INDUCTION OF TYROSINE KINEASE RECEPTOR B BY RETINOIC ACID ALLOWS BRAIN-DERIVED NEUROTROPHIC FACTOR-INDUCED AMYLOID PRECURSOR PROTEIN GENE EXPRESSION IN HUMAN SH-SY5Y NEUROBLASTOMA CELLS

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Abstract—Retinoic acid (RA) is a potent regulator of morphogenesis, growth and cell differentiation. Incubation with RA causes arrest of proliferation and neurite extension in SH-SY5Y cells, a neuroblastoma cell line of human origin. In these cells, RA regulates the expression of the β-amyloid precursor protein. The retinoid increases the levels of intracellular and secreted forms of APP (amyloid precursor protein), APP–mRNA levels and the activity of the APP promoter in transient transfection studies. These responses require long periods of exposure to the ligand, thus suggesting a direct effect of the RA receptors on the APP gene. Also in these cells, RA induces the expression of TrkB, the tyrosine kinase receptor for brain-derived neurotrophic factor (BDNF), and 4 days of pretreatment with retinoic acid confers BDNF responsiveness to the APP promoter. © 2003 Published by Elsevier Science Ltd on behalf of IBRO.

Key words: β-amyloid precursor protein, APP, promoter, neuroblastoma cells, retinoic acid, RA, brain-derived neurotrophic factor, BDNF, tyrosine kinase receptor, TrkB.

The β-amyloid protein (βA4), the major component of the Alzheimer-associated plaques is derived from a set of alternatively spliced β-amyloid precursor proteins (APP) which are encoded by a single gene located on human chromosome 21 (for a review see Selkoe, 2001). Although the biological function of this protein remains to be fully elucidated, at physiological levels APP appears to be involved in neurotrophic events and modulates functions such as neuronal excitability, synaptic plasticity, neurite outgrowth, synaptogenesis, or cell survival (for a review see Mattson, 1997). In contrast, its overexpression may contribute to increased production and deposition of βA4, and finally to activation of processes which cause cellular degeneration (Calhoun et al., 1999; Yoshikawa et al., 1992). APP is ubiquitously expressed in mammalian tissues and its expression can be regulated by a variety of stimuli, including ligands of the nuclear superfamily of steroid/thyroid hormone receptors (Belandia et al., 1998; Konig et al., 1990; Yoshikawa et al., 1990), and ligands of membrane tyrosine kinase receptors (Ruiz-Leon and Pascual, 2001; Villa et al., 2001). The 5' promoter region of the APP gene has been cloned and characterized for both humans (Lahiri and Robakis, 1991; Salbaum et al., 1988) and rodents (Hoffman and Chernak, 1994). The promoter lacks a TATA or CAAT boxes, but contains other consensus sequences for binding of a number of transcription factors. However, the molecular mechanisms that control its expression are not well understood.

Induction of APP expression by retinoic acid (RA) has been described in a number of cell lines of neuronal and non neuronal origin (Beckman and Iverfeldt, 1997; Hung et al., 1992; Lahiri and Nall, 1995). The effects of RA are mediated by nuclear receptors (RARs), which normally function as ligand-inducible transcription factors by binding to response elements (RAREs) located in the regulatory region of target genes. RARs are encoded by three different genes which give rise to three different isoforms (α, β and γ; Glass, 1994). As has been previously described, neuroblastoma primary tumor tissue and cell lines express RARα, and also RARγ to a lesser degree. RARβ is expressed at very low levels, but is strongly induced by treatment with RA (Carpentier et al., 1997; Lovat et al., 1993).

In SH-SY5Y cells, a clonal derivative of the human neuroblastoma SK-N-SH cell line which expresses high levels of RAR (Lovat et al., 1993), RA has been shown to increase APP mRNA levels. In agreement with these results, we found that RA not only increased mRNA levels but also the levels of intracellular and secreted APP protein. However, the effects of RA on APP gene expression are delayed when compared with other effects induced by this ligand on the same cells. In addition, a 48 h treatment with RA was unable to stimulate APP promoter activity in transient transfection assays. An increased activity of the promoter was only observed following 4–6 days of incubation in the presence of RA. These results again suggest an indirect mechanism, likely mediated by other APP promoter-interacting proteins.

Tyrosine kinase receptor B (TrkB) is the high-affinity Trk for brain-derived neurotrophic factor (BDNF), a neurotrophin that is known to promote the structure and function of the nervous system. TrkB mediates most of the BDNF-induced responses and a synergistic effect of RA and BDNF on neurite generation has been reported (Mey and
Rombach, 1999). Moreover, RA stimulates TrkB expression in neuroblastoma cells (Encinas et al., 1999; Kaplan et al., 1993; Kobayashi et al., 1994), and as we have described, exogenous BDNF induces APP promoter activity in SH-SY5Y cells transiently transfected with an expression vector for TrkB (Ruiz-Leon and Pascual, 2001).

To probe a potential cross-talk between the response induced on APP by RA and BDNF in SH-SY5Y cells, we have analyzed the effects of RA on APP and TrkB expression in SH-SY5Y cells, and the effects of BDNF in SH-SY5Y cells cultured in the presence or absence of RA.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

SH-SY5Y cells were cultured in RPMI medium containing 10% fetal bovine serum (Gibco Life Technologies Ltd., Paisley, Scotland, UK). The same medium was used to analyze the effect induced by RA, but not the effects induced by BDNF on the APP promoter activity, which were determined in experiments carried out in RPMI medium supplemented with 0.5% serum. At the times indicated in the text, media and cells were collected and processed, or stored frozen for posterior analysis.

**Chemicals**

Polyclonal antibody 369A against the cytoplasmic domain of APP was a generous gift of Dr. Samuel E. Gandy and the monoclonal antibody 22C11, which recognizes the amino terminus of APP, was purchased from Boehringer Mannheim (Germany). BDNF and “all trans” RA were from PeproTech Ltd. (London, UK). The anti-TrkB monoclonal antibody was from Transduction Laboratories (Lexington, USA), and the trk inhibitor K-252a from Calbiochem (Darmstadt, Germany).

**Reporter plasmids and expression vectors**

The chloramphenicol acetyl transferase (CAT) reporter plasmid containing the – 1099/+105 fragment of the human APP gene has been previously described (Belandia et al., 1998). The cDNA encoding the TrkB receptor was inserted into the EcoRI site of the expression vector CMV5, which contains the SV40 early promoter. The R-140-Luc construct containing the fragment – 124 to +14 of the human RARβ2 promoter has been described previously by Vivanco-Ruiz et al. (1991).

**DNA transfection**

SH-SY5Y cells were transfected in Dulbecco’s Modified Eagle medium (DMEM) containing 10% fetal calf serum. The 10% serum-containing RPMI growing culture medium was replaced by DMEM 4 to 6 hours before transfection, and the cells were cotransfected by the calcium phosphate coprecipitation method with 5 μg of the reporter plasmid (−1099CAT, or R-140-Luc) and 5 μg of carrier DNA (high molecular mass calf thymus DNA). After 18 h of incubation in the presence of calcium phosphate, the medium was replaced with fresh RPMI, growing medium was added and the cells incubated in the presence or in the absence of 1 μM RA for the time periods studied. In all cases, the total amount of DNA among different transfections was kept constant by addition of an empty noncoding expression vector. To analyze the effects of BDNF, RA-pretreated cells were cotransfected with the reporter plasmid −1099CAT and 2 μg of a TrkB expression vector, and transfection medium replaced with 0.5% serum-containing RPMI. The cells were then cultured in the presence or in the absence of 10 μg/ml BDNF. CAT activity was determined by incubation of [3H]-thymidine incorporation

DNA synthesis rate was determined by measuring the incorporation of [3H]-thymidine. Cells were plated in 96-well multiwells at an initial density of 4000 cells/well. Twenty-four hours later the growing medium was replaced by RPMI (200 μl) containing 10% serum and the cells were maintained at 37 °C by an additional 4 day period. [3H]-Thymidine (0.1 μCi) was added to the cultures during the last 6 h of incubation. The cells were then transferred to glass fiber filters and processed in a cell harvester. The radioactivity associated to filters was measured with a scintillation counter.

**Statistical analysis**

Results are expressed as means ± S.D. The differences between means were evaluated by a Student’s t-test and considered significant at P<0.05.

**RESULTS**

RA induces differentiation and decreases the proliferation of SH-SY5Y cells

RA plays a major role in neuronal cell differentiation, thus inducing neuroblastoma cells to differentiate in vitro by extending neurites; a positive relationship between the in vitro neuronal differentiation and APP expression has been quantified and the data expressed as the percentage of acetylated forms after each treatment. Each experiment was repeated at least two to three times with similar relative differences in regulated expression.

**RNA extraction and hybridization**

Total RNA was extracted from SH-SY5Y cells, cultured in the presence or in the absence of 1 μM RA, with guanidinium thiocyanate (Chomczynski and Sacchi, 1987). The RNA (30 μg) was electrophoresed in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes for Northern blot analysis. After staining with 0.02% Methylene Blue the blots were hybridized, as described by Church and Gilbert (1984), with a plasmid containing the human APP cDNA, or the rat TrkB cDNA, labeled by random oligonucleotide priming. Hybridization was at 65 °C in PSE buffer (0.3 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA). The quantification of APP-mRNA levels shown in Fig. 2 was carried out by densitometric scan of the autoradiograms, and corrected for the total amount of RNA applied.

**Western blot analysis**

Cellular proteins were extracted by lysis with a buffer (150 mM NaCl, 50 mM Tris pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) containing the protease inhibitors PMSF (1 mM) and leupeptin (10 μg/ml). Equal amounts, 40 μg, of cell extracts were electrophoresed in an 8% SDS-polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane and the cellular APP detected with the rabbit polyclonal antibody 369A. Secreted full-length APP isoforms were detected by the same method from 50 μl (1:100 from total) of conditioned medium using the monoclonal antibody 22C11 at a final concentration of 10 μg/ml. To determine TrkB, cellular proteins were extracted by lysis with a modified RipA buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% NaNAD, 0.1% SDS), in the presence of protease inhibitors, and the TrkB levels determined from 40 μg aliquots with a monoclonal antibody, which recognizes both the full-length 145 kDa, and the 95 kDa forms of TrkB.

**Proliferation assays: [3H]thymidine incorporation**

DNA synthesis rate was determined by measuring the incorporation of [3H]-thymidine. Cells were plated in 96-well multiwells at an initial density of 4000 cells/well. Twenty-four hours later the growing medium was replaced by RPMI (200 μl) containing 10% serum and the cells were maintained at 37 °C by an additional 4 day period. [3H]-Thymidine (0.1 μCi) was added to the cultures during the last 6 h of incubation. The cells were then transferred to glass fiber filters and processed in a cell harvester. The radioactivity associated to filters was measured with a scintillation counter.
previously described (König et al., 1990; Hung et al., 1992; Pan et al., 1993; Rossner et al., 1998; Yang et al., 1999). As shown in Fig. 1, a 4 day treatment of SH-SY5Y cells with 1 μM RA, promoted morphologic differentiation (panel A) and these results were not essentially different from those reported by other authors who used 10-fold higher doses of RA (König et al., 1990; Beckman and Iverfeldt, 1997). Interestingly, BDNF did not induce any morphologic change on cells when added alone, and did not further increase morphologic changes induced by RA when both agents were added together. In addition, 1 μM RA also induced a strong reduction of the cellular proliferation as determined by thymidine incorporation (panel B).

Induction of APP expression by RA in SH-SY5Y cells

RA treatment of SH-SY5Y cells increases APP–mRNA levels, as well as the accumulation of intracellular and secreted isoforms of APP. The mRNA levels were determined in SH-SY5Y cells, that were incubated for 2 to 6 days in the presence or the absence of 1 μM RA. As illustrated in panel A of Fig. 2, which shows the results obtained in a representative Northern blot analysis of RNA, as well as the quantization of APP mRNA levels obtained in two independent experiments, no changes were observed after 2 days of incubation. However, a small but significant increase of APP–mRNA levels was observed after 4–6 days of treatment with RA.

In parallel, the effects of RA on the cell-associated APP and the full-length soluble derivatives of APP released into the culture medium were analyzed by Western blot using the polyclonal antibody 369A or the monoclonal antibody 22C11, respectively. Results are depicted in panel B of Fig. 2. As occurred with the APP–mRNA levels, the presence of 1 μM RA in the culture medium for 4 days induced a significant increase of both the intracellular (left) and secreted (right) APP species.

Based on previous descriptions (Buxbaum et al., 1990; Weidemann et al., 1989), the immunoreactive bands detected with the antibody 369A contain at least six protein species corresponding to immature and mature isoforms of APP. Incubation of SH-SY5Y cells with RA for 4 days did not affect the pattern of isoforms but led to a generalized increase of the intracellular APP content. In addition, RA induced a generalized increase of the APP-derived isoforms released to the culture medium.

Effects of RA on APP promoter activity in transiently transfected SH-SY5Y cells

A stimulatory effect of RA on the APP promoter activity has been previously described in rat primary hippocampal neurons (Yang et al., 1998), and also in transiently transfected PC12 pheochromocytoma cells pretreated with the retinoid for 4 days (Lahiri and Nall, 1995). To prove a similar effect of RA on the APP promoter activity in neuroblastoma cells,
we have transfected the cells with the −1099CAT reporter plasmid, a construct which contains the −1099/+105 fragment of the human APP gene. As observed in the previous figures, the induction of APP by RA in SH-SY5Y cells requires at least 4 days, and this response appears to be very slow when compared with other responses induced by this ligand on these neuroblastoma cells. In agreement with those findings, Fig. 3 shows that in transient transfection assays, a 48 h incubation with RA strongly induces the activity of the RARβ2 promoter, which contains a well-

Fig. 2. Induction of APP expression by RA in SH-SY5Y cells. A. Autoradiogram (left) of a representative northern blot analysis of RNA extracted from cells incubated in the presence or the absence of 1 μM retinoic acid for the indicated times. APP mRNA levels (right) were determined by densitometric scan of autoradiograms and corrected by the amount of rRNA applied. Data are expressed as fold induction above the levels found in control cells, and represent mean ± S.D. values of four determinations (duplicates from two separate assays). Significance at P<0.005 is indicated *** compared with basal control B. Western blot analysis of intracellular (left) and secreted (right) APP isoforms in SH-SY5Y cells incubated for 4 days in the presence or the absence of 1 μM retinoic acid.

Fig. 3. Regulation of APP promoter activity by RA in SH-SY5Y cells. CAT activity was determined in SH-SY5Y cells transfected with a reporter plasmid containing the −1099 to +105 region of the APP promoter (left panel). The right panel shows the effects induced by retinoic acid on a control reporter plasmid (R-140-Luc) containing the fragment −124 to +24 of the RARβ2 promoter. CAT and luciferase activities were measured after a 48 h period of incubation in the presence or in the absence of 1 μM RA. Data are expressed as fold-induction over the values obtained in the corresponding untreated cells and are the mean ± S.D. of four data points obtained in two separate experiments. Significant reduction by RA compared with control groups are indicated P<0.05 (*); P<0.01 (**); P<0.001 (***).
defined RARE (right panel), but is unable to induce APP promoter activity at that time period. The retinoid was able to induce APP promoter activity only after 4–6 days of treatment (left panel). In addition, a computer-assisted study indicates that the APP gene promoter region does not contain a consensus RARE, thus supporting an indirect mechanism, likely mediated by the previous induction of other protein/s, but not directly mediated by binding of RA receptors to a RARE in the APP gene promoter.

**BDNF induces APP promoter activity in RA-pretreated cells**

As we have previously described, BDNF stimulates the APP gene promoter in TrkB expressing SH-SY5Y cells, whereas it is unable to induce a response in control TrkB-untransfected cells (Ruiz-Leon and Pascual, 2001). In addition, as has also been described, RA may regulate the expression of TrkB in neuroblastoma cell lines (Lucarelli et al., 1995), which have become dependent on BDNF for survival (Feng et al., 2001). To test whether or not RA treatment could induce TrkB and confer BDNF responsiveness to the APP, we first determined the levels of TrkB in SH-SY5Y cells incubated for 4 days in the presence or in the absence of 1 μM RA. As shown in panel A of Fig. 4, RA induces the expression of the TrkB gene, increasing both the TrkB–mRNA (left) and TrkB protein (right) levels in SH-SY5Y cells after 4 days of treatment.

In addition, we have also analyzed the effects of BDNF on APP promoter activity in SH-SY5Y cells pretreated with RA. In order to analyze whether or not the RA-induced expression of TrkB is enough to confer BDNF responsiveness to the APP promoter, cells were incubated in the presence or in the absence of 1 μM RA for 4 days, and then cotransfected with the 1099-CAT reporter plasmid (which contains the fragment -1099 to +105 of the APP gene) and a TrkB-expressing vector, or the corresponding empty noncoding control vector. As shown at the left of panel B, treatment of RA-untreated cells with 10 ng/ml BDNF for 48 h leads to a significant increase of CAT activity in TrkB expressing transfected cells, but is unable...
to increase the basal activity in TrkB-untransfected cells, which express very low levels of TrkB. In contrast, as shown at the right side of the panel, CAT activity was significantly induced by BDNF in cells preincubated for 4 days in the presence of 1 μM RA, even in the absence of transfected TrkB. Moreover, as illustrated in panel C of the figure, the levels of APP-mRNA induced by RA were markedly decreased in the presence of the trk inhibitor K-252a (0.2 μM). Altogether, these results suggest that treatment of cells with RA which, as shown in panel A, is able to induce TrkB expression, is sufficient to confer BDNF responsiveness in APP promoter.

**DISCUSSION**

In the present work we have demonstrated that treatment with RA can induce TrkB expression in SH-SY5Y cells, and confer BDNF responsiveness to the APP gene promoter.

The effects of RA are mediated by RAR, which belong to the subfamily of nonsteroid nuclear hormone receptors. SH-SY5Y cells, a neuroblastoma cell line of human origin, express RAR (Lovat et al., 1993), and the expression of a number of genes has been described to be directly modulated by RA in these cells. Also in SH-SY5Y cells, 10 μM RA has been reported to activate APP expression, specifically increasing the APP<sub>396</sub>-mRNA isofrom, as proved by S1 nuclease protection assay (Konig et al., 1990).

We have demonstrated that exposure to 1 μM RA, a smaller and more physiological dose, is able to induce the expression of the APP gene increasing not only the total APP mRNA levels, but also the intracellular and extracellular content of APP. However, this response requires a relatively long exposition to RA (4–6 days), and strongly suggests a non-direct mediated mechanism. Although with slight differences, these results are similar to those described by Beckman and Iverfeldt (1997) in the same cellular line. According to these authors, a higher dose of RA (10 μM) induces an increase of the APP messenger levels, which appears to be significant only after 6 days of treatment.

The delayed response of the APP gene could be explained by the presence of low levels of RA receptor. Nevertheless, such a possibility appears to be diminished since as shown in Fig. 3, the retinoid was unable to induce APP promoter activity in transient transfection assays at a time in which this compound is very effective in increasing the activity of the R-140-Luc construct (Vivanco-Ruiz et al., 1991) which contains the fragment -124 to +14 of the human RARβ2 promoter. In addition, a theoretical analysis of the APP gene, reveals that the APP promoter does not contain a consensus, or even an imperfect, RARE. These results strongly suggest that activation of APP by RA is not directly mediated by binding of RARs to sequences of the APP promoter, and may require the previous activation of other protein(s), which in turn could affect APP gene expression.

In this respect, it has been reported that RA effectively induces the expression of the BDNF receptor TrkB in neurons (Kaplan et al., 1993). Both BDNF and TrkB, are highly expressed in the adult hippocampus, one of the most affected tissues in Alzheimer’s disease, and we have previously described that BDNF induces APP expression in SH-SY5Y cells transiently expressing TrkB (Ruiz-Leon and Pascual, 2001). In addition, it has been suggested that RA may induce BDNF responsiveness of sympathetic neurons by alteration of Trk neurotrophin receptors expression (Kobayashi et al., 1994). Therefore, the effects induced by RA on the APP expression in SH-SY5Y cells could be mediated by the previous increase of TrkB levels. Although SH-SY5Y cells express very low amounts of TrkB receptor, RA has been described to induce a sustained increase in TrkB-mRNA that is already detectable at the second day of treatment and leads to a significant elevation of TrkB levels (Kaplan et al., 1993). In contrast, as has been also described, SH-SY5Y cells express TrkB, and RA specifically decreases TrkB-mRNA levels in the same cell line (Ehrhard et al., 1993). Those discrepancies could be secondary to the experimental conditions maintained by both groups, or even due to specific cellular properties associated to the cellular clones of SH-SY5Y used. In order to validate our model we first analyzed the effects induced by RA on TrkB expression in our cells. Although with minor differences, our results are in agreement with those described by Kaplan et al. (1993). We have found that 4 days of treatment with 1 μM RA significantly increases the levels of both TrkB mRNA and protein, as determined by Northern and Western blot, respectively. Moreover, we have also found that RA induces BDNF responsiveness of the APP promoter, thus proving that RA-induced TrkB is functionally active. Without RA treatment, APP promoter activity was very low and was minimally induced by BDNF. In contrast, the neurotrophin led to a significant increase of APP promoter activity in cells exposed to RA for 4 days. As a control, in cells not exposed to RA, this response was only found after transfection of TrkB. Finally, the effects induced by RA on APP mRNA were substantially diminished in the presence of the Trk inhibitor K-252a, further confirming a TrkB-mediated mechanism.

BDNF, a member of the neurotrophin family, protects neurons against cellular insult (Cheng and Mattson, 1994), and promotes survival in human neuroblastoma cells (Middlemas et al., 1999). Furthermore, it has been suggested that neurodegeneration may be, at least in part, secondary to an alteration in BDNF and/or TrkB levels. In agreement with this hypothesis, the use of BDNF as a potential therapeutic agent in patients with Alzheimer’s disease has been proposed (Ferrer et al., 1999; Mufson et al., 1999). Our results suggest that RA may substantially intensify the effects of BDNF by increasing the expression of its receptor TrkB; this would open the possibility of using both agents, RA and BDNF in a coordinate way. Unfortunately, APP overexpression is also considered a risk factor for Alzheimer’s disease. Therefore, both the beneficial effects induced by the neurotrophin on cellular survival, and the negative and toxic effects that accompany APP overexpression, should be considered. Moreover, although the molecular events that regulate the expression of TrkB and the effects induced by the BDNF/TrkB system in neurons...
are poorly understood, it results plausible that BDNF-induced effects are controlled by RA, through a very sensitive and accurate mechanism which determines the levels of TrkB and hence the BDNF-induced effects. An exhaustive analysis of the mechanisms involved in the responses induced by RA on TrkB and APP would result of maximal interest for the development of therapeutic strategies.

Our results suggest that RA may substantially intensify the effects of BDNF by increasing the expression of its receptor TrkB, and it results plausible that BDNF-induced effects are controlled by RA, through a very sensitive and accurate mechanism which determines the levels of TrkB and hence the BDNF-induced effects. These results open the possibility of using both agents, RA and BDNF, coordinately, and this would be of maximal interest for the development of therapeutic strategies. However, it is evident that new experiments should be carried out to evaluate the physiological relevance of these findings. First, an important effort should be made to elucidate whether these mechanisms are also observed in vivo, and definitely prove that RA and BDNF functionally interact in neurons and intact tissues.

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