Lane 16 & 17) were only minimally weaker.

## DISCUSSION

As shown by the results, as well as by other published data (Junod *et al.* 1985; Schraufstatter and Cochrane, 1991), it seems obvious that 100% oxygen has an inhibitory effect on the rate of DNA synthesis in HUVEC. In this experiment we used a 1 h pulse for [ $^3$ H]thymidine labelling in order to see the effect of 100% oxygen at that specific time point instead of the cumulative effect. In oxygen-exposed HUVECs, the rate of DNA synthesis decreased drastically in the first 24 h and gradually thereafter. However, there was no significant difference in protein content between air and oxygen-exposed groups, nor were there any microscopic morphological changes observed at 24 h. This suggests that 24 h exposure of 100% oxygen on HUVECs had an inhibitory effect on the rate of DNA synthesis, and a further inhibitory effect as the period of oxygen exposure increased, while there was not yet any change in the protein synthesis and the total amount of protein in the cells, which could reflect the cell number at the time. There may be a number of explanations for this finding. The first is that the rate of DNA synthesis does not reflect the total content of DNA in the cell or the total amount of DNA newly synthesized. This also means that the rate of

DNA synthesis does not correspond to the actual content of protein, but rather that the DNA content may correlate with the protein content and the cell number. The second is that there may be a delay in the synthesis of proteins following the transcription of DNA, possibly due to the interim steps such as RNA translation and post-translational modification. This is only a crude observation of the effect of hyperoxia on the overall rate of DNA synthesis, as well as the overall protein content, without specifying any individual gene or protein.

In the experiment in which the early effects (up to 24 h) of 100% oxygen were examined, the rate of DNA synthesis was significantly lower in oxygen-exposed cells at 24 h than in 0 h controls and 24 h air control. At 6 h the rate of DNA synthesis was lower in oxygen-exposed cells compared to 6 h air controls, but it was significantly higher than 0 h control, as well as 12 and 24 h oxygen-exposed cells ( $p < 0.05$ ). This trend is in agreement with the observation (Bjerkvig *et al.* 1992) that there was initially an increase in S phase cells in the first 24 h of hyperoxia exposure. We could speculate that during the early stages of hyperoxic injury, hyperoxia may stimulate gene expression of either proinflammatory mediators or adaptive protective antioxidant enzymes. Even though [ $^3$ H]thymidine uptake only reflects nonspecific DNA synthetic activity, it is suggestive of stimulatory activity at an early stage. This finding prompted the examination of the effect of hyperoxia on gene expression at an earlier time point, as early as 6 h, and at 24 h.

Although we used an arbitrary scoring system for the comparison of band intensities, which is liable to subjective misjudgement or bias, the results still provide a glimpse into what is happening in gene expression of cytokines in response to a very short-term exposure to hyperoxia.

On the basis of reports that IL-1 $\beta$  was implicated in the pathways of inflammation (PMN adhesion to endothelial cells, monocyte activation, acute phase protein synthesis, etc.), production of reactive oxygen species, and stimulation of antioxidant enzymes as an adaptive response (Lee *et al.* 1994) and that its gene expression was increased in keratinocytes by UV irradiation, a source of injury which is known to produce ROS-like hyperoxia (Kupper *et al.* 1987), we expected an increase in mRNA expres-

sion of IL-1 $\beta$  in HUVECs exposed to hyperoxic environment.

Without any drug treatment, oxygen-exposed cells expressed slightly more IL-1 $\beta$  mRNA than air control at 6 h, but slightly less at 24 h. The 100% oxygen alone did not seem to cause any substantial alteration in IL-1 $\beta$  mRNA expression at 6 or 24 h exposure. We expected that 20 mM and 20  $\mu$ M antioxidant NAC treatments would decrease the amount of ROS and possibly block the pathway leading to activation of NF- $\kappa$ B (Suzuki *et al.* 1994). At 6 h they did not effectively inhibit IL-1 $\beta$  mRNA expression, but 20 mM NAC almost completely blocked the expression at 24 h in both air and oxygen groups. We can speculate that the 20 mM concentration of NAC was high enough to block even the basal production of IL-1 $\beta$  mRNA, but at the same time we could not rule out the possibility of the direct toxic effect of 20 mM NAC, much the same as Kharazmi *et al.* (1988) demonstrated the cytotoxic effect of NAC at concentrations of 30 mM. As to the effect of glucocorticoid on IL-1 $\beta$ , Lew *et al.* (1988) showed that glucocorticoid suppressed the production of IL-1 $\beta$  in human peripheral mononuclear cells. In our experiment, dexamethasone seemed to have an inhibitory effect on IL-1 $\beta$  mRNA expression in both air control and oxygen-exposed cells, which was especially notable in the oxygen+dexamethasone group at 24 h.

IL-8, a neutrophil-activating and chemotactic cytokine, has recently been described in association with hyperoxia. Hyperoxia, especially pre-conditioned in anoxic conditions, augmented the production of IL-8 as well as steady state mRNA at 24 h (Metinko *et al.* 1992). Our result using HUVEC as a model showed that increases in IL-8 mRNA expression seemed to occur earlier than 24 h; that is, 6 h exposure to 100% oxygen slightly increased mRNA expression, whereas after 24 h of oxygen exposure, mRNA reverted to the baseline 0 h level. This could be in agreement with the fact that endothelial damage occurs much earlier than other cells, such as epithelial cells, macrophages and fibroblasts, possibly due to comparatively less antioxidant defence capacity (Stogner and Payne, 1992). Twenty mM NAC showed an inhibitory effect as did in IL-1 $\beta$  results, while 20  $\mu$ M NAC had no notable influence.

The role of dexamethasone in hyperoxia is controversial. Yam and Roberts showed that dexamethasone aggravated lung injury induced by hyperoxia in rat models and suggested the decrease in activities of antioxidant enzymes as a possible mechanism (Yam and Roberts, 1979). On the other hand, Deaton *et al.* showed that dexamethasone at concentrations of 10  $\mu$ M, 1  $\mu$ M, and 100 nM significantly reduced the release of IL-8 as well as mRNA expression in alveolar macrophages and U937 cells induced by hyperoxia (Deaton *et al.* 1994). This implies that dexamethasone could effectively block the neutrophil chemoattractant activity of IL-8 involved in the pathways of inflammation. Our results did not agree with these data. Dexamethasone seemed to increase IL-8 mRNA levels slightly rather than reducing them in oxygen-exposed cells treated with 10  $\mu$ M dexamethasone at 6 h compared with the corresponding oxygen group without drug treatment. There seemed to be a slight inhibitory effect of dexamethasone on IL-8 mRNA in the oxygen+dexamethasone group at 24 h compared with the oxygen-alone group at 24 h, which was at a similar level as 0 h control. This could possibly be the result of the differing effects of dexamethasone on different cell types.

As already mentioned, TNF $\alpha$ , in conjunction with IL-1, acts mainly as a proinflammatory mediator. Considering the fact that hyperoxic injury involves inflammatory responses, we would expect that TNF $\alpha$  would play a major role in the pathways of hyperoxic injury. This can be supported by reports showing that release of IL-8 as well as its mRNA (Metinko *et al.* 1992), which is closely related to IL-1 and TNF, was increased and that TNF $\alpha$  can endow cells with tolerance to hyperoxia by stimulating antioxidant enzyme systems, which can also be observed as an adaptive response to hyperoxia alone without exogenous administration of TNF $\alpha$ . In addition, Hallahan *et al.* showed that ionizing radiation increased the mRNA expression of TNF $\alpha$  and later suggested that it could be mediated by protein kinase C, either through direct DNA damage activating nuclear signals or by production of oxygen free radicals (Hallahan *et al.* 1989; Hallahan *et al.* 1991). However, the results we obtained in HUVECs did not show any sign of increased expression of TNF $\alpha$  mRNA either at 6 h or at 24

h of 100% oxygen exposure. On the contrary, the mRNA of the group treated with oxygen alone was reduced to a very low level at 24 h compared with 6 h oxygen control or 24 h air control. A possible explanation may be that endothelial cells are not, or need not be, as active in producing and releasing TNF $\alpha$  in response to hyperoxia as other cell types such as alveolar macrophages and blood monocytes. In *in vivo* conditions, vascular endothelial cells under hyperoxia are subject to various sources of stimulation including neutrophils, platelets, blood monocytes and tissue macrophages. Each of these cells produces a wide range of inflammatory mediators, free radicals and cytokines, which then secondarily act on endothelial cells to elicit the subsequent inflammatory responses in cells (Stogner and Payne, 1992). For this reason endothelial cells might be spared from actively producing the proinflammatory cytokine by themselves.

Unlike TNF $\alpha$ , TGF $\beta$  mRNA was profusely expressed on oxygen exposure for 6 h, which subsided to a lower level at 24 h. This implies that hyperoxia may stimulate endothelial cells to produce TGF $\beta$  mRNA at a very early stage but soon turns off its upregulating switch by an unknown mechanism. There have been findings on the prooxidant effects of exogenous TGF $\beta$  on endothelial cells that it enhances hydrogen peroxide production (Thannickal *et al.* 1993) and mimics the effect of hyperoxia (Das and Fanburg, 1991). On the contrary, TGF $\beta$  was found to have antioxidant effects (Lefer *et al.* 1990) as well as to stimulate the antioxidant enzyme Cu, Zn-SOD in endothelial cells (Wong and Goeddel, 1988). It cannot be answered by the results of our experiment whether TGF $\beta$  has prooxidant or antioxidant effects, but it is quite certain that TGF $\beta$  is involved in the pathways of hyperoxia-induced injury. It is also interesting to note that NAC did not have any inhibitory effect on TGF $\beta$  mRNA expression at an early stage when the effect of hyperoxia on TGF $\beta$  expression was most prominent. On the other hand, dexamethasone effectively inhibited the expression of TGF $\beta$ .

In conclusion, in using HUVEC as a model of hyperoxic injury, 100% oxygen exposure decreased the rate of DNA synthesis as early as 6 h and markedly so at 24 h compared with air control. As to the expression of mRNA of IL-1 $\beta$  and TNF $\alpha$ ,

there were no remarkable increases after 6 or 24 h or with 100% oxygen exposure, contrary to our expectation. However, IL-8 and TGF $\beta$  showed an increase in mRNA expression in response to hyperoxia at 6 h, both returning to near the 0 h control level at 24 h. Even though these results are only crude estimations of the changes occurring in mRNA expression of a number of cytokines implicated in the pathways of hyperoxic injury, they can definitely serve as a platform to expand research into this subject.

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