A new role for vascular endothelial growth factor and fibroblast growth factors: increasing endothelial resistance to oxidative stress

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Abstract Intracellular oxidative stress was measured in cultured human umbilical vein or bovine aortic endothelial cells exposed to hydrogen peroxide using an oxidation-sensitive vital dye, 2′,7′-dichlorofluorescein, and flow cytometry. Vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) caused both a 5–10-fold increase in resistance to hydrogen peroxide induced fluorescence and an increase in intracellular reduced glutathione concentration. Increased resistance to oxidative stress mediated by growth factors is likely to be biologically relevant, and may open new avenues for therapeutic protection against oxidative stress.

Key words: Endothelium; Oxidative damage; Vascular endothelial growth factor; Fibroblast growth factor, acidic; Fibroblast growth factor, basic; Glutathione

1. Introduction

Oxidative stress has been suggested as a possible cause of endothelial damage in a variety of situations including atheroma, reperfusion injury and endotoxic shock [1–3]. Endothelial defence against oxidative damage is mediated by a variety of mechanisms including glutathione peroxidase/reduced glutathione/NADPH, with NADPH being generated by the pentose phosphate pathway [4,5]. We measured endothelial intracellular peroxidative activity using flow cytometry and the vital dye 2,7-dichlorofluorescein diacetate (DCF-DA) [6] in cultured bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) exposed to low concentrations of hydrogen peroxide. Human endothelial cells in culture were apparently much more resistant to oxidative stress than bovine cells. Systematic analysis of differences in the culture conditions used for BAEC and HUVEC identified bovine brain extract (BBE) as a factor capable of upregulating bovine endothelial resistance to hydrogen peroxide-induced fluorescence. In this paper we show that the effect of BBE can be replicated by recombinant vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF), and correlate changes in resistance to hydrogen peroxide-induced fluorescence with changes in cellular reduced glutathione (GSH) content. Clinically, it may be possible to increase the resistance of endothelium to oxidative stress by upregulating 'active defence' mechanisms such as the glutathione/glutathione peroxidase/NADPH system as well as by exogenous antioxidant supplementation.

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2. Materials and methods

BAEC were obtained by collagenase digestion of fresh aortas, cloned to prevent contamination with smooth muscle cells or fibroblasts [7], and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (both from Gibco) unless otherwise specified. Cells were subcultured every 3–5 days using 0.05% w/v trypsin/1 mM EDTA (Clonetics) at a 1:3 or 1:4 split ratio. HUVEC were also obtained by collagenase digestion and were cultured in Clonetics EGM, which is an Eagles-based medium supplemented with insulin, hydrocortisone, heparin, selenium, bovine brain extract (BBE) and 10% fetal calf serum. HUVEC were subcultured at 3–7 day intervals with a 1:2 split ratio. Gas phase was 95% air/5% CO_2 for all cultures. Cells were characterised as endothelial by light and electron microscopic morphology, and by their expression of endothelium-specific antigens including von Willebrand factor.

Intracellular peroxide was measured by a modification of the technique of Carter et al. [6]. Endothelial monolayers were resuspended as single cells using trypsin-EDTA and washed. Cell suspensions at 10^6/ml in Hanks' saline were preincubated with 2 μM DCF-DA for 30 min at 37°C and 2 μg/ml propidium iodide (Sigma) for 10 min at 37°C. (Propidium iodide stains the nuclei of damaged cells so that they can be distinguished from those with intact cell membranes.) Hydrogen peroxide (diluted from 30% stock solution, Sigma) was added to different aliquots to produce final concentrations of 5–100 μM. DCF-DA is incorporated into lipid-rich regions of cells and hydrolysed to 2′,7′-dichlorofluorescein by cellular esterases. In the presence of peroxide this is oxidised to fluorescein, which emits green fluorescence at 514 nm on excitation at 490 nm. At times of 1–30 min after the addition of hydrogen peroxide samples were analysed in a Becton-Dickinson FACScan flow cytometer. An argon laser operating at 488 nm was used for excitation, fluorescein fluorescence was detected using a 530 ± 30 nm band-pass filter, and propidium iodide fluorescence using a 630 nm long pass filter. 10^4 cells were analysed per sample, of which 5–10% were gated out using propidium iodide fluorescence. Data are presented as mean fluorescence per cell (standard deviation), expressed as a percentage of mean fluorescence of cells cultured in DMEM+10% FCS exposed to 100 μM hydrogen peroxide for 30 min (as internal positive control).

Reduced glutathione (GSH) was measured by the method of Punchard et al. [8], which is a microplate adaptation of Tietze's method [9]. Briefly, endothelial cultures in 96-well plates were washed twice with isotonic phosphate-buffered saline (PBS), 0.1 ml 1% 5-sulphosalicylic acid added, and plates stored at −80°C. After thawing, samples and GSH standards (1–10 nM/ml) were diluted 1:4 with PBS/5 mM EDTA pH 7.5 and 0.1 ml aliquots added to an equal volume of PBS containing 1.5 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 0.5 mM NADPH. 50 μl glutathione reductase (Sigma, 1 U/ml) was added to each well and absorbance measured at 405 nm at 0–10 min at 2-min intervals. GSH content was measured by reference to the standard curve. All measurements were made in octuplicate. Control experiments showed that neither *N*-acetylcysteine nor ascorbate (0.2 mM) interfered with the assay under the conditions used.

GSH concentrations were expressed as pM GSH/ng cellular DNA. DNA content was measured in parallel cultures using fluorescence developed after incubation with Cy-QUANT-GR reagent (Molecular Probes, USA), using excitation at 485 nm and measuring emission at 530 nm.

To inhibit glutathione synthesis, L-buthionine *S,R*-sulphoximine (BSO, Sigma) was added to cultures at a final concentration of 0.1 mM, 24 h before they were harvested for flow cytometry. In other experiments, *t*-butyl hydroperoxide (tBHP) was added to cultures at a

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final concentration of 5 μM, 1 h before harvesting. The antioxidants ascorbic acid (final concentration 0.1 mM) or *N*-acetylcysteine (final concentration 0.1 mM) were added to cultures (where specified) 5 h or 1 h before harvesting, and were also added at the same concentration to the flow cytometry buffer.

Flow cytometry distributions were compared using the Kolmogorov-Smirnov statistic; other comparisons were by 2-way analysis of variance with post-hoc Newman-Keuls test; $P < 0.05$ was taken as the level of statistical significance. Experiments were repeated at least three times; data in Fig. 1 and Tables 1–3 represent typical experiments.

Recombinant human VEGF, aFGF, bFGF, t-BHP, DTNB and BSO were purchased from Sigma.

3. Results

Hydrogen peroxide caused a dose- and time-dependent increase in fluorescence of DCF-DA-loaded BAEC cultured in DMEM+10% FCS (Table 1A, Fig. 1). tBHP (5 μM), which is both a substrate for and inhibitor of glutathione peroxidase, or previous culture in BSO-containing medium (data not shown) increased the fluorescence observed after exposure to the same concentrations of hydrogen peroxide. DCF-DA-loaded HUVEC cultured in EGM+supplements+10% FCS also showed increased fluorescence when exposed to hydrogen peroxide, but much higher concentrations of hydrogen peroxide (50–200 μM) were required to produce equivalent fluorescence (data not shown). Neither ascorbate nor *N*-acetylcysteine pre-incubation for 1 h affected fluorescence in response to hydrogen peroxide exposure (Table 1B).

BAEC cultured in DMEM+EGM supplements before DCF-DA loading became resistant to hydrogen peroxide-induced fluorescence (Fig. 2). The factor conferring hydrogen peroxide resistance was identified by exclusion as BBE, and BBE added to DMEM+FCS increased oxidative resistance (Table 1C). VEGF (10 ng/ml), aFGF (10 ng/ml), or bFGF (2 ng/ml) increased hydrogen peroxide resistance to the same extent as BBE, whereas epidermal growth factor (10 ng/ml) was ineffective. Inhibiting cell growth by reducing serum content of the medium to 2% did not block the increase in hydrogen peroxide resistance (Table 2). An increase in hydrogen peroxide resistance could be detected within 24 h of adding BBE or VEGF to cultures; similarly the effect is lost within 24–48 h of transferring to DMEM+FCS (data not shown).

Bovine aortic endothelial cells cultured in DMEM+FCS with the addition of BBE, VEGF bFGF or aFGF ('growth factors') showed higher GSH contents than BAEC cultured in DMEM+FCS alone (Table 3). BSO lowered basal GSH levels and inhibited the increase in GSH content caused by growth factors, but tBHP had little effect on GSH levels at 1 h, and

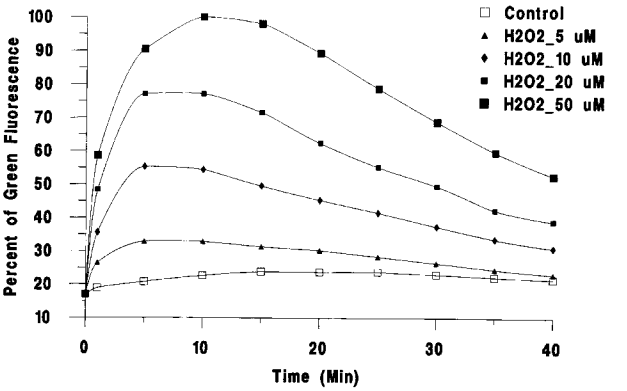

Fig. 1. Time-fluorescence curves for resuspended, DCF-DA-loaded BAEC exposed to hydrogen peroxide. Cell suspensions at 10^6/ml in Hanks' saline were preincubated with 2 μM DCF-DA for 30 min at 37°C and 2 μg/ml propidium iodide for 10 min at 37°C. Hydrogen peroxide was added to different aliquots to produce final concentrations of 5–20 μM. At times of 1–30 min after the addition of hydrogen peroxide samples were analysed in a Becton-Dickinson FACScan flow cytometer. 10^4 cells were analysed per sample, of which 5–10% were gated out as permeable or damaged cells using propidium iodide fluorescence. Vertical axis indicates fluorescence in arbitrary units.

Table 1
Fluorescence of DCFH-loaded endothelial cells exposed to H_2O_2 (expressed as % ± S.D. fluorescence of control cells 20 min after exposure to 0.1 mM H_2O_2)

Time:	1 min	10 min	20 min	
A. BAEC in DMEM/10% FCS; effect of H_2O_2 and tBHP				
No H_2O_2	19 ± 9	22 ± 10	24 ± 12	
H_2O_2 5μM	31 ± 15	56 ± 33	55 ± 32	
H_2O_2 20μM	54 ± 28	99 ± 46	100 ± 43	
tBHP 5μM	32 ± 19	68 ± 48	78 ± 56	
tBHP 5μM+H_2O_2 5μM	39 ± 23	92 ± 66	103 ± 76	
B. Effect of N-acetylcysteine and ascorbic acid; BAEC exposed to 0.1 mM H_2O_2				
10% FCS	39 ± 26	86 ± 48	100 ± 64	
NAC (0.1 mM)	37 ± 27	86 ± 35	99 ± 63	$P = 0.8$*
Ascorbic acid (0.1 mM)	32 ± 21	75 ± 45	85 ± 53	$P = 0.4$*
C. BAEC in DMEM/10% FCS+growth factors; exposed to 0.1 mM H_2O_2				
No growth factors	31 ± 19	79 ± 34	95 ± 54	
EGF (10 ng/ml)	37 ± 32	87 ± 51	102 ± 52	$P = 0.4$
BBE (4 μl/ml)	22 ± 10	41 ± 22	47 ± 28	$P < 0.001$
aFGF (10 ng/ml)	13 ± 9	39 ± 26	52 ± 25	$P < 0.001$
bFGF (2 ng/ml)	12 ± 8	35 ± 30	42 ± 15	$P < 0.001$
VEGF(10 ng/ml)	10 ± 7	33 ± 16	42 ± 18	$P < 0.001$

Each block represents a separate experiment. Figures represent mean fluorescence per cell (S.D.) of viable cells at 1, 10 and 20 min after adding hydrogen peroxide, as a percentage of mean fluorescence of internal positive control (see text).
A. Effect of different hydrogen peroxide concentrations+tBHP on fluorescence of BAEC grown in DMEM/10% FCS.
B. Effects of preincubation with ascorbate or *N*-acetylcysteine on DCF fluorescence of BAEC exposed to 100 μM hydrogen peroxide.
C. Effects of different growth factors added to DMEM+10% FCS 72 h before experiment.
* vs 10% FCS; vs no growth factors.

Table 2
Effect of cell growth rate on fluorescence of DCF-DA-loaded BAEC exposed to 100 μM H_2O_2

Culture condition	Cell density (cells/cm^2)	Fluorescence	
10% FCS	1.1×10^5	100 ± 44	
			} $P<0.01$
2% FCS+BBE	0.5×10^5	61 ± 36	
			} $P=0.7$
10% FCS+BBE	1.2×10^5	63 ± 32	

BAEC were plated at 0.4×10^5/cm^2 in DMEM ± BBE with either 2% or 10% FCS. Cells were harvested at 48 h, cell numbers were counted and mean fluorescence measured 20 min after hydrogen peroxide exposure. 2% FCS+BBE vs 10% FCS, $P<0.01$, 2% FCS+BBE vs 10% FCS+BBE, $P=0.7$.

caused only a modest fall at 5 h. Neither ascorbate nor *N*-acetylcysteine significantly increased intracellular GSH after 1 or 5 h preincubation, indeed ascorbate caused a fall in GSH content.

4. Discussion

In our experimental system, hydrogen peroxide-induced fluorescence in DCF-DA-loaded BAEC correlated inversely with GSH content. BBE, VEGF, bFGF and aFGF increased hydrogen peroxide resistance and simultaneously raised GSH levels, and both the increase in resistance and the rise in GSH were inhibited by BSO, which inhibits glutathione synthesis. This would be compatible with a mechanism whereby increased GSH led to a more rapid clearance of intracellular H_2O_2 by glutathione peroxidase, and thus to lower peak H_2O_2 concentrations. Conversely, fluorescence was enhanced by tBHP with little change in GSH level. tBHP is a substrate for glutathione peroxidase, and at high concentrations may cause GSH depletion [10], but our results at the low tBHP concentration of 5 μM would be more compatible with an action as an inhibitor of hydrogen peroxide reduction by glutathione peroxidase.

BBE greatly facilitates the growth of HUVEC in culture, particularly at low plating density [11], but is not essential for the culture of BAEC. It is not clear whether HUVEC are particularly prone to lethal oxidative damage in the absence of BBE, or conversely whether BAEC have alternative defence mechanisms: it was the ability of BAEC to survive and grow in the absence of BBE that made the experiments described above feasible.

BBE is a source of crude VEGF and FGF [11,12]. VEGF is a highly conserved peptide growth factor which occurs in several isoforms resulting from differential splicing of a single gene, circulates as a homodimer, and binds to two distinct endothelial cell receptors [13]. It is a powerful angiogenic stimulus and endothelial mitogen in several different models. VEGF also causes a marked increase in vascular permeability. In the present study the up-regulation by VEGF of resistance to oxidative stress appeared to be distinct from a mitogenic effect. VEGF expression can be induced in several cell types, including endothelium [14] and myocardium [15], by hypoxia, and in lung type II alveolar cells by oxidative damage [16]. It has been cited as a member of a family of 'stress-induced genes' [17]. Vascular endothelial growth factor may also be induced by cytokines such as platelet-derived growth factor or basic fibroblast growth factor [18,19]. Despite its expression by such a variety of cell types, VEGF receptors are confined to endothelium. aFGF is also produced by cardiac myocytes and is thought to play a role in myocyte proliferation and capillary angiogenesis [20]. In bovine epithelial lens cells, up-regulation of aFGF expression is important for the survival of quiescent cells, an effect which is also independent of mitogenesis [21].

Endothelial oxidative stress is known to be increased under conditions of hypoxia/reoxygenation [1,22,23], and may be further increased as a result of leukocyte activation [24]. Resistance to such stress may be passive, depending on other cells, on enzymes such as catalase and superoxide dismutase, or on exogenous antioxidants from dietary sources. Alternatively, 'active' defence involves the generation of reducing equivalents in the form of NADPH by glucose metabolism via the pentose phosphate pathway, in conjunction with glutathione and glutathione peroxidase [4,5,25]. Reduced glutathione is also important in resistance to nitric oxide induced cytotoxicity [26].

We have now shown that this active defence mechanism can be powerfully upregulated by specific growth factors, VEGF and basic or acidic FGF. The effect appears to be the physiological converse of the increased oxidative sensitivity and

Table 3
Effect of growth factors, inhibitors and antioxidants on BAEC reduced glutathione content

	Control	aFGF	bFGF	BBE	VEGF	
Control	5.2 (0.5)	9.1 (0.7)	10.0 (1.0)	9.1 (1.0)	8.0 (0.6)	$P<0.001$[a]
BSO (0.1 mM, 24 h)	2.0 (0.2)	1.9 (0.2)	2.6 (0.2)	2.5 (0.2)	3.2 (0.2)	$P<0.001$[b]
tBHP (5 μM, 1 h)	5.6 (1.0)	6.8 (0.8)	7.6 (0.8)	8.2 (1.0)	8.0 (0.7)	$P=0.06$[c]
tBHP (5 μM, 5 h)	4.2 (0.4)	6.2 (0.9)	6.6 (0.4)	7.1 (6.7)	6.5 (0.5)	$P<0.01$[c]
Ascorbate (0.2 mM, 1 h)	3.1 (0.4)	5.6 (0.7)	5.7 (0.7)	5.4 (0.5)	5.4 (0.4)	$P<0.01$[d]
Ascorbate (0.2 mM, 5 h)	3.3 (0.3)	5.0 (0.5)	5.2 (0.5)	5.3 (0.5)	3.9 (0.1)	$P<0.01$[d]
NAC (0.2 mM, 1 h)	5.3 (0.7)	9.1 (0.5)	10.9 (0.5)	10.2 (1.1)	8.2 (0.7)	$P=0.2$[e]
NAC (0.2 mM, 5 h)	5.6 (1.0)	6.8 (0.8)	7.6 (0.8)	8.2 (1.0)	8.0 (0.7)	$P=0.07$[e]

GSH content of confluent bovine aortic endothelial cells cultured in DMEM+FCS (control) or DMEM+FCS with the addition of aFGF, bFGF, VEGF (all at 10 ng/ml) or BBE (4 μl/ml) for 72 h. Where indicated cells were incubated before harvesting with ascorbate, *N*-acetylcysteine (NAC), BSO or tBHP for the times and at the concentrations specified. Results are expressed as mean ($n=8$) (S.E.) pM GSH per ng DNA.
[a]Control (no growth factors) vs growth factors. [b]BSO vs control. [c]tBHP vs control. [d]Ascorbate vs control. [e]NAC vs control.

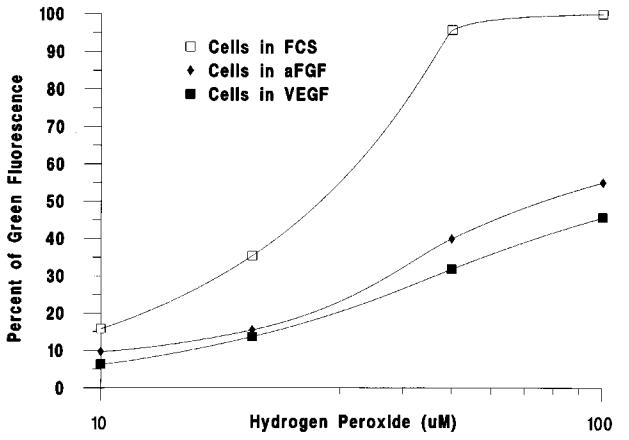

Fig. 2. Dose-response curve of DCF-DA-loaded BAEC fluorescence against log hydrogen peroxide concentration for control cells and for cells cultured for 72 h with 10 ng/ml VEGF or aFGF. Fluorescence was measured 10 min after the addition of hydrogen peroxide. Vertical axis indicates fluorescence expressed as percent fluorescence of control cells exposed to 100 μM hydrogen peroxide for 10 min.

glutathione depletion in response to tumor necrosis factor alpha described by Ishii and colleagues [4]. Fuks and colleagues have previously shown that bFGF increased endothelial resistance to radiation-induced damage in vivo and in vitro [27], and GSH upregulation may be one of the mechanisms involved. There is a clear teleological link between increased risk of oxidative damage, increased VEGF expression, and upregulation of the glutathione-glutathione peroxidase system in response to hypoxia. The system may have a role in the phenomenon of myocardial 'preconditioning' by prior ischaemic exposure protecting against ischaemia-reperfusion damage. Also of interest is the potential involvement of cell-cell cooperation, since many cell types produce VEGF but only endothelial cells appear to have VEGF surface receptors.

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