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Diallyl disulfide (DADS) increases histone acetylation and p21$^{waf1/cip1}$ expression in human colon tumor cell lines

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Diallyl disulfide (DADS) is a naturally occurring organosulfur compound, from garlic, which exerts pleiotropic biological effects. In rodents, DADS inhibits colon chemically induced carcinogenesis. DADS anti-promoting effect may partly result from its ability to inhibit tumoral cell proliferation *in vivo* and *in vitro*. As far as DADS may modulate the expression of a subset of genes, we investigated DADS effect on histone acetylation, in two human colon tumor cell lines. Our study demonstrates that in Caco-2 and HT-29 cells treated for 6 h, 200 μM DADS increases histone H3 acetylation (×2 and ×1.4, respectively). In Caco-2 cells, we also observed histone H4 hyperacetylation, preferentially at the lysine residues 12 and 16. We explored the effects of DADS and one of its metabolites, allyl mercaptan (AM), on histone deacetylase (HDAC) activity: using nuclear extracts of Caco-2 cells, 200 μM DADS decreased HDAC activity by 29% and AM at the same concentration was more efficient (92% inhibition). We also observed that DADS induced an increase in p21$^{waf1/cip1}$ expression, at mRNA and protein levels, in both cell lines. This effect was associated with an accumulation of cells in the G_2 phase of the cell cycle. Our results suggest that in Caco-2 and HT-29 cells, DADS could inhibit cell proliferation through the inhibition of HDAC activity, histone hyperacetylation and increase in p21$^{waf1/cip1}$ expression. The present study provides evidence for cellular and molecular responses triggered by DADS that could be linked to its effect on histone acetylation and play a role in its protective properties on colon carcinogenesis.

Introduction

Diallyl disulfide (DADS) is a naturally occurring organosulfur compound derived from crushed garlic, which accounts for 40–60% of the total lipid-soluble sulfides in garlic oil (1,2). Epidemiological studies suggest a protective effect of garlic intake against digestive cancers (3). These observations are supported by *in vivo* experiments carried out in rodents, concluding that garlic extract or garlic powder intake reduces chemically induced carcinogenesis in different organs (skin and mammary gland) (4–6). Further studies suggest that allyl sulfides, including DADS, may account for the anticarcinogenic properties of garlic (7–9). In particular, DADS inhibits the initiation and promotion phases of colonic, renal and stomachal chemically induced carcinogenesis in rodent models. Several mechanisms of action have been proposed to explain DADS anticarcinogenic properties. The anti-initiating action of DADS can result from its ability to modulate the activity of phase I and II drug metabolizing-enzymes, especially cytochrome P450 and glutathione *S*-transferase (10–12), and to reduce the genotoxicity of various carcinogenic compounds (13–15). The anti-promoting effects of DADS may be related to its ability to inhibit the proliferation of tumoral cells, *in vivo* and *in vitro* (16,17).

Recent data indicate that pleiotropic biological effects of DADS may involve the modulation of gene expression. With regard to drug-metabolizing enzymes for example, DADS enhances the expression of CYP 1A1, 2B1 and 3A1 genes at the mRNA and protein levels (18). Moreover, a study using the cDNA array technology in HCT-15 human colon tumor cells provides further evidence that DADS up- or down-regulates the expression of a wide range of genes (19). Interestingly, another one reveals that DADS up-regulates (H)-ferritin (20), a gene whose transcription is at least in part regulated by the histone acetylase activity of the p300/CBP transcriptional coactivator protein (21), suggesting that DADS may modulate the expression of specific genes through a modification of histone acetylation.

Histone acetylation is a process involved in the regulation of gene expression. In eucaryotic cells, the DNA is tightly wrapped around octamers composed of two copies of the H2A, H2B, H3 and H4 core histone proteins. All core histones undergo reversible post-translational modifications. Acetylation of specific lysine residues, which occurs within the N-terminal domain of core histones, is one of the mechanisms involved in the modification of the chromatin structure, and generally correlated with transcriptional gene activity. Although all core histones are acetylated, *in vivo*, modifications of histones H3 and H4 have been much more characterized: histone H3 is acetylated preferentially on lysines 9 and 14 and histone H4 on lysines 5, 8, 12 and 16. Steady-state levels of acetylation result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (22–25). Recently, increasing interest has been focused on molecules such as trichostatin A (TSA) and sodium butyrate, which can induce histone hyperacetylation via an inhibition of HDAC activity (26). The action of HDAC inhibitors, which activates the expression of several genes (27), including p21$^{waf1/cip1}$ gene (28), is associated with inhibition of proliferation, induction of differentiation and/or apoptosis of tumoral cells, *in vitro* (26) and *in vivo* (29–31). The relevance of these HDAC inhibitors for cancer therapy is pointed out, since some of them are the subjects of clinical trials (32,33).

Abbreviations: AM, allyl mercaptan; DADS, diallyl disulfide; DMSO, dimethyl sulfoxide; HAT, histone acetyltransferase; HDAC, histone deacetylase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TSA, trichostatin A.

It has been proposed previously that the differentiation of erythroleukemic cells induced by DADS was related to an enhancement of histone acetylation (34). Thus, we investigated DADS effect on the acetylation of the core histones H3 and H4 in two human colon tumor cell lines, Caco-2 and HT-29. In addition, we examined whether DADS could modify HDAC activity. As DADS inhibits HT-29 cell proliferation (35), we also studied the effect of DADS on cell distribution in the cell cycle phases. Since $p21^{waf1/cip1}$ is a down-regulating protein of the cell cycle progression (36), we further investigated the effect of DADS on the expression of $p21^{waf1/cip1}$ gene, which is partly regulated by histone acetylation (37).

Materials and methods

Reagents

Dimethyl sulfoxide (DMSO), TSA, sodium butyrate, DADS (purity 80%, the remaining 20% being diallyl trisulfide and diallyl sulfide) and allyl mercaptan (AM) (purity 80%, remaining 20% composed of sulfides) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). All reagents for cell culture were from Biomedia (Boussens, France). Protease inhibitor cocktail was from Roche Diagnostics GmbH, Mannheim, Germany.

Cell culture

Two human colon adenocarcinoma cell lines, parental Caco-2 cells, established in 1974 by Jorgen Fogh (Sloan Kettering Cancer Center, NY), and HT-29 $Glc^{-/+}$, established in permanent culture in 1975 and selected by Zweibaum *et al*. (38,39) from parental cells by growing them in glucose-free medium for 36 passages, were used.

Both cell lines were cultivated under 10% CO_2, at 37°C, in DMEM (with 4.5 g/l glucose), supplemented with 10 (HT-29) or 20% (Caco-2) heat-inactivated fetal calf serum, 1% penicillin (50 U/ml)/streptomycin (50 U/ml), 4 mM glutamine. Amino acids (1%) were added for Caco-2 media. Media completed with DMSO, DADS, TSA and butyrate were prepared as follows: DMSO, 200 mM DADS and 1 mM TSA solutions were diluted at 1:1000 in decomplemented fetal calf serum, sonicated 2 min, and then, DMEM was added to reach final concentrations of 0.1% DMSO, 200 μM DADS and 1 μM TSA. Sodium butyrate was added directly to medium to obtain a final concentration of 5 mM. For all experiments, cells were seeded at 0.2×10^6 cells in 100 mm Petri dishes. Three (for 24 h incubations) or four days later (for 3 and 6 h incubations), cells were submitted to the different treatments for 3, 6 or 24 h.

Histone extraction

Histones were extracted from 20×10^6 cells as described previously with few modifications (40). Briefly, cell pellets were resuspended in 10 ml cold lysis buffer pH 6.5 (10 mM Tris, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, 8.6% sucrose, protease inhibitor cocktail) and homogenized with a 20 G needle. Nuclei were collected by centrifugation at 700 *g* for 15 min, and then washed successively with lysis buffer and Tris–EDTA buffer, pH 7.4 (10 mM Tris, 13 mM EDTA, protease inhibitor cocktail). The pellet was resuspended in 100 μl of bidistilled water and H_2SO_4 was gradually added to reach a final concentration of 0.4 N. After homogenization by sonication and incubation for one night at +4°C, the suspension was centrifuged at 10 000 *g* for 15 min. Histones were precipitated overnight at −20°C, with 1 ml acetone. Pellets were neutralized with 10 μl NaOH 2 N and resuspended in loading buffer.

Histone acetylation analysis

Analysis of histone acetylation state was performed by western blotting. Five micrograms of histone preparation were loaded on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel for electrophoresis and transferred onto PVDF membrane (Amersham Biosciences, Saclay, France). Membranes were incubated with 1:1000 anti-acetylated H4 (directed against histone H4 acetylated at the lysine residues 5, 8, 12 and 16, indistinctly), anti-acetylated H4 lysine 8, anti-acetylated H4 lysine 12, anti-acetylated H4 lysine 16, anti-acetylated H3 lysine 14 or anti-acetylated H3 lysine 9 antibodies (Upstate, Lake Placid, NY), followed by 1:30 000 anti-rabbit peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Histones were detected using ECL (Enhanced ChemiLuminescence) kit (Amersham Biosciences). Acetylated histone signals were quantified using Lasplus camera (Fujifilm, Paris, France) and Aida software (Raytest, Courbevoie, France). To verify loading homogeneity, membranes were re-incubated with 1:2500 anti-H1 antibody (Upstate) followed by 1:12 000 anti-mouse peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories). Acetylated histone amounts were normalized towards histone H1 quantity. Stimulation factors were then calculated as ratios between treated versus control cell values.

$p21^{waf1/cip1}$ western blot analysis

$p21^{waf1/cip1}$ protein was extracted from 2×10^6 cells with 10 mM Tris, 20 mM NaCl, 5 mM $MgCl_2$, X-100 Triton, protease inhibitor cocktail, pH 7.4 buffer (41). Twenty micrograms total protein extracts were loaded on 15% SDS–polyacrylamide gel for electrophoresis. Membranes were incubated with 1:133 anti-$p21^{waf1/cip1}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by 1:12 000 anti-mouse peroxidase-conjugated antibody. $p21^{waf1/cip1}$ was detected using ECL Plus (Enhanced ChemiLuminescence Plus) kit (Amersham Biosciences). To verify loading homogeneity, membranes were re-incubated with 1:20 000 anti-α tubulin antibody (Sigma-Aldrich) followed by 1:12 000 anti-mouse peroxidase-conjugated antibody (Jackson Immuno-Research Laboratories).

$p21^{waf1/cip1}$ northern blot analysis

To obtain $p21^{waf1/cip1}$ probe, reverse transcription was performed from 2 μg total RNAs of butyrate-treated HT-29 cells, in a reaction volume of 20 μl with 2 μl of 10× RT buffer, 2 μl of 50 μM random decamers, 4 μl of dNTP mix (2.5 mM), 1 μl of RNase inhibitor and 1 μl of reverse transcriptase (Ambion kit, Austin, TX). The reaction mixture was incubated for 1 h at 42°C, then 10 min at 92°C, and the volume was adjusted to 100 μl with distilled water. PCR amplification was then performed during 35 cycles with 2 μl of the cDNA solution supplemented with 5 μl of 10× buffer 2 mM $MgCl_2$, 1.5 μl dNTP mix (10 mM), 1 μl of each 50 μM solution of primers (41), 0.3 μl *Taq* polymerase (Genaxis Biotechnology, Montigny le Bretonneux, France), and water to a final volume of 50 μl. Each PCR cycle consisted of 1 min at 92°C, 30 s at 58°C and 30 s at 72°C. Fifteen nanograms of $p21^{waf1/cip1}$ probe were ^{32}P-labeled using redi prime II kit (Amersham Biosciences). The specific activity of the probe was ~10^9 c.p.m/μg.

RNAs were extracted from 16×10^6 cells by guanidium–phenol–chloroform procedure, as described by Chomczynski and Sacchi (42). Northern blots were performed by loading 20 μg of total RNA on agarose-formaldehyde gel, separating by electrophoresis and transferring onto nylon membranes (Amersham Biosciences). Blots were pre-hybridized for 4 h in 10 ml of Quick hybrid solution (Stratagene, Cedar Creek, TX), and then hybridized with salmon sperm DNA (Sigma-Aldrich) and $p21^{waf1/cip1}$ ^{32}P-labeled probe for 1 h at 68°C. After two washes in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7), 0.1% SDS for 15 min at room temperature and one wash in 0.1× SSC, 0.1% SDS at 60°C for 30 min, blots were exposed to MP hyperfilm (Amersham Biosciences) at −80°C overnight. Signals were quantified using FLA 3000 (Fujifilm, Paris, France) and Aida software (Raytest). To assess RNA loading homogeneity, blots were re-hybridized with a murine ^{32}P-labeled 18S probe.

Cell cycle

After each treatment, cells were washed with phosphate-buffered saline (PBS), trypsinized and collected by centrifugation (800 *g*, 10 min). Cell pellets were washed with PBS and resuspended in nuclear isolation medium (100 mg/l RNase, 50 mg/l propidium iodide, 0.1% Triton X-100, in PBS). About 20 000 cells were analyzed by flow cytometry using a fluorescence-activated cell sorting (FACS) Scan (Becton-Dickinson, Pont de Claix, France). Cell distribution into cell cycle phases was calculated using ModFit (V3.0) software (Becton-Dickinson).

Preparation of nuclear protein extract from Caco-2 cells

Caco-2 nuclear extract was prepared from 40×10^6 cells. Cells were washed twice with PBS and incubated 10 min with 5 ml of nuclear extraction (NE)-1 buffer [10 mM HEPES–KOH pH 7.9, 2.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail]. Once scraped, and collected by centrifugation (250 *g*, 10 min), the cell pellet was resuspended in 1 ml of NE-1 buffer and lysed using a Dounce glass homogenizer then a 23 G needle. After centrifugation (250 *g*, 10 min), the nuclei pellet was resuspended by sonication in 1 ml of NE-2 buffer [20 mM HEPES–KOH pH 7.9, 2.5 mM $MgCl_2$, 0.42 M NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail]. After a 1 h incubation at +4°C, nuclear lysate was centrifuged (20 000 *g*, 45 min). The supernatant was then dialyzed against NE-3 buffer [20 mM HEPES–KOH pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail], one night at +4°C. The supernatant, containing nuclear proteins was collected. Protein concentration of the nuclear extract was determined by measurement of the absorbance (at 280 nm).

HDAC activity assay

Effects of DADS and AM on HDAC activity was assessed with the HDAC Fluorescent Activity Assay™ kit (Tebu, Le Perray en Yvelines, France). The

fluorescence signal is proportional to HDAC activity and decreases in the presence of HDAC inhibitors. 4.5 mg of HeLa cell protein nuclear extract (from the kit) were incubated with different concentrations of TSA (5 or 200 nM), taken as a reference inhibitor, DADS or AM (at 100, 200, 400, 660 μM or 1 mM) and 25 μl of substrate for 15 min at 25°C. After 10 min at 25°C with developer, fluorescence signals were assessed with SpectraFluor Plus (excitation filter: 360 nm, emission filter: 465 nm) (Spectrafluor Plus, Tecan, Trappes, France). In the same conditions, 4.5 mg protein nuclear extract prepared from Caco-2 cells (see above) was incubated with 200 nM TSA, DADS or AM at 200 μM. HDAC activity of extracts incubated with the different compounds (TSA, AM or DADS) was expressed as the percentage of the control (nuclear extract alone) fluorescence values.

Statistical methods

Statistical analyses were performed using *t*-test for histone acetylation and cell cycle experiments. Concerning HDAC activity experiments, we used ANOVA followed by Student Newman–Keuls method. Differences with $P < 0.05$ were considered significant.

Results

DADS increases the acetylation state of histones H4 and H3 in Caco-2 cells

The effect of 200 μM DADS on histone H4 and H3 acetylation status was studied by western blotting, for different incubation times. DADS induced histone H4 hyperacetylation after 3 and 6 h incubations with significant 1.8-fold and 2.5-fold increases, respectively (Figure 1A and B). In contrast to TSA and butyrate (positive controls), that increased acetylated histone H4 signals after 3, 6 and 24 h of treatment, no modification of acetylated H4 level was detected after a 24 h treatment with DADS (Figure 1A).

As core histones are mainly acetylated on specific residues (24), we further extended our investigations to particular lysine residues of histones H4 and H3, focusing on 3 and 6 h incubation times. For histone H4, we observed that the 3 h treatment with DADS significantly enhanced histone H4 acetylation status at lysines 12 (×2.6) and 16 (×5), but did not modify histone H4 lysine 8 acetylation (Figure 2A and B). For the 6 h treatment, comparable results were obtained: DADS induced significant 4.2- and 2.3-fold increases in the acetylation levels of histone H4 lysines 12 and 16, respectively (Figure 2C and D). For histone H3, DADS significantly increased the amount of histone H3 acetylated at lysine 14 (×1.8 and ×2 at 3 and 6 h, respectively; Figure 3A and B) and of histone H3 acetylated at the lysine 9 (×2.3 and ×1.5 at 3 and 6 h, respectively; Figure 3C and D).

Thus, DADS induced a transient increase in histone H4 and H3 acetylation status at specific lysine residues in Caco-2 cells.

DADS increases p21$^{waf1/cip1}$ protein and mRNA levels in Caco-2 cells

p21$^{waf1/cip1}$ protein is an essential regulatory protein of cell cycle progression (43,44). Expression of its gene is partly regulated by histone acetylation (37). Since we observed that 200 μM DADS induced histone hyperacetylation and inhibited Caco-2 cell proliferation (data not shown), we studied its effects on p21$^{waf1/cip1}$ protein and mRNA levels.

DADS markedly increased p21$^{waf1/cip1}$ mRNA amounts after 6 and 24 h incubations (Figure 4A). Considering two independent experiments, DADS induced significant 3.4- and 2.8-fold increases in p21$^{waf1/cip1}$ mRNA level after 6 and 24 h incubations, respectively. p21$^{waf1/cip1}$ protein amounts were

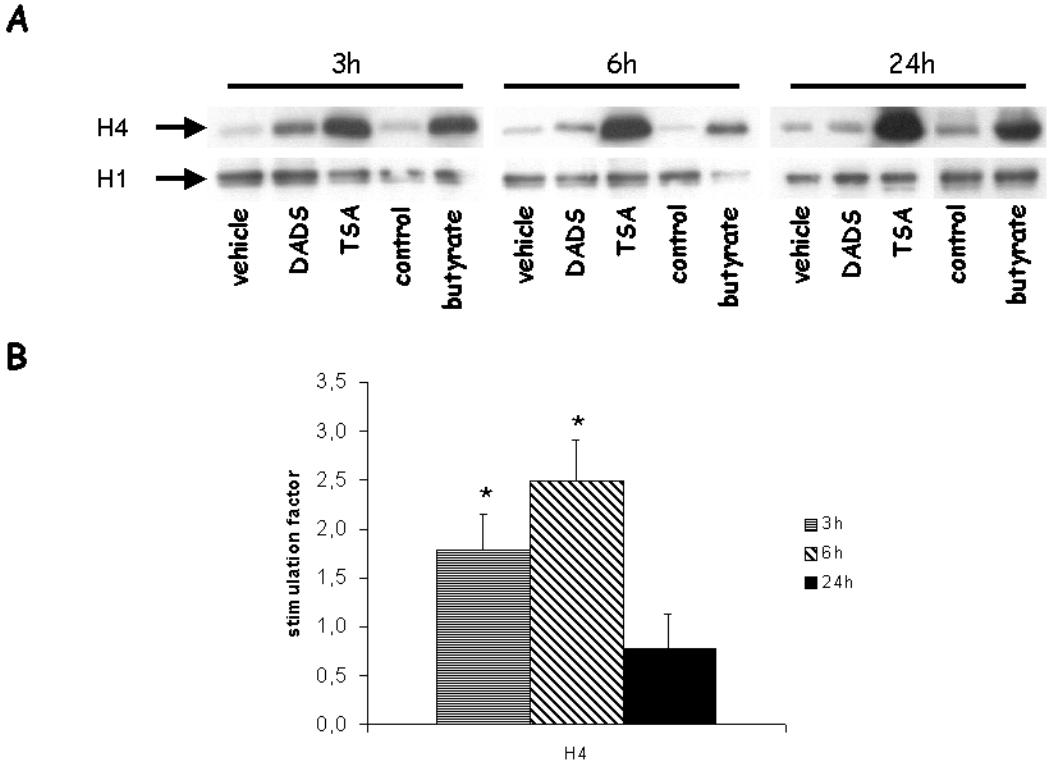

Fig. 1. Effects of DADS on histone H4 acetylation state in Caco-2 cells. Cells were treated with 0.1% DMSO (vehicle), 200 μM DADS, 1 μM TSA, medium alone (control towards butyrate treatment) or 5 mM butyrate for 3, 6 or 24 h. Western blot analysis was performed on a 15% SDS–polyacrylamide gel with 5 μg histone protein extracts. (**A**) A representative western blot. (**B**) Stimulation factors were determined as described in Materials and methods, and expressed as mean ± SEM ($n = 4$ independent experiments; $^{*}P < 0.05$).

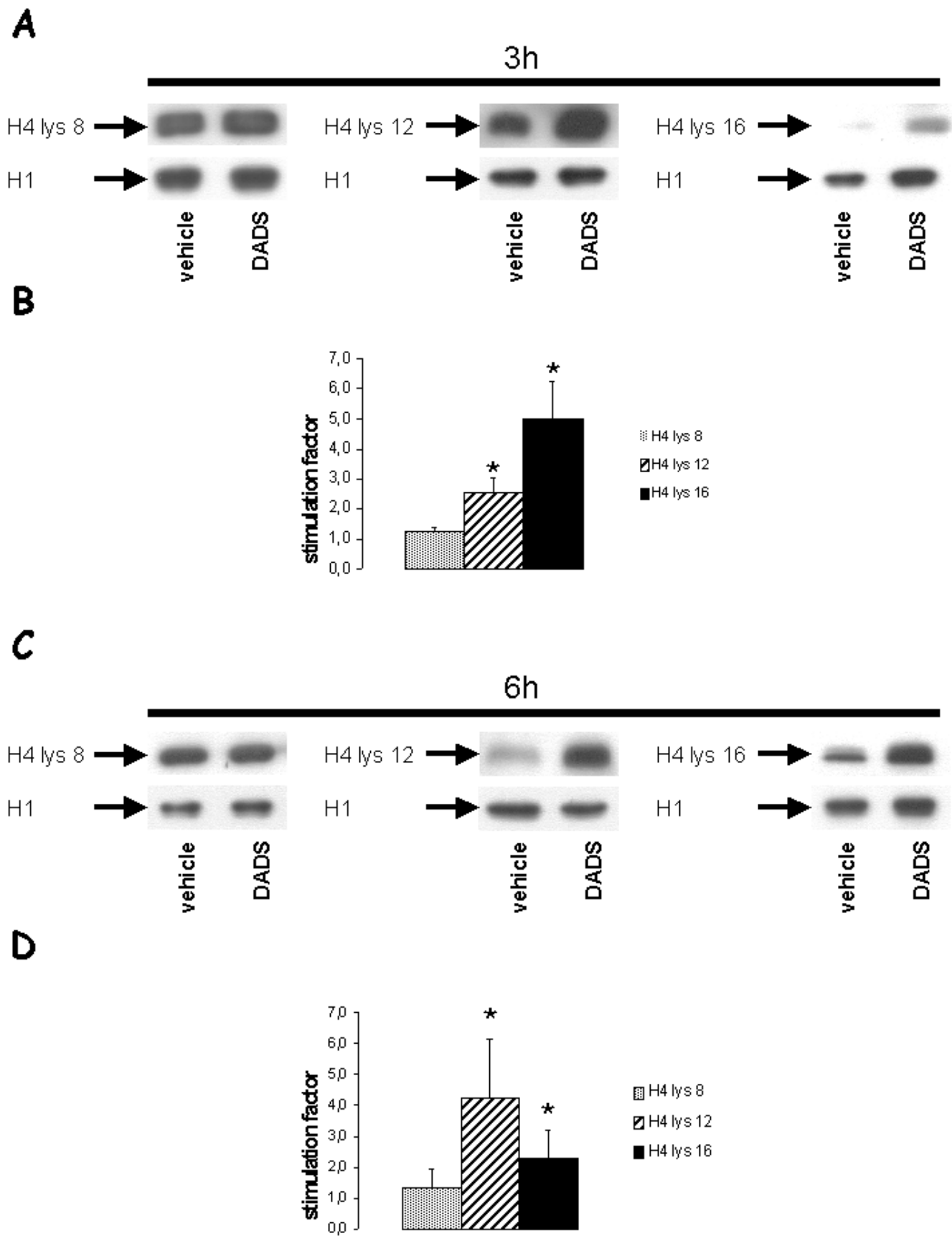

Fig. 2. Effects of DADS on histone H4 lysine 8, 12, 16 acetylation states in Caco-2 cells. (**A** and **C**) Western blot analysis was performed on a 15% SDS–polyacrylamide gel with 5 μg histone proteins extracted from untreated cells or cells treated by 200 μM DADS for 3 (A) or 6 h (C). Antibodies directed against histone H4 specifically acetylated at the lysine residues 8, 12 or 16 were used ($n = 3$ independent experiments). (**B** and **D**) Stimulation factors were determined as described in Materials and methods, and expressed as mean ± SEM ($n = 3$ independent experiments; $^{*}P < 0.05$).

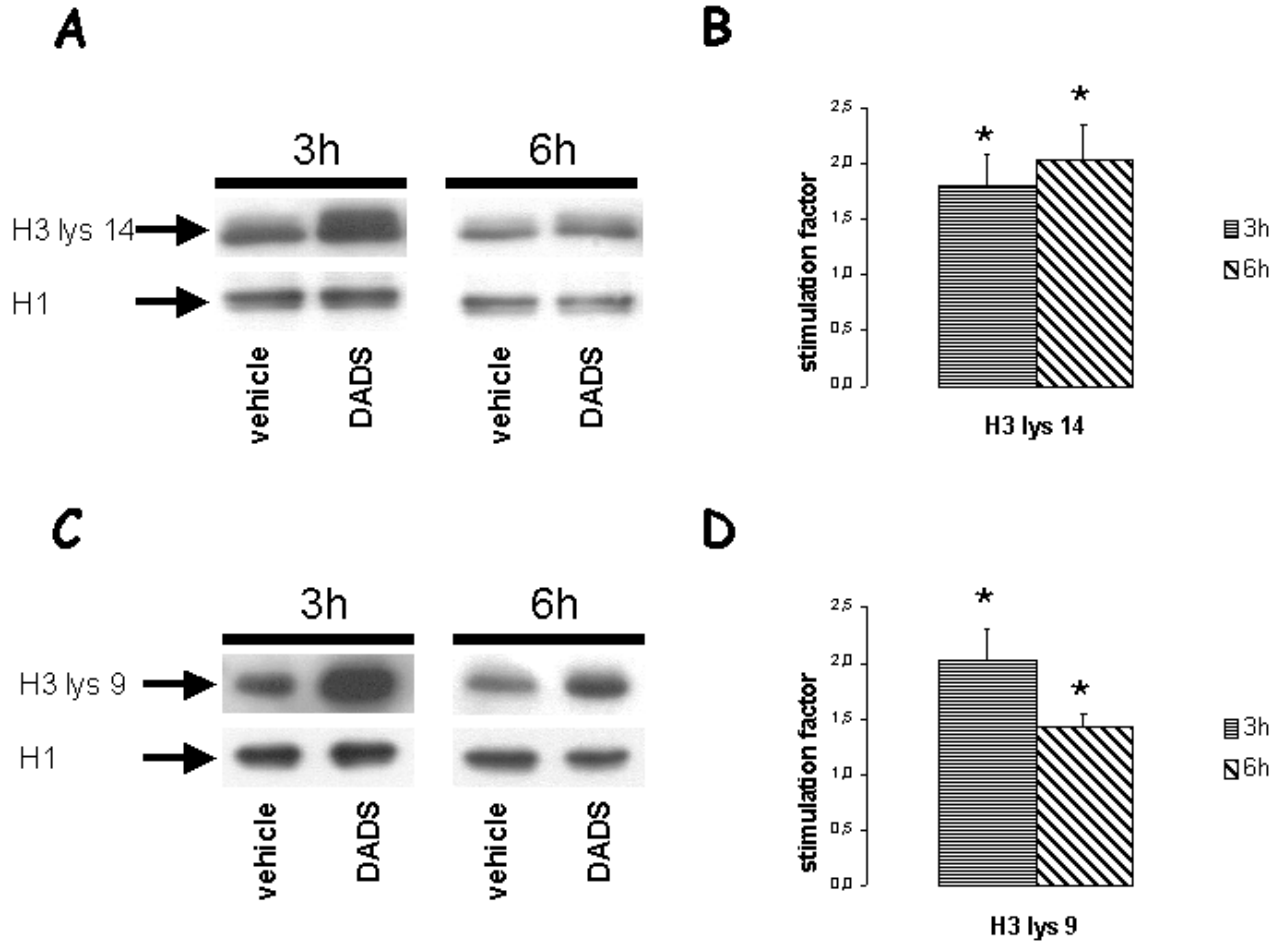

Fig. 3. Effects of DADS on histone H3 lysine 14, 9 acetylation states in Caco-2 cells. (**A** and **C**) Western blot analysis was performed on a 15% SDS–polyacrylamide gel with 5 μg histone proteins extracted from untreated cells or cells treated by 200 μM DADS for 3 (A) or 6 h (C). Antibodies directed against histone H3 specifically acetylated at the lysine residues 14 or 9 were used ($n = 3$ independent experiments). (**B** and **D**) Stimulation factors were determined as described in Materials and methods, and expressed as mean ± SEM ($n = 3$ independent experiments; $^{*}P < 0.05$).

also enhanced by DADS, after 6 (×3.5) and 24 h (×2.3), (Figure 4B).

DADS induces G_2 cell cycle arrest in Caco-2 cells

Since $p21^{waf1/cip1}$ protein, a known cyclin-dependent kinase inhibitor (36), induces G_1 or G_2 cell cycle arrests (45), the effect of DADS on Caco-2 cell distribution in the cell cycle phases was then assessed. After 6 and 24 h DADS treatments (Figure 5), the percentage of cells in G_2 phase reached values of 23 and 29%, respectively (13% in control conditions), at the expense of the G_1 phase. Thus, DADS treatments resulted in significant increases in the cell proportion in G_2 phase.

In HT-29 cells, DADS induces an increase of $p21^{waf1/cip1}$ protein and mRNA and acetylated histone H3 lysine 14 levels

Previous data obtained in our laboratory have shown that DADS inhibits HT-29 cell proliferation through a G_2 cell cycle arrest (35). Therefore, we explored whether this inhibition of HT-29 proliferation induced by DADS was associated with modifications of $p21^{waf1/cip1}$ expression or histone acetylation.

As shown in Figure 6A and B, 200 μM DADS enhanced $p21^{waf1/cip1}$ mRNA and protein levels, after 6 and 24 h incubations. Concerning histone acetylation, DADS increased by 40% the acetylation state of H3 lysine 14 after a 6 h incubation (Figure 6C and D) whereas it had no effect at 3 or 24 h. DADS did not modify significantly the acetylation of H4 (Figure 6C and D) for 3, 6 and 24 h of incubation nor the amount of histone H3 acetylated at the lysine 9 residue (Figure 6E). In summary, in HT-29 cells, DADS increased $p21^{waf1/cip1}$ protein and mRNA amounts and the acetylation of lysine 14 of histone H3.

DADS and AM inhibit HDAC activity of nuclear extracts from Hela and Caco-2 cells

Since TSA and butyrate modulate histone acetylation through an inhibition of HDAC activity, we examined HDAC activity in the presence of DADS, using firstly HeLa (provided in the kit) and secondly Caco-2 protein nuclear extracts. With HeLa extract (Figure 7A), TSA inhibited HDAC activity by 35 and 97%, at 5 and 200 nM, respectively. DADS inhibited HDAC activity only at high concentrations (from 400 μM). Interestingly, AM, a DADS metabolite (46), was more efficient in reducing the HDAC activity. Its effect was significant and dose dependent.

With Caco-2 extract (Figure 7B), 200 μM DADS significantly inhibited HDAC activity by 29%. In the presence of AM at the same concentration, the inhibition of HDAC activity was almost complete (92%). Thus, DADS significantly inhibited

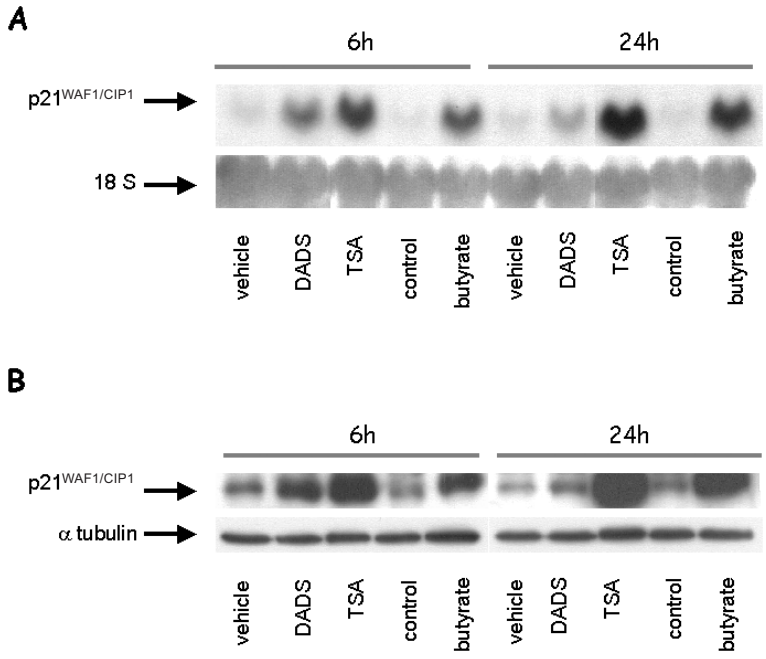

Fig. 4. Analysis of DADS effects on $p21^{waf1/cip1}$ mRNA and protein amounts in Caco-2 cells. Cells were treated with 0.1% DMSO (vehicle), 200 μM DADS, 1 μM TSA, medium alone (control towards butyrate treatment) or 5 mM butyrate for 6 or 24 h. (**A**) Northern blot analysis was performed with 20 μg of total RNAs ($n = 2$ independent experiments). (**B**) Western blot analysis was performed with 20 μg of cell protein extracts ($n = 3$ independent experiments).

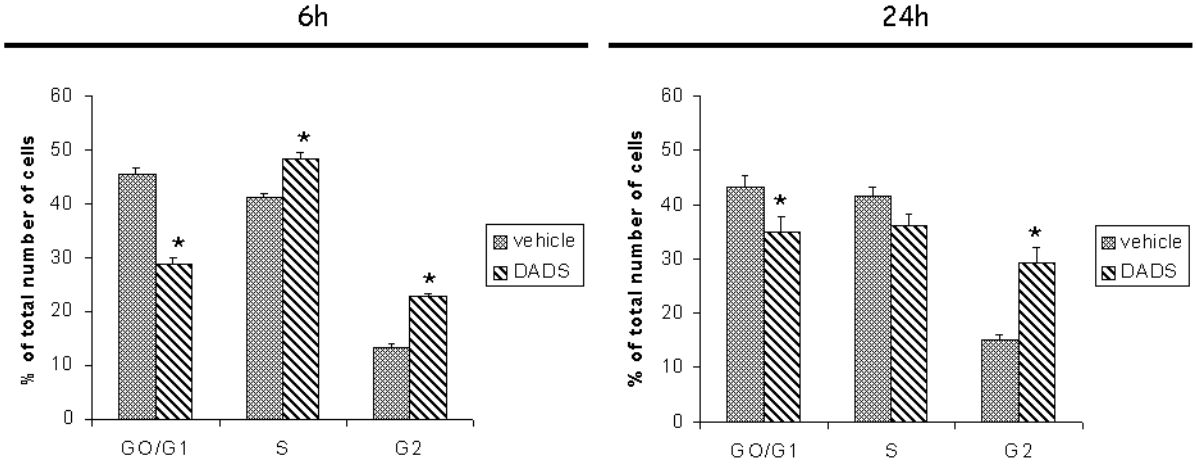

Fig. 5. DADS effects on cell cycle distribution of Caco-2 cells. The distribution of cells treated with 0.1% DMSO (vehicle) or 200 μM DADS for 6 or 24 h, in G_0–G_1, S and G_2 phases was analysed by flow cytometry as described in Materials and methods. Results are expressed as mean ± SEM ($n = 3$ independent experiments; $^*P < 0.05$ versus corresponding control).

HDAC activity from HeLa and Caco-2 extracts and one of its metabolites (AM) was even more efficient.

Discussion

To our knowledge, the present study reported for the first time the ability of DADS to increase histone acetylation status, in two human colon tumor cell lines, Caco-2 and HT-29. DADS increased the acetylation of histone H3 in both cell lines and of histone H4 only in Caco-2 cells.

In response to DADS, histone acetylation status increased rapidly after 3 (Caco-2) or 6 h (HT-29) of treatment and returned to the basal level after 24 h treatments. This kinetic is partially consistent with previous data obtained with erythroleukemic cells, showing that DADS-induced histone hyperacetylation was rapid and maintained up to 24 h (34). DADS transitory effect on histone acetylation in colon cell lines may come from its rapid metabolism. Another explanation could be that once disturbed by DADS treatment, the balance between HAT and HDAC activities is progressively restored. This hypothesis is supported by recent data indicating that TSA increases HDAC1 expression at a transcriptional level, suggesting a potential feedback loop controlling HDAC expression (47). Interestingly, the transient histone hyperacetylation due to DADS might be sufficient to induce cell cycle arrest, in Caco-2 and HT-29 cells, as reported for TSA, in HT-29 cells (48).

Histone H3 acetylation on lysine 14 was significantly increased by DADS in both cell lines, according to previous

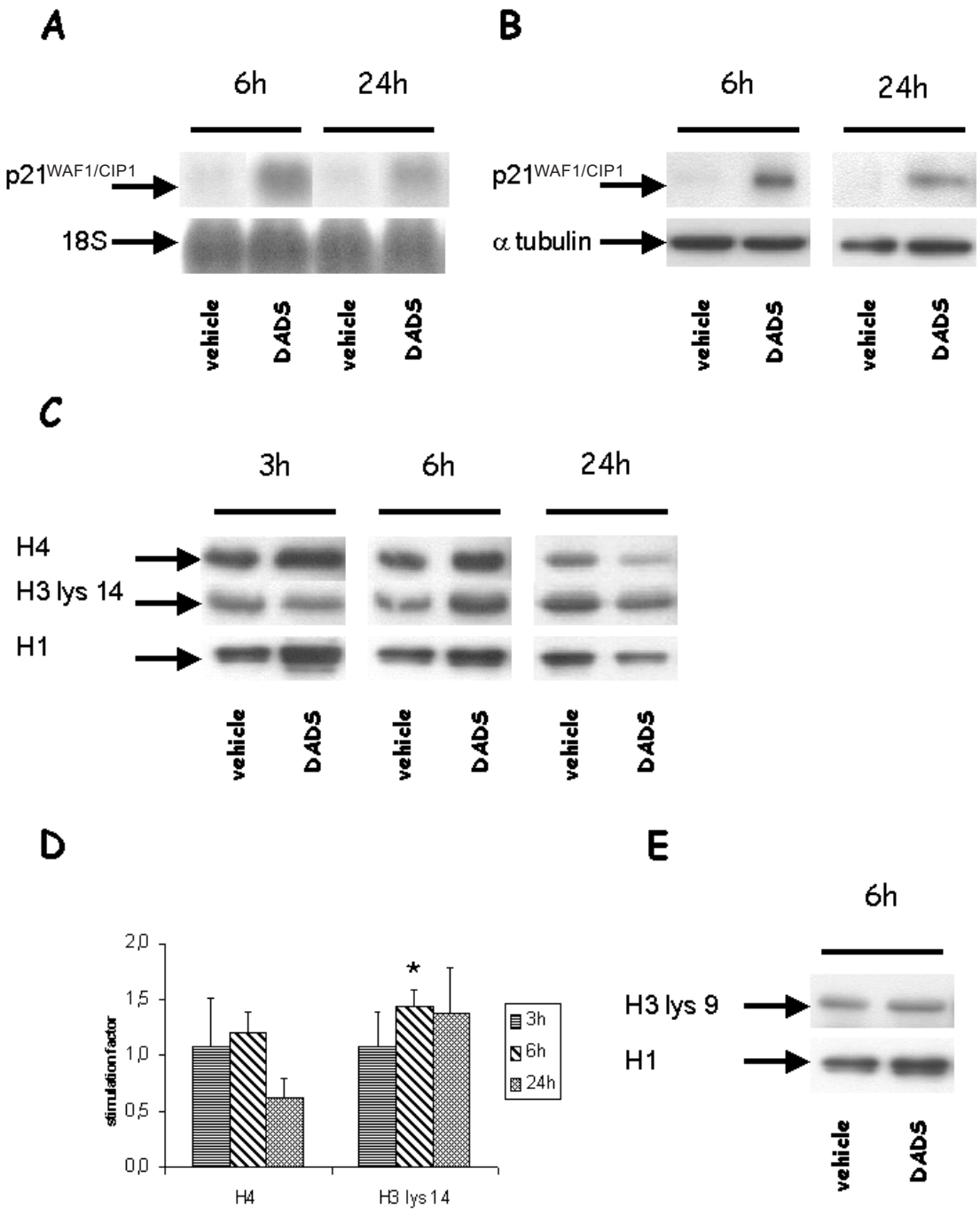

Fig. 6. Effects of DADS on $p21^{waf1/cip1}$ mRNA and protein levels and on the acetylation of histone H4, H3 lysine 14 and H3 lysine 9 in HT-29 cells. Cells were treated with 0.1% DMSO (vehicle) or 200 μM DADS for indicated times. (**A**) Effect of DADS on $p21^{waf1/cip1}$ mRNA amounts was assessed by northern blotting ($n = 2$ independent experiments). (**B**) Effect of DADS on $p21^{waf1/cip1}$ protein amounts was assessed by western blotting ($n = 3$ independent experiments). (**C** and **D**) Effects of DADS on histone H4 and H3 lys 14 acetylation were assessed by western blot analysis. (D) Stimulation factors obtained with DADS versus corresponding control are expressed as mean ± SEM ($n = 4$ independent experiments; $^{*}P < 0.05$). (**E**) Effects of DADS on histone H3 lys 9 acetylation was assessed by western blot analysis.

data indicating that lysine 14 is the preferential acetylation site linked to transcription (49). In Caco-2 cells, we also found that DADS increased H3 acetylation at lysine 9, indicating that acetylation of H3 could occur at other lysine residues (50). Therefore, it would be interesting to further analyse DADS effect on the acetylation of histone H3 at lysines 18 and 23, which may also be involved in the regulation of transcription.

Histone H4 hyperacetylation was significant only in Caco-2 cells when treated with DADS for 3 or 6 h. Hyperacetylation was located on lysines 12 and 16, as described previously for butyrate (51). As compared with Caco-2 cells, the absence of modification of H4 histones in HT-29 cells in response to DADS could come from differences in the various elements involved in acetylation regulation. As other studies showed that histone H4 hyperacetylation could be induced in HT-29

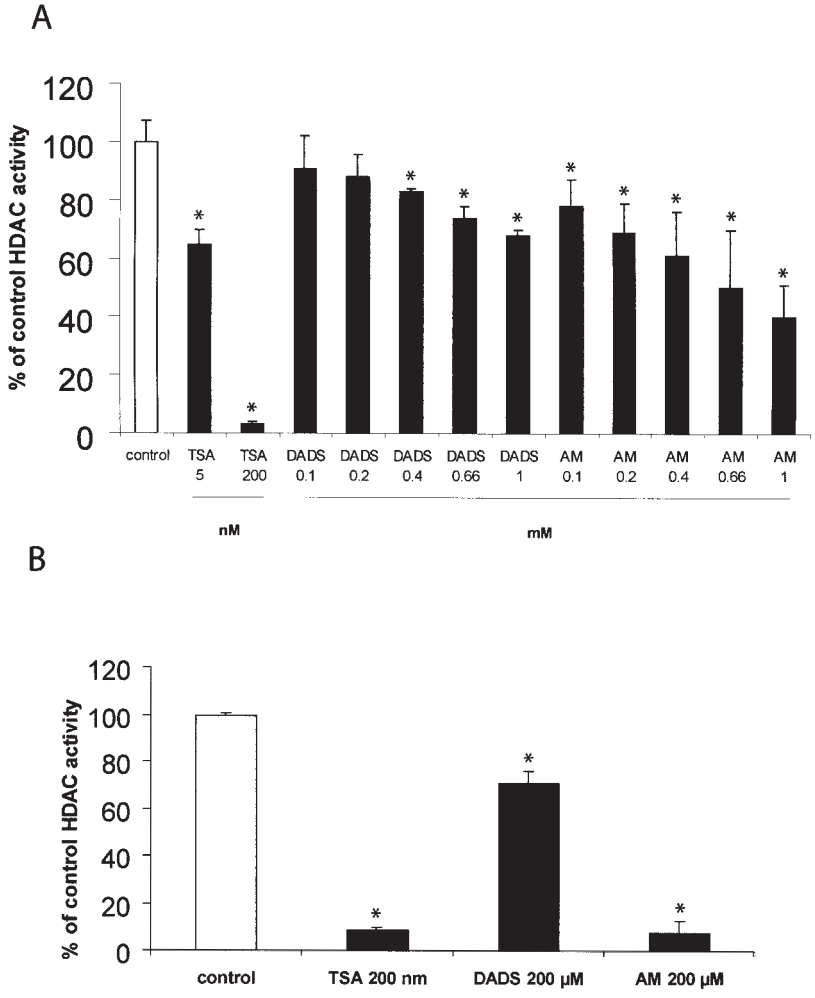

Fig. 7. Effects of DADS, AM and TSA on HeLa and Caco-2 HDAC activity. HDAC activity was assessed with protein nuclear extracts from HeLa cells (**A**) or Caco-2 cells (**B**) using histone deacetylase assay kit, as described in Materials and methods. Data were expressed as a percentage of the control fluorescence values. The results are mean ± SEM ($n = 3$ independent experiments for HeLa extract, or $n = 2$ for Caco-2 extract, $^{*}P < 0.05$).

cells by butyrate (41,52), we hypothesize that histone H3 might be a predominant target for DADS. From a general point of view, DADS thus appears to be able to enhance histone acetylation in the two studied colon tumor cell lines, as well as in erythroleukemic cell lines (34), hepatoma and normal rat liver (53).

DADS-induced histone hyperacetylation could result either from a stimulation of HAT activity or an inhibition of HDAC activity. To study DADS effect on HDAC activity, we used firstly the HeLa nuclear extract supplied by the HDAC Fluorescent Activity Assay^{TM} and secondly nuclear proteins extracted from Caco-2 cells. In both systems, DADS significantly inhibited HDAC activity, but to a lesser extent than TSA. As TSA physically interacts with HDAC catalytic sites (54,55), we hypothesize that DADS could also interact directly with HDAC, but with a lower affinity than TSA. Another explanation could be that DADS must be metabolized to inhibit HDAC activity. Indeed, a recent study has identified DADS metabolites (AM, allyl methyl sulfide, allyl methyl sulfoxide, allyl methyl sulfone) in the stomach, liver, plasma and urine after a single oral administration of DADS (46). In our study, whereas addition of DADS to nuclear extracts that are devoid of drug-metabolizing enzymes had a moderate inhibitory effect on HDAC activity, addition of AM inhibited by 92% HDAC activity with Caco-2 nuclear extract, suggesting that DADS metabolites would be more efficient than their precursor. DADS and AM effects were greater with Caco-2 nuclear extract than with HeLa one, suggesting that these two human cell types could have different expression patterns of the HDACs identified in humans (26). Although our experiments strongly suggest the ability of both sulfides to inhibit HDAC activity, a possible effect on HAT activity cannot be excluded.

DADS inhibits the proliferation of various human tumor cell lines (56,57), including human tumor colon cell lines, HCT-15, HCT-116 and HT-29 (35,58,59). Moreover, we found that DADS (200 μM) also inhibited Caco-2 cell proliferation (data not shown). Recently, it has been reported that in colon tumor cells, DADS modulates the expression of genes involved in the regulation of cell proliferation (19). Other studies have shown that in colon cell lines, the two HDAC inhibitors butyrate and TSA induce $p21^{waf1/cip1}$ expression (41,60). We pointed out that DADS (200 μM) increased $p21^{waf1/cip1}$ mRNA and protein amounts after 6 and 24 h incubations, in the two cell lines studied. As $p21^{waf1/cip1}$ protein is involved in the cell cycle progression and can induce cell cycle arrest in

G_1 or G_2 phases (45), increased $p21^{waf1/cip1}$ expression could explain the G_2 cell cycle arrest induced by DADS in both Caco-2 and HT-29 cells (35). We suppose that DADS effect on $p21^{waf1/cip1}$ expression was p53-independent: DADS did not modulate mutated protein p53 protein levels in HT-29 cells (data not shown) and p53 could not be detected by western blot analysis in Caco-2 cells (data not shown). Considering (i) that $p21^{waf1/cip1}$ expression is regulated by histone acetylation (37), (ii) that in both cell lines a p53-independent pathway is involved, (iii) that in both cells lines DADS induced hyperacetylation of histone H3 at lysine 14, we hypothesize that the DADS effect on $p21^{waf1/cip1}$ expression (protein and mRNA levels) could be due to histone H3 lysine 14 acetylation in these two cell lines. Acetylation on particular histone (H3) or lysine residue (lysine 14 of H3) within $p21^{waf1/cip1}$ promoter should be confirmed by chromatin immunoprecipitation assays, as done previously for other genes (61,62).

Finally, our study suggests strongly the involvement of histone acetylation state at preferential lysine sites in the modulation of gene expression by DADS. Thus, DADS-induced hyperacetylation through HDAC activity inhibition might be one of the mechanisms involved in its biological pleiotropic effects. Hence, these experiments have permitted to partially elucidate the DADS-induced inhibition of the proliferation of Caco-2 and HT-29 cells, suggesting that these cellular effects might be associated with its colon anticarcinogenic properties. Nevertheless, considering limited data on DADS bioavailability (63), additional studies are warranted to check similar effects using *in vivo* models of colon carcinogenesis.

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