DNA methylation affects cell proliferation, cortisol secretion and steroidogenic gene expression in human adrenocortical NCI-H295R cells

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Abstract

Aberrant DNA methylation may be involved in human adrenocortical tumorigenesis, which is often accompanied by abnormal hormone production. In this study, we aimed to clarify the effects of DNA methylation on steroidogenesis using the human adrenocortical NCI-H295R cell line as a model. Treatment with the DNA methylation inhibitor 5-aza-2’-deoxycytidine (Azad; 10 µM for 7 days) decreased the proliferation rate to approximately 20% and the cell number to 60% of the control, with a simultaneous increase in the expression of the cyclin-dependent kinase inhibitor p57KIP2 gene. In addition, Azad treatment increased cortisol secretion dose and time dependently, whereas dehydroepiandrosterone sulfate secretion was not affected. Azad treatment decreased basal and (Bu)2cAMP-induced expression of low- and high-density lipoprotein receptor, steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme, steroid 17α-hydroxylase/17,20-lyase and steroid 21-hydroxylase mRNA, as well as the STAR protein level. In contrast, Azad treatment increased the basal expression of steroid 11β-hydroxylase and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase genes, although it inhibited the (Bu)2cAMP-induced expression of these two genes. The expression of steroidogenic factor-1 (SF-1) and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome 1) genes (both harboring putative CpG islands in their promoters) and the methylation degree of the HpaII recognition site(s) in the SF-1 gene promoter region were reduced by Azad treatment. The immunostaining pattern of the methyl-CpG-binding protein MeCP2 was also modified by Azad treatment. These results suggest that DNA methylation may be implicated in the regulation of cell proliferation and steroidogenesis in human adrenocortical cells.

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Introduction

Modification of DNA by cytosine methylation is an important mechanism of epigenetic regulation of genomic functions (Zingg & Jones 1997, Jaenisch & Bird 2003). DNA methylation patterns are mosaics in various cell and tissue types of an organism. These cell- and tissue-specific methylation patterns are a result of an ordered demethylation and re-methylation process that occurs during development. Once a given sequence becomes methylated, it then becomes a target for the binding of a methyl-CpG-binding protein (MeCP), such as MeCP2. The binding of MeCPs can induce changes in the surrounding chromatin to affect gene transcription by interfering with the actions of transcription factors (Wade 2001, Prokhortchouk & Hendrich 2002). It is believed that activation of cell lineage-determining genes leads to activation and maintenance of a cell-type-specific network of gene expression. The genes involved in this determination are tightly regulated so that any expression of these genes is prevented in a different cell lineage. Cell-type-specific DNA methylation could be involved in establishing or maintaining the expression pattern of these determinative genes and thus contribute to the stability of a specific differentiation state (Zingg & Jones 1997, Jaenisch & Bird 2003).
In adrenocortical cells, steroid hormone production is controlled at two levels, i.e. substrate mobilization for acute control and gene transcription for long-term regulation of steroidogenesis. Cholesterol, taken up by low- or high-density lipoprotein receptors (LDL receptor or CLA-1 respectively), is first transported to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) and converted to pregnenolone by P450 scc (cholesterol side-chain cleavage enzyme). Production of steroid hormones in human adrenals is zonally and developmentally regulated. Pregnenolone may be hydroxylated to 17α-hydroxypregnenolone and further cleaved to dehydroepiandrosterone (DHEA) by P450c17 (steroid 17α-hydroxylase/17,20-lyase), augmented by cytochrome b5. Pregnenolone and 17α-hydroxypregnenolone are also metabolized to aldosterone and cortisol respectively, via successive reactions of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD), steroid 21-hydroxylase (P450c21) and steroid 11β-hydroxylase (P450c11) or aldosterone synthase (P450c18). Two orphan members of the nuclear receptor superfamily, steroidogenic factor-1 (SF-1) and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome 1), have been shown to play a central role in adrenocortical morphogenesis and in mediating transcriptional regulation of the steroidogenic genes (Mesiano & Jaffé 1997, Miller et al. 1997, Peter & Dubuis 2000).

The contribution of DNA methylation to the steroidogenic activity of adrenocortical cells has not been studied much. The methylation level of specific CpG sites in the 5′-flanking region of the P450c17 gene was positively correlated with P450c17 expression when compared in bovine adrenal cortex, cultured adrenocortical cells and fibroblasts, but not when compared in different bovine adrenocortical zones and white blood cells, suggesting that the methylation status of the P450c17 gene is not always related to its transcription (Hornsby et al. 1991, 1992). In contrast, the promoter methylation status of the P450c21 gene is inversely associated with its expression level in mouse adrenocortical Y-1 and normal mouse adrenocortical cells, and suppression of methyltransferase mRNA expression reduces the methylation degree but activates P450c21 expression in Y-1 cells and in transplanted Y-1 tumors. Nevertheless, the DNA methylation inhibitor 5-azacytidine cannot recover the expression of the P450c21 gene even though the methylation degree of the gene is reduced. In addition, loss of P450c21 expression temporally precedes the extensive methylation of the transfected P450c21 gene in Y-1 cells, indicating that methylation may not be the principal cause of P450c21 gene repression in this cell line (Szyf et al. 1990, Ramchandani et al. 1997).

Altered hormone production is often observed in human adrenocortical tumors (Dackiw et al. 2001, Ng & Libertino 2003), which have aberrant DNA methylation. In normal and pathological adrenocortical tissues, the H19 promoter methylation correlated negatively with the tumor suppressor H19 and positively with the nearby insulin-like growth factor-II (IGF-II) gene expression. A DNA methylation inhibitor 5-aza-2′-deoxycytidine (Azad) reduced proliferation of human adrenocortical cell line NCI-H295R (H295R) cells, probably on the basis of the induction of H19 and reduction of IGF-II gene expression (Gao et al. 2002). Azad incorporates into newly synthesized DNA and then inhibits DNA methylation. Thus, it has been widely used to demonstrate the association between methylation in specific gene regions and the expression of the associated genes (Christman 2002). A number of experiments have shown that altering DNA methylation patterns with Azad can change the differentiation state of cells (Zingg & Jones 1997). As knowledge about the effects of DNA methylation on steroidogenesis could increase our understanding of the precisely controlled gene expression during adrenocortical differentiation and tumorigenesis, we studied the effect of Azad on cortisol secretion and expression of steroidogenic genes in H295R cells.

Materials and methods

Cell cultures

H295R cells obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (Sigma) supplemented with 1% Insulin, Transferrin and Selenite (ITS)+1 liquid media supplement (Sigma), 2% Ultroser SF (Biosepra, Marlborough, MA, USA), 2 mM l-glutamine (Gibco) and antibiotics (125 µg/ml streptomycin and 125 IU/ml penicillin; Orion Pharmaceutical Co.,
Espoo, Finland) at 37 °C in a 5% CO₂ atmosphere. The medium was changed every other day and the cells were split at a ratio of 1:3 with trypsin after reaching confluence. Treatment with Azad and (Bu)₂cAMP (both from Sigma) was initiated on the second day after reseeding of the cells. Triplicate dishes were used for the experiments that were repeated at least three times. Trypan blue exclusion was routinely used to monitor cell viability.

Hormone measurements
Cortisol was measured from conditioned cell culture media with the Technicon Immuno 1 system (Bayer). This method is a magnetic separation competitive enzyme immunoassay (Letellier et al. 1996) with a detection limit of 20 nM. The intra- and interassay coefficients of variation were <4 and <6% respectively. Dehydroepiandrosterone sulfate (DHEA-S) was analyzed by competitive enzyme immunoassays with measurement kits from Diagnostic System Laboratories (Webster, TX, USA; product code DSL-10–3500). The detection limit of the DHEA-S assay was 40·7 nM. The intra- and interassay coefficients of variation were 5·1 and 7·2% respectively.

RNA analysis
Cytoplasmic RNA was extracted from the H295R cells as previously described (Voutilainen et al. 1986, Ilvesmäki & Voutilainen 1991). PolyA⁺ mRNA was prepared from the cytoplasmic RNA with Oligotex mRNA Spin-Column (Qiagen) according to the manufacturer’s instructions. The cytoplasmic RNA or polyA⁺ mRNA was analyzed by Northern blotting and the membranes were hybridized with cDNA or oligonucleotide probes as reported previously (Ilvesmäki & Voutilainen 1991, Liu et al. 1996). The probes for p57KIP2 (Liu et al. 1997), LDL receptor, CLA-1 (Liu et al. 2000), StAR (Liu et al. 1996), P450 scC, P450c17, P450c21, P450c11, γ-actin (Ilvesmäki & Voutilainen 1991) and 3β-HSD (Voutilainen et al. 1991) were the same as described previously. Additional 30 mer oligonucleotide probes were used to detect SF-1, DAX-1 and cytochrome b5 mRNAs in Northern hybridization analyses. The oligonucleotide sequences were 5′-TCA GGC ACT TCT GGA AGC GGC AGA AGG GAC-3′ for SF-1 (GenBank accession no. D84207), 5′-AGA AAG

CAC TTG ATG GCT TGG ACC TGG GAG-3′
for DAX-1 (GenBank accession no. U31929) and
5′-ATG GCA GGG ATC ACC CAG TTT GTC
CAC CAA-3′ for cytochrome b5 (GenBank
accession no. BC015182) mRNAs. The relative
intensities of the autoradiographic signals were
quantified by densitometric scanning. All RNA
data from Northern blotting prepared with
cytoplasmic or polyA⁺ RNA shown here were
normalized with the respective 28S ribosomal RNA
or γ-actin mRNA values.

PCR-based methylation analysis
PCR-based methylation analysis (Gao et al. 2002)
was used to analyze the SF-1 promoter and exon 1
area (GenBank accession no. D84206) which
contains numerous HpaII/MspI recognition
sequences CCGG. Briefly, genomic DNA (500 ng)
was first digested for 2 h with 10 U EcoRI and
HpaII (methylation sensitive) or MspI (methyla-
tion insensitive) enzymes (Boehringer Mannheim)
according to the manufacturer’s recommendations.
Optimal PCR conditions with a clean 383 bp PCR
product from undigested genomic DNA but no
product from MspI-digested DNA were found by:
denaturing at 95 °C for 5 min; 35 cycles of 95 °C
for 60 s, 65 °C for 30 s and 72 °C for 45 s; and then
final extension at 72 °C for 10 min. The PCR
reaction volume was 20 µl, containing 0·2 mM of
each dNTP, 0·5 µM of each primer, 1 × reaction
buffer, 2·5 mM MgCl₂, 1·5 U Taq DNA poly-
merase (Fermentas Tamro Corporation, Vantaa,
Finland) and 50 ng template DNA. The primer set
was 5′-TCA GCC CCC AGA TAG ATA GG-3′
(forward) and 5′-aag gag gct ggc cat tag ag-3′
(reverse). IGF-II exon 9 was used as an internal
control for the DNA amount because this
region has no HpaII/MspI cutting site (Gao et al.
2002). The PCR products were resolved on 2%
agarose gels. PCR-based analyses were performed
at least twice to ensure reproducibility of the
results.

Flow cytometric analysis
Flow cytometric analysis was performed on cells
treated with Azad. The cells were trypsinized,
collected into 70% ethanol and stained with
propidium iodide. DNA content was analyzed by a
flow cytometer. Cell cycle analysis was performed
using the Sync Wizard Model of ModFit LT software.

**Immunocytochemical staining**

For immunocytochemistry, H295R cells were cultured on two-well chamber plastic slides. The proliferation was demonstrated with a commercial cell proliferation kit (Amersham) to detect 5-bromo-2′-deoxyuridine (BrdU) incorporation into the replicating DNA. MeCP2 was detected with a polyclonal antibody (Upstate Ltd, Milton Keynes, UK). During the staining, the primary antibody diluted at 1:100 was added to the culture slides in PBS and incubation was performed overnight at 4 °C. The slides were then incubated with biotin-conjugated secondary antibody from the ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA), and thereafter with ABC solution according to the manufacturer’s instructions. Finally, light counterstaining was performed with hematoxylin, and the slides were dehydrated and mounted. For the negative control, the primary antibody was replaced with normal rabbit serum or PBS alone. To exclude the effect of possible endogenous biotin, biotin blocking (avidin–biotin blocking kit, Vector Laboratories) was performed before the addition of the primary antibody. The culture area was photographed and the nuclei were counted from each culture chamber. The positive staining percentage was calculated on the basis of positively stained nuclei relative to all cells.

**Western blotting**

Cells grown on 10 cm plates were collected and lysed with 160 µl protein sample buffer (63.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue). The mixture was sonicated briefly on ice and the total protein concentration in the supernatant was determined by the standard BCA protein assay kit (Pierce Boston Technology, Woburn, MA, USA). Subsequently, 4 µl β-mercaptoethanol were added to each sample before heating at 95 °C for 5 min. Twenty-five micrograms of total protein were loaded to each lane and separated in 10% SDS–PAGE. Non-specific binding was minimized by blocking with 5% non-fat milk in PBS–0.05% Tween 20. The membrane was then incubated with StAR (ABR Affinity BioReagents, Golden, CO, USA) and MeCP2 antibodies. The secondary antibody was a horseradish peroxidase-conjugated polyclonal antibody (DAKO, Glostrup, Denmark) and the immunoblots were developed with ECL films (Amersham). Monoclonal anti-actin antibody from Sigma (recognizing all actin isoforms) was used to control the protein loading.

**Statistical analyses**

The non-parametric Kruskal–Wallis test was used to reveal differences in cortisol secretion, the expression levels of the steroidogenic genes and the methylation status among the differently treated groups of H295R cells. If significant differences were found, the Mann–Whitney U test was subsequently used for statistical evaluation. The Mann–Whitney U test was also used to evaluate the changes in the proliferation of the cultured cells. The level of significance was chosen as \( P<0.05 \).

**Results**

Azad treatment reduced BrdU incorporation into H295R cells. This reduction was detectable from the 72-h time point and was maintained for at least 2 weeks when Azad was used at the concentration of 10 µM. At the 7-day time point Azad treatment (10 µM) reduced the proliferation rate to approximately 20% of the control (mean ± s.e. of proliferation percentage, 7.7 ± 2.8% for the treated cells and 41.7 ± 11% for the controls, \( n=5, P<0.01 \)). Direct cell counting showed decreased cell number in Azad-treated cultures (approximately 60% of the controls at the 7-day time point). In addition, Azad treatment enlarged the nuclear size, as analyzed by immunocytochemistry. To confirm this finding, we used flow cytometric analysis to measure the DNA content of the cell nuclei (Fig. 1a). Under normal growth conditions for 6 days after reseeding of the cells, about 60% of the H295R cells were in G0/G1 phase, and only 17% were in G2/M phase. In contrast, about 40% of the cells were in G2/M phase after 6 days of sustained treatment with Azad (10 µM), and the fraction of the G0/G1 cells decreased down to 43%. The fraction of S-phase cells was also reduced by about 10% with the Azad treatment. The average DNA content of the cells was increased by Azad
Treatment in G₀/G₁, G₂/M and S phases by about 12, 16 and 27% respectively. The cell viability after treatment with Azad (10 µM) for 7 days remained at more than 98%, as measured by trypan blue exclusion analysis. Higher doses of Azad induced cell death (data not shown). We further studied the effect of Azad on the expression of the cyclin-dependent kinase inhibitor p57^KIP2 gene, which is often lost in malignant adrenocortical tumors (Liu et al. 1997). In agreement with a previous report on various hematological cell lines (Li et al. 2002), Azad treatment increased the accumulation of p57^KIP2 mRNA in H295R cells (Fig. 1b).

To characterize the effect of Azad on steroidogenesis in H295R cells, we first measured cortisol concentration from the conditioned media. The basal steroid secretion depended on the cell number and culture time, with the cortisol production 36·1 ± 3·5 nmol/mg DNA and DHEA-S 16·9 ± 1·1 nmol/mg DNA during 1 day.
of culture. As the absolute steroid values do not have any particular significance for the regulatory effect of Azad, we expressed steroid concentrations as percentages of the respective control in each experiment. As shown in Fig. 2, Azad treatment increased cortisol secretion in a dose- and time-dependent manner. Since the cell number was decreased during Azad treatment, the increased cortisol concentration means enhanced cortisol secretion from individual cells. In contrast, DHEA-S secretion was not significantly affected by Azad treatment. In addition, (Bu)$_2$cAMP increased cortisol secretion dose and time dependently (data not shown), and this stimulatory effect of (Bu)$_2$cAMP was inhibited by pre- or co-treatment with Azad for 1 week (Table 1, and data not shown). DHEA-S secretion was also increased by (Bu)$_2$cAMP (1 mM for 7 days) incubation by about 35%, and this stimulatory effect was not affected by co-treatment with Azad (Table 1). Androstenedione secretion behaved similarly to that of DHEA-S in these experiments (data not shown).

In order to clarify the mechanism underlying the increased cortisol secretion during Azad treatment, we studied the expression of steroidogenic genes with Northern blot analyses. Unexpectedly, Azad treatment decreased basal and (Bu)$_2$cAMP-induced LDL receptor, CLA-1, StAR, P450 sc, P450c17 and P450c21 mRNA expression (Fig. 3a and b). Since StAR controls the rate-limiting step in steroidogenesis (Christenson & Strauss 2001), we measured StAR protein levels by Western blot analysis to find out if the mRNA and protein levels behave similarly after Azad treatment. As shown in Fig. 3c, the basal and (Bu)$_2$cAMP-induced StAR protein (including the 37 kDa preprotein and 30 kDa mature protein) levels were reduced by Azad treatment ($P<0.05$, $n=4$). The reduction of StAR mRNA accumulation by Azad treatment was time dependent (Fig. 3d), with the earliest statistically significant effect detected after 5 days of the treatment. Interestingly, Azad treatment slightly induced the basal expression of P450c11 and 3β-HSD mRNAs, but inhibited

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The effect of Azad (10 µM) and (Bu)$_2$cAMP (1 mM) treatments (for 7 days) on the production of cortisol and DHEA-S in H295R cells</th>
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<tbody>
<tr>
<td>Cortisol</td>
<td>Control: 100±6</td>
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<tr>
<td>DHEA-S</td>
<td>Control: 100±11</td>
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The values represent the means±S.E. from six experiments, with the controls adjusted to 100. *$P<0.05$; **$P<0.01$ as compared with the control; ***$P<0.01$ as compared with the (Bu)$_2$cAMP group.
Effect of Azad (10 µM for 7 days) and (Bu)2cAMP (1 mM for 1 day) treatment on the mRNA expression of LDL receptor (LDL-R), CLA-1, StAR, P450 scc (scc), P450c17 (c17), P450c21 (c21), P450c11 (c11) and cytochrome b5 (b5) genes in H295R cells. The bars represent the relative mRNA expression (mean±S.E.) in eight separate experiments when the control is adjusted to 100. *P<0.01 compared with the control and #P<0.01 compared with the (Bu)2cAMP group. (b) Representative Northern blot analysis showing mRNA expression of steroidogenic genes regulated by Azad (7 days) and (Bu)2cAMP (1 day) treatment at the indicated concentrations in H295R cells. The Northern blotting was performed with 20 µg cytoplasmic RNA on each lane. 28S ribosomal RNA expression is shown to demonstrate equal loading. (c) Representative Western blot analysis showing StAR expression in H295R cells treated with Azad (10 µM for 7 days) and/or (Bu)2cAMP (1 mM for 1 day or 3 h). Actin (all isoforms) was used as a loading control. (d) Time-dependent effect of Azad (10 µM) treatment on StAR mRNA accumulation in H295R cells. Each column represents relative StAR mRNA level (mean±S.E. of four experiments) with the control values adjusted to 100. *P<0.05 compared with the control.
(Bu)2cAMP-induced P450c11 and 3β-HSD gene expression (Fig. 4a). Since the basal expression level of these two genes was too low to be clearly shown by Northern blotting using cytoplasmic RNA, the effect of Azad on these two genes was also studied by Northern blotting with polyA+ RNA (Fig. 4b). These effects of Azad treatment were dose and time dependent (data not shown). In contrast, cytochrome b5 mRNA was not affected by Azad treatment (Figs 3a and b, and 4).

Since the expression of many steroidogenic genes was cooperatively down-regulated by Azad treatment, and there are no putative CpG islands in the promoter regions of these genes, we hypothesize that these genes were not directly regulated by Azad. We therefore studied the expression of SF-1 and DAX-1 genes, which have many CpG sites in their promoter areas. As shown in Fig. 4, the expression of both SF-1 and DAX-1 genes was inhibited by Azad treatment. Since DAX-1 was previously reported to be regulated by SF-1 (Parker & Schimmer 1997, Peter & Dubuis 2000), we concentrated on the mechanism of SF-1 regulation.

To study if Azad treatment affects the methylation status of the SF-1 promoter, we used PCR-based methylation analysis. As shown in Fig. 5, the methylation degree of the HpaII recognition site(s) in the SF-1 gene promoter region decreased after Azad treatment, as demonstrated by the weaker PCR product band for the Azad-treated sample than for the control after HpaII digestion of the cell line DNA. We also used several other methylation-sensitive restriction enzymes, including AvaII, FspI, HaeII and HhaI. Their restriction sites were unmethylated in H295R cells and thus Azad treatment had no effect on their methylation status (data not shown).

Figure 4 (a) Effect of Azad (10 µM for 7 days) on the mRNA expression of StAR, P450c11 (c11), 3β-HSD (HSD), SF-1, DAX-1 and cytochrome b5 (b5) genes in H295R cells. The data were from three Northern blots prepared with either polyA+ or cytoplasmic RNA. The bars represent the relative mRNA expression (mean±s.e.) when the control is adjusted to 100. *P<0.05 compared with the control. (b) Representative Northern blot showing mRNA expression of indicated genes in H295R cells treated with or without Azad (10 µM for 7 days). The Northern blot was prepared with 3 µg polyA+ RNA on each lane; γ-actin mRNA expression was used to control mRNA loading.

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To find out whether the machinery for modulating gene transcription by DNA methylation exists in H295R cells, we immunostained cultured H295R cells with a MeCP2 antibody. While weakly to moderately positive staining was detected in most nuclei in both control cultures and Azad-treated cells, a minority of cells showed strong staining. These strongly stained nuclei were generally smaller in size than the others, and they were more frequently observed in the control than in the Azad-treated cultures (Fig. 6). Western blotting analysis also showed expression of the 75 kDa MeCP2 protein in H295R cells and Azad treatment had no effect on the MeCP2 protein level (data not shown).

**Discussion**

In this study, we demonstrated that DNA methylation is implicated in the regulation of cellular proliferation and steroidogenesis in adrenocortical H295R cells. The presence of the methyl-CpG-binding protein MeCP2 in H295R cells confirms that a functional machinery to mediate the effect of DNA methylation is available in these cells (Lewis  et al. 1992). Using incorporated BrdU as a proliferation marker, we reproduced the previous finding that inhibition of DNA methylation by Azad reduces H295R cell proliferation (Gao  et al. 2002). In addition, we confirmed the result by direct cell counting. This reduced proliferation rate is unlikely to be due to potential general toxicity of Azad, since Azad increased coordinately the expression of p57KIP2 (this study), p21 (data not shown) and H19 (Gao  et al. 2002) RNAs in this cell line. This increased expression of cyclin kinase inhibitors and a tumor suppressor may lead to the inhibition of H295R cell proliferation by blocking the cell cycle mainly to the G2/M phase. Furthermore, the reduction of proliferation was accompanied by increased cortisol secretion during Azad treatment. Azad treatment caused three types of modulation in the expression of steroidogenic genes. First, it reduced basal and...
(Bu)$_2$cAMP-induced expression of most steroidogenic genes, including LDL receptor, CLA-1, StAR, P450 scc, P450c17 and P450c21 genes. Secondly, for some genes, such as P450c11 and 3β-HSD, it increased their basal expression, but inhibited their (Bu)$_2$cAMP-induced expression. The third type of modulation concerned the cytochrome b5 gene, which did not change its expression in response to Azad treatment. The basal expression of the first type of gene was usually high, easily detectable by Northern blotting with cytoplasmic RNA. After treatment with Azad alone, the abundant expression of these genes was reduced, but still allowed basal steroidogenesis. It is also possible that, despite the reduced gene expression, the enzyme activities of these genes were not reduced significantly during Azad treatment; dissociation of steroidogenesis and StAR gene expression has been described previously in different steroidogenic cells (King et al. 2000, Gambaryan et al. 2003). However, the basal expression of P450c11 and 3β-HSD genes in untreated cells was very low, almost undetectable by Northern blotting with cytoplasmic RNA. The low expression levels of these genes might limit the basal biosynthesis of cortisol in untreated cells, as reported in human fetal adrenals (Voutilainen et al. 1991, Staels et al. 1993). Steroidogenesis is thus directed via the Δ$^5$-steroid pathway, leading to the production of DHEA and DHEA-S, which are the major steroid products of this cell line. The basal expression level of the P450c11 and 3β-HSD genes was increased by Azad, resulting in increased cortisol biosynthesis. Interestingly, the global genomic methylation level is gradually reduced with aging (Richardson 2003), which is accompanied by slightly increased basal serum cortisol concentration (Ferrari et al. 2001). Whether this age-related change in DNA methylation is involved in the regulation of steroidogenic gene expression is not clear. However, the interpretation of this study should be cautious, since H295R cells originated from an adrenocortical carcinoma, which often have aberrant methylation status (Gao et al. 2002). Steroidogenesis in adrenocortical tumors is usually abnormal (Dackiw et al. 2001, Ng & Libertino 2003). Whether DNA methylation is directly implicated in aberrant steroidogenesis in adrenal tumors remains to be clarified.

There are several ways in which Azad might regulate the expression of the steroidogenic genes in H295R cells. Previous reports suggested that the expression of some steroidogenic enzyme genes could be directly regulated by DNA methylation in certain CpG sites, although their promoter areas have no typical CpG islands (Szyf et al. 1990, Hornsby et al. 1991, 1992). In addition to a direct change of promoter methylation status affecting gene expression, Azad may indirectly regulate steroidogenic gene expression by modulating transcription factors or signal transduction pathways. Generally, more than half of the genes induced by Azad treatment were supposed to be induced by an indirect effect (Karpf & Jones 2002). Since not all steroidogenic genes have typical CpG islands in their promoters, indirect regulation of these genes by Azad is likely. If this is the case, SF-1 is a good candidate since its promoter region has a CpG island, and the transcription of the main steroidogenic genes as well as that of the DAX-1 gene has been reported to be regulated by SF-1 (Mesiano & Jaffe 1997, Peter & Dubuis 2000). The present study showed that SF-1 is down-regulated by Azad treatment. The important transcription elements E box CACGTG and GC-rich Sp1-binding site in the SF-1 promoter area (Parker & Schimmer 1997, Woodson et al. 1997, Scherrer et al. 2002) within our PCR-amplified region but not recognized by HpaII, may be the target sites for the effects of DNA methylation (Griswold & Kim 2001). It was previously reported that most of the Azad down-regulated genes may contain a typical CpG island in their promoter regions in the human LD419 fibroblast cell line (Liang et al. 2002). However, the inhibitory effect of Azad on SF-1 expression could also be secondary to an induction of a negative modulator of SF-1 transcription, since inhibition of promoter methylation is usually associated with activation of gene expression (Zingg & Jones 1997, Jaenisch & Bird 2003). In addition, since the modulation of steroidogenic gene expression was not identical, there could be several factors/signal pathways simultaneously implicated during Azad treatment. For example, IGF-II expression is down-regulated by Azad treatment in this cell line (Gao et al. 2002) and IGF-II can preferentially affect biosynthesis of some steroid hormones probably by regulating steroidogenic enzyme gene expression in human adrenocortical cells (Fottner et al. 1998). Interestingly, the Azad-upregulated genes – such as H19, p57KIP2, 3β-HSD and P450c11 – are generally expressed at
low levels, whereas the down-regulated IGF-II and some steroidogenic genes are expressed at high levels in adrenocortical carcinomas (Gao et al. 2002, this study). Therefore, the changed expression ratio of these genes during Azad treatment is likely to play a role in H295R cell proliferation and steroidogenesis.

In summary, our results showed that cell proliferation and both basal and (Bu)2cAMP-stimulated cortisol secretion and steroidogenic gene expression are regulated by the DNA methylation inhibitor Azad in H295R cells, suggesting that DNA methylation is implicated in the regulation of human adrenocortical cell proliferation and steroidogenesis.

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