Ambient but not incremental oxidant generation effects intercellular adhesion molecule 1 induction by tumour necrosis factor α in endothelium

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Proinflammatory cytokines upregulate endothelial adhesion molecule expression, thereby initiating the microvascular inflammatory response. We re-evaluated the reported role of reactive oxygen metabolites (ROMs) in signalling upregulation of intercellular adhesion molecule 1 (ICAM-1) on endothelial cells by tumour necrosis factor α (TNF-α) *in vitro*. TNF-α upregulation of endothelial-cell ICAM-1 expression was inhibited by the cell-permeable antioxidants, or by the adenovirus-mediated intracellular overexpression of Cu,Zn-superoxide dismutase, but not by the exogenous (extracellular) administration of the cell-impermeable antioxidants, superoxide dismutase and/or catalase. This ICAM-1 upregulation was also inhibited by inhibitors of NADH dehydrogenase, cytochrome bc_1 complex and NADPH oxidase. However, a measurable increase in net cellular ROM generation in response to TNF-α was not seen using four disparate sensitive ROM assays. Moreover, the stimulation of exogenous or endogenous ROM generation did not upregulate ICAM-1, nor enhance ICAM-1 upregulation by TNF-α. These findings suggest that an ambient background flux of ROMs, generated intracellularly, but not their net incremental generation, is necessary for TNF-α to induce ICAM-1 expression in endothelium *in vitro*.

INTRODUCTION

Proinflammatory cytokines stimulate a variety of responses in endothelial cells (ECs), including the surface expression of MHC antigens and of vascular adhesion molecules, as well as the secretion of inflammatory mediators [1]. Because of their unique location at the interface between the bloodstream and parenchymal tissue, adhesion molecule upregulation by ECs can act as the initial trigger for the arrest, adhesion and activation of circulating leucocytes at the onset of the efferent inflammatory response [2], a stereotypical response first described as reperfusion injury [3,4]. For example, it is known that tumour necrosis factor α (TNF-α) induces intercellular adhesion molecule 1 (ICAM-1) expression on the surface of ECs, resulting in leucocyte adhesion, accumulation and consequent diapedesis [2]. Recently, reactive oxygen metabolites (ROMs) have been found to be important second messengers for signal transduction in general, and for cytokine induction of ICAM-1 expression in ECs in particular [5,6]. Antioxidants have been reported to inhibit cytokine-induced ICAM-1 expression, as well as the activation of nuclear transcription factor κB (NF-κB), a transcription factor for ICAM-1, *in vitro* [7–9], but the precise role of ROMs in mediating this response is not fully understood. We re-evaluated the putative role of ROMs in this signal-transduction pathway by using an *in vitro* model of TNF-induced ICAM-1 expression in endothelium. We attempted to clarify (1) whether TNF-induced EC ICAM-1 expression is dependent on oxidant generation, (2) which EC enzymes are sources of these ROMs, (3) whether ICAM-1 induction/inhibition is associated with the stimulation of EC net ROM generation, and (4) whether the exogenous generation of extracellular and/or intracellular ROMs could mimic the response to TNF-α. Our findings suggest modification of the conventional view that ROMs act as second messengers for this signalling response.

MATERIALS AND METHODS

Endothelial cells

Hybridoma Eahy926, which is derived from human umbilical vein ECs and human epithelial cell line A549 [10] and has been found to have characteristics similar to native human umbilical vein ECs with regard to expression of ICAM-1, vascular cell adhesion molecule 1 and E-selectin [11], was a gift from Dr. Roger Harrison (University of Bath, Bath, U.K.). These cells were cultured in plastic flasks in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, U.S.A.) containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Gibco), passaged at confluence using 0.05% trypsin and 0.53 mM EDTA (Gibco), and then grown to confluent monolayers in 96-well plates, in which all experiments and assays were performed.

Measurement of ICAM-1 expression

Expression of ICAM-1 on the surface of the EC monolayer was determined by a modified ELISA in the 96-well plates. After

Abbreviations used: TNF-α, tumour necrosis factor α; ROM, reactive oxygen metabolite; ICAM-1, intercellular adhesion molecule 1; EC, endothelial cell; SOD, superoxide dismutase; NF-κB, nuclear transcription factor-κB; K-R, Krebs–Ringer; NBT, Nitro Blue Tetrazolium; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; XO, xanthine oxidase; NAC, *N*-acetylcysteine; PDTC, pyrrolidine dithiocarbamate; DMTU, 1,3-dimethyl-2-thiourea; BOF 4272, sodium-8-(3-methoxy-4-phenylsulphinylphenyl)pyrazolo[1,5-α]-1,3,5-triazine-4-olate monohydrate; L-NMMA, *N*-monomethyl-L-arginine; ANOVA, analysis of variance; AP-1, activator protein 1.

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treatment of the EC monolayer with agonists and/or antagonists, the monolayers were fixed with 2% paraformaldehyde (J. T. Baker, Phillipsburg, NJ, U.S.A.) in sodium phosphate buffer, pH 7.4, including 0.1 M L-lysine monohydrochloride (Sigma, St. Louis, MO, U.S.A.) and 0.01 M sodium *m*-periodate (Sigma) for 20 min at 4 °C, and then blocked with 1% BSA in PBS containing 0.1 M glycine (Baker) overnight at 4 °C. The fixed monolayers were then probed with monoclonal mouse anti-human ICAM-1 antibody (PharMingen, San Diego, CA, U.S.A.) for 1 h at 37 °C, followed by incubation with a peroxidase-conjugated monoclonal anti-mouse IgG $F(ab')_2$ antibody (Sigma) for 1 h at 37 °C. After a wash, developing substrate (0.2% H_2O_2/0.4 mg/ml *o*-phenylenediamine; Sigma) was added for 4 min, and the reaction then stopped with 1 M H_2SO_4. The plates were read on a spectrophotometric plate reader at 520 nm. In preliminary studies, there was a linear correlation between the amount of secondary antibody and absorbance (*A*) from 0 to 0.3, indicating a proportional relationship between *A* and ICAM-1 expression over this range (results not shown).

Measurement of superoxide generation

Cytochrome *c* reduction

Confluent cell monolayers in 96-well plates were washed with PBS and the medium was replaced with Krebs–Ringer (K-R) buffer (99.01 mM NaCl, 4.69 mM KCl, 1.87 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.03 mM K_2HPO_4, 25 mM $NaHCO_3$, 20 mM Hepes, 11.1 mM D-glucose, pH 7.4) containing cytochrome *c* (80 μM) (final concentrations) (Sigma). Increasing concentrations of TNF (30–1000 units/ml) or menadione (3–100 μM) were then added and the A_{550} was monitored on a spectrophotometric plate reader using the millimolar absorption coefficient of 28.0 for reduced cytochrome *c* [12]. Paired values obtained in the presence of 300 units/ml superoxide dismutase (SOD) were then subtracted from the corresponding experimental values to control for any non-oxidant-specific reduction of cytochrome *c*.

Nitro Blue Tetrazolium (NBT) reduction

K-R buffer containing 0.5 mg/ml NBT (Sigma) and various concentrations of TNF or menadione were added to the EC monolayers in 96-well plates. After 60 min of incubation at 37 °C, the resulting NBT formazan precipitate was solubilized by 100% DMSO and A_{560} was read on a spectrophotometric plate reader. The paired values obtained in the presence of 300 units/ml SOD were then subtracted from the corresponding experimental values to control for any non-oxidant-specific reduction of NBT.

Lucigenin-enhanced chemiluminescence

EC monolayers were suspended by gently scraping with a rubber 'policeman', washed and resuspended in K-R buffer. Scintillation vials containing 2.5 ml of K-R buffer with 40 μM lucigenin (Sigma), 10^7 cells and TNF or menadione were placed into a scintillation counter (Berthold Biolumat LB9505), and the chemiluminescence was monitored at 37 °C for 60 min.

Measurement of net intracellular ROM generation

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, U.S.A.) was used to estimate the intracellular generation of ROMs [13,14]. The EC monolayers in 96-well plates were incubated with 200 μM DCFH-DA in K-R buffer for 20 min at 37 °C and then washed twice with 0.1% BSA/PBS. These stained cells were first preincubated with/without inhibitors for 30 min, then incubated with/without TNF-α, H_2O_2 or menadione for 60 min, and the plates then read on a fluorescence plate reader (CytoFluor 2300) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Some of the wells were fixed with formaldehyde immediately after DCFH-DA loading (but before treatment with inhibitors), and the fluorescence intensity of these fixed wells was subtracted from those of the other values as background. In inhibition experiments, data were presented as percentages of the corresponding control values (the DCF fluorescence of wells without TNF-α/inhibitor treatment).

Experimental protocols

Agonists

TNF-α (10–1000 units/ml) (R&D Systems Inc., Minneapolis, MN, U.S.A.) was used exclusively here as a prototypical pro-inflammatory cytokine. Both dose– and time–response curves were obtained (see Figure 1). In some experiments to evaluate the possible effect of increased ROM flux on ICAM-1 upregulation, EC monolayers were incubated with various concentrations of exogenous H_2O_2 (12.5–200 μM) (Baker), xanthine (10–300 μM) (Sigma) plus xanthine oxidase (XO) (5 m-units/ml) (Calbiochem) (to generate exogenous superoxide), 3-amino-1,2,4-triazole (to inactivate endogenous catalase) (1–30 mM) (Sigma) or menadione (1–30 μM) (Sigma) (to increase intracellular oxidant flux via redox cycling) [15] in the presence or absence of TNF-α for 4 h. Menadione and H_2O_2 were also used to assess the sensitivity of the four assays of ROM generation.

Antagonists

For most inhibition experiments (except those employing SOD overexpression induced by an adenovirus vector), the inhibitor was added exogenously to the EC monolayers 30 min before the addition of the TNF-α, and remained present throughout the incubation with TNF-α. In separate experiments to help determine indirectly the time when the generation of these ROMs takes place, the EC monolayers were preincubated with the antioxidant for 30 min, and then this medium was replaced, after washing with PBS, with another which contained TNF-α with/without the antioxidant, and the cells were incubated for various times, as indicated. The antioxidants administered exogenously were: *N*-acetylcysteine (NAC) (1–50 mM), pyrrolidine dithiocarbamate (PDTC) (3–100 μM), DMSO (0.03–3%, v/v) (all from Sigma) and 1,3-dimethyl-2-thiourea (DMTU) (1–50 mM) (Aldrich Chemical Company, Milwaukee, WI, U.S.A.). Bovine erythrocyte SOD and human erythrocyte catalase (30–300 units/ml) were obtained from Calbiochem (La Jolla, CA, U.S.A.) and Boehringer-Mannheim (Indianapolis, IN, U.S.A.) respectively. The inhibitors used to inhibit specific oxidant-generating enzymes were: allopurinol (12.5–100 μM) (Aldrich) and sodium-8-(3-methoxy-4-phenylsulphinylphenyl)pyrazolo[1,5-α]-1,3,5-triazine-4-olate monohydrate (BOF 4272) [16] (30–1000 nM) (from Dr. R. Harrison), to inhibit xanthine oxidase; rotenone (3–100 μM) (Sigma), to inhibit mitochondrial NADH dehydrogenase; antimycin A (3–100 μM) (Sigma), to inhibit mitochondrial cytochrome bc_1 complex; diphenyleneiodonium chloride (3–100 μM) (Sigma) and apocynin [17] (30–1000 μM) (Aldrich), to inhibit NADPH oxidase; indomethacin (3–100 μM) (Sigma), to inhibit cyclo-oxygenase; metyrapone (3–100 μM) (Sigma), to inhibit cytochrome *P*-450. To evaluate the possible role of nitric oxide synthase or mitochondrial energy-coupled phenomena, *N*-monomethyl-L-arginine (L-NMMA) (3–100 μM) or NaN_3 (3–100 μM) (a cytochrome oxidase

inhibitor) (all from Sigma) was used respectively. At the end of each experiment, the continued viability of the ECs was confirmed by continued firm adhesion to the culture plate, microscopic morphology and Trypan Blue exclusion.

Adenovirus-vector-mediated overexpression of SOD in ECs

Confluent EC monolayers were incubated with a replication-incompetent adenovirus (ad-SOD) containing the human Cu,Zn-SOD gene [18] or a control virus (ad-βgal) encoding only the *Escherichia coli lacZ* gene [19,20] (gifts from Dr. Ronald Crystal via Dr. Toren Finkel, National Institutes of Health, Bethesda, MD, U.S.A.) at multiplicities of infection between 5 and 50. After 24 h, the virus was washed off with PBS. The ECs were then incubated in RPMI medium for an additional 48 h until the experiments were performed. The expression of intracellular SOD by the ECs was measured by ELISA after permeabilization of the cells with 0.2 % Triton X-100 for 20 min. The permeabilized EC monolayers were then probed with monoclonal anti-(human SOD) antibody (Sigma), then with FITC-conjugated goat anti-mouse IgG (American Qualex, San Clement, CA, U.S.A.) and read on a fluorescence plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Statistical analysis

Values are expressed as means $\pm$ S.D. Apparent differences between normally distributed means were evaluated for significance by Student's t test. Apparent differences in dose–, time–response and similar curves were evaluated by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate statistical significance.

RESULTS

TNF-α induction of ICAM-1 expression on the EC surface

ICAM-1 was constitutively expressed at a low levels on Eahy926, and was strikingly upregulated by exogenously administered TNF-α in a dose- and time-dependent relationship (Figures 1A and 1B). A submaximal dose of TNF-α of 300 units/ml was chosen, and 4 h of incubation with TNF-α was chosen as a submaximal time for subsequent (inhibition) studies.

Effects of antioxidants on TNF-induced ICAM-1 expression

The effects of several antioxidants on this TNF-α-induced ICAM-1 expression were examined to evaluate the possible role of ROMs in this response (Figure 2). PDTC, a relatively non-specific antioxidant and iron chelator, the thiol compounds, DMTU and NAC (the latter serves also as a precursor for the synthesis of glutathione), and the hydroxy radical scavenger, DMSO, each inhibited the TNF-α induction of ICAM-1 expression in a dose-dependent manner when added to the medium (Figures 2A–2D). On the other hand, SOD, catalase or a combination of the two had no inhibitory effect whatsoever when added exogenously to the medium (Figure 2E). Therefore the exogenous administration of each of the lipid-soluble cell-permeable antioxidants blocked TNF-α-stimulated ICAM-1 induction, but the relatively cell-impermeable scavengers did not. Even at maximum concentrations of TNF-α and inhibitors (and in all experiments), EC viability exceeded 99 %, by Trypan Blue exclusion.

Effect of adenovirus-mediated overexpression of SOD in ECs on ICAM-1 induction

Because extracellular SOD and/or catalase added to the medium failed to block signal transduction in this model, we overexpressed intracellular SOD by using the replication-deficient recombinant adenoviral vector containing the SOD gene. This increased the expression of SOD protein more than 4-fold (Figure 3A). This overexpression of intracellular SOD inhibited ICAM-1 expression in a dose-dependent manner. Successful transfection with the control virus, confirmed by the expression of β-galactosidase activity (results not shown), failed to increase SOD expression, although the transfection itself appears to have inhibited ICAM-1 expression somewhat, presumably, a non-specific reflection of the viral infection. Such an effect is not unprecedented (Figures 3A and 3B) [18,19]. As in all experiments, EC viability exceeded 99 %.

Determination of the critical time for oxidation/reduction by ROMs/antioxidants after TNF-α stimulation

To determine indirectly the time period within which the necessary redox change of the target molecules induced by the

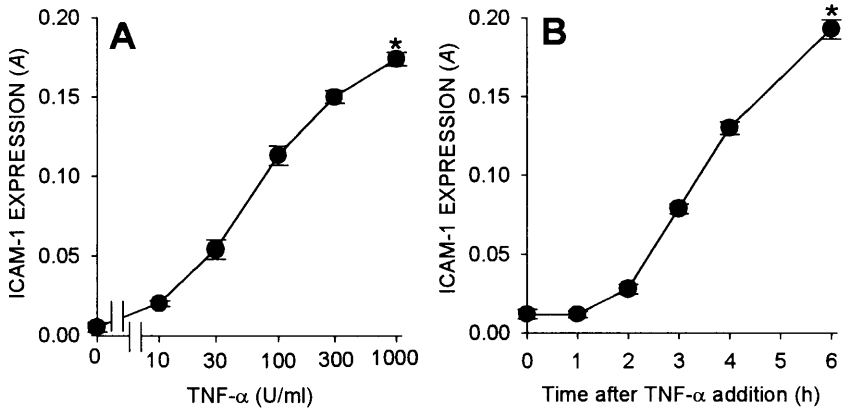

Figure 1 TNF-α induction of ICAM-1 expression on the surface of Eahy926 ECs

EC monolayers in 96-well plates were treated with various concentrations of TNF-α for 4 h (**A**) or with 300 units/ml TNF-α for various times (**B**), and then fixed with paraformaldehyde. ICAM-1 was upregulated on the monolayers by TNF-α in a dose- and time-dependent manner, as determined by modified ELISA. Values presented are means $\pm$ S.D. for 10 wells for each data point. $^*P < 0.001$ by ANOVA, compared with no change.

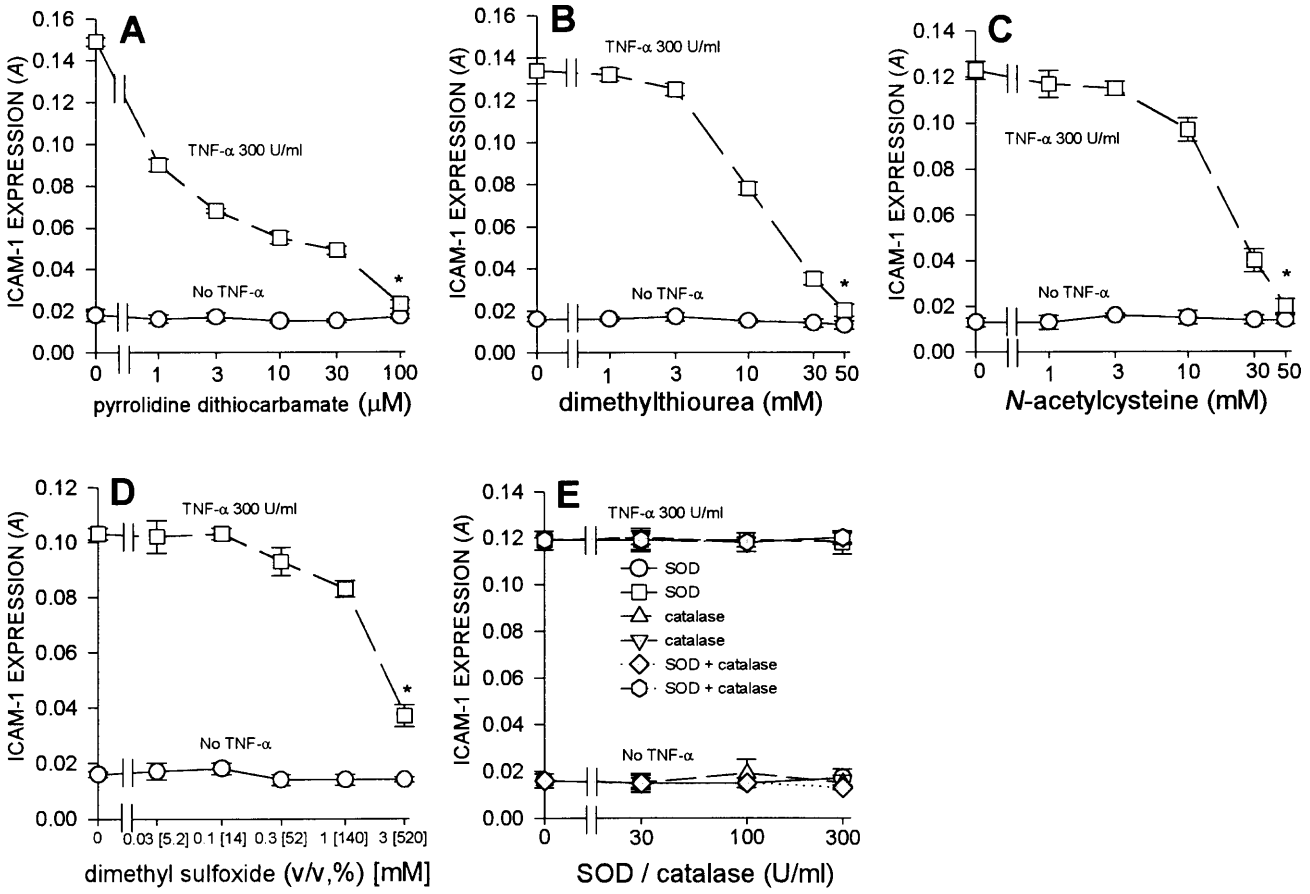

Figure 2 Effects of various exogenous antioxidants added to the medium on TNF-induced ICAM-1 expression

EC monolayers were preincubated with the specified antioxidant for 30 min and then stimulated with 300 units/ml TNF-α for 4 h. The cell-permeable antioxidants uniformly inhibited TNF-stimulated ICAM-1 expression. Exogenously administered SOD and/or catalase had no effect. Values are means $\pm$ S.D. for six wells for each data point. *$P < 0.001$ by ANOVA, compared with TNF-α alone.

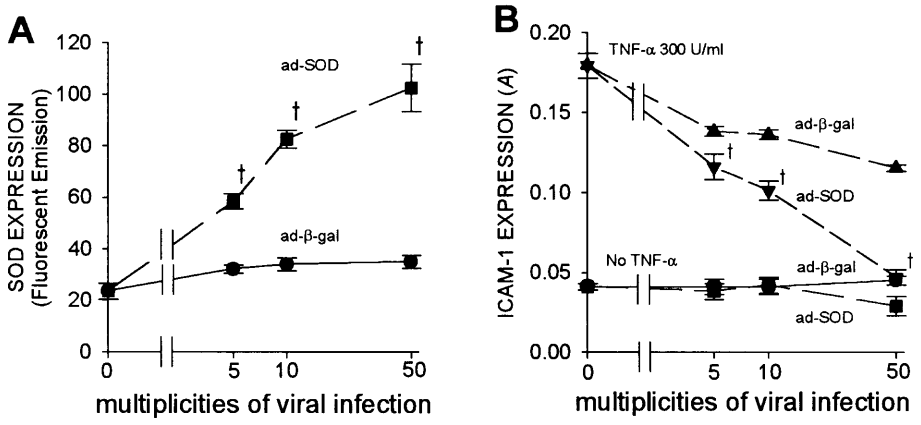

Figure 3 Effect of the adenovirus-mediated overexpression of (intracellular) SOD on ECs

Confluent EC monolayers were incubated with a replication-incompetent adenovirus (ad-SOD) containing the human Cu,Zn-SOD gene, or a control virus, ad-β-gal encoding only the *E. coli* reporter *lacZ* gene. (**A**) The expression of intracellular SOD by the EC was measured by ELISA after permeabilization of the formaldehyde-fixed cells with 0.2% Triton X-100 for 20 min. (**B**) ICAM-1 expression was determined after treatment of the infected EC monolayers with 300 units/ml TNF-α for 4 h. The infection of EC with ad-SOD increased SOD protein expression and inhibited ICAM-1 induction by TNF-α. †$P < 0.001$ by Student's *t* test, compared with the effect of the control virus.

intracellular ROMs takes place to permit TNF-α-stimulated ICAM-1 induction, we employed 50 μM PDTC, which we had found to be the most potent antioxidant inhibitor. EC monolayers were preincubated with PDTC for 30 min, and then stimulated with TNF-α for 4 h in the presence of PDTC for various periods. The inhibition of ICAM-1 expression by the PDTC when it was

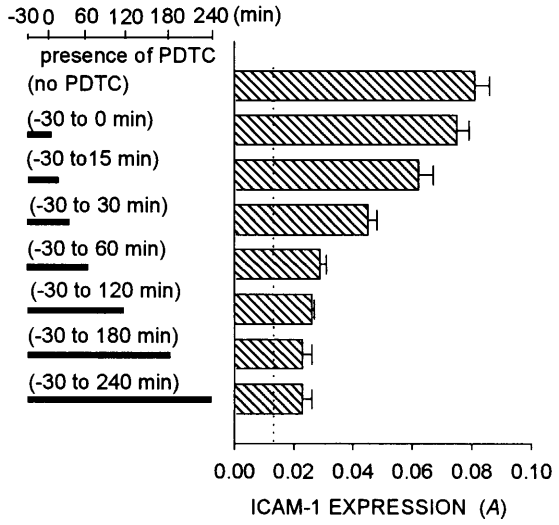

Figure 4 Determination of the critical period of oxidation/reduction by ROMs after TNF-α stimulation

EC monolayers in 96-well plates were preincubated with 50 μM PDTC for 30 min. After the plate had been washed, the ECs were incubated with 300 units/ml TNF-α for 4 h together with 50 μM PDTC for the indicated time. The presence of PDTC in only the early time period after TNF stimulation was sufficient to inhibit ICAM-1 induction. Data are means ± S.D. for six wells. The dotted line represents constitutive expression of ICAM-1.

present up to 60 min after TNF-α stimulation was almost equivalent to the effect of the full-time presence of PDTC, when the ECs had been stimulated with TNF-α continuously for 4 h (Figure 4), suggesting that the relevant oxidation/reduction of target molecules had taken place very early after the initial stimulation of TNF.

Effect of enzyme inhibitors on ICAM-1 induction by TNF-α

To help elucidate the enzymic source(s) of the ROM generation necessary for TNF-induced ICAM-1 expression, we examined the effect of specific inhibitors of various oxidant-generating candidate enzymes on TNF-induced ICAM-1 expression in ECs. Neither of the inhibitors of XO [allopurinol (12.5–100 μM), BOF 4272 (30–1000 nM)], or of cyclo-oxygenase [indomethacin (3–100 μM)] or cytochrome *P*-450 [metyrapone (3–100 μM)] significantly inhibited this ICAM-1 response to any degree whatsoever (results not shown). The inhibition of either mitochondrial NADH dehydrogenase (rotenone) or cytochrome bc_1 complex (antimycin A) did significantly inhibit TNF-induced ICAM-1 induction (Figures 5A and 5B) whereas NaN_3 (3–100 μM), a cytochrome oxidase inhibitor, had no effect (results not shown), suggesting that the effect of rotenone or antimycin A was not due to non-specific inhibition of energy generation. Diphenylene iodonium, which is known to be a potent 'neutrophil' NADPH oxidase inhibitor [12], but also inhibits the activity of a number of flavoenzymes, including nitric oxidase synthase and NADH dehydrogenase [21], markedly inhibited the ICAM-1 induction (Figure 5C), whereas the nitric oxide synthase inhibitor L-NMMA (3–100 μM) did not affect this response at all (results not shown). However, apocynin, another 'neutrophil' NADPH oxidase inhibitor in which activation was reported to require the presence of myeloperoxidase [17], did not significantly inhibit ICAM-1 induction at concentrations of 30–300 μM (results not shown). Taken together, the mitochondrial enzymes and NADPH oxidase emerged as possible sources of ROMs necessary for this EC response to TNF, although the effect of diphenylene iodonium could also be partially explained by its potential inhibition of NADH dehydrogenase.

Measurement of net ROM generation by ECs in response to TNF-α

Since our results suggested that ROMs generated intracellularly in the first hour after TNF stimulation might mediate the TNF upregulation of ICAM-1, we attempted to measure net cellular ROM generation in response TNF, and to correlate this with ICAM-1 induction. However, not one of these three separate quite sensitive assays for ROMs detected a significant increase in ROM generation in response to TNF. The sensitivity of each of these assays was confirmed by their response to the adminis-

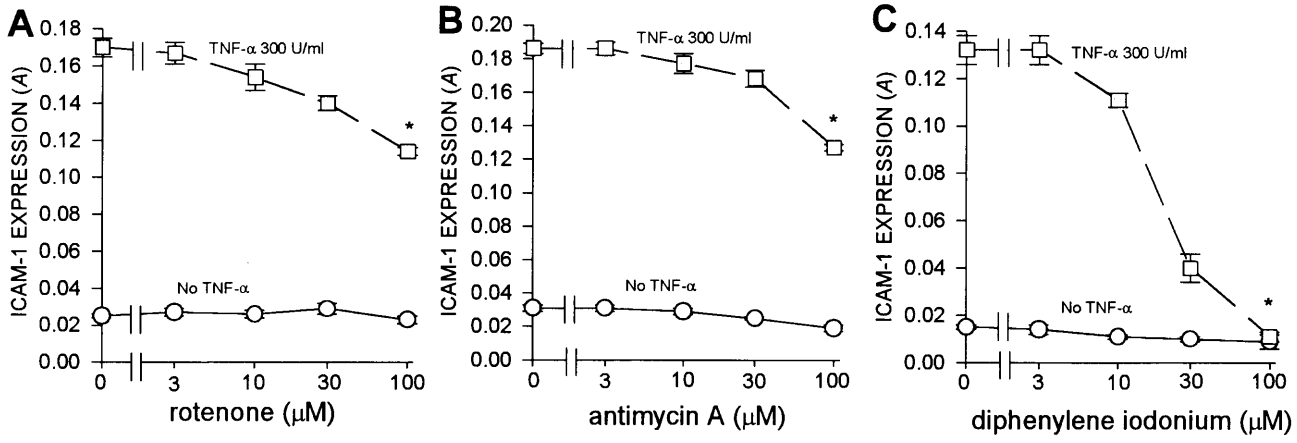

Figure 5 Effects of oxidant-generating enzyme inhibitors on TNF-induced ICAM-1 expression

EC monolayers were preincubated with the inhibitors for 30 min and then stimulated with 300 units/ml TNF-α for 4 h. The inhibitors of mitochondrial NADH oxidase (**A**) and cytochrome bc_1 complex (**B**) slightly, and that of NADPH oxidase (**C**) markedly, inhibited this ICAM-1 induction. Values are means ± S.D. for six wells for each data point. $^*P < 0.001$ by ANOVA, compared with TNF-α alone.

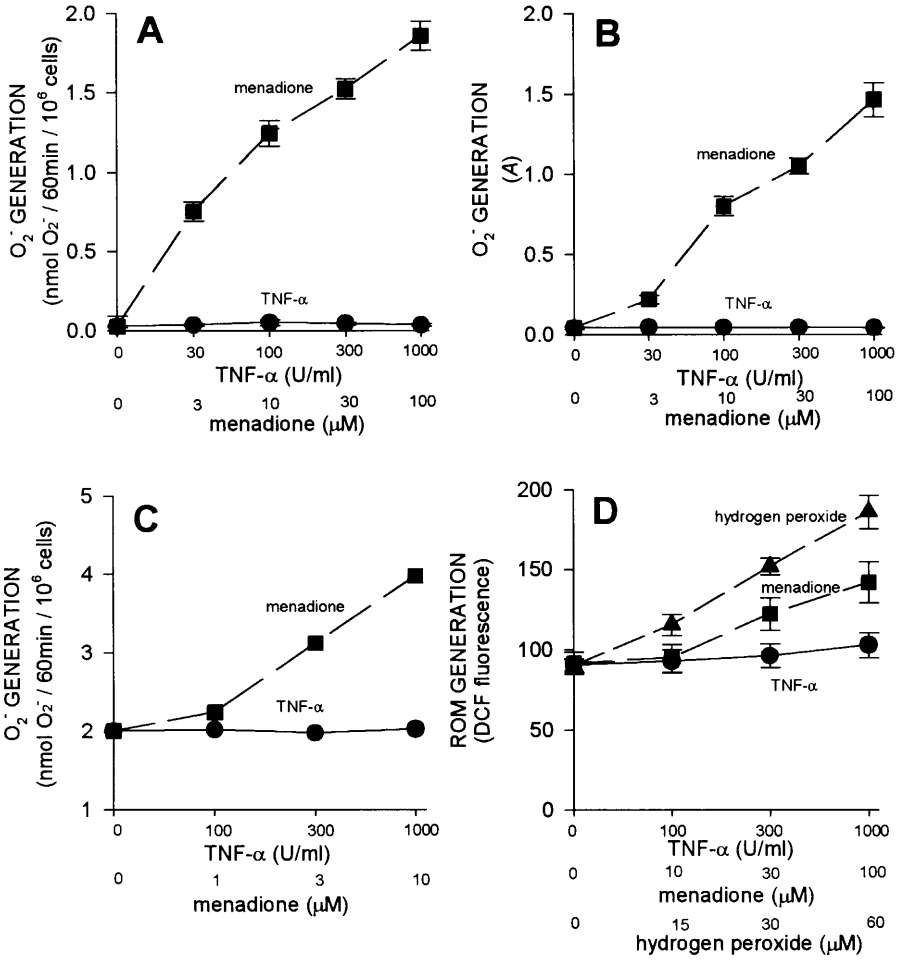

Figure 6 Measurements of net ROM generation by ECs in response to TNF-α

(**A**) Cytochrome *c* reduction: confluent EC monolayers in 96-well plates were incubated with TNF or menadione in the presence of cytochrome *c* for 60 min at 37 °C and the plate was read at 550 nm. The values obtained in the presence of 300 units/ml SOD were subtracted from corresponding values. Menadione, but not TNF, significantly increased measured net cellular superoxide (O_2^-) generation ($n = 6$ for each group). (**B**) NBT reduction: NBT and various concentrations of TNF or menadione were added to cell monolayers in 96-well plates. After 60 min of incubation at 37 °C, the resulting NBT formazan precipitate was solubilized by DMSO and the plate read on a spectrophotometric plate reader at 560 nm. The values obtained in the presence of 300 units/ml SOD were subtracted from corresponding values. The menadione, but not TNF, significantly increased measured net cellular superoxide generation ($n = 6$ for each group). (**C**) Lucigenin-enhanced chemiluminescence: the EC suspension was incubated with TNF or menadione in the presence of lucigenin, and the luminescence was monitored at 37 °C for 60 min. The values obtained in the absence of cells were subtracted as a background. The menadione, but not TNF, significantly increased net measured cellular superoxide generation. A representative of three experiments is shown. (**D**) DCFH-DA-loaded and then washed EC monolayers in 96-well plates were incubated with TNF-α, menadione or H_2O_2 for 60 min, and the plate was read on a fluorescence plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The exogenous H_2O_2 and menadione, but not TNF, significantly increased DCF fluorescence ($n = 5$ for each group).

tration of menadione (Figures 6A–6C). These assays, however, might not have reflected intracellular ROM generation (although they did respond to menadione, which generates superoxide both intracellularly and extracellularly [15]). Therefore we used the ROM-sensitive intracellular fluorophore DCFH-DA to measure the net intracellular generation of ROMs in response to TNF-α. Whereas exogenously administered H_2O_2, as well as menadione, did increase the measured level of intracellular fluorescence intensity (positive controls), TNF-α did not substantially stimulate measurable fluorescence emission over baseline levels (Figure 6D). However, the DCF fluorescence seen both at baseline and after the TNF stimulation was inhibited by either diphenylene iodonium or rotenone. Allopurinol, BOF4272, indomethacin and metyrapone, each of which had failed to inhibit ICAM-1 upregulation, did not affect this DCF fluorescence (Table 1). In preliminary studies we had found that antimycin A and apocynin increased DCF fluorescence directly, even in the absence of cells, and therefore could not be used in our system to evaluate ROM generation with these inhibitors.

Effect of increased ROM generation on the expression of ICAM-1

Because none of the above assays had detected any increase in net ROM generation in response to TNF-α, we evaluated the effect of exogenously administered ROMs on ICAM-1 expression, in both the presence and absence of TNF-α. The exogenous addition of H_2O_2 (12.5–100 μM) or the exogenous generation of superoxide with xanthine (10–100 μM) plus XO (5 m-units/ml) for 4 h did not stimulate ICAM-1 expression at all (Figures 7A and 7B). Moreover, each also failed to enhance the level of ICAM-1 expression induced by 30 or 300 units/ml TNF-α (Figures 7A and 7B). Moreover, similar results were found when ECs were incubated with aminotriazole (1–10 mM) (to inactivate endogenous catalase) or menadione (1–30 μM) (to

Table 1 Effect of enzyme inhibitors on ROM generation by ECs

DCFH-DA-loaded ECs were first incubated with various inhibitors for 30 min, then incubated with 300 units/ml TNF-α for 60 min, and the plate was read on a fluorescence plate reader. Data (means $\pm$ S.D.) are presented as percentages of control (the DCF fluorescence in the absence of TNF-α or inhibitors). The levels of both DCF fluorescence from baseline or TNF-stimulated cells was inhibited by either diphenyleneiodonium (DPI) or rotenone ($n = 5$ for each group). $^*P < 0.001$ compared with no inhibitor (Student's t test).

	ROM generation (% of control)	
	No TNF-α	TNF-α
No inhibitor	100	106.0 $\pm$ 8.5
PDTC (50 μM)	8.3 $\pm$ 1.6*	6.9 $\pm$ 2.5*
Allopurinol (100 μM)	104.1 $\pm$ 7.1	108.0 $\pm$ 6.1
BOF 4272 (1 μM)	106.4 $\pm$ 9.8	103.0 $\pm$ 12.2
Indomethacin (100 μM)	108.6 $\pm$ 8.2	112.0 $\pm$ 5.0
Metyrapone (100 μM)	120.2 $\pm$ 1.5	115.0 $\pm$ 4.0
DPI (10 μM)	40.3 $\pm$ 19.6*	41.0 $\pm$ 2.4*
DPI (100 μM)	4.0 $\pm$ 12.0*	3.5 $\pm$ 3.6*
Rotenone (100 μM)	43.5 $\pm$ 10.1*	38.0 $\pm$ 5.1*

increase intracellular superoxide generation) (Figures 7C and 7D). (At the higher concentrations of each reagent, ICAM-1 levels decreased slightly, consistent with non-specific toxicity.)

DISCUSSION

There is increasing evidence that ROMs are involved in signal transduction for the upregulation of leucocyte–endothelial interactions in general, and of the EC adhesion molecules in particular [22–24]. In this study we employed Eahy926 cells as representative venous ECs. These cells have been reported to have characteristics similar to human umbilical vein ECs, used to study TNF-induced adhesion molecule expression including ICAM-1, vascular cell adhesion molecule 1 and E-selectin [11], and also have the redox-sensitive promoter region for ICAM-1 containing binding sites for NF-κB and activator protein 1 (AP-1) [8]. Since the microvascular venules are the primary sites of interaction between leucocytes and endothelium *in vivo* [25–27], TNF-α induction of ICAM-1, the counter-receptor for the leucocyte β_2-integrins and a major mediator of tight adhesion, on venous endothelium is relevant. We focused on TNF-induced ICAM-1 protein expression on the surface of these ECs, using a modified ELISA, which proved highly useful with respect to quantification and reproducibility. More importantly, it provided for the assay of adhesion molecule expression from ECs that are still (physiologically) adherent to their substrate, avoiding the need for mechanical or biochemical disruption of the EC monolayer.

Our data suggest that the presence of endogenous ROMs, generated intracellularly, is necessary but not sufficient for the upregulation of ICAM-1 by TNF-α, since cell-permeable anti-

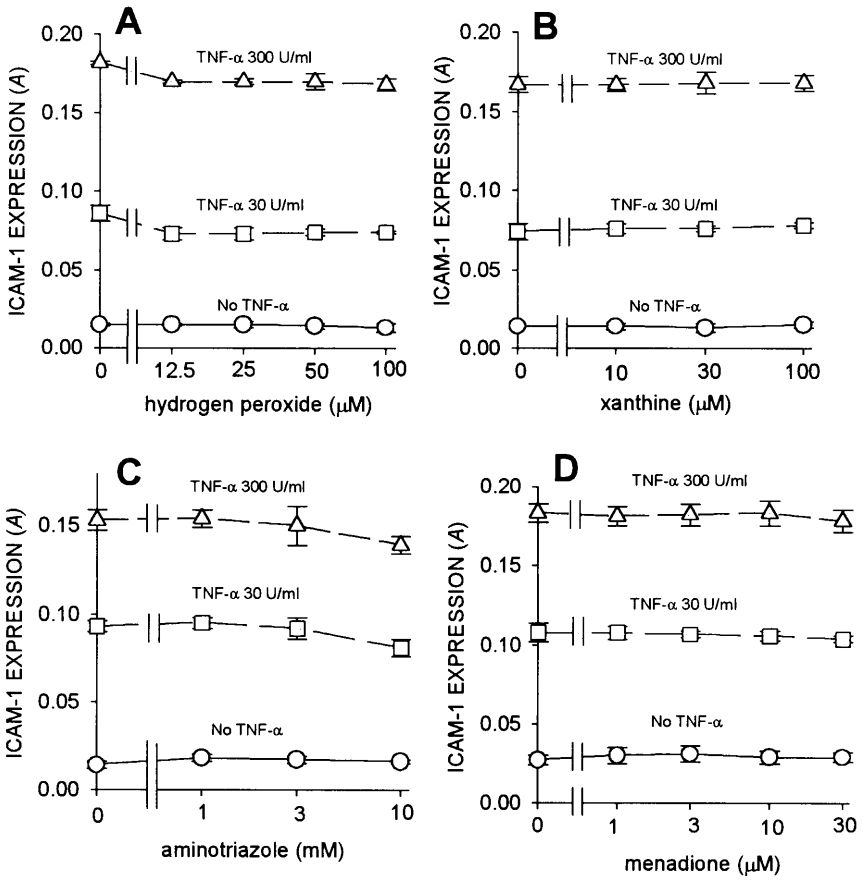

Figure 7 Effect of exogenously stimulated ROM generation on the expression of ICAM-1

EC monolayers were incubated with various concentrations of exogenous H_2O_2 (**A**), xanthine plus XO (5 munits/ml) to generate superoxide (**B**), 3-amino-1,2,4-triazole to inactivate endogenous catalase (**C**), or menadione to generate increased intracellular oxidant flux via redox cycling (**D**) in the presence or absence of TNF-α for 4 h. Not one of these induced increases in exogenous or endogenous ROM generation upregulated ICAM-1, or enhanced ICAM-1 upregulation by TNF-α. Data are means $\pm$ S.D. for four to six wells.

oxidants or the overexpression of intracellular SOD strikingly inhibited ICAM-1 induction. The findings that scavenging of intracellular superoxide by means of overexpressing SOD inhibited the induction of ICAM-1, but that exogenously added SOD failed to inhibit this response, also clearly suggest that intracellularly generated superoxide is important, either directly or as a source of other ROMs.

From our data, NADH dehydrogenase, cytochrome bc_1 complex and NADPH oxidase appeared to be the enzymic sources of the ROMs involved in this signal transduction. Although XO has been clearly implicated in the generation of superoxide by endothelium, especially after ischaemia/reperfusion *in vivo* [3,4], XO appears to not be a source of the operative ROMs in this mechanism of ICAM-1 expression. It seems unlikely that the effect of rotenone or antimycin A resulted from their non-specific inhibition of energy generation, as we found that azide had no effect on TNF-induced ICAM-1 upregulation.

It has been postulated that ROMs mediate signal transduction from various stimuli, including cytokines, endotoxin and PMA, through the activation of NF-κB and the consequent transcription of the mRNA for inflammatory mediators, including adhesion molecules, because in some cells the activation of NF-κB by such stimuli is blocked by antioxidants or by the overexpression of catalase within the cells [22,28]. Moreover, most of the agents that activate NF-κB, including cytokines, have been reported to trigger endogenous ROM generation [22,29]. It has also been reported that ICAM-1 is regulated by another redox-sensitive transcriptional factor, AP-1 [8], which is strongly activated by antioxidants and shows reduced activity in the presence of oxidants [30]. Indeed, it has been shown that antioxidant-induced upregulation of ICAM-1 involves AP-1 in endothelium [31], and the PDTC inhibits ICAM-1 induction by interleukin-1 without inhibiting NF-κB activation [32]. Schmidt et al. [28] reported that stable overexpression of SOD potentiated NF-κB activation by overproducing H_2O_2. On the other hand, transient overexpression of SOD in our study inhibited TNF-induced ICAM-1 expression. One possible explanation for this discrepancy might be the difference in the methods employed to overexpress SOD. The concentration of GSH has been reported to be significantly lower in the cell line genetically overexpressing SOD, compared with the parent cell line, probably because of a chronic pro-oxidant state [33]. It is also possible that differential activation of NF-κB and AP-1 by overexpression of SOD is quite sensitive to cell type and experimental conditions. Although accumulating data suggest the involvement of endogenous ROM generation in the signal transduction of cytokine upregulation of ICAM-1 mRNA and protein [8,9,34], the correlation of cytokine-stimulated specifically intracellular ROM generation and consequent ICAM-1 upregulation in endothelium has not been reported, to the best of our knowledge. As our data clearly indicate, intracellularly generated ROMs are necessary for the upregulation of ICAM-1. Nevertheless, our failure to detect substantial increments in net cellular ROM generation by TNF using four disparate, sensitive assays is a very clear finding in our experiment. Similar findings have been reported previously [35,36]. However, a failure to detect an increment in net cellular ROM generation does not rule out small local intracellular changes in oxidant flux that might not be detectable as a net change in overall cellular oxidant generation. On the other hand, our finding that enhanced extracellular or intracellular ROM generation did not enhance ICAM-1 expression, either at baseline or in response to TNF-α, suggests that the incremental generation of ROMs might not be involved in the upregulation of ICAM-1 by TNF-α. Although we have not addressed directly in this study whether such transcriptional factors are targets for the ambient ROMs, it seems reasonable to speculate that ambient ROM generation creates an ambient oxidative state which enables TNF to transduce a signal, depending on cell types [37], and that the oxidized state is reversed by cell-permeable antioxidants or SOD overexpression.

The data from this study suggest that the TNF-mediated upregulation of ICAM-1 in ECs is dependent on an intracellular oxidant mechanism, but that the incremental generation of ROMs by the cell does not appear to be the primary agonist for this signal-transduction pathway. It seems likely that an ambient flux of intracellular oxidants, generated by NADPH oxidase or by mitochondrial enzymes, is necessary but not sufficient for TNF-α-stimulated ICAM-1 expression by ECs.

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