Induction of γ-glutamylcysteine synthetase by cigarette smoke is associated with AP-1 in human alveolar epithelial cells

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Abstract Increased levels of glutathione (GSH) occur in the epithelial lining fluid (ELF) of chronic cigarette smokers. Therefore we investigated the effect of cigarette smoke condensate solution (CSC) on GSH synthesis and the regulation of γ-glutamylcysteine synthetase (γGCS) in human type II alveolar epithelial cells (A549). CSC exposure increased GSH levels, γGCS activity and γGCS heavy subunit (HS) mRNA, as well as increasing DNA binding of the activator protein-1 (AP-1) and the human antioxidant response element (hARE). Transfection of deletion constructs of the γGCS-HS promoter in a chloramphenicol acetyl transferase (CAT) reporter system revealed that an hARE, present within promoter, is not required for the CSC mediated induction. We conclude that CSC induction of γGCS-HS expression is associated with AP-1/AP-1-like responsive elements.

Key words: Glutathione; γ-Glutamylcysteine synthetase; AP-1; Cigarette smoke; A549 cell

1. Introduction

The capacity of mammalian cells to maintain homeostasis of cellular functions during oxidative stress depends on the rapid induction of protective antioxidant enzymes [1–3]. Reduced glutathione (GSH), a ubiquitous cellular non-protein sulphydryl, functions to maintain cellular redox balance and is also involved in the detoxification of xenobiotics, electrophiles, organic peroxides and heavy metals, either through direct thiol conjugation or in enzyme-catalysed reactions [4]. We and others have demonstrated increased levels of GSH in association with an increased oxidant burden in the epithelial lining fluid (ELF) of healthy chronic smokers [5,6]. The mechanism of this increase in GSH is currently unknown.

Glutathione is synthesised from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalysed by γ-glutamylcysteine synthetase (γGCS) and GSH synthase [7]. γGCS is the rate-limiting step in de novo GSH synthesis and is inhibited by a feedback mechanism. In cells, the γGCS, holoenzyme exists as a dimer composed of heavy (γGCS-HS; 73 kDa) and light (γGCS-LS; 28 kDa) subunits [8]. The heavy subunit possesses all of the catalytic activity [9].

Cigarette smoke contains 10^{14}–10^{16} free radicals/puff and a host of highly electrophilic chemical compounds [10]. Recently, we have shown that cigarette smoke condensate solution (CSC) rapidly depletes intracellular GSH, by a mechanism involving the formation of GSH-conjugates in the human alveolar type II epithelial cell line, A549 [11]. However, this effect was reversed after the cells were cultured in fresh medium, with a significant increase in GSH concentrations 12 h after exposure. We hypothesised that GSH synthesis may be induced in alveolar epithelial cells as an adaptive response to CSC exposure by upregulation of γGCS-HS gene expression. These findings may be relevant to the enhanced GSH levels which are present in the ELF of chronic smokers [5,6].

Mulcahy and Jipp [12] and Yao et al. [13] recently reported that the promoter (5′-flanking) region of the human γGCS-HS gene contained a consensus fos/jun heterodimeric complex-activator protein (AP-1) sequence, AP-1-like binding sites; an human-antioxidant response element (hARE), and several SP-1 and AP-2 binding sites. However there is no information so far available about the characteristics of the γGCS-HS promoter region and the regulation of GSH synthesis.

In this study we hypothesised that CSC induced γGCS-HS gene expression in airspace epithelial cells, which may be related to the increase in the DNA binding of transcription factors to the promoter regions of γGCS-HS gene. We therefore investigated the effect of CSC on the regulation of γGCS-HS mRNA and the DNA binding activities of AP-1 and hARE, transcription factors in alveolar epithelial cells. We also for the first time, partially characterised the 5′-region of the γGCS-HS gene by creating deletion constructs and analysed the involvements of various transcription factors sites in the regulation of γGCS-HS promoter.

2. Materials and methods

Unless otherwise stated, all of the biochemical reagents used in this study were purchased from the Sigma Chemical Co., Poole, UK; cell culture media and molecular biology reagents from Gibco-BRL (Paisley, UK); and the gel shift assay kit from Promega, Southampton, UK.

2.1. A549 epithelial cells

The type II alveolar epithelial cell line, A549 (ECACC No. 86012804), which was mycoplasma free, was maintained in continuous culture at 37°C, 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM, Gibco) containing penicillin/streptomycin mixture, L-glutamine, sodium bicarbonate, and 10% fetal bovine serum (FBS) (Gibco).

2.2. Preparation of CSC

CSC (100%) was produced from standard cigarettes each containing 23 mg of tar and 2.2 mg of nicotine (University of Kentucky UK2R1). CSC was produced by blowing smoke, generated by a smoking machine using a vacuum syringe system, over 3 ml of phosphate buffered saline (PBS) in a siliconised glass tonometer (Vitalograph, Buckingham, UK). The smoke-generating machine delivers a 37 ml puff of whole cigarette smoke, including particulates, every minute to the tonometer which was rotated gently [14]. The solution was made up fresh on the morning of each experiment and CSC (100%) was filtered

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using 0.22 μm filter (Millipore S.A., Molsheim, France) to remove large particles and bacteria.

2.3. A549 epithelial cell exposure to CSC

Confluent monolayers of A549 cells were rinsed twice with DMEM and exposed to CSC (1, 5, 10% dilutions with PBS) for 4 h in 5 ml of DMEM with 10% FBS at 37°C, 5% CO_2. After incubation, the monolayers were washed with fresh medium and incubated for a further 12 or 24 h. The monolayers were then washed twice with cold sterile PBS (Ca^{2+} and Mg^{2+} free) (pH 7.4), trypsinised and used for the GSH, γGCS enzyme and mRNA assays. For gel shift assays, the cells were scraped into ice cold PBS and used for the preparation of nuclear extracts. In some experiments, monolayers were washed with PBS and exposed to 10% CSC with fresh medium with 10% FBS at 37°C in 5% CO_2, for an additional 12 h. The cells were then harvested and used for the GSH, mRNA and electrophoresis mobility shift assays (EMSA).

2.4. GSH, GSSG and γGCS activity assays

Acid extracts of A549 cells, were spun and the supernatant immediately used in the soluble GSH assay by the 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB-GSSG reductase recycling method described by Tietze [15]. For the GSSG assay, acid extract was treated with 2-vinylpyridine and triethanolamine as previously described [16], and the treated supernatant was used in the assay [15]. γGCS activity was measured by the method described by Seelig and Meister [17].

2.5. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared by the method of Staal et al. [18]. The oligonucleotides used were: **hARE**: 5′-ATC CAG TCA CAG TGA CTC AG-3′, 3′-TAG GTC AGT GTC ACT GAG TC-5′, synthesised by Oswel DNA Services, Edinburgh, UK; and **AP-1**: 5′-CGC TTG ATG AGT CAG CCG GAA-3′, 3′-CGC AAC TAC TCA GTC GGC CTT-5′, obtained from Promega. The hARE and AP-1 oligonucleotides were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and poly(dI-dC). poly(dI-dC) according to the manufacturers instructions (Promega). Binding reactions were carried out as per the manufacturers instructions using 4 μg of nuclear-extract protein for AP-1 and 15–20 μg nuclear-extract protein for hARE EMSA assays. The protein-DNA complexes were resolved on non-denaturing polyacrylamide gels. The gels were then vacuum dried and autoradiographed with an intensifying screen overnight at −80°C.

2.6. Isolation of RNA and reverse transcription

RNA was isolated from A549 cells by the acid-guanidine method described by Chomczynski and Sacchi [19]. Total RNA was reverse transcribed using superscript II according to the manufacturer's instructions (Gibco-BRL). The resultant cDNA was stored at −20°C, until required.

2.7. Primers and polymerase chain reaction (PCR)

To quantitate γGCS mRNA expression the reverse transcriptase-PCR assay was used. Oligonucleotide primers were chosen using the published sequence of γGCS-HS cDNA [20], and β-actin (Stratagene, Cambridge, UK). The primers for γGCS-HS were synthesised by Oswel DNA Services, University of Edinburgh, UK, with the sequences:The reverse transcribed mRNA mixture (5 μg) was added directly to the PCR mixture which consisted of buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$), 200 μM dNTPs and 20 pmoles of each primer, 1 U of Taq DNA polymerase in a final volume of 55 μl. Thirty-five cycles were repeated for the amplification of β-actin and γGCS-HS. Conditions for the thermal cycles were: **β-actin**: denatured at 94°C for 60 s, annealing at 60°C for 60 s, extension at 72°C for 60 s and final extension at 72°C for 10 s; **γGCS**: denatured at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 120 s and final extension 72°C for 5 min. 25 μl of the amplified PCR products was applied to a 2% agarose gel containing ethidium bromide and electrophoresed in 0.5×Tris-borate-EDTA buffer. Bands were visualised by uv transilluminator and photograph negatives were scanned using a LKB-ultrascan XL enhanced laser densitometer. The relative levels of the γGCS-HS mRNA were estimated by comparison with the intensity of the β-actin bands and expressed as a percentage. The positive control for γGCS-HS was the pKS-hGCS plasmid (American type culture collection, Rockville, MD, Cat no. 79023). 50 fg was used for each reaction to check the specificity of the PCR. The γGCS-HS PCR product (531 bp) was checked by digestion with the restriction endonuclease *Bsp*HI (New England Biolabs) which produced in two fragments 314 bp and 217 bp in size, which is in agreement with γGCS-HS cDNA sequence [20]. Serial dilutions (1:10, 1:100) of first strand cDNAs of γ-GCS-HS and β-actin were used to check the specificity and sensitivity of RT-PCR assay.

2.8. Actinomycin D and cycloheximide treatments

Confluent cells were treated with 10% CSC together with 0.05 μg/ml actinomycin D or 0.5 μg/ml cycloheximide in culture medium for 4 h.

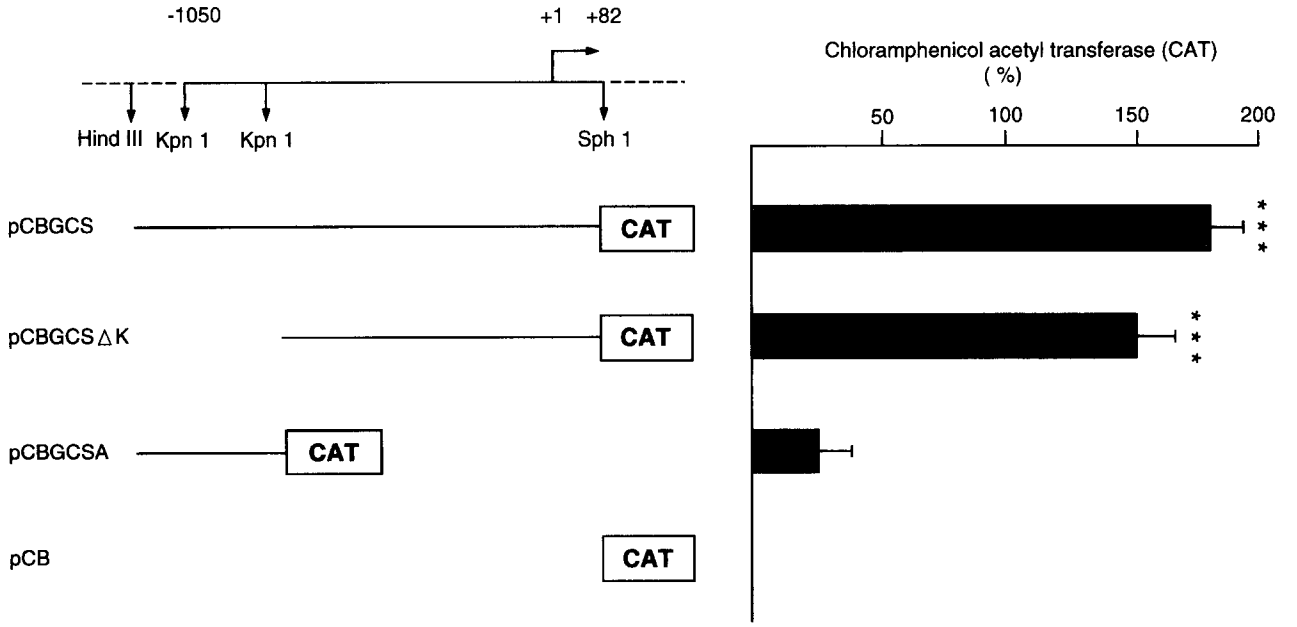

Fig. 1. Transient expression analysis of the human γGCS-HS gene. The line diagram at the top is a restriction map of the promoter region cloned in pCRII vector. Dotted line on the left indicates an additional 50 bp from multiple cloning sites of pCRII vector. Transcriptional start site is indicated by the bent arrow. The structure of the γGCS-CAT plasmids are showed on the left. Deletion mutants were ligated upstream of the CAT gene in pCAT Basic vector (pCB), transfected into A549 cells and exposed to CSC. After 24 h incubation the cells were harvested and assayed for CAT activity. The activities of the different constructs are expressed as means ± S.E.M. of triplicate transfection experiments, each performed in duplicates with the activity of pCBGCS set at 100%. ***$P < 0.001$ compared to pCBGCS.

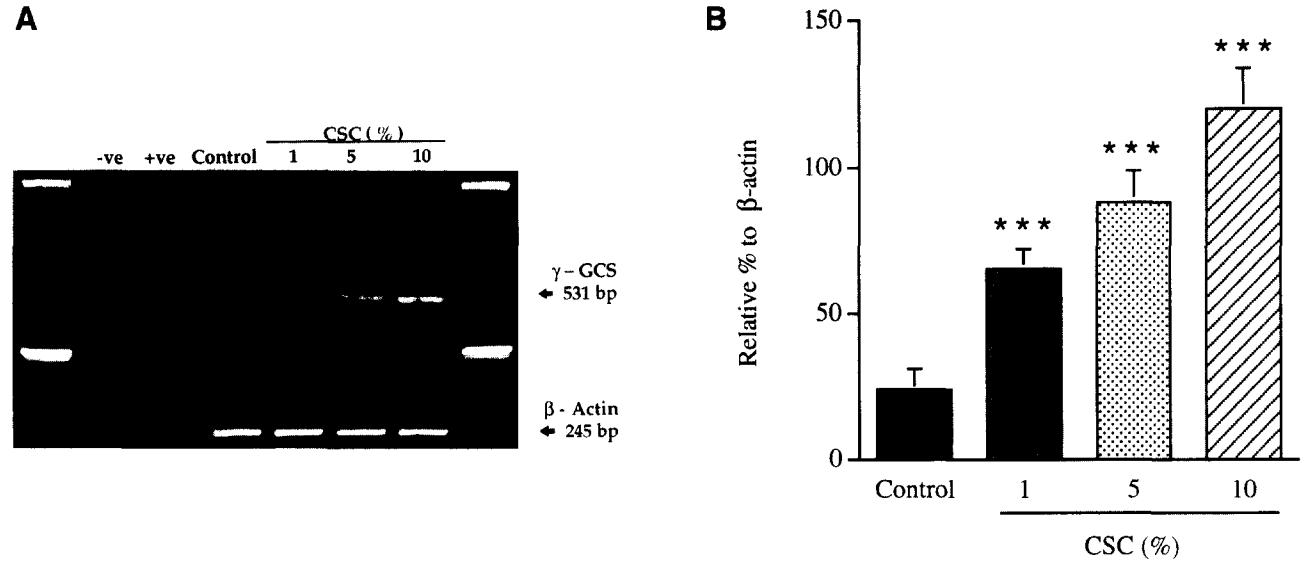

Fig. 2. Effect of cigarette smoke condensate solution (CSC) on γGCS-HS mRNA expression in A549 cells. Total RNA was isolated from control cells and cells exposed to different concentrations of CSC for (A) 24 h. RNA was reversed transcribed and used for PCR analysis of γGCS-HS mRNA. (B) The gel was scanned on a LKB ultrascan enhanced laser densitometer and the numeric estimates of γGCS-HS mRNA levels compared with the subsequent β-actin bands from the same sample. The relative percentages of γGCS-HS mRNA to β-actin were expressed as the mean ± S.E.M. of six experiments each performed in duplicate. $^{***}P < 0.001$ compared to control (C) values.

The cells were then washed as before and re-incubated with actinomycin D or cycloheximide alone, in fresh culture medium for an additional 12 h.

2.9. Promoter deletion constructs

The γGCS-HS promoter was isolated by PCR from human genomic DNA using the upstream oligonucleotide 5′- (+82) GGCGACATCCAATATGAAGGCTGTG-3′ and downstream oligonucleotides 5′-(−1050) TTCCTACTTGTGACCAAAACCTGCG-3′. The resulting promoter fragment (−1050 to +82 bp) was cloned into pCRII cloning vector (Invitrogen) and a *Hin*dIII and *Sph*I fragment (1138 bp) containing the promoter was isolated and subcloned into polylinker of the promoterless plasmid pCAT Basic vector (Promega) which was named as pCBGCS. Deletion fragments of the γGCS-HS promoter were generated using *Kpn*I restriction site present within the γGCS-HS promoter which resulted in the short fragment containing hARE (−1050 to −818 bp) (pCBGCSA) and remaining large fragment (−817 to +45 bp) (pCBGCSΔK) containing AP-1 and AP-1-like sites (Fig. 1). Both the fragments were subcloned into pCAT Basic vector.

2.10. Transient transfection and CAT assay

A549 cells ($\sim 0.8 \times 10^6$) per well were seeded into 6-well tissue culture plates and cultured at 37°C until the cell density reached ~60–70% confluence. Plasmid DNA transfections were performed using lipofectAMINE reagent (Gibco), according to the manufacturer's instructions. After CSC treatments, the cell extracts were prepared and assayed for protein content using BCA reagent (Pierce, Rockford, IL, USA). Chloramphenicol acetyl transferase (CAT) activity was quantitated by the CAT enzyme-linked immunosorbent assay (ELISA). β-galactosidase expression plasmid (PSVgal, Promega) was cotransfected as an internal control to normalise the transfection efficiency. In all transfection experiments, pCAT-Basic, pCAT-Control were used as negative and positive controls, respectively.

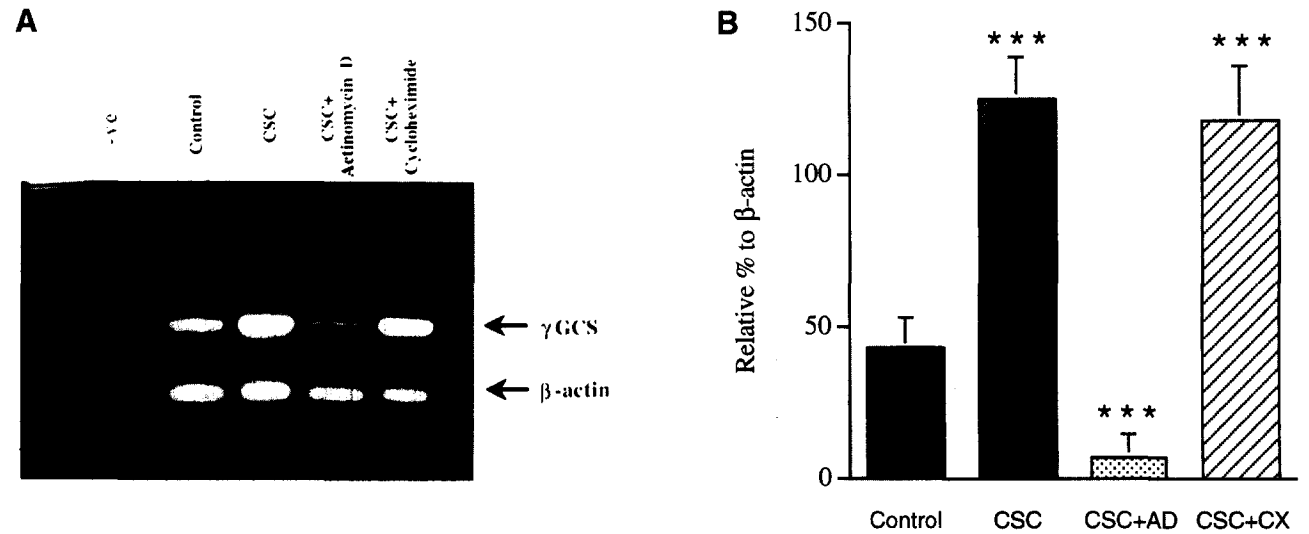

Fig. 3. Transcriptional regulation of γGCS-HS mRNA in A549 cells. (A) Effects of actinomycin D and cycloheximide on CSC-induced γGCS-HS mRNA expression. (B) Densitometric quantitation of γGCS-HS mRNA compared to β-actin mRNA. The data expressed as the mean ± S.E.M. of triplicate experiments each performed in duplicate. $^{***}P < 0.001$ compared to control (C) values.

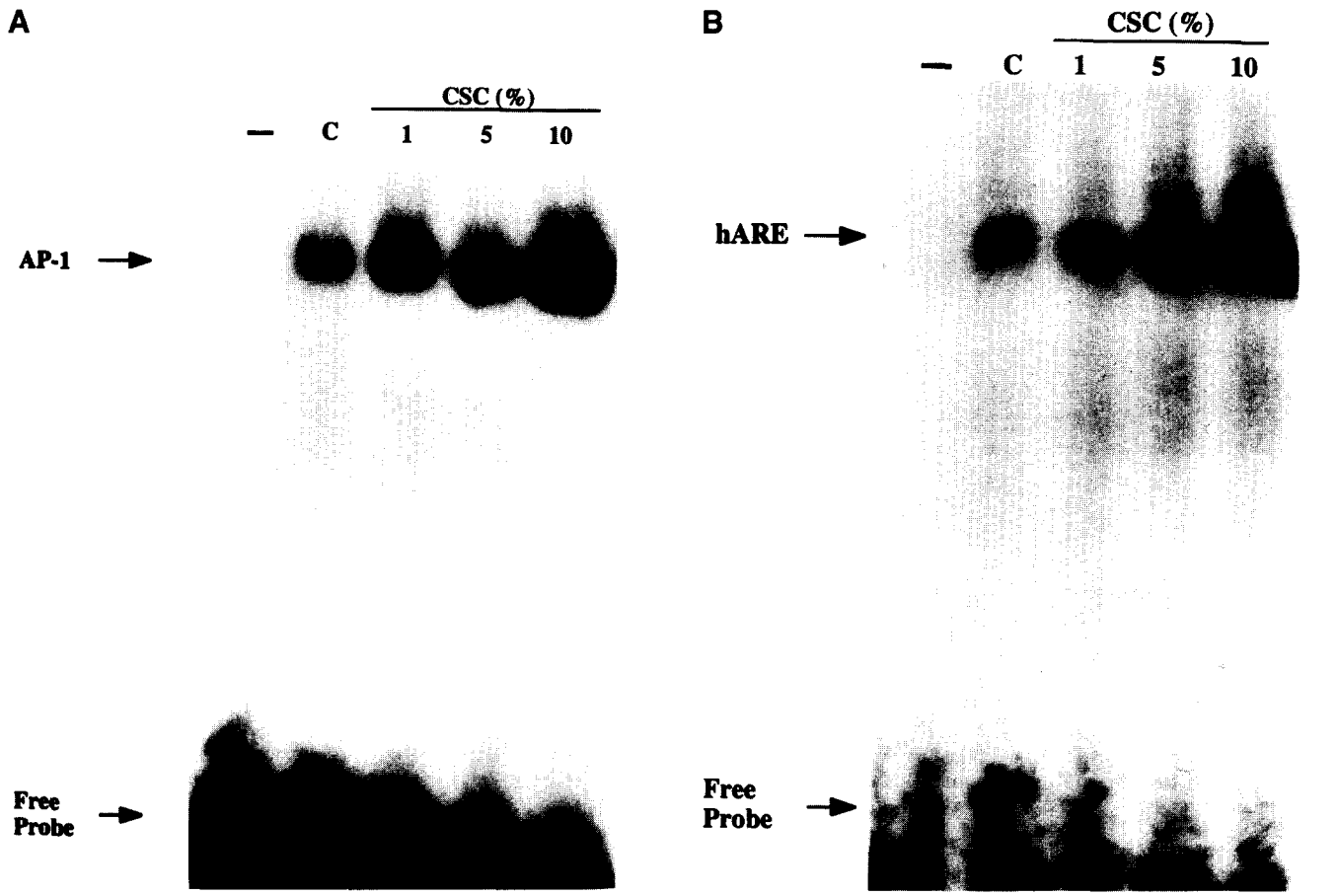

Fig. 4. Effect of CSC solution exposure on the induction of AP-1 and hARE binding activities in A549 cells. Nuclear extracts were prepared and analysed by the electrophoretic mobility shift assay. (A) AP-1, and (B) hARE binding activities in nuclear extracts from A549 cells untreated or exposed to CSC performed in quadriplicate. Nuclear extracts from HeLa cells were used as a positive control.

2.11. Statistical analysis

Results were expressed as mean (S.E.M.). Differences between values were compared by Duncan's multiple range test. Statistical analyses were performed using SPSS/PC$^+$ 4.0 package.

3. Results and discussion

We have previously shown that exposure of A549 cells to CSC produced a dose- and time-dependent depletion of intracellular GSH [11]. This was not due to oxidation of GSH or protein thiolation, but was associated with a decrease in γGCS activity and increased GSH-conjugate formation [11]. In the present study we show that after this initial depletion of GSH by CSC, prolonged re-culture (i.e. 24 h) results in an increase in GSH levels (control 15.6 ± 0.4, 10%; CSC 21.7 ± 1.4 nmoles/10^6 cells, $P < 0.05$, $n = 6$) with no change in GSSG concentrations (control 1.0 ± 0.3, 10%; CSC 1.2 ± 0.4 nmoles/10^{16} cells, P = NS, $n = 6$) and a concomitant increase in γGCS activity (control 0.24 ± 0.04, 10%; CSC 0.36 ± 0.06 U/mg protein, $P < 0.01$, $n = 6$). We show that these events are associated with a dose-dependent increase in the relative level of γGCS-HS mRNA (Fig. 2A,B) and that this induction occurs at the transcriptional level, since co-incubation with actinomycin D but not cycloheximide eliminated the increase of γGCS-HS mRNA (Fig. 3A,B). GSH levels have been shown to be twofold higher in the ELF [5,6] and erythrocytes [21] of chronic cigarette smokers so induction of γGCS-HS mRNA by CSC in epithelial cells is a possible mechanism for this elevation of GSH since intracellular GSH is exported from alveolar epithelial cells [22,23]. CSC also induces DNA-binding of the redox sensitive transcription factors AP-1 (fos/jun heterodimeric complex) and hARE in A549 cells (Fig. 4A,B). Response elements for these transcription factors, together with others such as AP-2 and SP-1, are present in the proximal 5′-promoter region of γGCS-HS [12,13], therefore our observation of induction of γGCS-HS mRNA may be related to the increased AP-1 and hARE binding activities.

γGCS-HS has been shown to be induced by various redox-regulating agents [24–27] but the precise stimulus for the induction of γGCS-HS mRNA following CSC exposure is not clear. Aqueous solution of CSC contains highly electrophilic compounds capable of generating H_2O_2 [28] and heavy metals, such as cadmium [10], which may modulate the AP-1 transcription factor [29,30]. In support of this, induction of the mRNAs of the AP-1 components (i.e. c-fos and c-jun) has recently been described in Swiss 3T3 cells exposed to CSC [31] and in tracheobronchial epithelial cells [32]. Induction of AP-1 may explain the upregulation of γGCS-HS mRNA in alveolar epithelial cells. This is further substantiated by γGCS-HS promoter deletion studies. The full proximal promoter (−1050 to +82 bp) linked to the CAT reporter system (pCBGCS) produced increased CAT activity on exposure to CSC. However, deletion of a 232 bp fragment containing the hARE responsive element, did not affect this induction (Fig. 1). This sug-

gests that this hARE is not involved in the regulation of γGCS-HS activity in response to CSC. Rather, downstream sequences, i.e. −817 to +1 bp containing AP-1/AP-1-like responsive elements are required. This is in good agreement with an earlier indirect observation that c-jun/c-jun homodimers (AP-1) regulate γGCS-HS gene expression induced by cisplatin [13] and cadmium [33]. However, there may be another hARE element present further upstream in the γGCS-HS gene which could affect enzyme induction. Further studies are in progress to characterise and identify other response elements as well as the specific region of γGCS-HS promoter which regulate basal γGCS-HS gene expression.

In summary, we have shown a dose-dependent elevation in steady state level of mRNA for the γGCS-HS in A549 cells in response to CSC. Analysis of γGCS-HS promoter revealed involvement of AP-1/AP-1-like responsive elements and not hARE in the regulation of γGCS activity. These observations provide a mechanism for the redox regulation of γGCS-HS mRNA at the transcription level, and may explain the increased GSH levels found in epithelial lining fluid of chronic cigarette smokers.

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