

Effect of 9-*cis* Retinoic Acid on Dopamine D2 Receptor Expression in Pituitary Adenoma Cells

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The dopamine receptor subtype 2 (D2R) promoter contains a functional retinoic acid response element involved in the control of D2R expression. The aim of the study was to evaluate the effect of 9-*cis* retinoic acid (9-*cis* RA) on D2R protein expression in human pituitary adenomas and GH3 cell line. Treatment with 9-*cis* RA (100 nM for 48 hrs) caused a $109 \pm 32\%$ increase of basal D2R levels in five of eight growth hormone (GH)-secreting adenomas (GH-omas), a $129 \pm 28\%$ increase in 7 of 11 nonfunctioning adenomas, and no effect in two resistant prolactinomas by Western blotting. The lack of D2R induction in some tumors was not associated with a different pattern of retinoid x receptor (RXR) and retinoic acid receptor (RAR) isoform expression that was similar in all tumors by immunohistochemistry. While the induction of D2R did not affect the slight but significant inhibitory effect exerted by dopamine (10 nM) on *in vitro* GH release by GH-oma cultured cells, in pituitary GH3 cell lines *cis*-9 RA enhanced the dopamine-induced inhibition of *in vitro* GH release (% inhibition: 16 ± 2 versus 26 ± 5 , $P < 0.05$), cell proliferation ($25 \pm 2\%$ versus $44 \pm 5\%$, $P < 0.05$) and cell viability ($16 \pm 0.8\%$ versus $29 \pm 1\%$, $P < 0.05$), likely by activating caspase-3 ($28 \pm 3\%$ versus basal, $P < 0.05$). In conclusion, this study provides novel evidence for a

permissive role of retinoids on the expression of D2R in a good proportion of pituitary tumors and on the generation of proapoptotic signals in GH3 cell line. *Exp Biol Med* 233:439–446, 2008

Key words: retinoic acid; D2 receptor; pituitary tumor; GH3 cell; apoptosis

Introduction

Dopamine exerts its inhibitory action on the release, secretion, and synthesis of different pituitary hormones through the interaction with the dopamine D2 receptor (D2R) (1). By coupling to different Gi/G0 proteins, D2R activation causes membrane hyperpolarization and reduces cAMP production, calcium mobilization and influx, and inositol phosphate metabolism (2). Moreover, D2R exerts antiproliferative actions, as indicated by the appearance of pituitary lactotroph hyperplasia and tumors in D2R-deficient mice (3, 4). Experiments carried out on pituitary cell lines expressing D2R suggest that dopamine may act as a pro-apoptotic agent to induce cell death (5–7).

The regulation of D2R gene expression has been only partially elucidated. Previous studies indicate that steroids, particularly estrogens, may affect the ratio between the two isoforms of the receptor (D2L and D2S) while growth factors, such as epidermal growth factor and nerve growth factor, may induce D2R expression in GH3 and in prolactinomas resistant to dopamine agonist treatment in term of both normalizing prolactin (PRL) levels and shrinking tumors (1). Sequence analysis of the D2R promoter has revealed features of a housekeeping promoter. The promoter lacks TATA and CAA boxes, whereas

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multiple Sp1 binding sites and consensus sequences for AP1 and AP2 are present (8). Interestingly, a retinoic acid (RA)-response element (RARE) that binds RA receptors (RAR α , RAR β and RAR γ) and retinoid X receptors (RXR α , RXR β and RXR γ) as RAR-RXR heterodimers in the promoter region of D2R has been identified (9). Consistent with a role of retinoids in D2R expression, previous studies demonstrated that D2R mRNA was induced by treatment of pituitary MMQ cells with retinoic acid ligands while a strong decrease of D2R expression was observed in the striatum of RA receptor-deficient mice, particularly RAR-RXR double mutants (9, 10).

Secreting and nonsecreting pituitary adenomas express D2R. Due to the antisecretory and antiproliferative actions of dopamine, dopamine agonists are the first choice therapy of prolactinomas and may represent an adjuvant treatment for growth hormone (GH)-secreting adenomas (GH-omas) and nonfunctioning adenomas. The efficacy of the treatment seems to be strongly related to the levels of D2R expression, as indicated by D2R mRNA and protein deficiency in tumors from patients resistant to dopaminergic drugs (11, 12).

The aim of this study was to evaluate the effects of retinoic acid treatment on D2R expression and pro-apoptotic signaling in cultured cells from human pituitary tumors and in pituitary cell line.

Material And Methods

Pituitary Tissue Samples and Cell Cultures. The study included 21 human pituitary adenomas, that is, 8 GH-omas, 11 nonfunctioning adenomas (NFPA), and 2 prolactin-secreting adenomas (PRL-omas), surgically removed by the transsphenoidal route. In particular, the two PRL-omas were considered to be resistant to medical therapy and consequently underwent surgical removal. Of the removed tumors, small fragments were fixed for light microscopy and immunohistochemistry (IHC) and a portion was placed in sterile culture medium for cell cultures. The cells were enzymatically dispersed, plated at the density required for the different assays, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin (Flow Laboratories, Mackenheim, Germany) at 37°C in an atmosphere of 95% air to 5% CO₂ in a humidified incubator, as described in Lania *et al.* (13). Similarly, GH3 cells were cultured in HAM's F-10 medium supplemented with 10% FCS. After 2 days, cell monolayers were washed and incubated at 37°C for 48 hrs in 0.5% FCS DMEM in the absence or presence of 100 nM 9-*cis*-retinoic acid (9-*cis* RA) (Sigma Chemical Co., St. Louis, MO) for the different determinations (see below). Ethics Committee approval was obtained for all studies.

In Vitro Hormone Secretion. Hormone release in the absence or presence of 100 nM 9-*cis* RA was evaluated in all cell cultures (5 × 10⁵ cells/ml) obtained from the GH-omas and PRL-omas included in the study. After incubating

for 48 hrs, medium was removed and the supernatant maintained at -20°C until hormone assays were performed. In two GH-omas and in GH3 cell preparations, cells were seeded in 24-well plastic cluster dishes (2 × 10⁵ cells/well) pretreated or not with 9-*cis* RA, washed, and subsequently cultured in 0.5% FCS DMEM with and without 10 nM dopamine (Sigma Chemical) for 1 hr; this process was done three times. Human GH and PRL were measured by an immunofluorimetric assay using a commercial kit (Perkin Elmer, Salem, MA) while rat GH levels were measured by a commercial rat growth hormone ELISA assay kit (Diagnostic System Laboratories, Webster, TX).

Determination of Dopamine Type 2 Receptor Protein Expression. The determination of D2R protein was performed after immunoprecipitation of lysates from cells incubated for 48 hrs at 37°C in the presence or absence of 9-*cis*-RA using a specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Western blotting, as previously reported (13). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a mouse monoclonal antibody (Ambion Europe Ltd., Huntingdon, UK) was used as an internal control for protein loading. The resulting bands were visualized and evaluated by an imaging densitometer (GS-700; BioRad, Richmond, CA) as described in Lania *et al.* (13). Experiments were repeated at least twice.

Determination of Dopamine Type 2 Receptor mRNA. RNA was extracted (TRIzol reagent; Invitrogen S.R.L., Milan, Italy) from GH3 cells cultured in the presence or absence of *cis*-9 RA for 48 hrs. To examine D2R gene transcription, 3 µg total RNA were subjected to reverse transcriptase-polymerase chain reaction (Promega Corporation, Madison, WI) and 5 µl of the cDNA was subjected to polymerase chain reaction (PCR) amplification using primers specific for the rat or for the human D2R gene (rat *drd2*: 5'-GGTCTACTCCTCCATTGTCT-3', as forward primer and 5'-CTCAAAGAAGCTGGCAATCC-3' as reverse primer; human *DRD2*: 5'-GGTCTACTCCTC-CATCGTCT-3' as forward primer and 5'-TCTCAAAGATCTTGGCAATC-3' as reverse primer). The hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene was used as internal standard to verify and normalize template concentration. Preliminary experiments were conducted to determine the PCR cycles corresponding to the exponential phase: 28 cycles of 45 secs each at 98°C, 58°C, and 72°C for D2R; 24 cycles of 45 secs each at 98°C, 58°C, and 72°C for HGPRT. Products of the PCR cycles were finally visualized on 1% agarose gel and evaluated by an imaging densitometer (GS-700; Bio-Rad). The specificity of RT-PCR products was confirmed by direct sequencing (data not shown).

Immunohistochemistry. Sections from paraffin-embedded tissues from surgically removed pituitary tumors were processed for IHC, as previously reported (13). Specific polyclonal antibodies for RXR α , RXR β , RXR γ and RAR α (Santa Cruz Biotechnology) were used under the

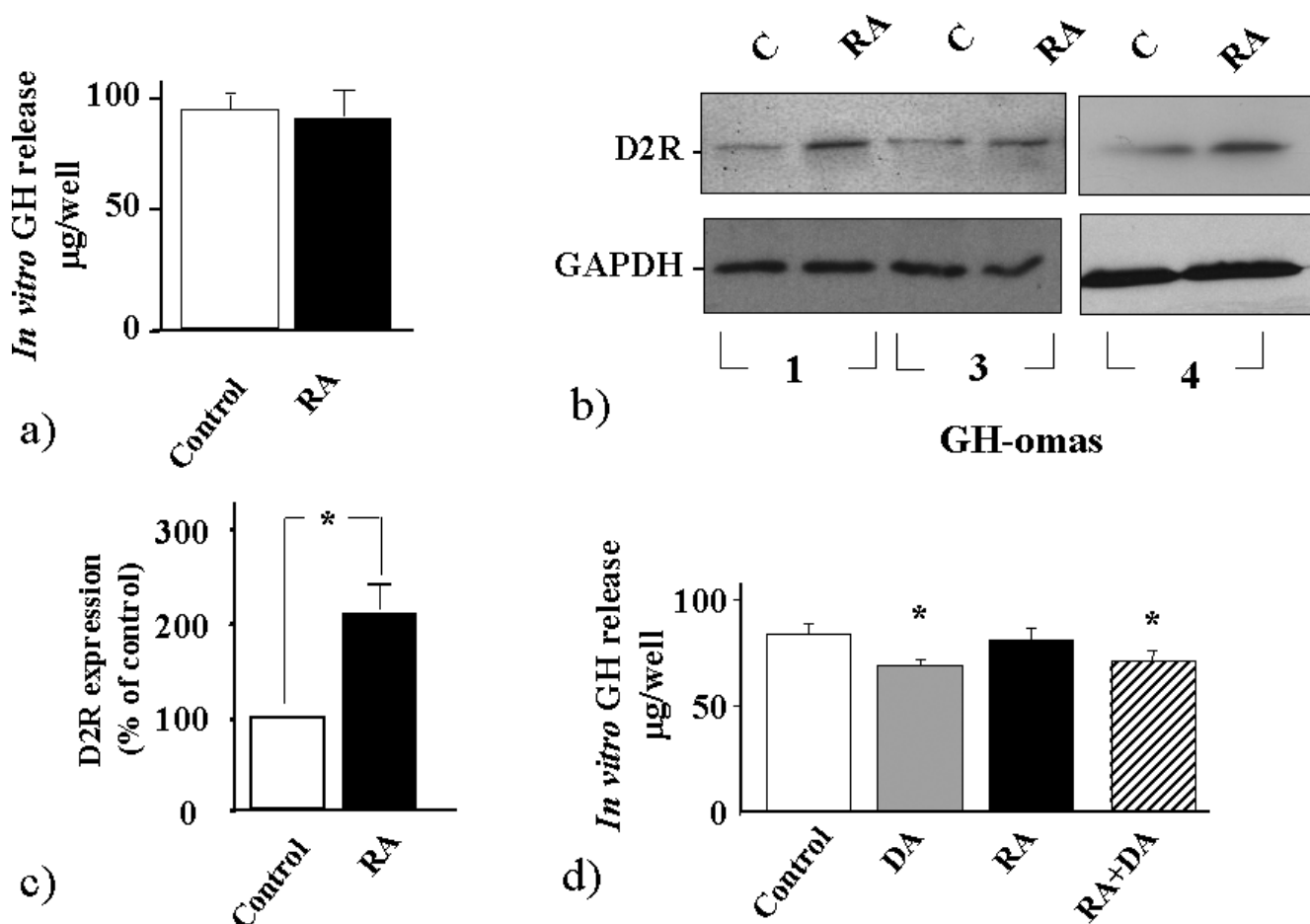


Figure 1. Effect of 9-*cis*-retinoic acid (9-*cis* RA) in GH-secreting tumors. a) In cultured cells obtained from the eight GH-omas included in the study, 9-*cis* RA treatment (100 nM for 48 hrs) did not affect *in vitro* GH release. Values given represent the means $\pm$ SD of GH determinations, carried out in duplicate, on each of the three wells. b) Immunoblotting performed with antibody raised against D2R in the presence (RA) or absence (C) of 9-*cis*-retinoic acid (100 nM for 48 hrs) in 3 responsive GH-omas, as representative example. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a mouse monoclonal anti-GAPDH antibody (Ambion) was used as an internal control for protein loading. c) Immunoblots were measured by an imaging densitometer and the values expressed in arbitrary units. Data represent the percent modification (mean $\pm$ SD) of D2R levels over basal values (control) arbitrarily expressed as 100% in the five GH-omas considered responsive to 9-*cis*-retinoic acid treatment. d) In cultured cells obtained from 2 responsive GH-omas, 9-*cis* RA treatment (100 nM for 48 hrs) did not affect the slight but significant inhibitory effect exerted by dopamine (10 nM) on *in vitro* GH release. Values given represent the means $\pm$ SD of GH determinations, carried out in duplicate, on each of the three wells. * $P < 0.05$

conditions specified by the manufacturer. Antigen-antibody detection was performed using the DAKO ChemMate En Vision detection kit (DAKO A/S, Glostrup, Denmark) according to the manufacturer's instructions. Sections were stained with 3,3'-diaminobenzidine substrate, counterstained with Meyer hematoxylin, and slides were prepared for light microscopy examination as reported by Lania *et al.* (13). Negative controls were obtained by occulting the primary antibody or by using an unrelated mouse monoclonal antibody. At least two blinded readers graded the specimens for all stainings. Briefly, RXR and RAR isoforms immunoreactivities were graded 0–3, with 0 = absence of immunoreactivity, 1 = <10%, 2 = 10–50%, and 3 = >50% in at least 400 cells in the main representative high power fields.

Cell Proliferation. The proliferation of the rat GH3 cells was assessed by colorimetric measurement of 5-bromo-

2'-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferating cells (Cell Proliferation Biotrak Elisa; Amersham Biosciences, Piscataway, NJ) as previously reported (13). Briefly, cells were cultured in 96-well plates (20,000 cells/well) in the presence of test substances for 72 hrs at 37°C and then with BrdU for 2 hrs to allow BrdU incorporation in newly synthesized cellular DNA. Proliferation was expressed as relative fluorescence units (RFU). All experiments were repeated at least three times on two different clones, and each determination was done five times.

Evaluation of Apoptosis. Apoptosis was assessed by caspase-3 activity determination. Caspase-3 enzymatic activity was measured using Apo-ONE Homogenous Caspase-3 Assay (Promega), a fluorescent assay based on cleavage of the nonfluorescent caspase substrate Z-DEVD-R110 by caspase-3 to create the fluorescent Rhodamine 110, as described by Lania *et al.* (13). Cells were seeded in 96-

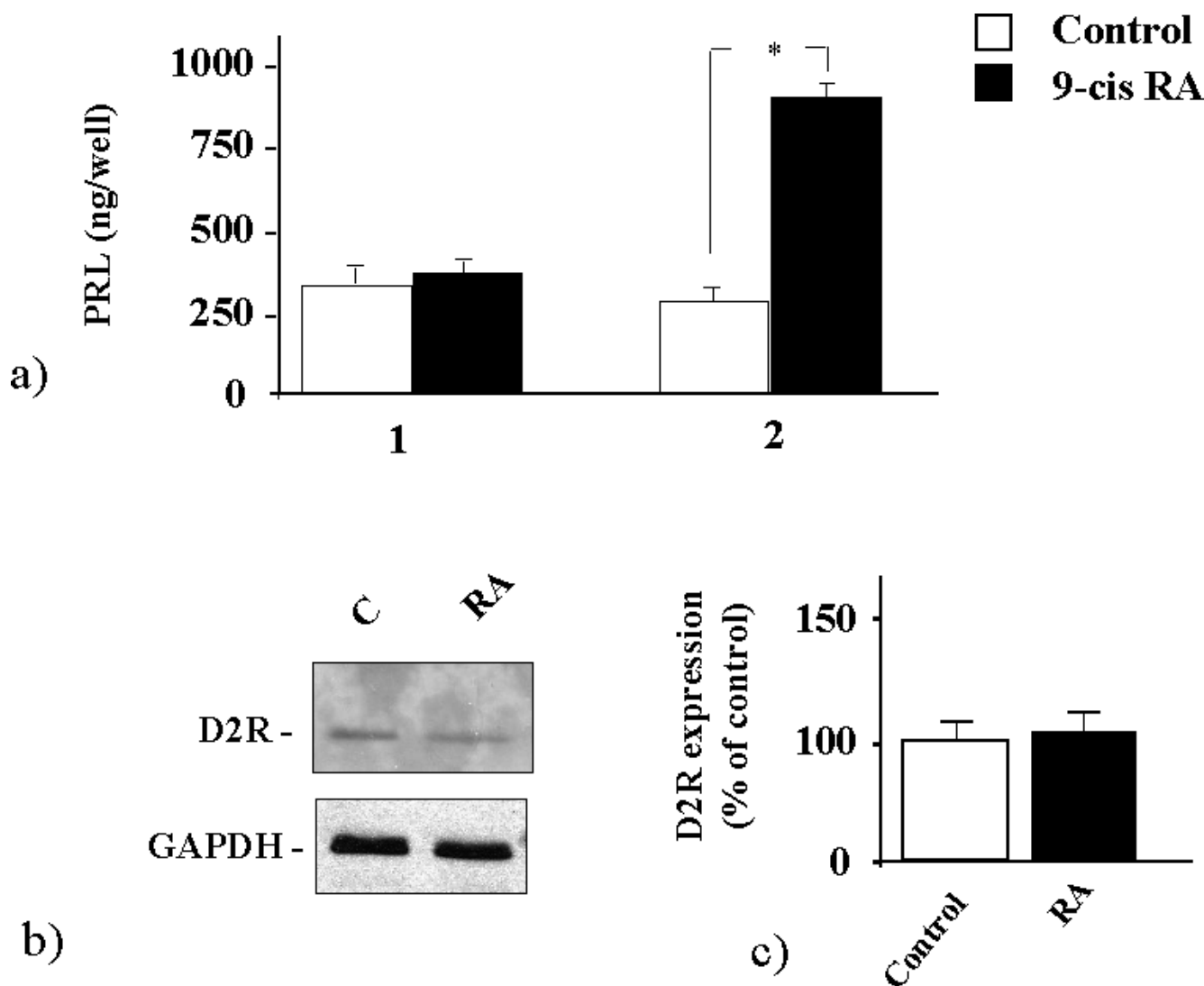


Figure 2. Effect of 9-*cis*-retinoic acid (9-*cis* RA) in PRL-secreting tumors resistant to medical treatment. a) In cultured cells obtained from 1 of 2 PRL-omas, 9-*cis* RA treatment (100 nM for 48 hrs) induced a significant increase in *in vitro* PRL release. Values given represent the means $\pm$ SD of PRL determinations, carried out in duplicate, on each of the three wells. b) Representative immunoblotting performed in the 2 PRL-omas with antibody raised against D2R in the presence (RA) or absence (C) of 9-*cis*-retinoic acid (100 nM for 48 hrs). The expression of GAPDH by a mouse monoclonal anti-GAPDH antibody (Ambion) was used as an internal control for protein loading. c) Immunoblots were measured by an imaging densitometer and the values expressed in arbitrary units. Data represent the percent modification (mean $\pm$ SD) of D2R levels over basal values (control) arbitrarily expressed as 100% in the 2 PRL-omas tested. * $P < 0.05$.

well plates (5×10^4 cells/well) and treated with different agents (10 nM dopamine, 100 nM 9-*cis*-retinoic acid, and 100 nM okadaic acid) for 24 hrs at 37°C in 10% FCS DMEM. Fluorescence was measured at an excitation at 485 ± 20 nm and an emission at 530 ± 25 nm. Caspase-3 activity was indicated by net fluorescence (assay RFU minus blank RFU). Experiments were repeated three to four times and each determination was done three times.

Cell Viability. The effects of dopamine and 9-*cis*-RA on cell viability *in vitro* were assessed by the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega), a dimethylthiazol-diphenyltetrazolium bromide-based colorimetric assay. Briefly, cells were seeded in 96-well plates (5×10^4 cells/well) and treated with different agents (10 nM dopamine and 100 nM 9-*cis*-retinoic acid) for 48 hrs at 37°C

in 10% FCS DMEM, and then treated with the staining solution provided in the CellTiter proliferation kit (Promega) for 3 hrs. Cell viability was expressed as RFU. All experiments were repeated three to four times and each determination was done five times.

Statistical Analysis. Results are expressed as the mean $\pm$ SD. A paired or unpaired two-tailed Student's *t* test was used to detect the significance between two series of data. $P < 0.05$ was accepted as statistically significant.

Results

Effect of 9-*cis* Retinoic Acid on Hormone Secretion and D2R Expression in Pituitary Tumor Cells. Exposure of cells obtained from eight GH-omas to 100 nM 9-*cis*-RA for 48 hrs did not induce changes on *in*

Table 1. Expression of Retinoic Acid Receptor Isoforms and D2R Protein Induction by 9-*cis*-Retinoic Acid in Pituitary Adenomas

No.	Tumor type	Retinoic acid receptor expression (IHC score) ^a				D2R protein increase (% of basal)
		RXR α	RXR β	RXR γ	RAR α	
1	GH-oma	2	1	0	2	160
2	GH-oma	3	2	0	3	5
3	GH-oma	2	2	0	2	80
4	GH-oma	3	1	0	3	120
5	GH-oma	3	2	0	2	5
6	GH-oma	2	2	0	2	95
7	GH-oma	2	1	0	2	90
8	GH-oma	3	1	0	3	15
9	PRL-oma	2	1	0	3	5
10	PRL-oma	3	2	0	2	2
11	NFPA	2	1	0	3	160
12	NFPA	3	2	0	3	5
13	NFPA	2	1	0	3	170
14	NFPA	3	2	0	3	180
15	NFPA	3	1	0	2	5
16	NFPA	2	2	0	2	125
17	NFPA	3	1	0	2	5
18	NFPA	3	2	0	3	115
19	NFPA	2	1	0	2	85
20	NFPA	2	1	0	3	15
21	NFPA	2	1	0	2	70

^a The pattern of RXR and RAR isoforms expression was graded with score 0–3: 0 = absence of immunoreactivity, 1 = <10%, 2 = 10–50%, and 3 = >50% in at least 400 cells in the main representative high power field.

vitro GH release (Fig. 1a). By contrast, in one prolactinoma this treatment was associated with a significant increase of *in vitro* PRL release (Fig. 2a).

Cell treatment with 9-*cis* RA for 48 hrs caused a significant increase in D2R expression at the protein level ($109 \pm 32\%$ over basal levels) (Table 1 and Fig. 1b, c) in five of eight GH-secreting adenomas. However, this treatment did not affect the slight but significant inhibition exerted by dopamine (10 nM) on *in vitro* GH release (19 ± 5 versus 19 ± 4 , $P = \text{NS}$; Fig. 1d). No correlation between basal D2R expression and the induction of the receptor after 9-*cis*-RA treatment was observed (data not shown).

In the two PRL-omas considered as resistant to medical treatment, the low expression of D2R was not increased by 9-*cis* RA at any concentration tested (from 100 nM to 1 mM) (Table 1, Fig. 2b, c, and data not shown). As far as NFPA were concerned, in the majority of these tumors (7 of 11) 9-*cis* retinoic acid treatment was associated with a $129 \pm 28\%$ increase in D2R protein levels (Table 1, Figure 3a).

Treatment of GH3 cells with 9-*cis* RA did not cause detectable increase in the expression of D2R neither at the protein nor at the mRNA levels (Fig. 4a and data not shown). By contrast, this treatment significantly enhanced the dopamine induced inhibition of *in vitro* GH release (% inhibition: 16 ± 2 versus 26 ± 5 , $P < 0.05$).

Expression of RXR Isoforms in Pituitary Tumors. The expression of RXR isoforms were investigated in all tumors of the present series. No immuno-

reactivity for RXR γ and a weak immunoreactivity for RXR β isoform was found in all secreting and nonsecreting pituitary tumors studied (Table 1). Conversely, all tumors showed a strong positivity for RXR α and RAR α that were detected in more than 50% of the total cell population in most cases. No differences in the pattern of RXR and RAR isoforms expression was observed between tumors found to be responsive or not responsive to 9-*cis* RA in term of D2R expression increase (Table 1).

Effect of 9-*cis* Retinoic Acid on Dopamine-Induced Proliferation, Cell Viability, and Apoptosis in Pituitary Tumor Cells. Due to the low proliferation rate of human pituitary tumor cells, the effects of 9-*cis* RA on cell proliferation, viability, and apoptosis were tested in GH3 cells. The exposure of the cells to 100 nM 9-*cis* RA for 48 hrs did not result in any significant change in cell proliferation and viability. However, 9-*cis* RA significantly enhanced the dopamine-induced inhibition of both cell proliferation (% inhibition: 25 ± 2 versus 44 ± 5 , $P < 0.05$; Fig. 4b) and cell viability (% inhibition: 16 ± 0.8 versus 29 ± 1 , $P < 0.05$; Fig. 4c).

In order to ascertain whether these effects were due to apoptosis activation, caspase-3 activity was measured. No effect on caspase-3 activity was observed in cells incubated either with 10 nM dopamine or 100 nM 9-*cis* RA for 48 hrs, while the contemporary treatment with both agents resulted in a significant enzyme activation ($28 \pm 3\%$ versus basal, $P < 0.05$; Fig. 4c). However, the effect of contemporary

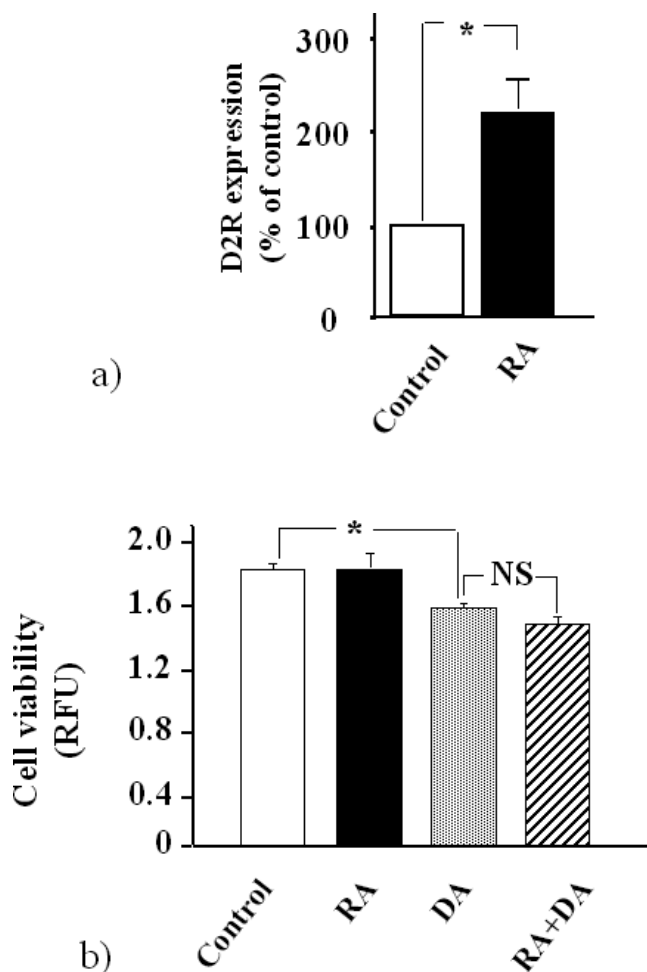


Figure 3. Effect of 9-*cis*-retinoic acid (RA) in nonfunctioning pituitary adenoma. (a) Immunoblotting was performed with antibody raised against D2R in the presence (RA) or absence (Control) of 9-*cis*-retinoic acid (100 nM for 48 hrs). Immunoblots were measured by an imaging densitometer and the values expressed in arbitrary units. Data represent the percent modification (mean $\pm$ SD) of D2R levels over basal values (control) arbitrarily expressed as 100% in the 7 tumors considered responsive to 9-*cis*-retinoic acid treatment. (b) In 2 NFPAAs dopamine 10 nM caused a significant decrease of cells viability, while a reproducible but nonsignificant potentiation of this inhibitory effect was observed by pretreating cultured cells with 9-*cis*-RA. Data are expressed as RFU and are the mean $\pm$ SD of cell number determinations carried out in triplicate for each clone tested. * $P < 0.05$.

treatment with 10nM dopamine or 100 nM 9-*cis* RA on caspase activity was lower than that induced by the aspecific pro-apoptotic agent okadaic acid used as control ($28 \pm 3\%$ and $58 \pm 4\%$ versus basal respectively, $P < 0.05$; Fig. 4d)

The effect of 9-*cis* RA on cell viability was tested in two NFPAAs and in two GH-omas. The exposure to this agent slightly but not significantly enhanced the dopamine induced inhibition of cell viability (inhibition %; 15 ± 0.5 versus 20 ± 1 , $P = 0.07$; Fig. 3b and data not shown).

Discussion

The study provided novel evidence for a permissive role of retinoids on the expression of D2R in a good

proportion of pituitary tumors. Retinoids, that include vitamin A, its metabolites and synthetic analogs, are involved in the control of different biological processes, such as embryonic development, cell differentiation, proliferation, and apoptosis (14, 15). Previous studies suggested a critical role of these transcription factors in pituitary development and differentiation. As far as pituitary development was concerned, it has been shown that a retinoid receptor RAR functionally interacts with *Pit-1* gene in a distal enhancer element and activates gene transcription (16). The physiopathological role of this morphogenic factor in pituitary development has been confirmed by the identification of a novel *Pit-1* mutation (K216E) that prevented retinoic acid induced *Pit-1* transcription in one patient with combined pituitary hormone deficiency (17).

Previous studies indicated that the D2R receptor may be considered another target of retinoic acid action. Indeed, the existence of a functional RARE in the promoter region of D2R that binds RAR and activates gene transcription has been reported (9). Consistent with the role of retinoids in D2R expression, previous studies demonstrated that D2R mRNA was induced by treating rat lactotroph MMQ cells with RAR ligands (9). In the present study we demonstrated that 9-*cis* RA, which is known to bind both the retinoic acid receptors RARs and RXRs, induced the expression of D2R in a consistent number of pituitary tumors, suggesting the existence of a similar interaction between 9-*cis* RA and D2R promoter in human pituitary cells. However, the main goal of the study, to induce D2R expression in prolactinomas (the tumor type that recognizes loss of D2R as a major cause of unresponsiveness to medical therapy), was not achieved. Therefore, although the number of tumors investigated was limited due to the rarity of prolactinomas requiring surgical removal, the present study seems to rule out the possibility of using these agents to improve sensibility to dopaminergic therapy. These data are partially in contrast with the persistent reduction of PRL levels observed in psoriatic patients during retinoid treatment and are probably related to the tumoral nature of prolactinoma cells (18). In the present study 9-*cis* RA even caused a significant increase of PRL release from one prolactinoma, this effect being consistent with the induction of the transcription of either *Pit-1* or PRL gene, as previously reported in lactotroph cell lines (17, 19).

Most cellular effects of retinoids are mediated by two receptor types, i.e. the retinoic acid receptors RARs, which bind both all-trans retinoids and 9-*cis* retinoic acid, and the retinoid receptors RXRs which bind specifically 9-*cis* retinoic acid (20). In agreement with previous data (21), all tumors showed a high expression of RXR α and RAR α associated with variably low and undetectable levels of RXR β and RXR γ , respectively. The homogeneous pattern of RAR and RXR isoforms expression observed in this series of tumors suggests that the absent D2R induction by 9-*cis* RA in individual tumors was not related to receptor deficiency. Admittedly, we did not investigate the expression of RAR β and RAR γ and therefore we cannot exclude a possible role of

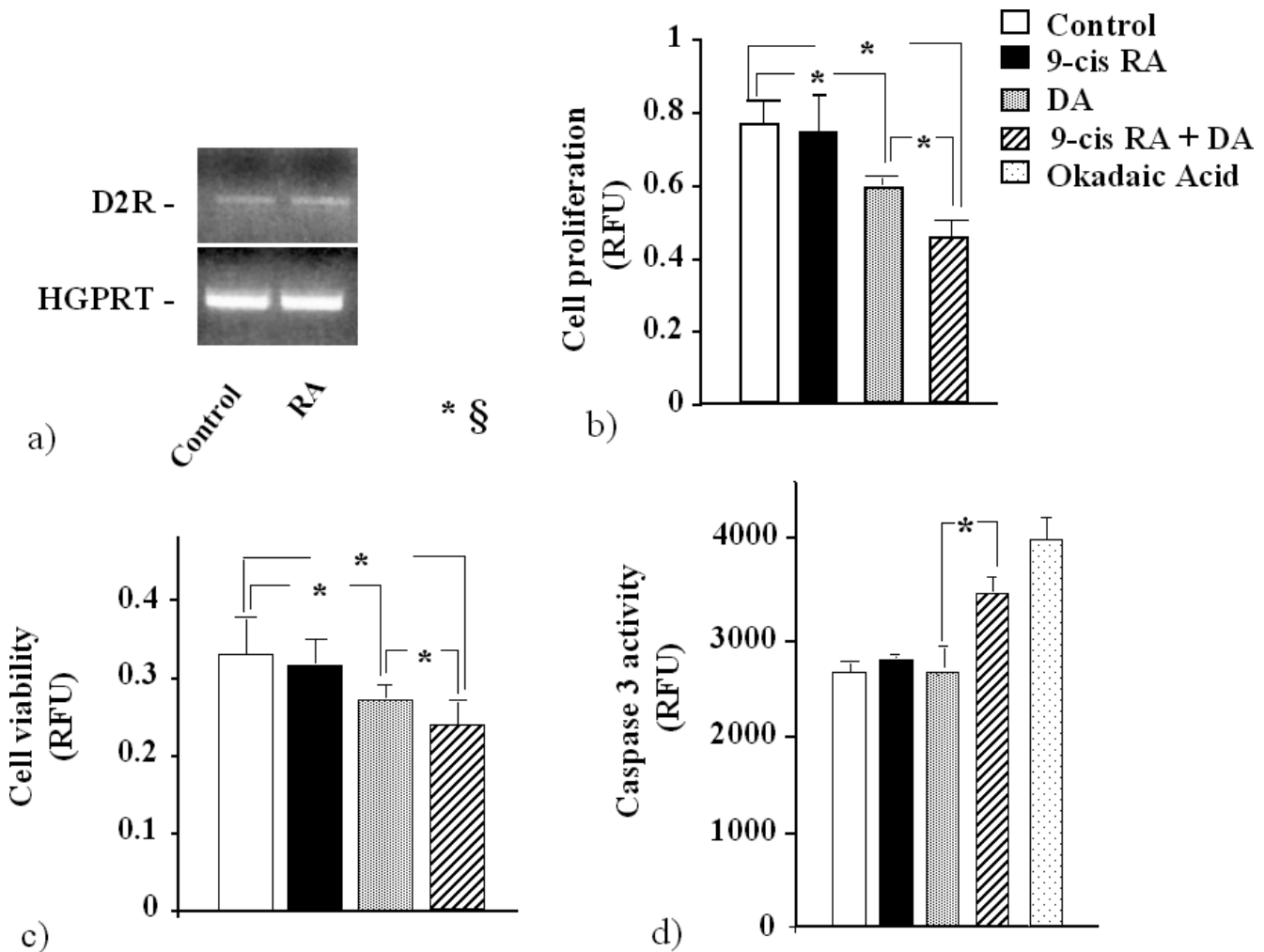


Figure 4. Effect of dopamine and 9-*cis* retinoic acid (9-*cis* RA) treatment on D2R mRNA expression, proliferation and apoptosis in GH3 cells. a) Agarose gel stained with ethidium bromide of RT-PCR products obtained using oligonucleotides specific for the amplification of D2R and HGPRT in GH3 cells in basal condition and after 48 hrs incubation with 9-*cis*-RA. b) In all samples dopamine 10 nM caused a significant decrease of GH3 proliferation, this effect being enhanced by pretreating cells with 100 nM 9-*cis* RA. Conversely, 9-*cis* RA alone did not affect cells proliferation. Data are expressed as RFU and are the mean $\pm$ SD of cell number determinations carried out in triplicate. c) In all samples dopamine 10 nM caused a significant decrease of GH3 proliferation, this effect being enhanced by pretreating cells with 9-*cis*-RA. Data are expressed as RFU and are the mean $\pm$ SD of cell number determinations carried out in triplicate * $P < 0.05$. d) Effect of dopamine and 9-*cis* RA on apoptosis. Neither 10 nM dopamine nor 100 nM 9-*cis* RA increased caspase-3 activity in GH3 cells. Conversely, dopamine induced a significant increase in caspase-3 activity in GH3 cells pretreated with 9-*cis* RA. The aspecific pro-apoptotic agent okadaic acid was used as control. Experiments were repeated at least twice and each determination was done in quintuplicate. Data are expressed as Relative Fluorescence Units (RFU) and are the mean $\pm$ SD of cell number determinations carried out in triplicate for each clone tested. * $P < 0.05$.

these isoforms on the different responsiveness. However, this possibility seems unlikely since it has been reported that pituitary tumors do not express these receptor isoforms (21). Moreover, the possible differential expression of cofactors required for the ligand-dependent transactivation mediated by retinoic acid receptors was not investigated.

The impact of retinoic acid on D2R functioning was investigated in pituitary adenomas and in GH3 cells, a cell line deriving from the lactotroph and somatotroph lineage. First, in cells obtained from GH-omas, the inhibitory action of dopamine on *in vitro* GH release was unaffected by 9-*cis* RA exposure, while this treatment enhanced the antiseecretory dopaminergic action in GH3 cells. This different

pattern might be related to different regulation of secretory activity in the two models of tumoral somatotrophs used in this study. Indeed, the main genetic alteration in GH3 cell lines is the loss of p27, while the activation of the cAMP dependent pathway is the main secretory and proliferative signal in human GH-omas (13, 22, 23).

Second, 9-*cis* RA exerted a permissive action on the inhibition of proliferation and viability of GH3 cells induced by dopamine. This effect was at least in part mediated by the activation of apoptotic signals, as indicated by the increased levels of cleaved caspase-3, a major protease mediator of the extrinsic and intrinsic apoptotic pathway. These data obtained in GH3 cells were reminiscent of what observed in cortico-

troph tumor cells (24). In fact, retinoic acid treatment was effective in inhibiting cell proliferation and, after prolonged treatment, increasing caspase-3 activity in ACTH-secreting cells (24). It is interesting to note that the permissive effects of 9-*cis* RA on the antiseecretory, antiproliferative, and proapoptotic actions of dopamine occurred in the absence of detectable modification of D2R expression in this cell type. It is tempting to speculate that, as reported for several nuclear receptors, 9-*cis* RA might activate nongenomic signaling pathways independent on transcriptional activity, thus resulting in the generation of intracellular signals favoring dopamine action (25, 26). Alternatively, the possible effect of 9-*cis* RA on the induction of postreceptor components involved in dopamine signal transduction might be implicated.

Probably due to the low rate of cell proliferation and apoptosis that characterize human pituitary tumors, the effects of 9-*cis* RA observed in GH3 were only partially replicated in GH-omas and nonfunctioning tumors. Indeed, 9-*cis* RA exposure resulted in a tendency to increase the inhibition of cell viability induced by dopamine. Alternatively, the different pattern of responsiveness may be related to different postreceptor signaling in the two models of tumoral somatotrophs used in this study.

In conclusion, the present study provides novel evidence for the induction of D2R expression by 9-*cis* RA in a good proportion of pituitary tumors. Moreover, based on the observation that 9-*cis* RA enhanced the effects of dopamine on GH secretion, cell proliferation and viability, and apoptosis in GH3 cells line, it tempting to hypothesize that retinoic acid might be regarded as an interesting antitumorigenic agent in pituitary tumors.

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