Apocynin increases glutathione synthesis and activates AP-1 in alveolar epithelial cells

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Abstract Apocynin (4-hydroxy-3-methoxy-acetophenone) is a potent intracellular inhibitor of superoxide anion production in neutrophils. In this study, we studied the effect of apocynin on the regulation of the antioxidant glutathione (GSH) and activation of the transcription factor AP-1 in human alveolar epithelial cells (A549). Apocynin enhanced intracellular GSH by increasing γ-glutamylcysteine synthetase activity in A549 cells. Apocynin also increased the expression of γ-GCS heavy subunit mRNA. This was associated with increased AP-1 DNA binding as measured by the electrophoretic mobility shift assay. These data indicate that apocynin displays antioxidant properties, in part, by increasing glutathione synthesis through activation of AP-1.

Key words: Glutathione; Antioxidant; Apocynin; Activator protein-1; A549

1. Introduction

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of root extracts of the medicinal herb *Picroria kurroa* which grows in the Himalayan mountains [1]. Apocynin is known to be a potent intracellular inhibitor of superoxide anion ($O_2^{-\bullet}$) production by activated neutrophils and eosinophils [2,3]. It also protects secretory leukocyte protease inhibitor (SLPI) from oxidative inactivation [3]. Furthermore, apocynin improves the efficacy of SLPI in lipopolysaccharide-induced emphysema in hamsters [2]. Apocynin possesses anti-inflammatory properties and can decrease the adhesion of monocytic U937 cells to human umbilical vein endothelial cells (HUVEC) induced by tumour necrosis factor-α (TNF-α) through suppression of the adhesion molecule VCAM-1 [4]. It also decreases collagen-induced plasma interleukin-6 levels in rats [5]. These effects may be mediated by a transcriptional mechanism involving transcription factors such as activator protein-1 (AP-1). Furthermore, recent study has indicated that apocynin inhibits endotoxin-induced bronchial hyperresponsiveness to substance P and plasma levels of lipid peroxides in guinea pigs [6].

The tripeptide glutathione (GSH) or L-γ-glutamyl-L-cysteinylglycine is a ubiquitous cellular non-protein sulphydryl which has an important role in maintaining intracellular redox balance and is involved in the detoxification of peroxides, free radicals, heavy metals and xenobiotics [7].

GSH synthesis is controlled by two rate-limiting factors. One is the enzyme γ-glutamylcysteine synthetase (γ-GCS), which catalyses the first reaction of de novo GSH synthesis. The other is the availability of cysteine [7], a breakdown product of extracellular GSH by the membrane-bound enzyme γ-glutamyltranspeptidase (γ-GT) [8]. We have recently shown that GSH synthesis is induced in cells exposed to oxidative stress [9,10], as an adaptive defence response. We have also recently demonstrated that a putative AP-1 transcription factor binding site is necessary for the regulation of the catalytic γ-GCS-HS gene promoter [11,12].

In this study, we hypothesise that the antioxidant properties of apocynin may result from an increase in the GSH synthesis in human alveolar epithelial cells. Therefore, we studied the levels of GSH, γ-GCS and γ-GT activities and γ-GCS-HS mRNA expression in response to apocynin in a human type II alveolar epithelial cell line (A549). We also investigated the effects of apocynin on the DNA binding of AP-1 in these cells.

2. Materials and methods

Unless otherwise stated, all of the biochemical reagents used in this study were purchased from Sigma Chemical Co. (Poole, UK); cell culture media were purchased from Gibco-BRL (Paisley, UK). Apocynin was purchased from Aldrich Chemical Co. (Dorset, UK).

2.1. Epithelial cell exposure to apocynin

The human type II alveolar epithelial cell line, A549 (ECACC No. 86012804), was maintained in continuous culture at 37°C, 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM) containing L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 μg/ml) and 10% foetal bovine serum (FBS).

Monolayers of confluent A549 epithelial cells were prepared by seeding 1×10^6 cells/well in a 6-well plate and reculturing in DMEM with 10% FBS at 37°C, 5% CO_2 for 24 h. Confluent monolayers were rinsed twice with DMEM and exposed to apocynin (100 or 500 μM) for 1 and 24 h in 2 ml of full medium at 37°C, 5% CO_2. Thereafter, the monolayers were washed twice with cold PBS, detached with trypsin-EDTA (0.05% w/v) solution and washed with full DMEM at $250\times g$ for 5 min, after which the cells were resuspended in 1 ml PBS. Cell viability was determined by trypan blue exclusion.

2.2. GSH and GSSG assays

The epithelial cells were centrifuged at $250\times g$ for 5 min at 4°C, and the cell pellets were resuspended in 1 ml of cold 0.6% (w/v) sulphosalicylic acid and 0.1% (v/v) Triton X-100 in PBS, sonicated on ice, homogenised with a Teflon pestle, and vortexed vigorously. The cells were then centrifuged at $4000\times g$ for 10 min at 4°C. The supernatant was immediately stored at −20°C or used immediately in the soluble GSH assay by the 5,5′,dithiobis-(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling method described by Tietze [13]. In the GSSG assay, supernatant was treated with 2-vinylpyridine and triethanolamine as previously described [14], and thereafter was used in the assay for GSH as described above.

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2.3. γ-GCS and γ-GT assays

Cell extracts for γ-GCS activity were prepared 1 or 24 h after apocynin exposure. Cultured cells were washed with ice-cold PBS, scraped into PBS and collected by centrifugation at 250×*g* for 5 min at 4°C. After two additional washes with PBS, the cells were resuspended in 100 mM potassium phosphate buffer, pH 7.4, sonicated and homogenised using a Teflon pestle on ice with Triton X-100, to a final concentration of 0.1% (v/v). The extracts were spun at 13 000×*g* for 15 min at 4°C. The supernatants were recovered and then stored at 4°C. Protein concentrations were determined using the bicinchoninic acid reagent assay (Pierce, Chester, UK).

γ-GCS activity was assayed by the method described by Seelig and Meister [15] using a coupled assay with pyruvate kinase and lactate dehydrogenase. The rate of decrease in absorbance at 340 nm was followed at 37°C. Enzyme specific activity was defined as μmol of NADH oxidised/min/mg protein, which is equal to 1 IU.

In the γ-GT activity assay, whole cells were suspended in 100 mM Tris-HCl buffer. γ-GT activity was assayed with the method of Tate and Meister [16] using 0.1 M glycyl-glycine and 5 mM L-γ-glutamyl-*p*-nitroanilidine. The rate of formation of *p*-nitroaniline was recorded as the change in absorbance at 410 nm, 37°C for 1 min. Purified γ-GT was used as a standard, and 1 IU of γ-GT was defined as the amount in μmol of *p*-nitroaniline released from L-γ-glutamyl-*p*-nitroaniline/min/mg protein.

2.4. Isolation of RNA and reverse transcription

RNA was isolated from A549 cells using the Trizol reagent (Life Technologies, Paisley, UK). Total RNA was reverse transcribed according to the manufacturer's instructions (Life Technologies, cat. no. 8025SA). The resultant cDNA was stored at −20°C until required.

2.5. Analysis of γ-GCS-HS mRNA by the polymerase chain reaction (PCR)

Oligonucleotide primers were chosen using the published sequence of human γ-GCS-HS cDNA [17] and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18] (Stratagene, Cambridge, UK). The primers for γ-GCS-HS were synthesised by Oswel DNA Services, University of Southampton, UK [11,12,19]. The sequences of the primers used in the PCR were as follows: γ-GCS-HS (sense 5′-GTG GTA CTG CTC ACC AGA GTG ATC CT-3′) and (anti-sense 5′-TGA TCC AAG TAA CTC TGG ACA TTC ACA-3′); GAPDH (sense 5′-CC ACC CAT GGC AAA TTC CAT GGC A-3′) and (anti-sense 5′-TC TAG ACG GCA GGT CAG GTC AAC C-3′). 5 μl of the reverse transcribed mixture (cDNA) was added directly to the PCR mixture and used for the PCR reactions, which we have previously described [9,10,19]. Bands were visualised by a UV transilluminator and photograph negatives were scanned using a white/ultraviolet transilluminator, UVP (Orme Technologies, Cambridge, UK). The relative levels of the γ-GCS-HS mRNA (531 bp) were normalised as a percentage of the intensity of the GAPDH bands (600 bp). The pKS-hGCS plasmid (American Type Culture Collection, ATCC, Rockville, MD, USA., cat. no. 79023) was used as a positive control for γ-GCS-HS. 50 fg was used for each experiment to check the specificity of the PCR.

2.6. Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared by the method of Staal et al. [20]. The consensus oligonucleotides used were commercial AP-1-containing sequences (5′-CGC TTG ATG AGT CAG CCG GA-3′, 3′-CGC AAC TAC TCA GTC GGC CTT-5′), which were obtained from Promega. The oligonucleotides were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and [γ-^{32}P]ATP (Promega). Binding reactions were carried out using 5 μg of nuclear extract protein and 0.25 mg/ml poly(dI-dC).poly(dI.dC) (Pharmacia Biotech, St. Albans, UK), in a 20 μl binding buffer (Promega). The protein-DNA complexes were resolved on 6% non-denaturing polyacrylamide gels at 100 V for 3–4 h. The gels were then vacuum dried and autoradiographed overnight with an intensifying screen at −80°C. The gel was scanned and bands analysed on a white/ultraviolet transilluminator UVP densitometry (Orme Technologies, Cambridge, UK).

2.7. Statistical analysis

The data expressed as means ± S.E.M. Differences between values were compared by ANOVA.

Table 1
Effects of apocynin on γ-GT activity in A549 alveolar epithelial cells

Treatment	γ-GT activity (U/mg protein)	
	1 h	24 h
Control	0.09 ± 0.01	0.09 ± 0.01
Apocynin 100 μM	0.09 ± 0.02	0.11 ± 0.02
Apocynin 500 μM	0.10 ± 0.03	0.09 ± 0.02

A549 epithelial monolayers were treated with apocynin (100, 500 μM) for 1 and 24 h. Data are expressed as mean ± S.E.M. (n=3). There are no significant differences compared with control values.

3. Results

3.1. Effect of apocynin on GSH and GSSG levels in alveolar epithelial cells

Exposure to 100 and 500 μM of apocynin significantly increased GSH levels after 24 h in A549 epithelial cells by 71% and 111%, respectively (Fig. 1). A similar increase was observed after 1 h of treatment with both concentrations of apocynin. GSSG levels were not affected by either 100 or 500 μM apocynin treatment for 1 h (1.74 ± 1.16, 2.05 ± 1.51 nmol/10^6 cells, n = 4) and 24 h (1.81 ± 0.61, 2.08 ± 0.84 nmol/10^6 cells, n = 4), respectively, in A549 cells, compared to control values (1 h 0.88 ± 0.36 and 24 h 1.17 ± 0.73 nmol/10^6 cells, n = 4). Cell viability remained >95% after all of the above treatments.

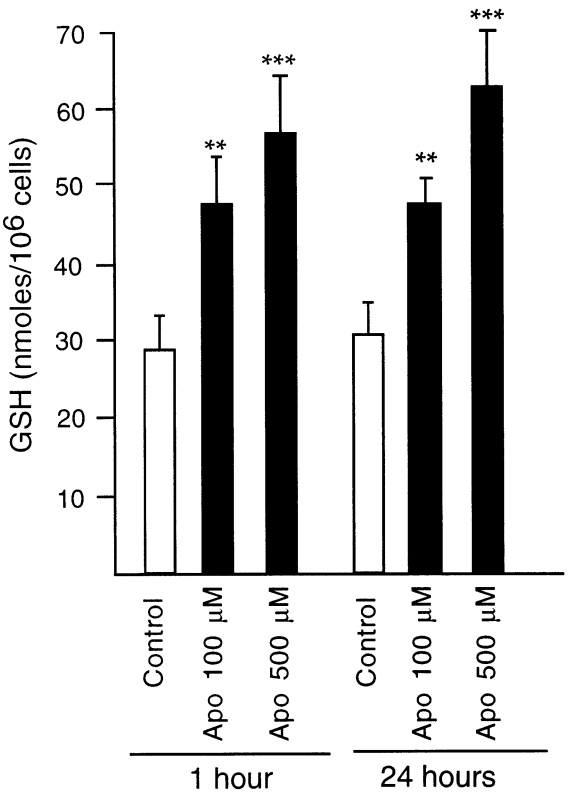

Fig. 1. Effect of apocynin on GSH levels in A549 type II alveolar epithelial cells. A549 epithelial cells were incubated with 100 and 500 μM apocynin for 1 and 24 h. Each histogram represents the mean and the bars the S.E.M. of 3–5 experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control values.

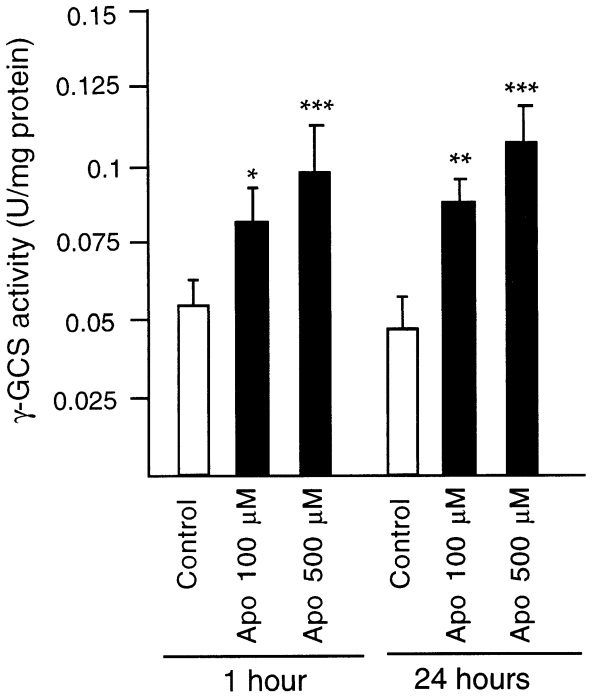

Fig. 2. Effect of apocynin on γ-GCS enzyme activity in A549 alveolar epithelial cells. A549 epithelial cells were incubated with 100 or 500 μM apocycin for 1 and 24 h. The histograms represent the means and the bars the S.E.M. of 3–5 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control values.

3.2. *Effect of apocynin on γ-GCS and γ-GT activities in alveolar epithelial cells*

γ-GCS activity was significantly elevated by 81% and 124% by apocynin 100 and 500 μM treatment, respectively, at 24 h in A549 cells, compared to control values (Fig. 2). A similar increase was observed after 1 h of treatment with both concentrations of apocynin. The activity of γ-GT was not affected by either 100 or 500 μM apocynin treatment for 1 and 24 h (Table 1).

3.3. *Effect of apocynin on γ-GCS-HS mRNA expression*

We investigated the mechanism of the effect of apocynin on GSH level and γ-GCS activity. Apocynin (100, 500 μM) exposure significantly increased γ-GCS-HS mRNA expression after 24 h without any significant increase at 1 h, compared to GAPDH gene expression (Fig. 3A,B). The level of γ-GCS-HS expression was increased by 142%, 183% by apocynin 100 and 500 μM treatment, respectively, at 24 h in A549 cells.

3.4. *Role of AP-1 in the apocynin-mediated regulation of γ-GCS-HS*

To determine if AP-1 plays an important role in apocynin-mediated γ-GCS-HS gene regulation, nuclear proteins were isolated after 1 h of treatment with apocynin, and were incubated with the DNA probe containing an AP-1 site. We found that apocynin exposure increased AP-1 DNA binding activity using this probe at 1 h exposure (Fig. 4A,B), in A549 cells, compared to untreated cells. The specificity of the binding was checked using 100-fold excess unlabeled AP-1 oligonucleotides, and non-specific oligonucleotides for NF-κB (data not shown).

4. Discussion

Apocynin is a potent inhibitor of membrane-bound NADPH oxidase and therefore it inhibits the production of superoxide and hydrogen peroxide from stimulated neutro-

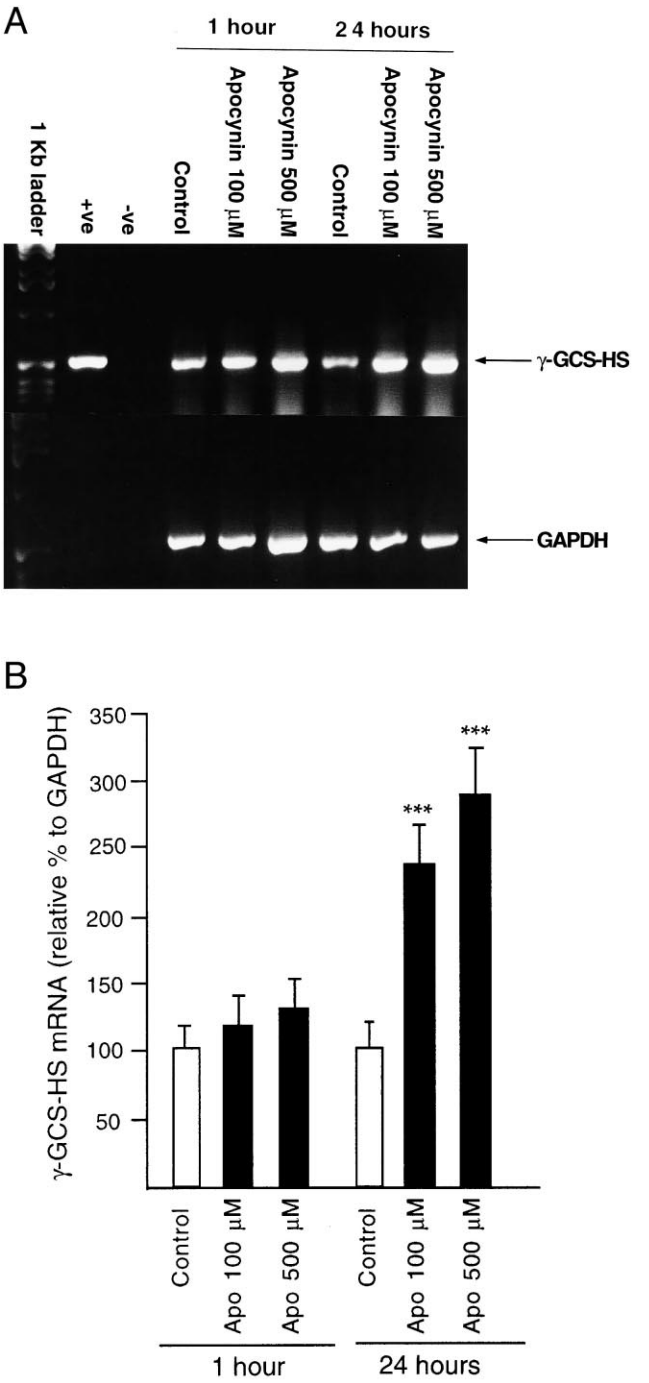

Fig. 3. Effect of apocynin on γ-GCS-HS mRNA expression in A549 alveolar epithelial cells. Total RNA was isolated from control cells and cells exposed to apocynin for 1 and 24 h. A: RNA was reverse transcribed and used for PCR analysis of γ-GCS-HS mRNA as described in Section 2. B: The numeric estimates of γ-GCS-HS mRNA levels compared with the subsequent GAPDH bands from the same sample. The histograms represent the relative intensities of γ-GCS-HS mRNA to GAPDH bands, which were expressed as the mean ± S.E.M. of three experiments each performed in duplicate. $^{***}P < 0.001$ compared with control levels.

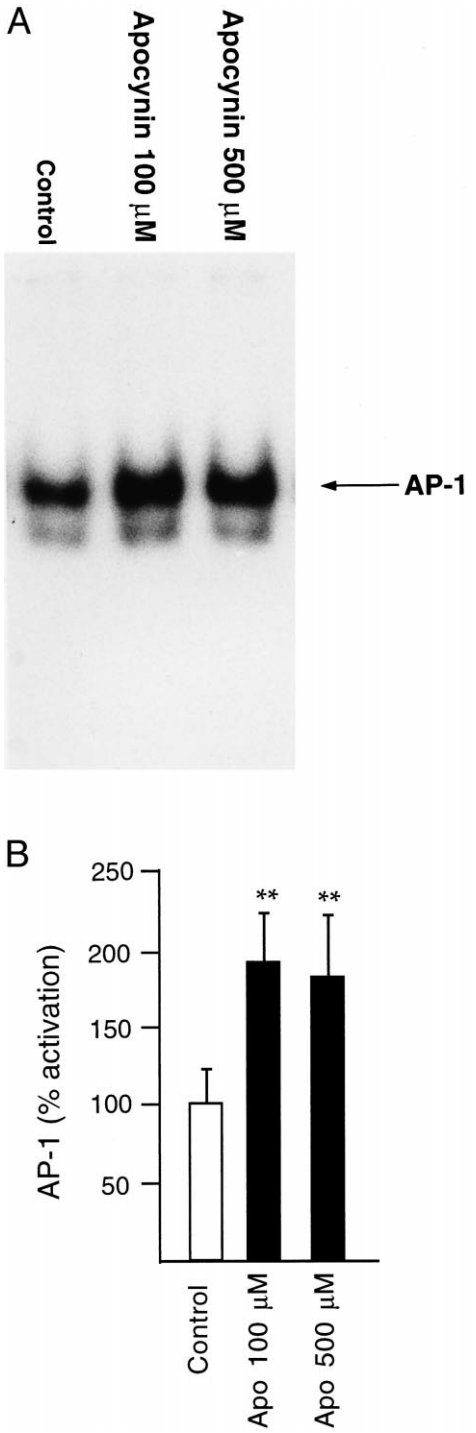

Fig. 4. Effect of apocynin on the regulation of AP-1 binding activity in A549 alveolar epithelial cells. A549 cells were treated with apocynin (100–500 μM) at the times indicated. A: Nuclear extracts were prepared and analysed by the electrophoretic mobility shift assay (EMSA) using ^{32}P-labeled synthetic double-stranded oligonucleotides containing AP-1. The DNA-protein complexes formed are indicated (arrow) for AP-1. B: The numeric estimates of DNA binding levels were compared with the control value set at 100%. The histograms are expressed as the means and the bars the S.E.M. of three experiments. $^{**}P < 0.01$, compared with control values.

phils [3,21]. The therapeutic potential of apocynin (10 mg/kg) in the lungs has been demonstrated in vivo in which apocynin improved the efficacy of elastase inhibitors to prevent the development of experimental emphysema in animal models, possibly by increasing the antioxidant potential of the lungs [2]. In this study, we show that apocynin concentrations of 100 and 500 μM, which were easily achievable in in vivo studies [2], increase the synthesis of the antioxidant GSH in alveolar epithelial cells without altering GSSG levels suggesting that apocynin itself does not cause oxidative stress. One mechanism we considered to account for the elevation in GSH was an increase in γ-GCS activity, the rate-limiting enzyme for GSH biosynthesis. The results of this study demonstrated that the increase in GSH levels was associated with an increase in γ-GCS activity in A549 epithelial cells.

Although the enzyme γ-GT has been considered to be important in maintaining intracellular GSH, we found that apocynin had no effect on γ-GT activity in epithelial cells. Thus our data suggest that γ-GT may not be involved in apocynin-mediated GSH synthesis in alveolar epithelial cells. Indeed we have previously shown that the increase in GSH levels in epithelial cells is mainly due to transcriptional upregulation of γ-GCS-HS mRNA in response to TNF-α and oxidative stresses [10–12,22].

The levels of GSH and γ-GCS activity are regulated by the expression of the γ-GCS-HS mRNA [23]. Recently, Rahman et al. and others have demonstrated that phenolic antioxidants, oxidants and chemotherapeutic agents can increase GSH concentrations by induction of γ-GCS-HS expression in various cell lines [9–12,24–27]. In this study, we show, for the first time, that in human alveolar epithelial cells, γ-GCS-HS gene expression is induced by apocynin. Similar increases in GSH levels have been shown in bronchoalveolar lavage fluid in rats treated with apocynin, which protected their lungs against the deleterious effects of ozone [28]. In addition, Chander et al. reported that the herb *Picroria kurroa* (apocynin) protected against *Plasmodium berghei*-infected *Mastomys natalensis* by a mechanism involving activation of GSH synthesis in the liver [29]. The mechanism of this antioxidant protection was not investigated.

We have recently shown the critical role of an AP-1 site in the regulation of the γ-GCS-HS [11,12]. To understand the molecular mechanism of the transcriptional induction of glutathione and γ-GCS-HS in response to apocynin, we studied the activation of putative transcription factors AP-1 in A549 epithelial cells in response to apocynin. Activation of AP-1 is known to be regulated by a variety of pro- and anti-oxidant agents including quinones and acetophenones/catechols [30]. Exposure of alveolar epithelial cells to apocynin produced a significant increase in the DNA binding activity of nuclear protein, using AP-1 as consensus probe. Thus AP-1 transcription factor is activated by apocynin treatment.

In conclusion, the present study demonstrates that apocynin caused an increase in intracellular GSH content in human type II alveolar epithelial cells, γ-GCS activity and induction of γ-GCS-HS. Our results also indicate that this increased GSH synthesis appears to be regulated by an AP-1-mediated mechanism leading to the up-regulation of γ-GCS-HS. This data may have implications for apocynin treatment in patients with inflammatory lung diseases, since such treatment may increase synthesis of the protective antioxidant GSH.

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