Hyperoxia, unlike phorbol ester, induces glutathione peroxidase through a protein kinase C-independent mechanism

Lan JORNOT¹ and Alain F. JUNOD

Respiratory Division, University Hospital, 1211 Geneva 14, Switzerland

Human selenium-dependent glutathione peroxidase (GP) is implicated as a mechanism of resistance against oxygen free radicals. The 5' flanking sequence upstream from the coding region of GP contained an oxygen-responsive element termed ORE1 that is responsive to hypoxia, as well as several copies of the activator protein-1 (AP-1)- and AP-1-like-binding sites. In this study, we sought to define the molecular events that lead to GP gene transcription in response to hyperoxia in human umbilical-vein endothelial cells, and asked whether such induction is mimicked and sustained by activation of protein kinase C (PKC) by phorbol esters. Treatment of cells with 100 nM phorbol 12,13-dibutyrate (PdBu) induced a delayed (24–48 h) but significant (2-fold) increase in steady-state GP mRNA levels. Steady-state GP mRNA levels also rose after exposure to 95% O₂, again after considerable delay (48–72 h). For both PdBu and oxygen, induction was transcriptionally regulated, as demonstrated by nuclear run-on experiments. The stimulations by PdBu and oxygen were additive. In contrast with PdBu, hyperoxia did not stimulate translocation of PKC from

the cytosol to the particulate fraction, although the specific activity of both cytosolic and particulate-associated PKC was increased 2-fold in cells exposed to 95% O₂ for 5 days. In addition, gel mobility-shift assays using double-stranded tumour-promoting-agent-responsive element (TRE) and nuclear extracts derived from phorbol- and oxygen-treated cells revealed that PdBu, but not hyperoxia, increased AP-1 DNA-binding activity. On the other hand, the up-regulation of GP expression by oxygen could not be accounted for by the ORE1 core sequence, since no specific protein–DNA binding activity could be detected using nuclear extracts from hyperoxic cells and ORE1. Taken together, these results suggest that there may be different molecular mechanisms controlling GP expression. After exposure to PdBu, GP undergoes transcriptional activation via a process that can be readily explained by a classic AP-1 interaction with the TRE sites in the GP promoter. During hyperoxia, GP also undergoes transcriptional activity, but via a process that appears to involve neither TRE nor ORE1.

INTRODUCTION

Human cellular glutathione peroxidase (GP) belongs to a family of selenium-dependent GPs, which includes a second novel cytosolic form of the enzyme [1], a plasma GP [2] and a phospholipid hydroperoxide GP [3]. These enzymes protect cells against oxidative damage by reducing H₂O₂ and a wide range of organic hydroperoxides with GSH as the hydrogen donor [4,5]. Selenium-dependent GPs may also participate in arachidonate metabolism by reducing key intermediates in the prostacyclin and leukotriene metabolism [4]. Of the four members of this family, GP remains the predominant intracellular form. The enzyme exists as a homotetramer, with each 22 kDa subunit containing a selenium atom incorporated within a catalytically selenocysteine residue [6,7]. The expression of GP is complex as it is found to be regulated developmentally and hormonally [8], as well as by oxygen tension [9,10], diet and some xenobiotics [8]. GP may also have a role in anti-neoplastic drug resistance [8]. Drug-resistance studies have shown that, in several instances, increased levels of GP are associated with increased resistance of tumour cells to free-radical-producing anthracycline drugs such as adriamycin or doxorubicin.

The gene encoding GP, *hgp1*, resides on chromosome 3 regionally assigned to 3qll-3qll1; however, two loci assumed to be processed pseudogenes are known to exist on chromosomes

21 and X [11–13]. Analysis of the *hgp1* promoter and 5'-flanking region has revealed two distinct regions upstream from the transcription start site, designated ORE1 and ORE2, that are responsive to the oxygen tension in culture [14]. On the other hand, several tumour-promoting-agent-responsive elements (TREs) have been identified. TRE is the binding site for the transcription factor activator protein-1 (AP-1), which represents a family of transcription factors comprised of homo- and heterodimers of individual members of the Fos- and Jun-related families of proteins. The binding of AP-1 to the TRE is observed early in response to treatment of cells with phorbol esters, which are activators of protein kinase C (PKC). Interestingly, it was demonstrated that the DNA-binding activity of AP-1 is sensitive to changes in its oxidation–reduction status [15,16], and that PKC-specific activity is increased in response to active oxygen species [17–19].

We [9] and others [10] have observed a positive correlation between oxygen tension and GP activity in cultured endothelial cells and cardiomyocytes. The differences reported for GP activity induced by oxygen have been found to be paralleled at the mRNA and gene transcription levels. The presence of ORE1 and TRE, along with the well-established redox regulation of AP-1 DNA-binding activity and sustained activation of PKC by oxidants, prompted us to investigate the regulation of GP expression by oxygen and phorbol ester, an activator of PKC,

Abbreviations used: AP-1, activator protein-1; GP, glutathione peroxidase; HUVEC, human umbilical-vein endothelial cell; ORE, oxygen-responsive element; PdBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; TRE, tumour-promoting-agent-responsive element; DTT, dithiothreitol.

¹ To whom correspondence should be addressed.

and to evaluate the potential role of PKC in the activation of GP by hyperoxia in human endothelial cells.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical grade and came from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Fluka (Buchs, Switzerland). RPMI 1640 medium was from Biological Industries (Beth Haemek, Israel). Fetal calf serum was from Seromed (Berlin, Germany), and endothelial cell growth supplement was from UBI (Lake Placid, NY, U.S.A.). [α - 32 P]UTP (800 Ci/mmol), [γ - 32 P]ATP (5000 Ci/mmol) and Hyperfilm were from Amersham International (Amersham, Bucks, U.K.). The SP6 transcription kit and the DNA 5' end-labelling kit were obtained from Promega (Madison, WI, U.S.A.). Poly(dI-dC)·(dI-dC) and DNase-free BSA were obtained from Pharmacia Biotech. (Piscataway, NJ, U.S.A.).

Cell culture

Human endothelial cells were obtained from umbilical cord veins (HUVECs) using 0.1 % collagenase in Krebs–Ringer bicarbonate buffer. The cells were plated in 60 mm gelatin-coated (0.1 %) Petri dishes and grown in RPMI 1640 supplemented with 25 mM Hepes, 50 units/ml penicillin, 50 μ g/ml streptomycin, 10 % fetal calf serum, 15 μ g/ml endothelial cell growth supplement and 90 μ g/ml heparin. Second- and third-passage cells in monolayer culture were used for all experiments at confluence. Endothelial cells were characterized using morphological (phase-contrast microscopy) and functional (measurement of the angiotensin-converting enzyme activity) criteria as previously described [9].

Experimental conditions

Exposure to hyperoxia

At confluence, the cells were exposed to 5 % CO₂/95 % O₂ for 1, 2, 3 and 5 days in a humidified chamber. The percentage of ambient oxygen in the chamber was always checked by an oxygen analyser (OM-11; Beckman) at the beginning and end of the exposure period.

Exposure to phorbol 12,13-dibutyrate (PdBu)

At confluence, the culture medium was removed, and the cells were incubated with fresh medium containing 100 nM PdBu for 1, 2, 3 and 5 days.

Exposure to hyperoxia and PdBu

At confluence, the cells were exposed to 5 % CO₂/95 % O₂ for 3 or 5 days, and 100 nM PdBu was added for the last 24 h of incubation.

RNA preparation and Northern-blot analysis

A single-step method of RNA isolation using acid guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi [20] was used to isolate total RNA from the cultured endothelial cells. Northern-blot analysis for GP mRNA was performed as previously described [9] except that we used in this study storage phosphor screens (Molecular Dynamics) for autoradiography. The density of the hybridization signal was quantified using a PhosphorImager (Molecular Dynamics). As a loading control, the density of the 28 S and 18 S

RNA was quantified by scanning the negatives of the prints of the ethidium bromide-stained gel. All the quantitative data were presented as the ratio of the hybridization signal to the 28 S + 18 S RNA intensity.

Nuclear transcription assay

In vitro transcription assays using isolated nuclei were performed as previously described [21]. Purified [α - 32 P]RNA transcripts were resuspended in 2 ml of hybridization buffer, and equal amounts of incorporated counts were hybridized to 5 μ g of unlabelled cDNA probes immobilized on Biodyne membranes by dot-blotting. Probes included glyceraldehyde-3-phosphate dehydrogenase, GP and pSPT19, the plasmid vector into which GP cDNA was cloned.

Preparation of nuclear extracts and electrophoretic mobility-shift assay

Cell nuclear extracts were prepared as described by Dignam et al. [22]. Briefly, the cells were washed twice with ice-cold PBS, pH 7.4, and collected by scraping with a rubber 'policeman' into cold PBS. After centrifugation at 1000 *g* for 10 min at 4 °C, cell pellets were resuspended in 1 ml of hypotonic buffer [10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.2 mM PMSF, 1 μ g/ml antipain, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 0.5 mM dithiothreitol (DTT)]. The samples were allowed to stand on ice for 15 min, then homogenized with a Dounce homogenizer. The nuclei were then pelleted at 1000 *g*, and resuspended in 100 μ l of extraction buffer (10 mM Hepes, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 5 % glycerol, 0.2 mM PMSF and 0.5 mM DTT) with constant mixing at 4 °C for 30 min. The samples were centrifuged at 8000 *g* for 15 min at 4 °C, and the high-salt supernatant was dialysed on ice for 2 h against a buffer containing 20 mM Hepes, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 20 % glycerol, 0.2 mM PMSF and 0.5 mM DTT. The dialysed extracts were quickly frozen in liquid nitrogen and stored at –70 °C. Protein concentrations were determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.). Nuclear extracts were analysed for ORE1- and TRE-binding activity by gel mobility-shift assays. The binding reaction mixture contained 5 μ g of protein, 2 μ g of poly(dI-dC)·(dI-dC) and 10 μ g of BSA in a final volume of 20 μ l of binding buffer (20 mM Hepes, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20 % glycerol). Each reaction mixture contained 5×10^5 c.p.m. of a double-stranded ORE1 oligonucleotide (5'-ATACACTTCTTGAGGATGACAG-3' and its complementary strand) or TRE (5'-CTTGTGAGTCATTCC-3') which was 5' end-labelled using T4 polynucleotide kinase and [γ - 32 P]ATP. The reaction mixtures were incubated for 30 min at room temperature, and then free and bound DNA were separated by electrophoresis on a 4 % non-denaturing polyacrylamide (acrylamide/*N,N'*-methylene-bisacrylamide, 30:1) in 50 mM Tris, pH 8, containing 380 mM glycine and 2 mM EDTA. Gels were run at 100 V (20–15 mA) at room temperature, dried under vacuum and exposed for autoradiography at –70 °C using Hyperfilm and intensifying screens. The specificity of binding to ORE1 and TRE was determined by competition with a 100-fold molar excess of non-radioactive ORE1 or TRE.

PdBu binding to intact HUVECs and Scatchard-plot analysis

Binding of [3 H]PdBu to intact cells after exposure to hyperoxia was determined at 37 °C in duplicate samples with a final [3 H]PdBu concentration of 5 nM in the absence or presence (non-specific binding) of 1 mM unlabelled PdBu. After 5 min of

incubation, the monolayers were rinsed ten times with 20 mM Tris/HCl, pH 7.4, containing 10 mM MgCl₂ and 2 mM CaCl₂, and scraped into 0.1 M NaOH/1 % Na₂CO₃. One aliquot was taken for measurement of radioactivity, and another for determination of protein. Binding of [³H]PdBu was expressed as pmol/mg of protein. For Scatchard analysis, cells were incubated with various concentrations (0.97–62.5 nM) of [³H]PdBu for 5 min at 37 °C.

Partial purification of PKC from HUVECs and determination of PKC activity

Cells were washed with ice-cold buffer A (20 mM Tris/HCl, pH 7.4, 2 mM EDTA, 10 mM EGTA, 2 mM DTT), collected by scraping into the same buffer, and sonicated on ice. The resulting homogenates (0.5 ml) were centrifuged at 100 000 *g* for 30 min, and the supernatants were collected for determination of cytosolic PKC activity. The pellets were resuspended in 0.25 ml of buffer A containing 0.5 % (v/v) Triton X-100 using a glass homogenizer. The resuspended pellets were then centrifuged at 100 000 *g* for 30 min. The supernatants obtained were used as the solubilized particulate fraction. The various cell fractions were incubated with a batch of 0.5 ml of DEAE-cellulose pre-equilibrated in buffer B (20 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA and 2 mM DTT) in 1.5 ml Eppendorf tubes for 10 min on ice [23]. After a 5 min centrifugation at 8000 *g* for 10 min at 4 °C the supernatant was discarded, and the DEAE-cellulose mass with the enzyme bound was washed three times with 1 ml of buffer B. The final DEAE-cellulose mass, having bound PKC, was treated with 0.3 ml of buffer B containing 0.15 M NaCl, gently shaken, incubated for 10 min in ice, and centrifuged as described above. A portion of the supernatant was immediately assayed for PKC activity by measuring the transfer of ³²P from [γ -³²P]ATP to histone III-S, as described by Godson et al. [24]. The final reaction mixture (70 μ l) contained 1 μ Ci of [³²P]ATP, 50 μ M ATP, 10 mM MgCl₂, 1.5 mM CaCl₂ and 50 μ g of histone III-S, with or without 5 μ g of phosphatidylserine and 0.5 μ g of diacylglycerol, in 20 mM Tris/HCl, pH 7.4. The reaction was carried out at 30 °C for 10 min. After incubation, 60 μ l aliquots were spotted on to 1.5 cm $\times$ 1.5 cm phosphocellulose strips (Whatman P81) and immersed in 75 mM phosphoric acid to terminate the reaction. The strips were swirled gently for 10 min, the phosphoric acid was decanted, and the strips were washed three times more in phosphoric acid with gentle agitation. After drying, the radioactivity was determined by scintillation counting in 6 ml of Ultima Gold (Packard, Groningen, The Netherlands). PKC activity was determined from the specific radioactivity of ATP and by correcting for the protein content of the eluates. One unit of PKC is defined as 1 pmol of ³²P transferred to histone/10 min in the presence of Ca²⁺, phosphatidylserine and diacylglycerol.

Measurement of cytotoxicity

Cell number was assessed by measuring total protein and DNA contents at various times up to 5 days after exposure to 95 % O₂ or 100 nM PdBu. Cellular integrity was assessed by the release of lactate dehydrogenase into the medium, as previously described [9].

Data analysis

Data are expressed as means $\pm$ S.E.M. and, where indicated, *n* is the number of experiments performed. The significance of the difference between two groups was assessed using the non-parametric Wilcoxon matched-pairs test, with a *P* value of < 0.05 considered to be significantly different.

RESULTS

Hyperoxia and PdBu induce GP mRNA levels and activate GP gene transcription

To determine the effects of O₂ and PKC activity on GP expression, HUVECs were exposed to 95 % O₂ or incubated with 100 nM PdBu for 1, 2, 3 or 5 days, and the cells were harvested to examine the accumulation of GP mRNA by Northern-blot analysis. As shown in Figure 1, an increase in GP mRNA levels was detectable after a 48 h exposure to hyperoxia (3.1 ± 0.6 -fold increase; *n* = 4; *P* < 0.05 compared with normoxic control cells), and became maximal after 72–120 h of induction (4.8 ± 1.1 - and 5.1 ± 0.3 -fold increase respectively; *n* = 4; *P* < 0.05). When HUVECs were treated with 100 nM PdBu as a function of time (Figure 2), PdBu significantly enhanced GP mRNA levels after a 24 h exposure (1.7 ± 0.2 -fold increase; *n* = 10; *P* < 0.05 compared with untreated control cells). This high level was sustained for another 48 h (1.9 ± 0.3 -fold increase; *n* = 6; *P* < 0.05), and then returned to baseline level after 120 h of PdBu treatment (1.1 ± 0.2 -fold increase; *n* = 6). The difference in the kinetics of enhancement of GP mRNA by O₂ and PdBu suggested that different mechanisms were involved in the activation of GP by these two conditions.

To establish whether the induction of GP mRNA by O₂ and PdBu was due to an increased rate of transcription, nuclear run-on experiments were performed using nuclei isolated from cells exposed to 95 % O₂ for 3 or 5 days, or to 100 nM PdBu for 1 day. As shown in Figure 3, both O₂ and PdBu significantly induced an elevation in GP gene transcription rate, which was increased 2.2 ± 0.7 -, 3.0 ± 1.2 - and 2.8 ± 1.2 -fold in nuclei from cells exposed to 100 nM PdBu for 24 h and to 95 % O₂ for 3 and 5 days respectively (*n* = 4; *P* < 0.05).

Hyperoxia and PdBu enhance GP expression in an additive manner

To determine whether PKC participates in hyperoxia-induced GP gene expression, the effect of PdBu in combination with O₂ on GP mRNA level and gene transcription rate was examined.

Figure 1 Effects of O₂ on GP mRNA levels

At confluence, cells were exposed to 95 % O₂ for 1, 2, 3 or 5 days. The cells were lysed, and GP mRNA levels determined by Northern blot.

Figure 2 Effects of PdBu on GP mRNA levels

At confluence, cells were exposed to 100 nM PdBu (P) for 1, 2, 3 or 5 days. The cells were lysed, and GP mRNA levels determined by Northern blot.

Figure 3 Effects of PdBu, O_2 and O_2 plus PdBu on GP gene transcription rate

At confluence, cells were exposed to 95% O_2 for 3 or 5 days (d), and PdBu (final concentration 100 nM) was added to the culture medium for the last 24 h of O_2 exposure. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pSPT19, the plasmid vector into which GP cDNA was cloned.

Figure 4 Effects of PdBu, O_2 and O_2 plus PdBu on GP mRNA levels

At confluence, cells were exposed to 95% O_2 for 3 or 5 days, and PdBu (final concentration 100 nM) was added to the culture medium for the last 24 h of O_2 exposure.

Figure 5 (A) $[^3H]$ PdBu binding to intact cells exposed to 21% (control) or 95% O_2 for 1, 3 or 5 days and (B) Scatchard analysis of $[^3H]$ PdBu binding to intact cells

In (A) the values indicate the means $\pm$ S.E.M. for five independent experiments. (B) After exposure to 21% (control) or 95% O_2 for 5 days, cells were incubated with various concentrations of $[^3H]$ PdBu for 5 min, thoroughly washed and scraped into 0.1 M NaOH/1% Na_2CO_3 . Aliquots were withdrawn for determination of bound $[^3H]$ PdBu and protein. Data points represent means of duplicate samples for a single experiment. Four separate experiments yielded similar results.

Table 1 Effects of a 3- and 5-day exposure to 95% O_2 and a 30 min treatment with 100 nM PdBu on *in vitro* phosphorylating activity of PKC

The results ($n = 3-4$) represent specific activity and are expressed as units/mg of protein. The values in parentheses represent the PKC activity given as a percentage of the appropriate control. *Significantly different from 21% O_2 -exposed cells.

Treatment	Cytosolic PKC activity	Particulate PKC activity
21% O_2 3 days	10.3 $\pm$ 3.9	1.4 $\pm$ 1.0
95% O_2 3 days	10.6 $\pm$ 2.6	0
21% O_2 5 days	11.8 $\pm$ 3.2	1.8 $\pm$ 1.5
95% O_2 5 days	25.2 $\pm$ 10.2 (210 $\pm$ 44%)*	4.3 $\pm$ 3.7 (229 $\pm$ 10%)*
Control	73.0 $\pm$ 5.3	16.6 $\pm$ 3.5
PdBu (100 nM)	35.3 $\pm$ 7.8 (48 $\pm$ 9%)	46.7 $\pm$ 5.8 (316 $\pm$ 56%)

An additive effect of PdBu and O_2 on the induction of GP message and gene transcription would suggest that two different mechanisms are responsible for the induction of GP expression by PdBu and hyperoxia. Cells were exposed to 21% O_2 (control cells) or 95% O_2 for 3 or 5 days, and PdBu (final concentration 100 nM) was added to the culture medium for the last 24 h. The effects of O_2 and PdBu were additive for both GP mRNA levels and gene transcription rate: a 3- and 5-day exposure to O_2 combined with a 1-day exposure to PdBu led to a 5.3 ± 1.2 - and 6.3 ± 2.2 - ($n = 6-8$) fold increase in GP mRNA (Figure 4), and a 4.3 ± 0.5 - and 5.9 ± 2.5 - ($n = 4$) fold increase in GP gene transcription rate (Figure 3).

Hyperoxia induces binding of $[^3H]$ PdBu to intact cells

To determine whether O_2 was capable of activating PKC in HUVECs, binding of the phorbol ester derivative PdBu to intact cells was assessed as a measure of the effect of hyperoxia on the ligand capacity of cellular PKC. As shown in Figure 5(A), there was no detectable difference in $[^3H]$ PdBu binding between normal cells and cells exposed to 95% O_2 for 3 days. Exposure of HUVECs to 95% O_2 for 5 days resulted in a 2.1 ± 0.3 -fold increase in $[^3H]$ PdBu binding (from 0.72 ± 0.01 pmol/mg of protein in normoxic cells to 1.5 ± 0.2 pmol/mg of protein in hyperoxic cells; $n = 5$; $P < 0.05$). Scatchard analysis (Figure 5B) yielded similar K_d values of 22.4 ± 5.9 and 19.0 ± 3.9 nM ($n = 4$) for phorbol ester binding to normoxic and hyperoxic cells respectively. However, hyperoxia increased the number of binding sites made available for the phorbol ester by 1.6-fold (from 2.8 ± 0.4 pmol/mg of protein in normoxic cells to 4.5 ± 1.0 pmol/mg of protein in cells exposed to 95% O_2 for 5 days; $n = 4$; $P < 0.05$).

Hyperoxia enhances total PKC activity, but does not induce PKC translocation

The potential role of PKC in mediating the expression of GP in response to hyperoxia was further documented by experiments measuring the specific activity of cytosolic and particulate-associated PKC. After a 3-day exposure to 95% O_2 , there was no significant change in cytosolic and particulate-associated PKC specific activity. When cells were exposed to 95% O_2 for 5 days, a 2-fold increase in both cytosolic and particulate-bound PKC specific activity was observed (Table 1). However, hyperoxia did not cause a redistribution of PKC from the cytosol to the membrane fraction. As a positive control, we examined the activation of PKC by phorbol esters. As expected, treatment of cells with 100 nM PdBu for 30 min caused a decrease in cytosolic

Figure 6 Gel mobility-shift assay using ORE1 oligonucleotide and nuclear proteins extracted from cells exposed to 21% O₂ (C) and 95% O₂ for 1, 2, 3 or 5 days (d)

The specificity of binding was determined by competition experiments using a 100-fold molar excess of unlabelled oligonucleotide.

PKC activity and an increase in PKC activity associated with the microsomal fraction. The variation in basal PKC specific activity observed in control cells might reflect the difference in the age of the cell cultures used for the PdBu (cells at confluence) and hyperoxia (cells at 3 and 5 days after confluence) experiments.

PdBu activates TRE whereas hyperoxia activates neither TRE nor ORE1

It is an established fact that activation of PKC by phorbol esters involves induction of AP-1 TRE-binding activity. On the other hand, ORE1, contained within the 5' flanking region of the GP gene, has been identified and found to be responsive to O₂ tension in culture. To determine whether the ORE1 and TRE found within the GP proximal promoter are likely to account for the O₂- and PdBu-induced up-regulation of GP expression, gel mobility-shift experiments were performed using nuclear extracts from HUVECs treated with O₂, PdBu or both. We first performed a time-course experiment. Cells were exposed to 95% O₂ for 24, 48, 72 or 120 h, or to 100 nM PdBu for 2, 4, 8, 12 or 24 h. As illustrated in Figure 6, the ORE1 probe demonstrated two shifted bands when incubated with nuclear extracts derived from

Figure 7 Gel mobility-shift assay using TRE oligonucleotide and nuclear proteins extracted from control cells and cells exposed to 100 nM PdBu for 2, 4, 8, 12 and 24 h

C, control cells; P, PdBu-treated cells.

Figure 8 Gel-mobility-shift assay using TRE oligonucleotide and nuclear proteins extracted from cells exposed to 21% O₂ (control) and 95% O₂ for 3 or 5 days (d) plus 100 nM PdBu added during the last 24 h

The specificity of binding was determined by competition experiments using a 100-fold molar excess of unlabelled TRE or ORE1 oligonucleotide.

Table 2 Effects of O₂ and PdBu on total cellular protein and DNA contents

Confluent cells in 35 mm gelatin-coated Petri dishes were exposed to 95% O₂ or 100 nM PdBu for 3 and 5 days, and then harvested for determination of total protein and DNA contents. Values are means $\pm$ S.E.M. for five experiments. *Significantly different from 21% O₂-exposed cells.

Treatment	Protein (μ g/Petri dish)	DNA (μ g/Petri dish)
21% O ₂ 3 days	170.1 $\pm$ 9.5	4.0 $\pm$ 0.3
95% O ₂ 3 days	136.1 $\pm$ 6.7*	3.1 $\pm$ 0.3*
100 nM PdBu 3 days	145.2 $\pm$ 8.5	3.8 $\pm$ 0.2
21% O ₂ 5 days	193.0 $\pm$ 4.4	4.5 $\pm$ 0.2
95% O ₂ 5 days	114.8 $\pm$ 6.0*	2.6 $\pm$ 0.2*
100 nM PdBu 5 days	149.8 $\pm$ 4.5*	4.9 $\pm$ 0.2

either normoxic or hyperoxic cells. However, neither complex was 'competed away' with the addition of a 100-fold molar excess of unlabelled ORE1. On the other hand, as shown in Figure 7, 100 nM PdBu rapidly induced the formation of AP-1-TRE complexes, with a maximum binding activity at 8 h. Figure 8 shows the effect of a 3- and 5-day exposure to 95% O₂, a 1-day treatment with 100 nM PdBu, and a combined treatment with O₂ and PdBu on binding activity of nuclear proteins to the TRE oligonucleotide. O₂ did not activate significantly the AP-1 DNA-binding activity, whereas a 2.4 $\pm$ 0.5-fold increase ($n = 7$; $P < 0.05$ compared with untreated cells) in protein-binding activity to the AP-1 site was observed using nuclear extracts from PdBu-treated cells under normoxic conditions. The addition of PdBu to O₂-exposed cells further augmented the induction of AP-1 TRE-binding activity compared with PdBu alone: 3.5 $\pm$ 0.9- and 5.6 $\pm$ 1.3-fold increase for O₂+PdBu at day 3 and 5 of exposure respectively ($n = 7$; $P < 0.05$ compared with PdBu-treated cells).

Effects of hyperoxia and PdBu on cell number and lysis

As shown in Table 2, there was a progressive loss of total cellular protein and DNA content after exposure to hyperoxia: a 3-day exposure to 95% O₂ resulted in a 17.0 $\pm$ 3.0% and a

$21.0 \pm 5.1\%$ decrease in total protein and DNA content ($n = 5$; $P < 0.05$ compared with normoxic cells) respectively; and a 5-day exposure to hyperoxia led to a $40.5 \pm 3.1\%$ and a $41.0 \pm 5.0\%$ loss of cell protein and DNA content respectively ($n = 5$; $P < 0.05$ compared with normoxic cells). For PdBu-treated cells, there was a $15.0 \pm 6.3\%$ and $22.4 \pm 1.8\%$ decrease in protein content after a 3- and 5-day treatment with PdBu, but no significant change in DNA content when compared with control cells. Exposure to O_2 did not significantly increase cell lysis, whereas PdBu was slightly toxic: the percentage of cellular lactate dehydrogenase released during a 5-day exposure to normoxia, hyperoxia and PdBu was $35.8 \pm 1.4\%$, $33.0 \pm 2.0\%$ and $42.9 \pm 2.2\%$ respectively. Thus the decrease in total protein and DNA content in the absence of cytolytic injury probably represents a loss of proliferative capacity of hyperoxic cells.

DISCUSSION

In this study, we sought to define the molecular events that lead to GP gene transcription in response to hyperoxia in HUVECs, and asked whether such induction is mimicked and sustained by activation of PKC by xenobiotics such as phorbol esters. Our results show that the increased GP expression after exposure to O_2 and PdBu occurs mainly through transcriptional up-regulation, and we provide indirect evidence that at least two separate signal-transduction pathways are involved in the regulation of GP by hyperoxia and phorbol esters.

The molecular mechanism(s) that promoted GP expression in hyperoxic cells did not appear to involve PKC and AP-1 activation, whereas phorbol ester affected GP gene expression through a process that could be ascribed to the TREs present in the 5' flanking region of the gene. Several lines of evidence support this notion. First, O_2 and PdBu each elicited a distinct temporal pattern of response in terms of steady-state GP mRNA levels. The effect of PdBu was also quantitatively less pronounced. Secondly, combined treatment with O_2 and PdBu resulted in additive effects on GP gene transcription and mRNA levels. This finding is highly suggestive that more than one signalling pathway exists. Thirdly, although the increased GP transcriptional activity after PdBu treatment was accompanied by activation of PKC and a rise in AP-1 DNA-binding activity, no difference in PKC translocation and AP1 TRE-binding activity could be detected in hyperoxic cells compared with normoxic cells.

A growing number of genes have been found to respond to phorbol esters. In many different cell types, treatment with PdBu or PMA leads to the activation of PKC and subsequently to the activation of AP-1-responsive gene expression [25]. AP-1 is a representative of a ubiquitous family of transcription factors comprised of homo- and hetero-dimers of individual members of the Jun- and Fos-related families of proteins. Any of these proteins can associate through leucine zipper domains to form Fos-Jun or Jun-Jun dimeric complex, which can bind to TRE. The GP gene proximal promoter region contains several AP-1-binding sites, suggesting potential regulation of this gene by the PKC signalling pathway. Our results indicate that this is indeed the case and further that the mechanism by which GP expression is activated by phorbol ester appears to involve the AP-1 transcription factors family. The increase in AP-1 DNA-binding activity was even greater in the case of combined treatment with PdBu and O_2 compared with PdBu treatment alone. This can be attributed to the increase in the number of binding sites for PdBu in hyperoxic cells. These data are thus consistent with those of earlier studies, which demonstrate that AP-1, like nuclear factor κB and antioxidant-responsive element, responds directly to the intracellular concentration of reactive O_2 species [26]. Indeed,

many different oxidative-stress-inducing stimuli, such as UV light or γ -rays and interleukin 1, activate AP-1. Low levels of H_2O_2 also activate this factor. In keeping with this notion, newly synthesized AP-1 binds DNA target sequences in many promoters, among them, interestingly, four enzymes known to be up-regulated by oxidants: haem oxygenase [27], Mn²⁺-dependent superoxide dismutase [28], NAD(P)H-quinone oxidoreductase [29] and glutathione S-transferase [30]. Thus so far, it appears that expression of genes encoding proteins involved in the antioxidant defence may be co-ordinately regulated by AP-1. The delayed increase in GP mRNA levels during PdBu treatment stands in contrast with the activation of PKC and AP-1-DNA interaction, which is a short-lived phenomenon. In this regard, several reports provide other examples of different mRNAs [31–33], including GP mRNA [34], the stimulated expression of which by phorbol esters reached maximum levels after 24–48 h of treatment. Perhaps the delay in mRNA induction reflects the time necessary for the cells to acquire the appropriate machinery for signal transduction of an AP-1 response, or even for the production of different transcription factors.

In contrast with phorbol ester treatment, there was no good temporal correlation between GP expression and PKC activation under hyperoxic conditions. In fact, hyperoxia failed to induce PKC translocation and to increase AP-1 DNA-binding activity, despite the apparent increase in both cytosolic and particulate-bound PKC specific activity, which correlated well with the increased number of PKC enzyme molecules. It is therefore unlikely that PKC and AP-1 are involved in the activation of GP expression by O_2 , although direct evidence supporting a causal relationship between these processes would require studies with PKC inhibitors. The delayed time course for the increase in GP mRNA during hyperoxia did not allow us to conduct such experiments. It may be questioned whether experiments conducted on cells exposed to hyperoxia for 3–5 days can confidently address any role played by PKC, the activation of which is a short-lived phenomenon followed by enzyme degradation [35]. However, down-regulation may not occur with physiological activators [36]. For example, activated PKC has been documented in the glomeruli of rats with 2 weeks of diabetes [36] and in bovine retinal endothelial cells exposed to high glucose for up to 10 days [37]. On the other hand, in our hands, the up-regulation of GP expression by O_2 could not be accounted for by the ORE1 core sequence, which has been previously shown to be responsive to hypoxia. In fact, we were unable to detect in nuclear extracts from hyperoxic cells any specific protein-DNA binding activity using ORE1. The reason for the differential effect of hypoxia and hyperoxia at the ORE1-protein interaction level remains unclear. It is reasonable to hypothesize that this interaction could be cell-type-specific.

The difference in the kinetics and magnitude of response in terms of steady-state GP mRNA levels might be expected to relate to the extent of cell injury elicited by O_2 and PdBu. Hyperoxia, and not PdBu, clearly interfered with cell proliferation, since a loss of total cellular protein and DNA content occurred in the absence of cytolytic injury. This conclusion is in agreement with our previous reports demonstrating that O_2 severely compromises DNA and protein synthesis in endothelial cells [38,39]. It is thus plausible that an oxidant-induced modification of cell cycle kinetics and/or inhibition of protein synthesis might activate GP gene expression. The mechanism of such an effect is of interest and requires further investigation. Our findings might also conceivably imply that all the surviving cells have entered a resistant stage by up-regulating their antioxidant defence.

In summary, we have indirect evidence that, although PdBu is

one of the potent inducers of GP expression, events other than activation of PKC may bridge high ambient O₂ concentration to changes in GP gene expression. An understanding of the regulation of GP gene expression by hyperoxia will require deletion mapping and footprinting of the GP promoter region experiments to identify the *cis*-acting elements as well as the corresponding *trans*-acting factors.

We thank G. Laverniaz and H. Petersen for excellent technical assistance. This research was supported by grant 31.37799.93 from the Swiss National Science Foundation.

REFERENCES

- 1 Akasaka, M., Mizoguchi, J. and Takahashi, K. (1990) *Nucleic Acids Res.* **18**, 4619
- 2 Takahashi, K., Akasaka, M., Yamamoto, J., Kobayashi, C., Mizoguchi, J. and Koyama, J. (1990) *J. Biochem. (Tokyo)* **108**, 145–148
- 3 Maiorino, M., Chu, F. F., Ursini, F., Davies, K. J. A., Doroshow, J. H. and Esworthy, R. S. (1991) *J. Biol. Chem.* **266**, 7728–7732
- 4 Flohe, L. (1989) in *Glutathione Chemical, Biochemical and Medical Aspects*, (Dolphin, D., Avramovic, O. and Poulson, R., eds.), pp. 644–731, John Wiley and Sons, New York
- 5 Spallholz, J. E. and Boylan, L. M. (1991) in *Peroxidases in Chemistry and Biology*, vol. 1, (Everse, J., Everse, K. E. and Grisham, M. B., eds.), pp. 259–291, CRC Press, Boca Raton, FL
- 6 Sukenaga, Y., Ishida, K., Takeda, T. and Takagi, K. (1987) *Nucleic Acids Res.* **15**, 7198
- 7 Mullenbach, G. T., Tabrizi, A., Irvine, B. D., Bell, G. I., Tainer, J. A. and Halliwell, R. A. (1988) *Protein Eng.* **2**, 239–246
- 8 Moscow, J. A., Morrow, C. S., He, R., Mullenbach, G. T. and Cowan, K. H. (1992) *J. Biol. Chem.* **267**, 5949–5958
- 9 Jornot, L. and Junod, A. F. (1992) *Am. J. Respir. Cell. Mol. Biol.* **6**, 107–115
- 10 Cowan, D. B., Weisel, R. D., Williams, W. G. and Mickle, D. A. (1992) *J. Mol. Cell. Cardiol.* **24**, 423–433
- 11 McBride, O. W., Mitchell, A., Lee, B. J., Mullenbach, G. and Hatfield, D. (1988) *Biofactors* **1**, 285–292
- 12 Chada, S., LeBeau, M. M., Casey, L. and Newburger, P. E. (1990) *Genomics* **6**, 268–271
- 13 Diamond, A. M., Cruz, R., Bensics, C. and Hatfield, D. (1992) *Gene* **122**, 377–380
- 14 Cowan, D. B., Weisel, R. D., Williams, W. G. and Mickle, D. A. (1993) *J. Biol. Chem.* **268**, 26904–26910
- 15 Abate, C., Patel, L., Rauscher, F. J. and Curran, T. (1990) *Science* **249**, 1157–1161
- 16 Xanthoudakis, S., Miao, G., Wang, F., Pan, Y. C. and Curran, T. (1992) *EMBO J.* **11**, 3323–3335
- 17 Gopalakrishna, R. and Anderson, W. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6758–6762
- 18 Palumbo, E. J., Sweatt, J. D., Chen, S. and Klann, E. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1439–1445
- 19 Kass, G. E., Duddy, S. K. and Orrenius, S. (1989) *Biochem. J.* **260**, 499–507
- 20 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 21 Jornot, L., Mirault, M. E. and Junod, A. F. (1991) *Am. J. Respir. Cell. Mol. Biol.* **5**, 265–275
- 22 Dignam, J. P., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- 23 Block, C., Freyermuth, S., Beyersmann, D. and Malviya, A. N. (1992) *J. Biol. Chem.* **267**, 19824–19828
- 24 Godson, C., Weiss, B. A. and Insel, P. A. (1990) *J. Biol. Chem.* **265**, 8369–8372
- 25 Rahmsdorf, H. J. and Herrlich, P. (1990) *Pharmacol. Ther.* **48**, 157–188
- 26 Pahl, H. L. and Baeuerle, P. A. (1994) *BioEssays* **16**, 497–502
- 27 Alam, J. and Zhining, D. (1992) *J. Biol. Chem.* **267**, 21894–21900
- 28 Fujii, J. and Tanuguchi, N. (1991) *J. Biol. Chem.* **266**, 23142–23146
- 29 Li, Y. and Jaiswal, A. K. (1994) *Eur. J. Biochem.* **226**, 31–39
- 30 Pinkus, R., Weiner, L. M. and Daniel, V. (1995) *Biochemistry* **34**, 81–88
- 31 Hu, Z., Shi, X., Sakaue, M. and Hoffman, B. B. (1993) *J. Biol. Chem.* **268**, 3610–3615
- 32 An, G., Tesfaijzi, J., Chuu, Y. and Wu, R. (1993) *J. Biol. Chem.* **268**, 10977–10982
- 33 Trejo, J., Massamiri, T., Deng, T., Dewji, N. N., Bayney, R. M. and Brown, J. H. (1994) *J. Biol. Chem.* **269**, 21682–21690
- 34 Shen, Q., Chada, S., Whitney, C. and Newburger, P. (1994) *Blood* **84**, 3902–3908
- 35 Ballester, R. and Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 15194–15199
- 36 Craven, P. A. and DeRubertis, F. R. (1989) *J. Clin. Invest.* **83**, 1667–1675
- 37 Lee, T. S., Saltzman, K. A., Ohashi, H. and King, G. L. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5141–5145
- 38 Clément, A., Hübscher, U. and Junod, A. F. (1985) *J. Appl. Physiol.* **59**, 1110–1116
- 39 Jornot, L., Mirault, M. E. and Junod, A. F. (1987) *J. Appl. Physiol.* **63**, 457–464