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Biochemical and Biophysical Research Communications 354 (2007) 140–147
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In vivo treatment by diallyl disulfide increases histone acetylation in rat colonocytes

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Received 11 December 2006
Available online 28 December 2006

Abstract

Diallyl disulfide (DADS) is an organosulfur compound from garlic which exhibits various anticarcinogenic properties including inhibition of tumor cell proliferation. DADS antiproliferative effects were previously associated with an increase in histone acetylation in two human tumor colon cell lines, suggesting that DADS-induced histone hyperacetylation could be one of the mechanisms involved in its protective properties on colon carcinogenesis. The effects of DADS on histone H4 and H3 acetylation levels were investigated *in vivo* in colonocytes isolated from non-tumoral rat. Administrated by intracaecal perfusion or gavage, DADS increases histone H4 and H3 acetylation in colonocytes. Moreover, data generated using cDNA expression arrays suggest that DADS could modulate the expression of a subset of genes. These results suggest the involvement of histone acetylation in modulation of gene expression by DADS in normal rat colonocytes, which might play a role in its biological effects as well as in its anticarcinogenic properties *in vivo*.
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Keywords: Diallyl disulfide; Gene expression; Histone acetylation; Rat colonocytes

Diallyl disulfide, an organosulfur compound from garlic, inhibits rodent chemically induced carcinogenesis in various organs including colon [1–3]. In the colon, DADS anti-initiating effects were associated to increases in the activity of phase II drug metabolizing-enzymes such as glutathione *S*-transferase, quinone reductase, and UDP-glucuronosyltransferase [2]. DADS anti-promoting effects may partly result from its ability to inhibit tumoral cell proliferation, as shown *in vitro* or in a xenograft [4,5]. Indeed, several studies focusing on DADS anti-proliferating effects in different tumor colon cell lines indicate that DADS inhibits tumor cell proliferation through G_2/M cell cycle arrest and/or apoptosis induction [6–9]. Recently, increasing interest has been focused on DADS ability to modulate gene expression. In particular, DADS increases the expression of several glutathione *S*-transferases in mouse colon [10]. Moreover, *in vitro* in HCT-15 cells, DADS anti-proliferating effects are associated to up- or down-modulation of the expression of a wide range of genes, including genes implicated in cell proliferation regulation [11]. Nevertheless, the mechanisms mediating modulation of gene expression in response to DADS remain to be determined.

Posttranslational histone modifications are mechanisms involved in the modification of the chromatin structure and consequently in the regulation of gene expression. Especially, acetylation of specific histone lysine residues within the N-terminal domain of the core histones, H2A, H2B, H3, and H4, reducing interactions between DNA and histones, may partly activate gene expression through an increased accessibility of DNA to transcription factors [12].

In previous studies, we reported DADS ability to increase histone acetylation status in two human colon tumor cell lines, Caco-2 and HT-29 [13,14]. This effect was associated to $p21^{waf1}$ mRNA and protein level increases, and cell cycle arrest in the G_2/M phase. Moreover, DADS inhibited HDAC activity of Caco-2 [13] and HT-

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.158

29 nuclear extract (unpublished data). These results obtained *in vitro* suggested strongly the involvement of histone hyperacetylation in the modulation of gene expression and the inhibition of tumor cell proliferation in response to DADS. Thus, the effect of DADS on histone acetylation might play a role in its colon anticarcinogenic properties. Before verifying this hypothesis using *in vivo* models of colon carcinogenesis, it was necessary to determine whether DADS could modulate histone acetylation, *in vivo*, in non-tumoral colon. Indeed, previous data have shown that DADS could induce histone hyperacetylation in hepatoma as well as in control rat liver [15]. Thus, modulation of histone acetylation by DADS might occur in normal cells and be linked to its chemopreventive effects.

The main objective of the present study was to investigate DADS effect *in vivo* on the acetylation state of histones H3 and H4 in colonocytes isolated from rat colon. Since limited data are available on DADS bioavailability [16], especially with regard to colon cells, rats were treated with DADS (200 mg/kg of body weight) using two different protocols: (i) intracaecal perfusion ensuring a direct exposure of colonocytes to DADS, and (ii) oral feeding by gavage mimicking exposure to DADS after garlic or DADS supplement intake.

Materials and methods

Reagents. DADS (purity 80%, the remaining 20% being diallyl trisulfide and diallyl sulfide) was purchased from Sigma–Aldrich (St. Quentin Fallavier, France).

Animals and experimental design. Two-months-old Wistar male rats weighing approximately 290 g were fed ad libitum with M25 pellets (Dietex, Saint-Gracien, France). DADS, diluted in rapeseed oil, was administered either by perfusion, using a caecal catheter connected to a peristaltic pump, or by gavage. In the case of intracaecal administration, a catheter was inserted into the caecum 8 days before DADS perfusion as previously described for gastric administration [17]. Rats were perfused during 6 h and sacrified 1 or 21 h after the end of the perfusion (Fig. 1A).

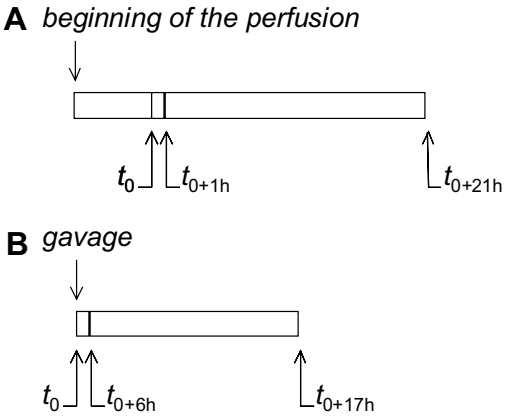

Fig. 1. Experimental design. Rats received DADS at 200 mg/kg body weight or rapeseed oil as vehicle. (A) Intracaecal perfusion: 8 days after the catheter installation, rats were treated by perfusion for 6 h and sacrified 1 h (t_{0+1h}) or 21 h (t_{0+21h}) after the end of the perfusion (t_0). (B) Gavage: rats received DADS in one dose and were sacrified 1 h (t_{0+1h}) or 17 h (t_{0+17h}) after gavage (t_0).

In the case of oral administration, DADS was given in one dose using a cannula. Then, rats were sacrified 6 or 17 h after gavage (Fig. 1B). Time points for intracaecal perfusion and gavage experiments were chosen to allow the investigation of DADS effects approximately (i) 6 h after colonocytes exposition to DADS (1 h after the 6 h perfusion or 6 h after gavage), since DADS was shown to induce histone hyperacetylation after a 6 h treatment in colon cell lines [13], (ii) 24 h after DADS treatment (21 h after the end of the perfusion or 17 h after gavage), while taking into account technical and experimental constraints. All aspects of the protocols conformed to the International Guiding Principles for Biomedical Research Involving Animals. For both administration protocols, rats were treated with DADS at 200 mg/kg body weight or with the vehicle alone (rapeseed oil).

Colonocyte isolation. Rat colons were removed under anaesthesia of animals and colonocytes isolated as previously described [18]. Briefly, epithelial cell fractions were collected from colonic mucosa after luminal perfusion of a 10 mM EDTA buffer (20 min at 37 °C). Then, cell suspensions were washed twice with phosphate-buffered saline. After centrifugation (5 min at 100*g*), cell pellets were frozen in liquid N_2 and stored at −80 °C before analysis.

Histone acetylation analysis. Histone extraction and acetylation analysis were performed as previously described [13]. Briefly, 5 μg of histone preparation were loaded on a 15% sodium dodecyl sulphate (SDS)–polyacrylamide gel. After electrophoresis and transfer onto PVDF membrane (Amersham Biosciences, Saclay, France), membranes were incubated with 1:1000 anti-acetyl H4, anti-acetyl H4 lysine 8, anti-acetyl H4 lysine 12, anti-acetyl H4 lysine 16, anti-acetyl H3 lysine 14 or anti-acetyl H3 lysine 9 antibodies (Upstate, Lake Placid, NY), followed by 1:30,000 anti-rabbit peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Acetylated histones were detected using ECL + kit (Amersham Biosciences) and acetylated histone signals were then quantified using Lasplus camera (Fujifilm, Paris, France) and Aida software (Raytest, Courbevoie, France). Acetylated histone amounts were normalized towards histone H1 quantity. Stimulation factors were then calculated as ratios between treated versus control cell values.

Statistical analysis. Statistical analyses were performed using *t*-test. Differences with $P < 0.05$ were considered significant.

Analysis of gene expression profiles. Total RNA was extracted from rat colonocytes isolated 1 or 21 h after the end of the intracaecal perfusion, as previously described [12]. For control and treated animals, 60 μg of total RNA were used. RNA from two control or treated animals were pooled to limit the observation of differences in gene expression resulting from animal heterogeneity. After Poly A^+ RNA enrichment, ^{33}P-cDNA probes were synthesized and purified using γATP^{33} (Perkin-Elmer, Villebon sur Yvette, France), Atlas™ Pure Total RNA Labelling System and Atlas Rat cDNA Array kits developed by Clontech according to the manufacturer's instructions (Palo Alto, CA). Atlas Rat cDNA Arrays were then hybridised with ^{33}P-cDNA probes from control or treated rats. After exposition, the signals obtained from the two arrays were quantified using a fla-3000 phosphoimager (Fujifilm, Paris, France) and Aida software (Raytest). Signals were then normalized towards housekeeping gene signals and fold increase or decrease were calculated between treated versus control rat values.

Results

DADS effects on histone acetylation after intracaecal perfusion

DADS was administrated during 6 h by an intracaecal perfusion ensuring the direct exposure of colon epithelial cells to DADS, as well as the delivery of the total dose of 200 mg/kg body weight. One hour after the end of the intracaecal perfusion, DADS rapidly induced histone H4 hyperacetylation with a significant 2.2-fold increase (Figs.

2A and B). This effect remained significant (1.9×) up to 21 h after the end of the perfusion (Figs. 2C and D). We further extended our investigation to different lysine residues of histones H4 and H3. For histone H4, 1 h after the end of the perfusion, DADS also significantly enhanced histone acetylation status at lysines 8 (1.5×), 12 (3.9×), and 16 (3.9×) (Figs. 2A and B). Twenty one hour after the end of the intracaecal perfusion, DADS effect remained significant for lysine 8 (2.3×) but not for lysines 12 and 16 of histone H4 (Figs. 2C and D). In the case of histone H3, 1 h after the end of the perfusion, DADS induced significant 1.5- and 2-fold increases in the acetylation of histone H3 lysine 14 and 9, respectively (Figs. 3A and B). Twenty one hour after the end of the intracaecal perfusion, the amounts of acetylated histone H3 lysines 14 and 9 were no more increased (Figs. 3C and D).

DADS effects on histone acetylation after oral administration

Since humans are generally exposed to DADS through garlic or supplement consumption, the effect of DADS on histone acetylation in colon epithelial cells was also assessed after DADS oral administration. In colonocytes isolated 6 h after DADS administration, we observed a significant 3-fold increase in the acetylation of histone H4 (Figs. 4A and C). Compared to control levels, histone H4 acetylation was no more increased 17 h after gavage (Figs. 4B and C). For lysine 14 of histone H3, 6 h after gavage, similar results were obtained: DADS significantly enhanced histone H3 acetylation at the lysine 14 (1.4×) (Figs. 5A and C). Acetylation state of lysine 14 of histone H3 was not significantly different from control level 17 h after gavage (Figs. 5B and C). In the case of histone H3 lysine 9, DADS-induced hyperacetylation (2.5×) was observed 6 h after treatment (Figs. 5D and F) but not 17 h after DADS gavage (Figs. 5E and F). Thus, DADS induced an increase in histone H4 and H3 acetylation status in colonocytes after both types of *in vivo* administration.

DADS effects on gene expression profiles after intracaecal perfusion

Since in DADS-treated rats histone hyperacetylation was observed in colonocytes, we further examined DADS

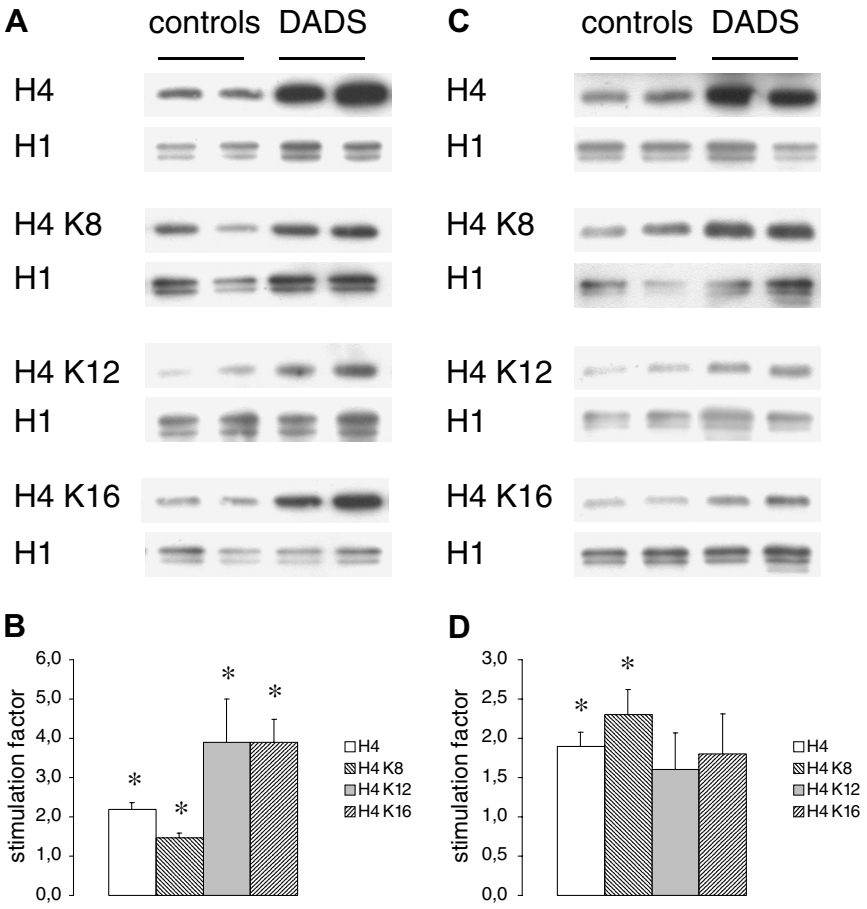

Fig. 2. Effects of DADS intracaecal perfusion on lysines 8, 12, and 16 and total histone H4 acetylation states in colonocytes isolated from control and treated rats. Rats were sacrified 1 h (A, B) or 21 h (C, D) after the end of the perfusion and colonocytes extracted as described in Materials and methods. (A, C) Representative Western blots. Western blot analysis was performed with 5 μg of protein extracts using antibodies directed against specifically acetylated lysine or acetylated histone H4. (B, D) Acetylation signals were normalized as described in Materials and methods and expressed as mean ± SEM (n = from 3 to 8 animals; $^{*}P < 0.05$).

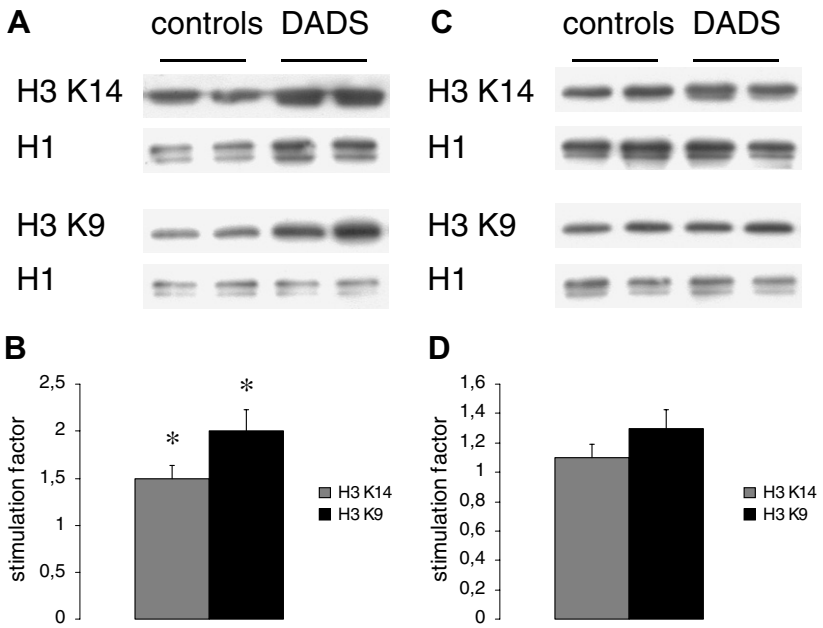

Fig. 3. Effects of DADS intracaecal perfusion on histone H3 lysines 14 and 9 acetylation states in colonocytes isolated from control and treated rats. Rats were sacrified 1 h (A, B) or 21 h (C, D) after the end of the perfusion and colonocytes extracted as described in Materials and methods. (A, C) Representative Western blots. Western blot analysis was performed with 5 μg of protein extracts using antibodies directed against histone H3 specifically acetylated at lysine residues 14 or 9. (B, D) Acetylation signals were normalized as described in Materials and methods and expressed as mean ± SEM ($n = 6$ animals; $^{*}P < 0.05$).

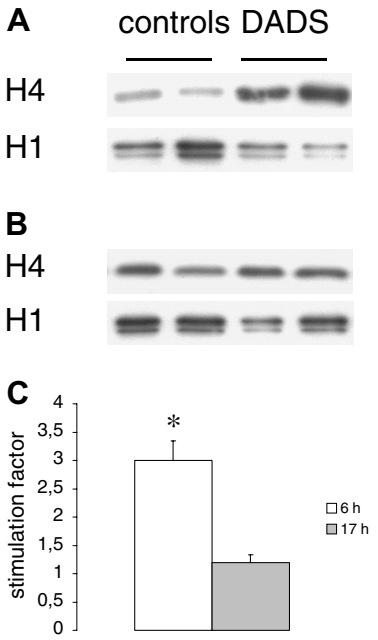

Fig. 4. Effects of DADS gavage on histone H4 acetylation state in colonocytes isolated from control and treated rats. Rats were sacrified 6 h (A) or 17 h (B) after DADS administration and colonocytes extracted as described in Materials and methods. (A, B) Representative Western blots. Western blot analysis was performed with 5 μg of protein extracts using antibodies directed against acetylated histone H4. (C) Acetylation signals were normalized as described in Materials and methods and expressed as mean ± SEM ($n = 4$ animals; $^{*}P < 0.05$).

effects on gene expression profiles in colonocytes isolated 1 or 21 h after the end of the intracaecal perfusion using Atlas Rat cDNA arrays. Gene expression modulations observed after DADS exposure are summarized in Table 1. One hour after the end of the intracaecal perfusion, the expression of only three genes upon 588 differed by at least 2-fold in colonocytes from DADS-treated rats compared to controls. Twenty one hour after the end of the intracaecal perfusion, we observed a modification of gene expression for ~8% of the genes tested: 45 genes were up-regulated whereas four genes were down-regulated in response to DADS. For some of them, the modulation factor is relatively high (up to 48-fold).Thus, in these experimental conditions, DADS treatment *in vivo* induced modulations of gene expression in colonocytes.

Discussion

To our knowledge, the present study reports for the first time the ability of DADS to increase *in vivo* histone acetylation in rat colonocytes. Whereas DADS effects on histone acetylation have been investigated in various tumor cell lines [13,14,19], only one *in vivo* study concerning rat liver and hepatoma was available until now [15].

Here, we show that DADS (200 mg/kg body weight) induces hyperacetylation of histones H4 and H3 in colonocytes after *in vivo* administration. Precisely, increases in histone acetylation status could be observed 1 h after the end of intracaecal perfusion and 6 h after gavage. This kinetic is consistent with previous data obtained in colon tumor cell lines, as well as in hepatoma and liver of tumor-bearing rats, showing a rapid DADS-induced hyperacetylation, after 3 or 6 h treatments in colon cell lines, and 2 and 5 h

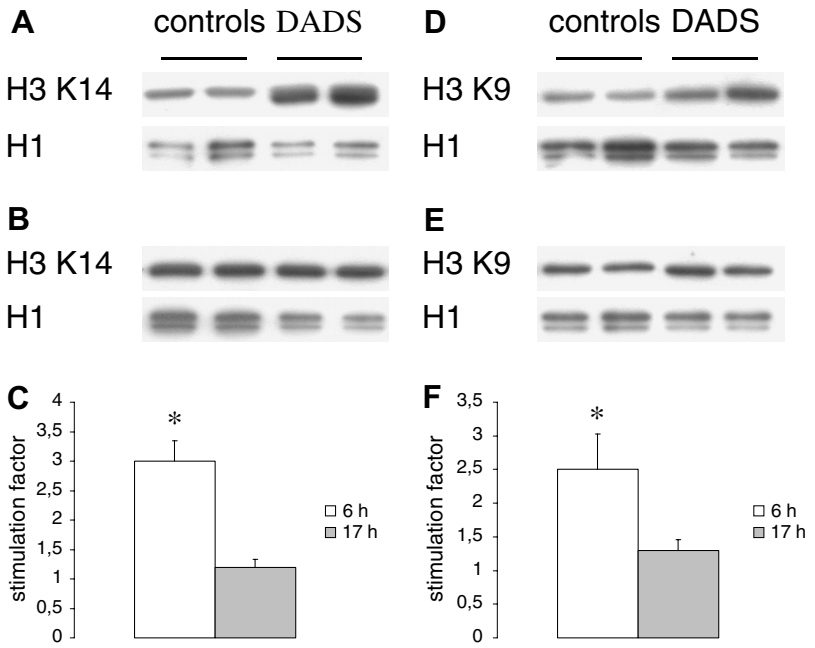

Fig. 5. Effects of DADS gavage on histone H3 lysines 14 and 9 acetylation states in colonocytes isolated from control and treated rats. Rats were sacrified 6 h (A, D) or 17 h (B, E) after DADS administration and colonocytes extracted as described in Materials and methods. (A, B, D, and E) Representative Western blots. Western blot analysis was performed with 5 μg of protein extracts using antibodies directed against histone H3 specifically acetylated at lysine residues 14 or 9. (C, F) Acetylation signals were normalized as described in Materials and methods and expressed as mean ± SEM ($n = 4$ animals; $^{*}P < 0.05$).

after *in vivo* administration in rats [13,15]. In our study, 21 h after the end of DADS perfusion, hyperacetylation of histone H4 and specific lysine 8 of histone H4 remained significant whereas acetylation levels of lysines 12 and 16 of histone H4, as well as lysines 14 and 9 of histone H3 were not significantly different from controls. Moreover, 17 h after gavage, histone H4 and H3 acetylation status was similar to control levels. Our results show a transitory effect on histone acetylation in response to DADS *in vivo* administration suggesting that repetitive exposures might be necessary for the expression of DADS biological or chemopreventive properties.

When given by gavage, DADS induced histone hyperacetylation. Considering limited data available on *in vivo* DADS metabolism, especially in regard to colon cells, this result provides evidence that in these experimental conditions DADS or one of its metabolites is delivered to colon cells.

Since steady-state levels of acetylation result from the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), DADS-induced histone hyperacetylation could involve either a stimulation of HAT activity or inhibition of HDAC activity. Considering that DADS was previously shown to inhibit HDAC activity in tumor colon and erythroleukemic cell lines and that none HDAC inhibitor has also the ability to increase HAT activity, we hypothesize that DADS effect on histone acetylation in rat colonocytes could be due exclusively to HDAC inhibition [13,19].

Recent studies have shown in rodents, that DADS modulates the expression of some genes *in vivo* in different organs. DADS modified the expression of genes encoding phase I and II drug metabolizing-enzymes, glutathione *S*-transferases in mice colon, liver, stomach, and intestine [10], and cytochrome P450 monooxygenases in rat liver [20]. Interestingly, a study from our laboratory revealed that in liver cells *in vitro* and *in vivo* DADS increases ferritin expression [21], a gene whose transcription is at least in part regulated by the histone acetylase activity of p300/CBP [22].

In the present study, using the Clontech Atlas Rat cDNA expression arrays, we explored the ability of DADS to modulate gene expression in colonocytes *in vivo*, after intracaecal perfusion. Although these data need to be confirmed using quantitative methods, they show that in colonocytes *in vivo* DADS modulated mRNA level of numerous genes. Our results suggest that the expression of a few genes could be rapidly modulated by DADS. Interestingly, 1 h after the end of the perfusion, DADS increased the mRNA level of the drug metabolizing-enzyme glutathione *S*-transferase pi; this observation is in agreement with previous results obtained by real time PCR in mouse colon [10]. Twenty one hours after the end of the intracaecal perfusion, the expression of 49 genes seemed to be modified by DADS. Until now, the analyse of DADS effects on gene expression profile using macro arrays (Clontech Atlas Human Cancer cDNA expression arrays) had been performed only *in vitro*: in the colon

Table 1
Genes up- or down-regulated by DADS in colonocytes isolated from rats 1 or 21 h after the end of the intracaecal perfusion

Gene name	Accession No.	Fold
One hour after the end of the intracaecal perfusion		
Inhibitor of DNA binding 1	D10862	↓ 2
Mitogen-activated protein kinase 3	M61177	↓ 4
Glutathione *S*-transferase pi 2	X02904	↑ 2.4
Twenty one hour after the end of the intracaecal perfusion		
Oncogenes and tumor suppressors		
MAD homolog 1	U66478	↑ 4.3
Neurofibromin	D45201	↑ 5.1
Maspin	U58857	↑ 2.2
Wilms' tumor protein homolog 1	X69716	↑ 48.2
c-myc proto-oncogene	Y00396	↑ 2.4
fyn proto-oncogene	U35365	↑ 2.7
B-cell leukemia/lymphoma protein 2	L14680	↑ 4.2
Cell cycle proteins		
G2/M-specific cycline G	X70871	↑ 3.2
Cycline-dependent kinase 7	X83579	↑ 2.6
Metabolism		
Cytochrome P450 IA1	X00469	↑ 10.3
Glutathione reductase	U73174	↑ 2.4
Carboxyl ester lipase	X16054	↑ 13.1
2-Hydroxyacylsphingosine 1-β-galactosyltransferase	U07683	↑ 3.6
Transcription activators and repressors		
Growth arrest and DNA damage-inducible protein 153	U30186	↑ 4.9
Nerve growth factor-induced protein I-B	U17254	↑ 9.1
Signal transduction		
Signal transducer and activator of transcription 3	X91810	↑ 2.7
fps/fes-related tyrosine–protein kinase	X13412	↑ 2.2
Spleen tyrosine kinase	U21684	↑ 2.2
Mitogen-activated protein kinase kinase 2	D14592	↑ 3
G protein-coupled receptor kinase 2	M87854	↑ 16.2
Phospholipase C β1	M20636	↑ 2
Calcium-independent phospholipase A2	U51898	↑ 25.8
Ral B	L19699	↑ 4.2
Muscle/brain cAMP-dependent protein kinase inhibitor	L02615	↑ 7.8
Receptor protein–tyrosine phosphatase ζ/β	U09357	↑ 4.4
Adenosine A2b receptor	M91466	↑ 4.4
Parathyroid hormone 2 receptor	U55836	↑ 2.7
Follicle-stimulating hormone receptor	L02842	↑ 4
Prostaglandin F2 α receptor	U47287	↓ 7.6
Hormones, growth factors, cytokines, and chemokines		
Endothelin 1	M64711	↑ 9.6
Somatoliberin	U10156	↑ 8.4
Thymosin β-like protein	U25684	↑ 4
Nerve growth factor 8A	M60525	↑ 2.4
Fibroblast growth factor 10	D79215	↑ 3.3
Transporters		
Organic anion transporter	AB004559	↑ 2.2
High affinity L-proline transporter	M88111	↑ 2.2
Organic cation transporter 1A	U76379	↑ 2.1
Proton-coupled dipeptide cotransporter	D50306	↑ 4.2
Sodium–glucose cotransporteur 1	U03120	↑ 4.7
Solute carrier family 14 member 2	U77971	↑ 4
Aquaporin 3	D17695	↓ 8
Aquaporin 8	AF007775	↓ 4.6
Thymosin β-like protein	U25684	↑ 4
Others		
Telomerase protein component 1	U89282	↑ 4.0
Urokinase-type plasminogen activator	X63434	↑ 2.9
Tripeptidylpeptidase II	U50194	↑ 3.6
Mast cell protease 1	U67915	↑ 5.1
Angiotensin-converting enzyme	U03734	↑ 3.3
Proteasome activator 28 α subunit	D45249	↑ 3.3
Fibrinogen β subunit	U05675	↓ 11.3

Values represent fold increase or fold decrease in gene expression by DADS compared with control signals.

tumor cell line HCT-15, DADS modified the expression of 36 genes involved in several processes including cell cycle, DNA repair and cellular adhesion factors [11]. Using different arrays, we show for the first time DADS ability to modify gene expression profile in colon cells *in vivo*. Interestingly, our observations suggest that DADS modulates *in vivo* the expression of genes encoding proteins involved not only in cell cycle and proliferation but also in other cellular processes such as metabolism, detoxication, signal transduction and transport.

We observed *in vivo* a correlation between the modulation of gene expression and histone hyperacetylation in response to DADS. Nevertheless, further investigations are needed to identify the genes whose expression modification involves at least in part histone hyperacetylation. The expression of some genes up-regulated by DADS, i.e., cytochrome P450 1A1 and maspin, is known to be regulated partly by histone acetylation [23–26]. Thus, the involvement of histone acetylation in the expression of such DADS target genes remains to be confirmed by chromatin immunoprecipitation experiments.

Interestingly, DADS enhanced the expression of genes encoding drug metabolizing-enzymes or tumor suppressor proteins such as glutathione *S*-transferase pi, cytochrome P450 1A1, glutathione reductase, neurofibromin, maspin..., that may account for DADS chemopreventive effects. Thus, it would be interesting to investigate *in vivo*, in colonocytes, the various biological effects associated to these modulations of gene expression and to determine whether histone acetylation plays a role in DADS colon anticarcinogenic properties using *in vivo* models of colon carcinogenesis.

Finally, the present study suggests the involvement of histone H4 and H3 hyperacetylation in the modulation of gene expression by DADS *in vivo*. Histone hyperacetylation might be one of the mechanisms involved in DADS biological and chemopreventive effects as recently suggested [27]. These results provide evidence that dietary compounds such as butyrate, sulforaphane and diallyl disulfide, recently identified as HDAC inhibitors, might subtly modulate histone acetylation and gene expression through a life time exposure and be effective in cancer prevention [28,29].

Acknowledgments

We are grateful to Claire Maudet and Patrice Dahirel for care of the animals.

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