Retinoic acid-induced nNOS expression depends on a novel PI3K/Akt/DAX1 pathway in human TGW-nu-I neuroblastoma cells

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NATURALLY OCCURRING AND SYNTHETIC analogs of vitamin A, termed retinoids, are critical for the maintenance of epithelial growth and differentiation (5, 30). The biologically active form of vitamin A, retinoic acid (RA), induces differentiation in various cell and tissue models, accompanied by profound changes in gene expression and induction of a more mature phenotype (2). Vitamin A homeostasis is an important determinant for the development of the central (62) and peripheral nervous system (35). In the nervous system, RA-induced signaling controls neuronal proliferation and differentiation (1, 23). RA acts through nuclear receptors, obligate heterodimers of a RA receptor with a retinoid X receptor. By binding to specific DNA sequences (RA response elements), these receptors act as ligand-dependent transcription factors of RA-responsive genes (24). In addition to the regulation of a complex genetic program, RA can act via novel nongenomic signal transduction mechanisms (22). For example, RA has been shown to modulate the activity of PKC by direct binding (36). Furthermore, phosphatidylinositol 3-kinase (PI3K)/Akt signaling is activated by RA (3, 51).

PI3K/Akt signaling controls various biological processes, including proliferation and differentiation (9). A regulatory subunit, p85, and catalytical p110 subunits, compose PI3K (9). In neuroblastoma cells, RA activates PI3K signaling which is required for RA-induced neural differentiation (21). At the molecular level, RA induces binding of the catalytical p110 subunits to a complex, containing the regulatory subunit p85 and RA receptor, thereby promoting PI3K activity (25). Target genes of the RA/PI3K signaling pathway have not been elucidated so far.

Nitric oxide (NO) is synthesized by nitric oxide synthases (NOS) during the oxidation of L-arginine to L-citrulline. In the central and peripheral nervous system, neuronal NOS (nNOS) is the predominant enzyme for generation of NO. NO acts as a neurotransmitter and intracellular signaling molecule hereby regulating processes like synaptic plasticity, neuronal development, and regeneration (10). Furthermore, NO is a mediator of neurotoxicity in stroke and neurodegenerative disorders (31). Although nNOS is responsible for the largest proportion of NO in the body, the transcriptional control of the human nNOS gene is not completely understood. Regulation of the nNOS gene by the transcription factor families SP, ZNF, NF-κB, CREB, and Oct is documented (6, 17, 20, 42, 44). Twelve first exons, exon 1a–1l, transcribed from distinct promoters, have been recently described, leading to nNOS variants with different 5′-untranslated regions. Transcriptional control of the nNOS gene is not understood in detail. To investigate regulation of nNOS gene expression by retinoic acid (RA), we used the human neuroblastoma cell line TGW-nu-I as a model system. We show that RA induces nNOS transcription in a protein synthesis-dependent fashion. We identify the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and the atypical orphan nuclear receptor DAX1 (NROB1) as critical mediators involved in RA-induced nNOS gene transcription. RA treatment increases DAX1 expression via PI3K/Akt signaling. Upregulation of DAX1 expression in turn induces nNOS transcription in response to RA. These results identify nNOS as a target gene of a novel RA/PI3K/Akt/DAX1-dependent pathway in human neuroblastoma cells and stress the functional importance of the transcriptional regulator DAX1 for nNOS gene expression in response to RA treatment.
protocol using 625,000 cells in 10-cm plates. The following siRNA sequences were used (target sequence, sense strand): scramble control siRNA 5'-CAGTCGGTGTTGCGACTGTGdTdT-3', DAX1 siRNA 5'-GGCAGGGCAGCATCCTCAGTdTdT-3', siRNAs were purchased from Qiagen (Hilden, Germany) and stored in a 40 μM stock at −80°C.

**MTT assay.** Cell proliferation was analyzed using the Cell Proliferation Kit I (MTT; Roche, Mannheim, Germany) as recently described (45). Cells were seeded on 96-well plates at 4 × 10^3 cells/well. After 24 h, cells were treated with RA or DMSO as control and incubated for 4 h with 0.5 mg/ml MTT dye in complete cell culture medium. Membranes were blocked in phosphate-buffered saline (PBS) supplemented with 5% skim milk and 0.1% NP-40 and incubated with antibodies against nNOS (BD Biosciences, Heidelberg, Germany), phospho-S^777^-Akt (Cell Signaling Technology, Beverly, MA), and β-actin (Sigma-Aldrich). Proteins recognized by the antibodies were detected and quantified by the Odyssey Infrared Imaging System (LI-COR, Bad Homburg, Germany) using Alexa680-coupled (Molecular Probes, Leiden, Netherlands) or IRDye800-coupled (Rockland, Gilbertsville, PA) secondary antibodies.

**Quantitative reverse transcriptase PCR.** TGW-nu-I cells were harvested and total RNA was isolated using the RNeasy kit (Qiagen) following the manufacturer’s instructions. A panel of pooled total RNA samples prepared from normal human tissues of at least three individuals of Caucasian origin was purchased from Clontech (Mountain View, CA). RNA samples were prepared at least three times. Pooled total RNA samples prepared from normal human tissues of at least three individuals of Caucasian origin was purchased from Clontech (Mountain View, CA). RNA samples were prepared at least three times.

**Preparation of total cell lysates.** Whole cell lysates were prepared by incubating cell pellets for 30 min at 4°C in immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 mM NaF). Insoluble material was removed by centrifugation, and lysates were aliquoted and stored at −80°C.

**Western blot analysis.** Extracts were normalized for protein and heated at 95°C for 5 min in Lämmli buffer. Proteins were resolved on 7.5–10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in a semi-dry blotting system. Membranes were blocked in phosphate-buffered saline (PBS) supplemented with 5% skim milk and 0.1% NP-40 and incubated with antibodies against nNOS (BD Biosciences, Heidelberg, Germany), phospho-S^777^-Akt (Cell Signaling Technology, Beverly, MA), and β-actin (Sigma-Aldrich). Proteins recognized by the antibodies were detected and quantified by the Odyssey Infrared Imaging System (LI-COR, Bad Homburg, Germany) using Alexa680-coupled (Molecular Probes, Leiden, Netherlands) or IRDye800-coupled (Rockland, Gilbertsville, PA) secondary antibodies.

**Transfections and luciferase assays.** TGW-nu-I cells were stably transfected with TGA expression plasmid pCMV-TVA essentially as described previously (52). TGW-nu-I cells (2 × 10^5) were transiently cotransfected with the different pGL3 firefly luciferase reporter gene plasmids (950 ng/well) and pRL-TK Renilla luciferase control plasmids (50 ng/well) in six-well plates using Lipofectamine (Invitrogen) as described previously (46). After 48, 72, and 96 h, cells were incubated in lysis buffer (Promega) for 15 min, harvested, and cleared by centrifugation for 15 min. Firefly and Renilla luciferase activities were determined in a LB 9501 luminometer (Berthold, Bad Wildbad, Germany) using a dual-luciferase assay system (Promega). At least three independent transfection experiments were performed in triplicate. Relative light units of firefly luciferase were normalized against relative light units of Renilla luciferase. Fold luciferase induction was calculated by comparison of treated (RA) and untreated (DMSO) cells.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (Chip) assays were performed as recently described (47). Equal amounts of chromatin (50–100 μg) were used for each precipitation.

| Table 1. Genes upregulated (>10-fold) in TGW-nu-I cells after 24 h of retinoic acid treatment |
|---------------------------------------------|---------------------------------|---------------|
| Symbol | RefSeq Transcript ID | Fold Induction |
| CYP26B1 | NM_019885 | 219 |
| CMKOR1 | NM_020311 | 158 |
| CKN1 | NM_016083/NM_033181 | 117 |
| ENPP2 | NM_006209 | 113 |
| NR0B1 | NM_000475 | 97 |
| NTRK2 | NM_00107097/NM_006180 | 74 |
| SPP1 | NM_005852 | 66 |
| CRABP2 | NM_001878 | 62 |
| PTPRR | NM_002849/NM_130846 | 61 |
| NTRK2 | NM_00107097/NM_006180 | 46 |
| RARB | NM_000965/NM_016152 | 37 |
| LOC492304 | NM_00107139 | 18 |
| PELD | NM_019546 | 17 |
| HEBP2 | NM_014320 | 14 |
| PRICKLE1 | NM_153026 | 14 |
| FLJ21986 | NM_024913 | 13 |
| RAB38 | NM_022337 | 13 |
| FLJ37266 | NM_175892 | 13 |
| DHRS3 | NM_004753 | 12 |
| TPS3NPI | NM_053285 | 11 |

**RAB38** NM_022337 13

**FLJ37266** NM_175892 13

**DHRS3** NM_004753 12

**TPS3NPI** NM_053285 11

**CYP26B1** NM_019885 219

**CMKOR1** NM_020311 158

**CKN1** NM_016083/NM_033181 117

**ENPP2** NM_006209 113

**NR0B1** NM_000475 97

**NTRK2** NM_00107097/NM_006180 74

**SPP1** NM_005852 66

**CRABP2** NM_001878 62

**PTPRR** NM_002849/NM_130846 61

**NTRK2** NM_00107097/NM_006180 46

**RARB** NM_000965/NM_016152 37

**LOC492304** NM_00107139 18

**PELD** NM_019546 17

**HEBP2** NM_014320 14

**PRICKLE1** NM_153026 14

**FLJ21986** NM_024913 13

**RAB38** NM_022337 13

**FLJ37266** NM_175892 13

**DHRS3** NM_004753 12

**TPS3NPI** NM_053285 11

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The antibody used for immunoprecipitation was directed against RNA Polymerase II (Santa Cruz Biotechnology, Santa Cruz, CA). One-twentieth of the precipitated chromatin was used for each PCR reaction. To ensure linearity, 28 to 38 cycles were performed, and one representative result out of at least three independent experiments is shown. Sequences of the promoter specific primers are as follows: nNOS exon 1f promoter forward 5'-AATGCTATCCCAACTGATGTAGAG-3', nNOS exon 1f promoter reverse 5'-ACGTGCACAACCCTCTATTAAATA-3', nNOS exon 1g promoter forward 5'-AGGGATCTTGGAGGCACAAAG-3', and nNOS exon 1g promoter reverse 5'-CCGCCTCGTACTCCTCCTTT-3'. A detailed experimental protocol is available on request.

β-Galactosidase staining. β-Galactosidase staining of cultured TGW-nu-I cells was done as described recently (48). Briefly, cells were fixed 10 min in 4% paraformaldehyde at 4°C and washed three times in lacZ wash buffer (2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS) for 5 min. β-Galactosidase activity was detected using lacZ staining buffer [35 mM potassium ferrocyanide, 0.1 M Na2HPO4, 0.5% Triton X-100, and 0.5% sodium deoxycholate] for 2 h at 37°C. Cells were then washed and stained with 1% (w/v) X-gal stock solution in PBS for 2 h at 37°C. Samples were then washed and analyzed for β-galactosidase activity.

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Retroviral gene transfer. Replication competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (RCASBP) with Bryan-RSV polymerase gene and subgroup A envelope [RCASBP(A)] vectors containing the sequence of an activated form of the PT3K p110 subunit [RCASBP(A)-p110K11047R] (19) and myristylated active Akt [RCASBP(A)-caAkt-myr] (28) were kindly provided by P. K. Vogt (The Scripps Research Institute, La Jolla, CA). RCASBP(A)-dnAktK179M plasmid with the coding sequence for dominant negative AktK179M was generated by subcloning of dnAktK179M from pUSE-dnAktK179M (Upstate, Lake Placid, NY) into the blunted ClaI restriction site of RCASBP(A) (a gift from S. Hughes, National Cancer Institute, Frederick, MD). RCASBP(A) viruses were generated as described previously (26, 48). Briefly, 2.5 × 10⁵ DF-1 chicken fibroblasts (American Type Culture Collectin, Manassas, VA) were transfected with 2.5 μg of the respective RCASBP(A) plasmids using 25 μl Superfect (Qiagen). After 2 wk, supernatants were filtered through a 0.22-μm filter and were used to infect DF1 cells for virus titer determination by limiting dilution or to infect TGW-nu-I cells with stable expression of the avian retroviral receptor TVA, under the control of the CMV promoter. Transduced TGW-nu-I cells were subsequently treated with RA (5 μM) or DMSO as control over a period of 3 days.

Gene expression profiling. Microarray analysis was performed as recently described (40, 52, 53). In brief, TGW-nu-I cells were treated with 5 μM retinoic acid or DMSO as control. Duplicates of total RNA were prepared using the RNeasy kit. Labelled cRNA was produced and hybridized onto the Affymetrix GeneChip Human Genome U133 Plus 2.0 set (Santa Clara, CA) according to Affymetrix standard protocols. Expression data were analyzed using Microarray Suite 5.0. Genes upregulated by retinoic acid treatment more than 10-fold are shown in Table 1.

Data analysis. Unless otherwise indicated, all data were determined from at least three independent experiments and are expressed as mean values ± SD. Normal distribution of data was tested and comparison among data sets was made with analysis of variance, followed by Student’s t-test using Sigmastat 3.0 (Systat Software, San Jose, CA). Values of P < 0.05 were considered to be statistically significant. The power of all statistical tests with P values <0.05 was >0.9 (Sigmastat).

RESULTS

Retinoic acid induces nNOS expression in TGW-nu-I neuroblastoma cells. To determine the effect of RA on nNOS protein expression, we treated TGW-nu-I neuroblastoma cells with different concentrations of RA (100 pM–5 μM) or DMSO as control for 1 or 3 days. As shown in Fig. 1A, RA treatment induced nNOS protein expression starting from day 1 in a concentration-dependent manner. Highest nNOS expression was induced by treatment with 5 μM RA. Therefore, all subseuent experiments were performed using this concentration. To investigate nNOS expression in response to RA over time, TGW-nu-I cells were treated with RA or DMSO for up to 7 days (Fig. 1B). RA induced nNOS protein expression in a time-dependent manner with a day-by-day increase until day 7 (Fig. 1B). In line with increased nNOS protein expression, RA also upregulated nNOS mRNA expression with a ~14,000-fold induction after 7 days (Fig. 1C).

Regulation of the nNOS gene is complex. At least 12 different untranslated first nNOS exons are known that are transcriptionally regulated by alternative promoters and different stimuli (6, 7, 12, 17, 43, 44, 56). To determine which alternative nNOS first exon variants are predominantly upregulated by RA, we performed quantitative real-time RT-PCR. As shown in Fig. 1D, RA treatment mainly induces nNOS exon 1c, 1f, and 1g mRNA variants, which are the predominant forms in TGW-nu-I cells (44). Upregulation was most pronounced for the nNOS exon 1g variant with a 105-fold induction after 6 days of RA treatment (Fig. 1D).

RA blocks proliferation of TGW-nu-I cells by NO-dependent and -independent pathways. Since RA inhibits proliferation and induces differentiation of neuroblastoma cells (21, 25), we investigated the potential role of nitric oxide within this process using MTT proliferation assays. As shown in Fig. 1E, 5 μM RA treatment of TGW-nu-I cells for 2 and 3 days reduced proliferation to ~70% and ~65%, respectively. Blockade of NOS activity by L-NAME partially reversed this RA-induced phenotype to ~85 and 80%, respectively, indicating that NO plays a role within this process.

RA induces nNOS transcription in TGW-nu-I cells. To investigate the mechanism involved in RA-mediated nNOS induction, we examined nNOS mRNA stability in TGW-nu-I cells. As shown in Fig. 2A, RA stimulation does not alter nNOS mRNA degradation in actinomycin D-treated cells compared with controls. To examine RA-induced transactivation of the different nNOS promoters, we used luciferase reporter gene plasmids containing the investigated first nNOS exons and their promoter regions as well as exon 2 and its recently characterized 5’-regulatory region (13, 57). In contrast to the strong induction of nNOS mRNA expression, NO luciferase reporter gene expression was only marginally upregulated by RA using various different nNOS promoter constructs (Fig. 2, B and C, and data not shown). Strongest induction was observed for the −10110/exon2nNOS-Luc construct, revealing a 1.92-fold transactivation 48 h after RA treatment (Fig. 2C). RA treatment for 72 or 96 h did not further increase transactivation of nNOS promoter confi...
Transcriptional control of \textit{nNOS} after RA treatment was further analyzed using ChIP assays, investigating exon 1f and 1g promoters, since these promoters demonstrate the strongest mRNA induction upon RA treatment in TGW-nu-I cells. As shown in Fig. 2D, RA induces binding of RNA polymerase II to the proximal promoter of \textit{nNOS} exon 1f and 1g. These results prove that RA induces \textit{nNOS} transcription in TGW-nu-I cells.
To investigate whether RA-induced nNOS expression requires protein de novo synthesis, TGW-nu-I cells were treated over a time course of 3 days with RA and cycloheximide or RA alone. As demonstrated in Fig. 2E, RA-induced nNOS mRNA expression is completely blocked by cycloheximide cotreatment, pointing to a protein de novo synthesis-dependent mechanism.

RA-induced upregulation of nNOS depends on PI3K/Akt signaling. Since RA is known to activate PI3K, MAPK, and JNK signaling, we tested the contribution of these pathways toward nNOS expression in TGW-nu-I cells (3, 51, 60, 61). The PI3K inhibitor LY294002 blocked dose-dependently RA-induced nNOS mRNA (Fig. 3A) and protein expression (Fig. 3, B and C). In contrast, RA-induced nNOS protein expression was further increased by cotreatment with the MAPK inhibitor PD98059 (Fig. 3D). The JNK inhibitor SP600125 showed only minimal effects on RA-mediated nNOS induction (Fig. 3E).

Furthermore, RA-induced nNOS expression was blocked by an Akt inhibitor (Fig. 3F). These data demonstrate that PI3K/Akt signaling contributes significantly to RA-induced nNOS expression. In line, we observed Akt activation after RA treatment toward RA-induced signaling contributes significantly to RA-induced nNOS expression. These data demonstrate that PI3K/Akt signaling upregulates transcription of nNOS in TGW-nu-I cells.

To further validate the contribution of PI3K/Akt signaling toward RA-induced nNOS expression, we used the avian RCAS-TVA retroviral gene transfer system (37). After transduction of TGW-nu-I cells, which stable express the TVA receptor, with RCASBP(A)-lacZ retrovirus, transduction efficiencies of ~65% were observed (Fig. 4A). RA treatment induced nNOS mRNA expression in RCASBP(A)-lacZ-transduced TGW-nu-I control cells as expected. In contrast, this induction was distinctly reduced in cells transduced with dominant negative AktK179M [RCASBP(A)-dnAktK179M] (Fig. 4B). In addition, RA-induced nNOS mRNA expression was further upregulated in TGW-nu-I cells transduced with retroviruses carrying constitutively active PI3K [RCASBP(A)-p110H1047R] or constitutively active myristylated Akt [RCASBP(A)-caAkt-myr] expression cassettes (Fig. 4B), validating the data obtained with LY294002 and the Akt inhibitor at the genetic level.

RA induces DAX1 mRNA expression via PI3K signaling. To identify transcription factors induced by RA in TGW-nu-I cells, we performed transcription profiling using microarray analysis. Genes upregulated more than 10-fold 24 h after RA treatment are presented in Table 1. Among the upregulated genes, a 97-fold induction of the nuclear receptor transcriptional regulator DAX1 (NR0B1) was observed. Induction of DAX1 mRNA by RA was confirmed by real-time RT-PCR. Here a ~450-fold maximal induction was found after 96 h of treatment (Fig. 5A). To test whether increased DAX1 expression is mediated by RA/PI3K signaling, we cotreated TGW-nu-I cells with LY294002 and RA for 24 h. As shown in Fig. 5B, PI3K inhibition effectively blocks RA-induced DAX1 mRNA expression.

RA-induced DAX1 expression is essential for nNOS upregulation. To investigate the role of DAX1 for RA-induced nNOS expression, we transfected TGW-nu-I cells with DAX1-specific siRNA. Knockdown of DAX1 was verified by quantitative real-time RT-PCR at different time points after transfection. The most profound reduction of DAX1 mRNA expression to ~20% compared with control siRNA-transfected cells was observed 48–72 h after transfection (Fig. 6A). At later time points, DAX1 mRNA abundance increased rapidly due to the transient siRNA effect (data not shown). After RA treatment, DAX1 mRNA is induced in both control and DAX1 siRNA-transfected TGW-nu-I cells. However, DAX1 siRNA treatment results in a reduction of DAX1 mRNA to ~35% compared with control siRNA-transfected cells after 48 h of RA treatment (Fig. 6B). The effect of DAX1 downregulation on RA-induced nNOS expression was subsequently analyzed by quantitative RT-PCR and Western blotting. As shown in Fig. 6, C and D, nNOS mRNA and protein expression is induced 48 h after RA treatment in untransfected and control siRNA-transfected TGW-nu-I cells. In contrast, RA-induced nNOS mRNA (Fig. 6C) and protein (Fig. 6D) expression is clearly reduced in DAX1 siRNA-transfected TGW-nu-I cells. These data demonstrate that DAX1 is critically involved in RA-induced nNOS expression.

DISCUSSION

Although NO and RA are key players in the process of neuronal differentiation, molecular pathways linking RA-induced signaling to NO formation are not entirely understood (11, 14, 16, 39). Previous studies demonstrated that PI3K/Akt signaling pathways are activated by RA leading to the induction of neuronal differentiation (21, 25). In the current study we provide evidence in human neuroblastoma cells that PI3K/Akt activation is needed for RA-induced transcriptional upregulation of human nNOS, which is the predominant enzyme for the generation of NO in the central and peripheral nervous system. Whether this pathway is also relevant for cells and tissue types other than neuroblastoma cells is unclear at the moment and awaits further investigation. RA-induced nNOS expression and differentiation has been observed in a variety of cells like neural precursors, neurons and neuron-like cells, embryonic carcinoma cells, and chondrocytes in vitro (14, 18, 39, 49, 50). However, involvement of PI3K/Akt signaling was not ad-

Fig. 2. RA induces nNOS transcription. A: analysis of nNOS mRNA stability. TGW-nu-I cells were treated with RA or DMSO as control. Cells were harvested at the indicated time points after actinomycin D treatment (2 μg/mL), and mRNA levels were quantified using real-time RT-PCR. B and C: firefly luciferase (Luc) reporter-gene plasmids containing the regulatory regions of nNOS exon 1a–c (B) and exon 1f–k and exon 2 (C) were cotransfected with Renilla luciferase control plasmid. Twenty-four hours after transfection, cells were treated with 5 μM RA or DMSO for an additional 48 h and luciferase activities were determined. Data are expressed as fold induction of luciferase activities calculated by comparison of RA-treated and -untreated (DMSO) cells and represent means ± SD of three independent experiments in triplicate. D: TGW-nu-I cells were transfected with RA or DMSO as control as indicated. Chromatin of TGW-nu-I cells was immunoprecipitated with an RNA-polymerase II-specific antibody (RNA-PoII). Precipitated DNA or 10% chromatin input was amplified with primers specific for the proximal promoter regions of nNOS exon 1f and 1g. E: TGW-nu-I cells were treated with RA (5 μM) over a time period of 72 h with or without cycloheximide (10 μM). Medium containing RA ± cycloheximide was changed every 24 h. Quantitative nNOS exon 6/7 mRNA expression analysis was performed at the indicated time points using real-time RT-PCR.
dressed by these studies. Our data demonstrate that RA-dependent PI3K/Akt signaling induces expression of the orphan nuclear receptor DAX1 in TGW-nu-I neuroblastoma cells. Importantly, upregulation of DAX1 is critically involved in the transcriptional induction of the nNOS gene and therefore, we show for the first time that the transcriptional regulator DAX1 links RA and NO signaling.

Human DAX1 (NR0B1), which stands for dosage-sensitive sex-reversal (DSS), adrenal hypoplasia congenita (AHC) locus on the X-chromosome, gene 1, has an established role in the
development and maintenance of steroid-producing tissues (34). This atypical orphan nuclear receptor homodimerizes with other nuclear receptors, including steroidogenic factor 1 (SF1), ligand-activated estrogen receptor-α, or small heterodimer partner (SHP; NR0B2), usually leading to transcriptional repression (15, 27). In contrast to this well-characterized repressor function, we demonstrate that RA-induced activation of nNOS gene transcription depends on DAX1 expression. This is in line with in vivo data demonstrating DAX1-dependent Sox9 and Amh gene expression in fetal testis, although a repressive function for the Amh gene was characterized in vitro (8, 32, 38). Furthermore, reduced expression of the SF1 target gene Dhh was observed in the testes of SF1+/− heterozygous knockout mice and Dhh expression was even further reduced in SF1+/−/Dax1−deleted mice, suggesting cooperation of SF1 and Dax1 toward Dhh gene activation in vivo (38). Together, these data argue for a promoter context-dependent activator or repressor function of DAX1. Whether the observed upregulation of the nNOS gene is due to a DAX1 function in cis is unclear at the moment, and a rather indirect molecular mechanism involving other transcription factors and action on non-nNOS gene elements cannot be excluded. Since no DAX1 antibodies are available working in ChiP assays, we cannot prove direct binding of DAX1 to the different nNOS first exon gene promoters.

A variety of human nNOS mRNA variants have been described recently (7, 43, 54, 56, 57, 59). Among these, transcripts with different untranscribed first exons are generated by alternative promoter usage (12, 43, 44, 49). Since these transcripts differ only in their 5′-untranslated region, they encode for identical nNOS proteins with the same catalytic functions and activities (see supplemental Fig. S1; supplemental data for this article can be found online at American Journal of Physiology-Cell Physiology website). We and others demonstrated a cell- and tissue-specific expression pattern of the 12 so far known nNOS first exons (7, 43, 44, 46, 56), nNOS exon 1c, 1f, and 1g transcripts are the predominant forms in human brain, the peripheral nervous system, and TGW-nu-I neuroblastoma cells (41, 44, 46). Here we show that nNOS exon 1g transcripts which are highly induced by RA treatment are the most abundant nNOS mRNA variant in fetal brain, lung, trachea, heart, and placenta (see supplemental Fig. 2). These findings point to a role of nNOS exon 1g in the developing human brain and suggest that this mRNA variant represents an important nNOS transcript in vivo. Interestingly, expression of nNOS exon 1c is significantly reduced in enteric nerves innervating the pyloric sphincter of infants with hypertrophic pyloric stenosis (46). Reduced nNOS exon 1c expression is due to genetic alterations in the 3′-regulatory region and proposed to contribute to the pathogenesis of the disease, which is characterized by a hypertrophy and hyperplasia of enteric smooth muscle cells (46). Whether smooth muscle hyperplasia is due to decreased NO production resulting in increased cell proliferation is unclear at the moment. In TGW-nu-I cells, we demonstrate blockade of cell proliferation due to RA treatment which can be partially reverted by NOS inhibition. Therefore, NO-dependent and -independent mechanisms mediate the antiproliferative effects of RA in TGW-nu-I cells. In addition to the broadly expressed 12 different first exon nNOS mRNA forms, Wang and colleagues (54, 55) showed expression of testis-specific nNOS variants in vitro and in vivo. Recently, it was also demonstrated that the 5′-untranslated region of human nNOS exon 2 and its 5′-flanking sequence exerts promoter activity (13, 57). However, in TGW-nu-I cells, we were not able to detect nNOS exon 2 mRNA variants and observed only a 2-fold induction of the −10110/exon2nNOS-Luc reporter gene construct due to RA treatment. In addition, no significant promoter activity of the 5′-regulatory region of exon 2 was found with and without RA treatment (Ref. 43, Fig. 2C; and unpublished observations). This is in accordance with recent reports demonstrating neuronal expression of human exon 2 variants only in response to hypoxia in vitro and in a transgenic reporter mouse line where lacZ is expressed under the control of the 2.5-krb 5′-regulatory region of human nNOS exon 2 in vivo (57). No significant lacZ expression was observed under normoxic conditions in the central nervous system in the lacZ reporter mouse line in vivo (57). A nuclear hormone receptor binding site for SF1 is present in the 5′-untranslated region of exon 2 of the human and mouse nNOS gene, and SF1 has been shown to upregulate nNOS transcription in murine aT3-1 pituitary gonadotrope cells. Interestingly, SF1-induced nNOS expression is negatively regulated by DAX1 in these cells (58). Therefore, the results of our study support a cell-specific dual role of DAX1 for the transcriptional regulation of the nNOS gene elements.
DAX1 represses nNOS transcription in pituitary cells by interference with SF1 on the exon 2 promoter, while it upregulates nNOS first exon transcripts in response to RA treatment in TGW-nu-I neuroblastoma cells.

There are only a few functions of DAX1 characterized, in addition to the role in the establishment and maintenance of steroid-producing tissues. The demonstration that the EWS/FLI1 oncoprotein upregulates DAX1 in Ewing sarcoma cells suggests a role of DAX1 in carcinogenesis (29). Furthermore, DAX1 was shown to maintain an undifferentiated state of embryonic stem cells, demonstrating a role in differentiation processes (33). The observation that RA-induced nNOS expression depends on DAX1 implicates a role of DAX1 in neuronal differentiation, since RA and NO are well-established mediators of this process (1, 14, 23).

Although we observed only marginal stimulation of transiently transfected nNOS reporter gene constructs, we provide clear evidence that RA induces nNOS transcription in TGW-nu-I cells: 1) protein and mRNA level are simultaneously upregulated, 2) nNOS mRNA stability is not changed by RA treatment, and 3) RNA polymerase II is recruited to the promoters of nNOS exon 1f and exon 1g. The lack of a strong induction of the nNOS reporter genes by RA argues for a nNOS enhancosome outside the investigated genomic region.

In summary, we have characterized molecular mechanisms linking RA signaling and nNOS gene expression. Our results demonstrate a previously unknown PI3K/Akt/DAX1 pathway that is important for RA-induced nNOS transcription and highlight the dual role of DAX1 as an activator and repressor of nNOS gene expression in a promoter context-dependent way.

REFERENCES


Fig. 6. RA-induced DAX1 expression is essential for nNOS upregulation. A: TGW-nu-I cells were transfected with a control or a DAX1-specific small interfering (si)RNA. Real-time RT-PCR analysis of DAX1 mRNA expression was performed 48 h after siRNA transfection (Student's t-test: **P < 0.001 vs. control). B: TGW-nu-I cells were transfected with a control or a DAX1-specific siRNA. Twenty-four hours after the transfection, TGW-nu-I cells were treated with RA (5 μM) for an additional 48 h. Real-time RT-PCR analysis of DAX1 mRNA expression demonstrates DAX1 knockdown (Student’s t-test: **P < 0.001 vs. control). C: TGW-nu-I cells were transfected with a control or a DAX1-specific siRNA. Twenty-four hours after the transfection, TGW-nu-I cells were treated with RA (5 μM) or DMSO as control for an additional 48 h. Real-time RT-PCR analysis of total nNOS mRNA expression demonstrates that upregulation of nNOS mRNA depends on DAX1 (Student’s t-test: **P < 0.001 vs. control). D: TGW-nu-I cells were transfected with a control or a DAX1-specific siRNA. Twenty-four hours after the transfection, TGW-nu-I cells were treated with RA (5 μM) or DMSO as control for an additional 48 h. Western blot of nNOS protein level demonstrates that DAX1 mediates nNOS upregulation. β-Actin served as a control for equal protein loading. NS, nonspecific bands.


