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Hydrogen Peroxide Induces mRNA for Tumour Necrosis Factor α in Human Endothelial Cells

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Reactive oxygen intermediates are important mediators of inflammation. We investigated if hydrogen peroxide (H_2O_2) induces tumour necrosis factor α (TNFα) expression in cultured human cells from umbilical vein endothelium (HUVEC), aortic smooth muscle cells (SMC), peripheral blood mononuclear cells (PBMC), or the cell line Mono Mac 6. Cultures were stimulated with 200 µmol/L H_2O_2 for 15 min. After 4 h cells were harvested, mRNA extracted, and amplified by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) with histone (H3) as reference gene. In HUVECs, mRNA for TNFα increased with a factor of 4 after stimulation ($p < 0.001$), in PBMC with a factor of 2 ($p < 0.05$), while mRNA from SMC and Mono Mac 6 did not increase significantly. Cellular TNFα protein in HUVECs was measured with flow cytometry (FACS) before and 6, 12, and 24 h after stimulation. TNFα protein was detectable in small, but reproducible amounts 12 h after stimulation, and increased further after 24 h. However, no secretion of TNFα was detected by ELISA. FACS analysis of the passaged HUVEC cultures did not reveal any contamination with non-endothelial cells. In conclusion, H_2O_2 induces TNFα mRNA in HUVECs and PBMC. In HUVECs an increase of intracellular TNFα protein was also detected, indicating that endothelial cells can produce small amounts of TNFα.

Keywords: Endothelial cells, hydrogen peroxide, tumour necrosis factor α, mononuclear blood cells, Mono Mac 6, reactive oxygen intermediates, smooth muscle cells

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry; FCS, foetal calf serum; H_2O_2, hydrogen peroxide; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; NFκB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; ROI, reactive oxygen intermediates; RT-PCR, reverse transcription polymerase chain reaction; SMC, smooth muscle cells; TNFα, tumour necrosis factor alpha; UEA 1, Ulex Europeus Agglutinin 1

INTRODUCTION

The endothelium is sensitive to injury caused by reactive oxygen intermediates (ROI), including hydrogen peroxide (H_2O_2).[1–3] Many pathophysiological states are associated with increased endothelial exposure to ROI, among others ischaemia-reperfusion, diabetes, hypertension, atherosclerosis, and sepsis.[4] ROI can be derived

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from sources as different as activated inflammatory cells, oxidized lipoproteins, cigarette smoke and cells in the vessel wall.[4,5]

Vascular cells are both a target for and a source of cytokines.[6] Tumour necrosis factor α (TNFα) in cooperation with interleukin 1β triggers a variety of inflammatory actions; facilitates thrombus formation, induces production of eicosanoids, platelet activating factor, inducible nitric oxide synthase, leukocyte adhesion molecules and chemotactic cytokines.[6–9] The major source of TNFα is macrophages, but other cells such as smooth muscle may also generate TNFα.[11] It is presently not clearly determined if endothelial cells can produce TNFα. Stimuli inducing TNFα include lipopolysaccharide and other bacterial products, cytokines, immuncomplexes, superantigens, as well as ischaemia-reperfusion.[6–8] TNFα production is transcriptionally regulated by the redox sensitive nuclear factor kappa B (NFκB).[10] It has recently been suggested that ROI mediate NFκB activation by a wide range of stimuli.[12] However, it has not been shown if ROI can induce TNFα production.

The present study investigates whether exogenous H_2O_2 induces TNFα in cultures of human umbilical vein endothelial cells (HUVEC), human aortic smooth muscle cells (SMC), human peripheral blood mononuclear cells (PBMC), or the human cell line Mono Mac 6.

MATERIALS AND METHODS

Cell Culture

All culture media were purchased at Life Technologies, Paisley, UK.

1. Endothelial Cells

HUVECs were isolated as described by Jaffe *et al.*[13] Each culture was derived from pooled cells from 3 to 5 cords, and the cells were seeded out on 9 cm culture dishes in M199 medium with 20% foetal calf serum (FCS) (Life Technologies), 100 U/mL penicillin, 10 µg/mL streptomycin, 0.25 µg/mL amphotericin (Sigma, St. Louis, Mo, USA) and 25 µg/mL endothelial cell growth supplement (Sigma). When confluent, the cells were subcultured in gelatin coated (0.5%) T-75 flasks for propagation, 6-well plates for RNA isolation, and T-25 flasks for FACS measurements in M199 containing 10% FCS, 10% pooled human serum, 50 µg/mL gentamicin (Life Technologies), 10 U/mL heparin (Pharmacia, Stockholm, Sweden), 2.5 mg/mL glutathione/ascorbic acid (Boehringer Mannheim GmbH, Mannheim, Germany), 25 µg/mL endothelial cell growth supplement, 1 mmol/L HEPES and 1× non-essential amino acids (both Life Technologies). Confluent cells at fourth to sixth passage were used for the experiments.

Twenty-four hours prior to the experiments, the culture medium serum content was reduced to 0.5% pooled human serum. On start of the experiments the medium was removed, the cells were washed, and serum-free M199 containing 200 µmol/L H_2O_2 was added for 15 min unless otherwise stated. Thereafter M199 was replaced by culture medium for 4 h (triplicate samples from 2 cultures run at least two times each), or cells were harvested after 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h in culture medium (triplicate samples from 1 culture run at least twice) for evaluation of relative mRNA. For FACS analysis of TNFα, cells were harvested after 6 h and compared to unstimulated controls (2 cultures each), after 12 h (2 cultures each), after 24 h (4 cultures each), or 6, 12, and 24 h were compared simultaneously to unstimulated controls (1 culture). In an additional culture 15 min stimulation with 200 µmol/L H_2O_2 was compared to 30 min stimulation, and cells harvested for FACS after 6 or 24 h. Two different cultures were employed for assessment of HUVEC purity (see below). Control stimulation with lipopolysaccharide (LPS from *E. Coli*, 10 µg/mL) (Sigma) was performed for 2, 4, and 6 h for cells destined for ELISA, and compared with medium and cell homogenates collected serially 0–24 h after H_2O_2 stimulation.

2. Smooth Muscle Cells

Human aortic SMCs were purchased from Clonetics Corporation (Walkersville, MD, USA) and grown in Waymouth medium with 15% FCS and 0.50 μg/L gentamicin. Cells were passed every 1–2 weeks and seeded at a density of 5000 cells per cm^2 for propagation in T-75 flasks, thereafter seeded in 6-well plates, and experiments were performed between passage 4 and 8. Twenty-four hours prior to the experiments the serum content in the culture medium was reduced to 0.5%. Hydrogen peroxide (200 μmol/L) was given for 15 min in serum-free Waymouth medium, thereafter replaced with culture medium until cell harvesting after 4 h for evaluation of relative mRNA (triplicate samples from 2 different cultures run at least twice). Additional 6-well plates were stimulated with H_2O_2 or LPS, and medium and cell homogenates collected serially 0–24 h afterwards for ELISA analysis of TNFα.

3. PBMC

PBMCs were freshly obtained from healthy donors and isolated by Ficoll–Paque gradient centrifugation (Pharmacia, Stockholm, Sweden). PBMC for FACS were used immediately. PBMC for reverse transcription polymerase chain reaction (RT-PCR) were seeded in 6-well plates (5–10 million cells per well), and grown in RPMI with 10% FCS, 2 mmol/L L-glutamine, penicillin 100 U/mL, and streptomycin 100 μg/mL (all from Sigma). After 2 days in culture, experiments were performed with H_2O_2-stimulation for 15 min (200 μmol/L in serum-free RPMI), thereafter culture medium for 4 h until cell harvesting in triplicate samples from two healthy donors.

4. Mono Mac 6

Mono Mac 6 cells[14] (a kind gift from Dr. Shu Ye, University of Oxford, UK) were cultured in RPMI supplemented with 10% FCS, penicillin 100 U/mL, and streptomycin 100 μg/mL. Approximately one million cells were aliquoted into 25 mL tubes. M199 with 200 μmol/L H_2O_2 was added for 15 min. After that the cells were resuspended in RPMI with 10% FCS until cell harvesting after 4 h for RT-PCR (triplicate samples from 1 culture run at least twice). For analysis of TNFα secretion with ELISA, 500,000 cells per well were seeded in 6-well plates, and the medium collected serially after stimulation with H_2O_2 for 15 min.

Semiquantitative RT-PCR

mRNA Extraction and cDNA Synthesis

At the end of the experiments, cells from 6-well plates were washed twice with PBS and scraped off with a cell scraper, while Mono Mac 6 were washed and centrifuged twice. All cells were stored at −80°C in test tubes containing 500 μL PBS until analysis. mRNA was extracted using a Dynabeads mRNA direct kit (Dynal A.S., Oslo, Norway), with the procedure according to the manufacturer. RNA was extracted twice into a final volume of 40 μL. Single stranded cDNA synthesis was performed by Superscript II (Life Technologies, Paisley, UK) according to the manufacturer, using random hexamers (Life Technologies) as primers in the prescence of RNasin (Promega, Madison, USA).

PCR Reaction

Each reaction was run in a volume of 25 μL. A mastermix consisting of all reaction components apart from cDNA and primer was first prepared. After dividing the mastermix and adding cDNA, the samples were aliquoted in separate PCR tubes. Primers were added to a final concentration of 0.2 μmol/L to the reaction mixture consisting of cDNA (1 μL per reaction), dNTP (6.25 mmol/L), $MgCl_2$ (1.5 mmol/L), 2.5 μL 10× PCR buffer, 0.02 U Taq polymerase (all Life Technologies) and 5 μCi ^{33}P-dATP (NEN, DuMedical Scandinavia). Histone (H3), which is expressed at the same level independent of cell cycle, was selected as a reference gene.[15] The primer for H3, 5′ CCA CTG AAC TTC TGA TTC GC (base position 282–301)

and 3′ (5′–3′) GCG TGC TAG CTG GAT GTC TT (base position 476–495), resulted in an amplification product of 215 base pairs. The TNFα primer, 5′ GAG TGA CAA GCC TGT AGC CCA TGT TGT AGCA (base position 337–368) and 3′ (5′–3′) GCA ATG ATC CCA AAG TAG ACC TGC CCA GACT (base position 749–780), resulted in a product of 444 base pairs (Clonetech, Palo Alto, CA, USA). The PCR reaction was performed as follows: (i) 94°C for 2 s: 30 min, 60°C for 30 s, 72°C for 45 s followed by cycles of (ii) 30 s at 94°C, 30 s at 60°C, 45 s at 72°C. The linear phase of the PCR reaction was determined, and thereafter 23 cycles were selected for H3, while TNFα was run for 36 cycles. Control PCR of mastermix with the H3 primer were routinely done in all samples, while control reactions on RNA were performed randomly in order to evaluate possible contaminations. All PCR reactions were run at least twice.

A radiolabelled DNA ladder was synthetized using the Gibco 100 base pair DNA ladder and T4 DNA polymerase kit according to the manufacturers method description (Life Technologies), with ^{33}P-dATP as the incorporated marker. The PCR-products were separated by electrophoresis on a 5% polyacrylamide gel, and evaluated in a phosphoimager (BioImaging Analyzer System BAS 1000, Fuji). The ratio between optical density of test gene band and reference gene was calculated in order to evaluate changes in the relative amount to test gene. RNA in the samples was not measured (too little material), so differences in density of the H3 band reflect that the samples contain different amounts of RNA.

TNFα Protein Measurement by ELISA

Samples of culture medium before and serially after stimulation with H_2O_2 were taken from HUVECs, SMC, and Mono Mac 6 for analysis of TNFα protein by the high sensitivity TNFα ELISA kit with a detection level of 0.1 pg/mL (Amersham Pharmacia, Stockholm, Sweden). Concomitant samples were taken of cell homogenates of HUVEC or SMC cultures in the same 6-well plates at different time points up to 24 h after stimulation; the cells were lysed with PBS containing 1% Triton-x for 10 min, thereafter collected with a cell scraper. As an alternative lysing method, three freeze–thaw cycles in 0.25 mol/L Tris-HCl, pH 8.0, were performed.

FACS Analysis

A. Cell-bo und TNFα Protein

After stimulation with H_2O_2, HUVECs were incubated with 3 mL culture medium for the times described under endothelial cell culturing, with addition of 2 μL Golgi-stop (PharMingen Cytofix/Cytoperm Plus Kit, San Diego, CA, USA) for the last 6 h. The cells were trypsinized, washed, resuspended in 50 μL PBS, and fixed with 250 μL Cytofix at 4°C. After 20 min, cells were washed with Cytoperm and resuspended in 250 μL Cytoperm with or without mouse anti-TNFα monoclonal antibody (mAb) (PharMingen) for 30 min at 4°C. After washing twice with Cytoperm, the cells were incubated with FITC-conjugated $F(ab')_2$ fragment rabbit anti-mouse IgG (DAKO, Glostrup, Denmark). Finally, cells were washed with PBS (0.5% FCS), and analysed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software. An acquisition gate was set to exclude cellular debris or aggregates, and 10,000 cells per sample were analysed. Mouse anti-(CD30 (DAKO), HLA-DR (DAKO), and Bcl-2 (Boehringer Mannheim)) mAb were used as negative controls before incubation with FITC-conjugated secondary Ab. Control analysis of unstimulated cells with Golgi-stop for 6 h was also performed.

B. Assess ment of Endot helia l Cell Culture Purity

Trypsinized endothelial cells (500,000 cells per tube) were resuspended in PBS containing 1% bovine serum albumin (Sigma), and incubated with mouse monoclonal anti-CD45/CD14 (Beckton Dickinson, Mountain View, CA), or

mouse monoclonal anti-CD3/CD19 (Beckton Dickinson) alone or with the FITC-conjugated lectin Ulex Europeus Agglutinin 1 (UEA 1, Vector Laboratories, Burlingame, CA) at 4°C for 30 min. (Dilutions: UEA 1 1 : 40, the others 1 : 20). Double labelling with phycoerythrin-conjugated anti-CD14 and FITC-conjugated anti-CD45 was performed to evaluate possible leukocyte and monocyte contamination, while double labelling with phycoerythrin-conjugated anti-CD19 and FITC-conjugated anti-CD3 were employed for evaluating B- and T-lymphocytes. The stained cells were washed twice in PBS, and fixed in PBS with 1% paraformaldehyde. For location of the different cell populations of interest, freshly prepared PBMC alone or in mixture with endothelial cells were used as positive controls before final endothelial cell analysis. The gating technique described by de Benschop *et al.* was employed in the forward- and sidescatter for visualizing all cell populations of interest.[16] All events were accepted for fluorescent analysis.

Statistics

For RT-PCR data, the calculated ratio between test gene and control gene was evaluated with a Wilcoxon Signed Rank test; $p < 0.05$ was considered significant. Values are shown as mean ± SEM.

RESULTS

Expression of TNFα after H_2O_2 stimulation on the mRNA level was assessed by RT-PCR, and on the protein level by ELISA and FACS analysis.

RT-PCR

mRNA was reverse-transcribed to cDNA and amplified by PCR using primers for TNFα and the histone H3 as a control gene. A representative polyacrylamide gel with PCR-products from all investigated cell types before and 4 h after stimulation with H_2O_2 is shown in Figure 1. The time course of mRNA induction was followed in one HUVEC experiment, where mRNA peaked 2 h after H_2O_2 stimulation, thereafter gradually decreased reaching a level less than initial value 24 h afterwards (results not shown). When comparing several experiments, TNFα mRNA in HUVECs increased with a factor of 4.1 4 h after stimulation with H_2O_2 ($p < 0.001$). In SMCs TNFα mRNA increased with a factor of 2 ($p < 0.09$), in PBMC with factor 2 ($p < 0.05$), and in Mono Mac 6 with 1.6 ($p < 0.15$) (Figure 2).

TNFα Protein Measurement by ELISA

No TNFα was detectable in samples of culture medium from HUVEC or SMC. When lysing cells by freezing and thawing, no TNFα protein was detectable at any time point from 0 up to 24 h after stimulation in neither HUVECs nor SMC. Stimulation of HUVECs or SMC with LPS did not increase the protein to a detectable level. To evaluate cell type capacity for TNFα secretion with the present stimulus, medium was collected from H_2O_2-stimulated Mono Mac 6. Also in this cell type the H_2O_2-induced TNFα secretion was below the assay detection limit, with the exception of 12 h after stimulation where the levels came up to about detection limit of 0.1 pg/ml. Twenty-four hours after stimulation the levels were beyond detection again.

TNFα Protein by FACS

Cell-associated TNFα was analysed by FACS after treatment with a monensin containing protein transport inhibitor (Golgi stop) and permeabilization. Cells stained with negative control primary Ab before incubation with FITC-conjugated secondary Ab did not exhibit increased fluorescence compared to labelling with FITC-conjugated secondary Ab alone. The latter showed a minor increase in fluorescent activity when compared to autofluorescence of unlabelled cells (results not shown). We found a slight, but reproducible

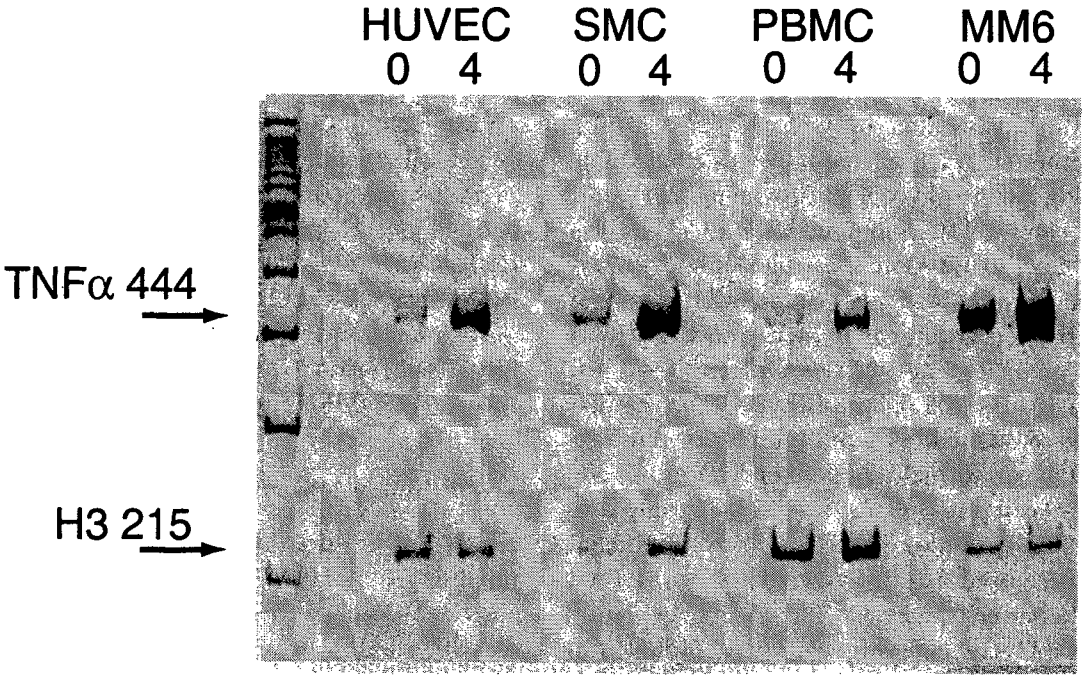

FIGURE 1 Representative polyacrylamide gel with RT-PCR products of unstimulated cell cultures (0) and 4 h (4) after stimulation with 200 μmol/L H_2O_2. The effect on HUVEC, SMC, PBMC, and Mono Mac are shown. The PCR-products of control gene histone H3 are 215 base pairs, while TNFα is 444 base pairs. The amount of RNA is not the same in all samples. The ratio between TNFα and H3 was calculated and employed for statistical evaluation (Figure 2).

staining for TNFα protein 12 h after stimulation with H_2O_2, which increased further after 24 h (Figure 3). Stimulation for 30 min increased TNFα protein compared to the 15 min stimulation (not shown).

Assessment of HUVEC Purity

Fifty thousand events within the endothelial cell gate were counted. All cells expressed UEA 1, indicating a homogenous endothelial cell population (Table I). This was supported by the lack of lymphocytes detectable by CD3/CD19 staining. Very few CD14/CD45 positive cells could be seen but they were all expressing UEA 1, implying that they were likely to be of endothelial origin (Table I). The frequency of such double-positive cells was below that considered to be the lower limit for detecting a significant cell population with the FACS instrument (1%).

DISCUSSION

The main findings of the present study were that H_2O_2 induced expression of TNFα mRNA in HUVEC and PBMC. In SMC and Mono Mac 6 the mRNA tended to increase, but without reaching significance. TNFα protein could be visualized in HUVECs by FACS at low but reproducible levels 24 h after H_2O_2 stimulation. However, TNFα was not detected in HUVEC or SMC by an ELISA kit with the lowest detection level 0.1 pg/mL, implying that TNFα protein synthesis was low and secretion negligible under these conditions. In comparison secretion of TNFα by Mono Mac 6 barely reached the assay detection level 12 h after stimulation, indicating that hydrogen peroxide is not a strong stimulus for TNFα production.

An abundance of literature suggests TNFα might be induced by H_2O_2. Firstly, ischaemia-reperfusion of the human myocardium increases

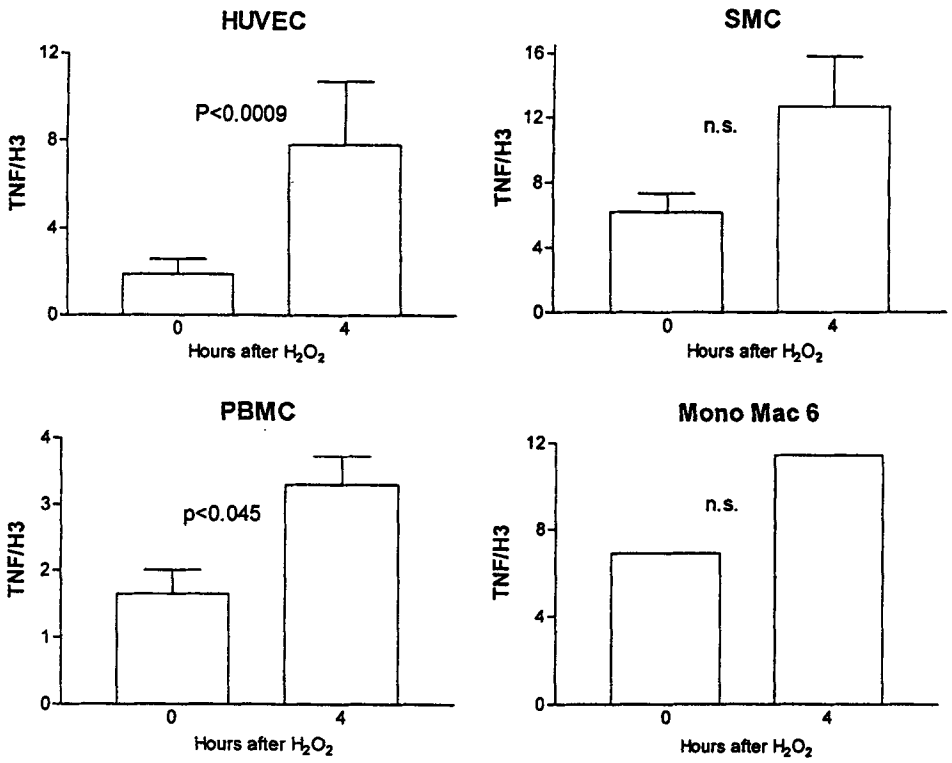

FIGURE 2 The calculated ratio between PCR products of TNFα and H3 before and 4 h after stimulation with 200 μmol/L H_2O_2 in cultured HUVEC, SMC, PBMC, and Mono Mac 6. Mean ± SEM values are shown.

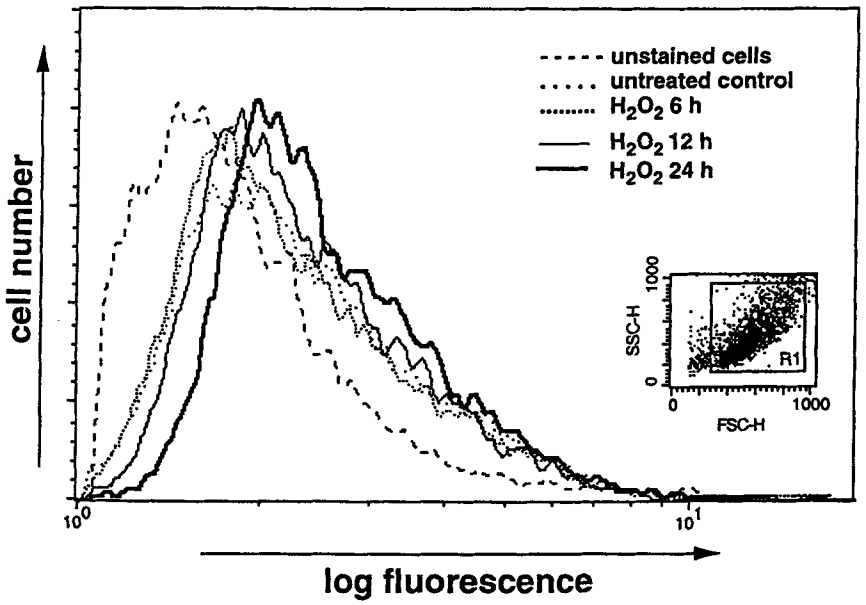

FIGURE 3 FACS analysis of TNFα protein in HUVECs stimulated with H_2O_2 200 μmol/L for 15 min and harvested after 6, 12, and 24 h as indicated in the illustration. The signal for untreated controls and 6 h incubation partially overlap. Inset shows a typical forward- and sidescatter dot blot with the region of analysed cells indicated (R1).

TABLE I Flow cytometric analysis of human umbilical vein endothelial cell culture purity. The cells were stained with UEA 1 to detect endothelial cells. Double staining with anti-CD3 and anti-CD19 was employed for detection of B-cells and T-cells, while double staining with anti-CD14 and anti-CD45 was employed for detection of possible contaminating leukocytes and monocytes. Mean values of two experiments are shown as total number of cells or percentage of total number

Gate	Total	Percent
Endothelial cell	51789	100
T-cell	3	0
B-cell	30	0.06
Leukocyte	103	0.20
Monocyte	29	0.06
Monocyte*	2	0

*UEA 1 was added to anti-CD14/CD45 Ab.

myocyte TNFα accumulation.[9] ROI are believed to be important components of reperfusion injury,[4,5] and are produced in the human heart as measured in coronary sinus blood by electron spin resonance upon reperfusion.[17] Secondly, transcriptional regulation of TNFα involves the redox sensitive transcription factor NFκB,[10] which may translocate to the nucleus after stimulation with ROI.[12] Cytosolic NFκB is bound to its inhibitors IκB, but after activation by ischaemia-reperfusion or other stimuli, phosphorylation of IκB results in disruption of the complex, nuclear translocation of NFκB, and binding to TNFα promoter sites.[9,18] H_2O_2 in similar concentrations to that employed here has previously induced nuclear translocation of NFκB in cultured endothelial cells as assessed by electromobility shift assay,[19,20] although this finding is controversial.[21] Thirdly, hydrogen peroxide activates mitogen-activated protein kinase and protein kinase C,[22,23] which in turn may influence TNFα production.[9] However, although we found TNFα mRNA to increase after H_2O_2 stimulation in several cell types, this was not accompanied by protein secretion, with the exception of in a Mono Mac 6 cell line where the levels came above the detection limit 12 h after stimulation. Thus, H_2O_2 appears to be a weak stimulus for TNFα.

The concentration of H_2O_2 employed was selected to be in the physiological range. Activated polymorphonuclear leukocytes *in vitro* can generate H_2O_2 concentrations of up to 200 μmol/L.[24,25] *In vivo* the radical production would be expected to be lower due to endogenous antioxidant defence. However, *in vivo* the free radical production would also occur simultaneously from extra- and intracellular sources, and thus reach a higher total level.[4,5] The time span of stimulation was selected in order to achieve a biological response, without killing the cells. We have previously observed that treatment with 200 μmol/L H_2O_2 induces a transient contraction of HUVECs, which spontaneously reverses 1 h after stimulation with no increase of cell detachment and no release of lactate dehydrogenase into the supernatant 1–120 h afterwards.[26]

TNFα production in endothelial cells has been addressed in several previous studies. One work reports TNFα protein in the supernatant of LPS-stimulated porcine pulmonary artery endothelial cells by the WEHI bioassay.[27] The detection level is the same as the ELISA employed in the present study, and levels as high as 400 pg/mL were measured.[27] Another paper reports that stimulating HUVECs with interferon gamma or platelet activating factor alone did not increase TNFα protein in the supernatant, but addition of antibodies against ICAM-1 or E-selectin resulted in TNFα accumulation in the supernatant at levels up to 500 pg/mL as measured by ELISA.[28] However, TNFα production by endothelial cells was not confirmed by other approaches, nor were the cultures' purity assessed.[27,28] We could not detect TNFα protein in supernatants or cell homogenates after stimulation with H_2O_2 or LPS with our ELISA kit, indicating that the protein release from HUVEC was minimal. Therefore, FACS was employed to detect intracellular TNFα protein after staining with a mAb against TNFα. A monensin-containing inhibitor of protein export, Golgi-stop, was employed to increase protein accumulation. A small, but consistant increase of TNFα was detected in permeabilized HUVEC

using this technique. Together with the data showing increased mRNA, these findings indicate that endothelial cells can express small amounts of TNFα upon stimulation with H_2O_2. This is in line with the findings of Amore *et al.*[29] who stimulated a murine endothelioma cell line with glycated albumin, and detected TNFα protein as well as mRNA. The levels in that paper are given as relative changes rather than concentrations, and comparisons with detection levels in the present study are therefore not possible.[29]

As TNFα production by endothelial cells is not a completely resolved issue, and the increase at the mRNA level could theoretically be due to contaminating cells, a FACS analysis of HUVEC purity was performed. It confirmed that our passaged HUVEC cultures were >99.8% pure. The minute numbers of suspected B-cells and monocytes could not account for the homogenous TNFα protein expression detected in the FACS. Furthermore, at the mRNA level the largest increase (factor of 4) was observed in HUVECs, while PBMC and SMC increased with a factor of 2, and Mono Mac 6 only with a factor of 1.6.

In conclusion, the present work demonstrates that H_2O_2 at a physiologically relevant concentration can increase mRNA for TNFα in human cultured cells. In HUVECs the increased mRNA is accompanied by an intracellular increase of TNFα protein, but no protein secretion. Thus, H_2O_2 appears to be a weak stimulus of TNFα production.

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