Cyclic AMP-Mediated Upregulation of the Expression of Neuronal NO Synthase in Human A673 Neuroepithelioma Cells Results in a Decrease in the Level of Bioactive NO Production: Analysis of the Signaling Mechanisms that Are Involved†

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ABSTRACT: The expression level of neuronal nitric oxide synthase (nNOS) can vary depending on the (patho)physiological conditions. Here we document a marked induction of nNOS mRNA, protein, and total NO production in response to dibutyryl cyclic AMP (db-cAMP) in human A673 neuroepithelial cells. However, the upregulation of nNOS was associated with a decreased level of production of bioactive NO and by an increase in the level of generation of reactive oxygen species (ROS). ROS production could be prevented by the NOS inhibitor L-NAME, suggesting nNOS itself is involved in ROS generation. Sepiapterin supplementation of db-cAMP-treated A673 cells could restore full bioactive NO production, most likely by preventing the uncoupling of nNOS. nNOS was upregulated by other stable analogues of cAMP, by the activator of adenylyl cyclase forskolin, by isoproterenol or by dopamine through activation of D1 receptors, and by inhibitors of phosphodiesterase. cAMP did not change the half-life of the nNOS mRNA. Inhibitors of protein kinase A (PKA), H-89 and R_p-cAMPS, produced a partial inhibition of basal and cAMP-induced nNOS expression. cAMP response element binding and modulator transcription factors (CREB and CREM), typical target proteins of PKA, were expressed in A673 cells, as was the coactivator CREB binding protein (CBP). cAMP-stimulated induction of nNOS was significantly enhanced in A673 cells stably transfected with wild-type CREB and almost abolished in cells transfected with KCREB (containing a mutation of the DNA binding domain). In A673 cells transfected with $CREB_{133}$ (containing a mutation of the phosphorylatable serine 133), the overall level of nNOS expression was reduced, but the expressional stimulation by cAMP remained. This suggests that CREB bypasses, in part, the classical requirement for phosphorylation and association with CBP. Three members of the recently described four-and-a-half-LIM-domain proteins (FHL1−FHL3) were found to be expressed in A673 cells; FHL-1 and FHL-3 were upregulated by cAMP. These proteins can provide direct activation function to both CREB and CREM, and may be responsible for the PKA-independent component of CREB and CREM activity.

NO generated by the neuronal nitric oxide synthase (nNOS,[1] EC 1.14.13.39) is involved in a wide variety of functions such as neurotransmission, neuroplasticity, neuroprotection, and neurotoxicity (*1*). nNOS was initially considered a constitutively expressed protein. However, more recent evidence demonstrates that the expression of the nNOS gene can be regulated by a large variety of physiological and pathological stimuli (*2*, *3*). Excessive NO production, due to an increased level of expression and/or activity of nNOS, can result in damage to the mitochondrial electron transport chain, leading to cellular energy deficiency (*4*). In addition to synthesizing NO, nNOS can produce reactive oxygen species (ROS) under certain conditions (*5*−*9*). ROS react rapidly with NO to form peroxynitrite, a highly reactive oxidant. This can worsen the outcome of ischemia/reperfusion injury and may be an important factor in the development of neurodegenerative pathologies such as Huntington's, Parkinson's, and Alzheimer's diseases (*10*, *11*).

A diverse array of stimuli has been shown to modulate nNOS expression. These include neuronal activity, neurotransmitters, peptide hormones, growth factors, and stress signals. These stimuli activate a variety of protein kinases, including protein kinase A (PKA), mitogen-activated protein

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[1] Abbreviations: ACT, activator of CREM in testis; cAMP, cyclic adenosine monophosphate; CBP, CREB binding protein; CRE, cAMP-responsive element; CREB, cAMP response element binding protein; CREM, cAMP response element modulator protein; db-cAMP, dibutyryl-cAMP; FHL, four-and-a-half-LIM-domain protein; IBMX, 3-isobutyl-1-methylxanthine; L-NAME, N^G-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MEK 1 and 2, mitogen-activated protein kinase kinase 1 and 2, respectively; nNOS, neuronal nitric oxide synthase; pCPT-cAMP, 8-(4-chlorophenylthio)-cAMP; PKA, protein kinase A; ROS, reactive oxygen species; R_p-cAMPS, adenosine 3′,5′-cyclic monophosphothioate (R_p isomer); RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean; UTR, untranslated region.

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Table 1: Plasmids Used To Generate the Various Antisense RNA Probes

gene	linearized primers[a]	plasmid	insert size (nucleotides)	Genbank accession no. (residues)	AS[b]
nNOS	ATGTCCATGGAAGAATATGACA (F) GTTGAATCGGACCTTGTAGCTC (R)	pmu285	285	XM_006698 (2792−2974)	T3 (by *Eco*RI)
CREB	CACTGATGGACAGCAGATCTTAGT (F) TTCTTCATTAGACGGACCTCTCTC (R)	pCREB	198	XM_054039 (775−972)	SP6 (by *Xba*I)
CREM	CCTCCACCAGGTGCTACAATTGTA (F) CTGTGGCAAAGCAGCAGTAGGAGC (R)	pCREM	153	NM_001881 (604−756)	T7 (*Hin*dIII)
CBP	AGACGACAATTTCAAAGGATCAGT (F) CTGAATTTGTTTTCTTTTCGAGGT (R)	pCBP	199	XM_036668 (3974−4172)	SP6 (by *Xba*I)
ACT	TTTTGAGAAGGAGTTTGCTCACTA (F) CTTGTTGGCATAAAGATGGTTGTA (R)	pACT	202	NM_020482 (608−809)	SP6 (by *Xba*I)
FHL1	ACTCCAAGGAGGTGCACTATAAGA (F) CCTTGTACTCCACGTTTTGATCTC (R)	pFHL1	207	XM_042931 (175−381)	T7 (by *Hin*dIII)
FHL2	GAACTGCTTCTGTGACTTGTATGC (F) TGTGTTGAATTCAGATGTCTTTCC (R)	pFHL2	221	XM_030011 (476−696)	SP6 (by *Xba*I)
FHL3	CTGACACAGGGTGGATTGACATAC (F) GTCGGTCTTCAAAGGACACATACT (R)	pFHL3	223	BC001351 (683−905)	T7 (by *Hin*dIII)

[a] F, forward primer; R, reverse primer. [b] RNA polymerase used to generate the specific antisense RNA probes.

kinases (MAP kinases), and Ca^{2+}/calmodulin-dependent protein kinases. Among other targets, these kinases phosphorylate the cAMP response element binding protein (CREB) at serine 133. This phosphorylation leads to the recruitment of the coactivator CREB binding protein (CBP), and the resulting protein complex binds to the cAMP-responsive element (CRE) in the promoter of responsive genes.

CREB was originally identified as a target of the cAMP signaling pathway. The cAMP transduction cascade plays an important role in many physiological and pathological conditions. Its deregulation has been reported in many diseases, whose pathophysiology may involve nNOS, such as acute cerebral ischemia (*12*), Alzheimer's and Parkinson's diseases (*13*, *14*), or chronic alcoholism (*15*). The level of nNOS expression is increased in alcoholic brain (*16*).

Therefore, this study was designed to investigate the interaction of the cAMP signal transduction pathways with nNOS expression and to document the signaling mechanisms controlling cAMP-induced nNOS gene expression.

EXPERIMENTAL PROCEDURES

Reagents. Dibutyryl-cAMP, pCPT-cAMP, 8-bromo-cAMP, dibutyryl cGMP, 8-bromo-cGMP, SKF-38393, *R*-(+)-SCH-23390, quinpirole, okadaic acid, and actinomycin D were purchased from Sigma (Deisenhofen, Germany). Dopamine, the D2-selective antagonist haloperidol, isoproterenol, forskolin, the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), the inhibitor of the cAMP-specific phosphodiesterase IV Ro-20-1724, the PKA inhibitor H 89, the selective inhibitor of Ca^{2+}/calmodulin kinase II KN-62, the inhibitors of the MAP kinase kinase (MEK 1/2) U 0126, PD 98059, and the inhibitor of the p38 MAP kinase SB 203580 were obtained from Calbiochem (Bad Soden, Germany). Sepiapterin and the PKA inhibitor adenosine 3′,5′-cyclic monophosphothioate, R_p isomer (R_p-cAMPS), were from Alexis (Grünberg, Germany). Isotopes were from New England Nuclear/DuPont (Bad Homburg, Germany). Restriction enzymes were obtained from New England BioLabs (Frankfurt am Main, Germany). Polynucleotide kinase, NTPs, and dNTPs were from Amersham Biosciences (Freiburg, Germany). Oligonucleotides were synthesized by MWG Biotech (Heidelberg, Germany). T3, T7, and SP6 RNA polymerases, DNase I, RNase A, RNase T1, Expand polymerase, proteinase K, and FuGENE 6 were from Roche Diagnostics (Mannheim, Germany). The QuantiTect Probe RT-PCR mix was obtained from Qiagen (Milden, Germany).

Cell Culture. Human cell lines A673 (neuroepithelioma) and SK-N-MC (neuroblastoma) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom). Human testis teratocarcinoma cell line NT2 was purchased from Stratagene (Heidelberg, Germany). Human keratinocyte-like cell line HaCat (*17*) was kindly provided by S. Frank (Center of Pharmacology, Johann Wolfgang Goethe University Hospitals, Frankfurt am Main, Germany). A673 cells, SK-N-MC cells, and HaCat cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen/GIBCO, Groningen, The Netherlands) with 10% fetal bovine serum, 2 mmol/L L-glutamine, and a 1% penicillin/streptomycin mixture. NT2 cells were cultivated in a 1:1 (v:v) mixture of Dulbecco's minimal essential medium and Ham's F12 Nutrient mix, supplemented with 2 mM glutamine and 10% fetal bovine serum. All cells were kept at 37 °C in 10% CO_2.

Human Brain Tissue. Tisssue from human cortex was supplied by T. J. Feuerstein [Albert-Ludwigs-University, Freiburg, Germany; see our previous publication (*18*)]. Human cerebellar tissue was obtained from P. Riederer (Julius-Maximilians-University, Würzburg, Germany; see ref *18*).

Cloning of cDNA Fragments and Generation of Antisense RNA Probes. The human nNOS cDNA was obtained by reverse transcription (RT) of 2 μg of total RNA isolated from human quadriceps muscle, using SuperScript reverse transcriptase (Invitrogen) and a combination of oligo$(dT)_{14}$ and random hexamers as primers. The obtained cDNA served as the template in a polymerase chain reaction (PCR) using Expand polymerase and the primers indicated in Table 1. The PCR fragment that was obtained was cloned into plasmid pXcmI (*19*), resulting in the pmu285 plasmid. The plasmid containing a fragment of human β-actin cDNA (108 nucleotides) (pCR_βactin_hu_Δ*Bst*EII_*Hin*dIII) has been generated previously (*20*). All the other human cDNA fragments used in this study were obtained by the same RT-PCR

method using total RNA extracted from A673 cells (from human testis for ACT). The PCR products were cloned into pCRII plasmids (TOPO TA cloning kit, Invitrogen). The respective oligonucleotides used as sense and antisense primers, the resulting PCR products, and plasmid names are listed in Table 1. All constructs were verified by sequencing. Radiolabeled antisense RNA probes for RNase protection assays were generated as previously described (*21*, *22*). The restriction enzymes and RNA polymerases that were used and the expected sizes of the protected fragments are given in Table 1.

Ribonuclease Protection Assays. The levels of the different mRNAs were determined from the amount of total cellular RNA by the RNase protection assay as previously described (*21*, *22*). Densitometric analyses were performed using a Phospho-Imager (Bio-Rad, Munich, Germany). The densities of the protected gene-specific bands were normalized using the corresponding protected β-actin bands.

Determination of the GTP Cyclohydrolase I mRNA Level by Reverse Transcription RealTime PCR. Quantification of the GTP-CH I message was achieved by RealTime PCR using the iCyler (Bio-Rad). One-step RT-PCR was performed on 500 ng of total RNA, from untreated cells or cells treated with 0.5 mM db-cAMP for 12 h, with the QuantiTect Probe RT-PCR mix (Qiagen) as recommended by the manufacturer. For the detection of GTP-CH I expression, oligonucleotides 5′-CCT CGG CCA TGC AGT TCT T-3′ and 5′-CTT CAT CAA ATA TAG CAT CGT TTA GGA C-3′ were used as the sense and antisense primer, respectively, and 5′-6FAM-CCA AGG GCT ACC AGG AGA CCA TCT CA-TAMRA-3′ was used as the fluorescent TaqMan probe. Levels of GTP-CH I were normalized by amplification of the constitutive GAPDH mRNA using oligonucleotides 5′-AGC CTC AAG ATC ATC AGC AAT G-3′ and 5′-CAC GAT ACC AAA GTT GTC ATG GA-3′ as the primer pair and 5′-6FAM-CTG CAC CAC CAA CTG CTT AGC ACC C-TAMRA-3′ as the Taqman probe. PCR conditions were initial reverse transcription at 50 °C for 30 min, denaturation of the reverse transcriptase and/or activation of the polymerase at 95 ° C for 15 min, and 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The fluorescence was monitored at each cycle during the annealing and extension step. For each sample, the amount of GTP-CH I and GAPDH was determined from standard curves generated by serial dilutions of the total RNA sample with a high content of GTP-CH I and GAPDH.

Creation of Stable Cell Lines Overexpressing Murine CREB, KCREB, and CREB$_{133}$. Vectors pCMV-CREB (wild-type murine CREB), pCMV-KCREB (mutations in the DNA-binding domain) (*23*), and pCMV-CREB$_{133}$ (serine-to-alanine mutation at position 133) (*24*) were purchased from BD Biosciences Clontech (Heidelberg, Germany). A673 neuroepithelioma cells were transfected with these vectors using FuGENE 6. Two days after transfection, the cells were split and exposed to G418 (600 μg/mL). Cell clones expressing wild-type CREB, KCREB, or CREB$_{133}$ were singled out and grown to confluence in G418-containing medium.

Western Blots. For the assessment of nNOS protein expression, total protein was isolated and Western blots were performed as previously described (*21*, *22*). Extracts were prepared from untreated cells or from cells induced for 24 h with 0.5 mM db-cAMP. For the detection of the nNOS protein, a mouse monoclonal antibody (directed to the C-terminal nNOS) was used (BD Transduction Laboratories, Heidelberg, Germany) at a 1:500 dilution. Levels of CREB protein expression were determined using a rabbit polyclonal antibody (Cell Signaling Technology/New England BioLabs) at a 1:500 dilution. For normalization, blots were also probed with a mouse monoclonal antibody against β-tubulin (Sigma) at a 1:250 dilution. Immunoreactive proteins were visualized with NBT/X-phosphate and quantified with a Video-Imager (Bio-Rad) (*21*, *22*).

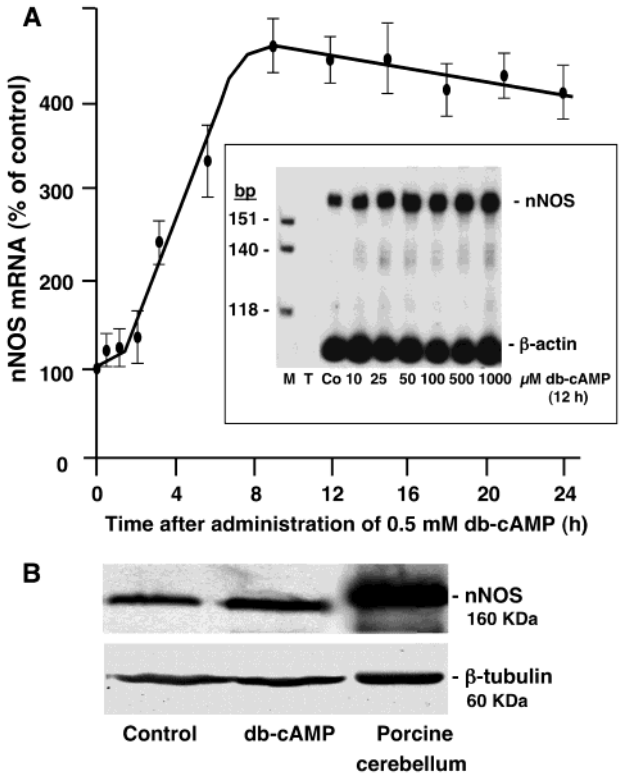

FIGURE 1: Upregulation of human nNOS mRNA and protein by dibutyryl-cAMP (db-cAMP) in A673 cells. (A) Time course of induction of nNOS mRNA in A673 cells following induction with 0.5 mM db-cAMP. nNOS mRNA levels were determined by RNase protection analyses. The nNOS mRNA level at time zero was set at 100%. Values are means ± SEM ($n = 4$). The inset shows an RNase protection assay (representative of six experiments) performed with total RNA from human A673 cells incubated for 12 h with medium alone (control) or with medium containing increasing concentrations of db-cAMP. Experiments were performed using antisense RNA probes for human nNOS and β-actin (for normalization): M, molecular weight standard; T, tRNA control. (B) Western blot using a monoclonal anti-nNOS antibody and a monoclonal anti-β-tubulin antibody (for normalization). Protein extracts were prepared from untreated cells (Co) or from cells exposed for 24 h to 0.5 mM db-cAMP. Aliquots corresponding to 100 μg of total protein were loaded onto a 7.5% SDS−polyacrylamide gel. A positive control (porcine cerebellum) was run in parallel on the same gel. Densitometric analyses of six different blots demonstrated an increase in nNOS immunoreactivity to 280 ± 39% of the control value (mean ± SEM).

Determination of the Total and Bioactive Amounts of NO in Cell Culture Supernatants. To determine the total amount of NO synthesized, the levels of nitrite and nitrate were measured in the supernatant of A673 cells. Confluent cultures were left untreated or were induced with 0.5 mM db-cAMP for 24 h. Then the supernatants were removed, and the cells were further incubated for 10 min at 37 °C in Locke's solution containing 2 mM L-arginine. NO_2^- and NO_3^-

Table 2: Effect of cAMP and cGMP Analogues on nNOS mRNA Expression in A673 Cells[a]

analogue	nNOS mRNA (% of control, mean ± SEM)	significance (vs control)
control (none)	100	
8-bromo-cAMP	275.8 ± 34.9	$p < 0.001$
pCPT-cAMP	348.9 ± 51.8	$p < 0.001$
db-cGMP	89.2 ± 15.9	ns
8-bromo-cGMP	102.7 ± 16.7	ns

[a] A673 cells were incubated for 12 h with medium alone (control) or with medium containing the respective cAMP or cGMP analogue (100 μM), 8-bromo-cAMP, pCPT-cAMP, db-cGMP, or 8-bromo-cGMP. RNase protection assays were performed using antisense RNA probes to human nNOS and β-actin (for standardization). ns means nonsignificant.

contents in supernatants were determined as NO_2^- after enzymatic reduction with NO_3^- reductase (NOA 280 nitric oxide analyzer, Sievers, Boulder, CO) (*22*). NO_2^- levels were normalized for the total amount of cell protein.

Bioactive NO produced by A673 cells was bioassayed using RFL-6 rat lung fibroblasts as reporter cells (*25*). Cells were initially incubated for 24 h with or without 100 μM sepiapterin. After a further 24 h incubation in medium (with or without 0.5 mM db-cAMP, 10 μM isoproterenol, or 100 μM dopamine), A673 cells were incubated for 30 min with Locke's solution containing 200 units/mL superoxide dismutase (SOD) and 1 mM L-arginine with or without 100 μM sepiapterin. Thereafter, A673 cells were stimulated for 2 min with 10 μmol/L A23187 (with or without sepiapterin), and the conditioned media from the cells were transferred to the RFL-6 cells as previously described (*26*). The cGMP content of the RFL-6 samples was determined with a radioimmunoassay.

Measurement of the Level of Intracellular Reactive Oxygen Species (ROS). The determination of the extent of intracellular oxidant formation was based on the oxidation of 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Leiden, The Netherlands) to yield a fluorescent compound trapped intracellularly. The fluorescence was measured in a FluoroCount Reader (Perkin-Elmer Life Sciences, Zaventem, Belgium). Confluent A673 cells in 96-well plates were cultivated for 24 h in medium alone or in medium containing 0.5 mM db-cAMP, then washed with Hank's buffered saline solution (HBBS, Invitrogen, Groningen, The Netherlands), and incubated for 30 min with 10 μM CM-H_2DCFDA in HBBS. Thereafter, cells were washed twice with HBBS and incubated for an additional 2 h before the fluorescence was measured at excitation and emission wavelengths of 488 and 530 nm, respectively (*27*).

Statistics. Data represent means ± SEM. Differences were tested for statistical significance using the Student's *t* test (Figure 1) or ANOVA and Fisher's protected least-significant-difference (PLSD) test (Figures 2, 3, and 5).

RESULTS

cAMP Analogues Enhanced nNOS mRNA and Protein Expression in Human A673 Cells. Treatment of A673 cells with db-cAMP resulted in an enhancement of nNOS mRNA expression in a concentration- and time-dependent manner (Figure 1A). A similar time course was observed when A673 cells were treated with 10 μM forskolin ($n = 3$, data not shown). A comparable induction of nNOS mRNA in response to cAMP was found in other human cell lines, such as SK-N-MC neuroblastoma cells, NT2 teratocarcinoma

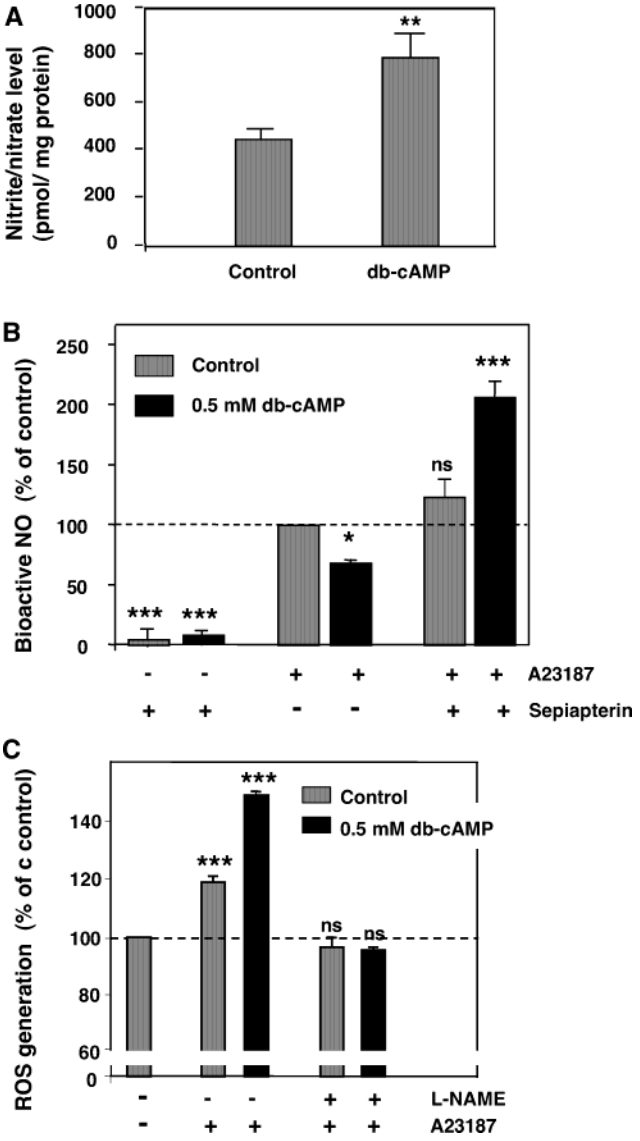

FIGURE 2: Effect of cAMP on NO and ROS production. (A) Effect of a 24 h incubation with 0.5 mM db-cAMP on the level of total NO production by A673 cells. NO production was determined as the amount of NO_2^- and NO_3^-. Control corresponds to untreated A673 cells. Cells were stimulated for 10 min with 10 μM Ca^{2+} ionophore A23187, and the amount of cumulative NO_2^- and NO_3^- production was determined during this period of time. Data represent means ± SEM ($n = 6$) [$p < 0.01$ vs control (two asterisks)]. (B) Effect of a 24 h incubation with 0.5 mM db-cAMP on the bioactive NO production by A673 cells. The amount of bioactive NO was determined with an RFL-6 reporter cell assay; 100% corresponds to 10.23 ± 0.21 pmol of cGMP/10^6 RFL-6 cells. When sepiapterin was used, the cells were pretreated for 24 h with the compound (100 μM). Data represent means ± SEM ($n = 4$) [$p < 0.05$ (one asterisk) and $p < 0.001$ (three asterisks) vs A23187-stimulated control cells without sepiapterin]. ns means not significant. (C) cAMP enhances the generation of ROS in A673 cells. Cells were treated for 24 h with 0.5 mM db-cAMP, and ROS production was assayed by CM-H_2DCFDA oxidation-based fluorescence; 100% corresponds to 1.2×10^4 arbitrary light units. For cells treated with L-NAME (100 μM), the inhibitor was present during the last 30 min of the incubation. Data represent means ± SEM ($n = 4$) [$p < 0.001$ vs nonstimulated control cells without L-NAME (three asterisks)]. ns means not significant.

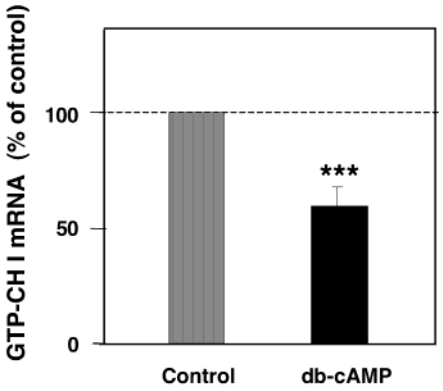

FIGURE 3: Effect of cAMP on GTP cyclohydrolase I expression in A673 cells. Total cellular RNA (500 ng) from control and A673 cells treated with 0.5 mM db-cAMP (12 h induction) was analyzed by RealTime PCR. Levels of GTP cyclohydrolase I message were normalized by amplification of the constitutive GAPDH mRNA. The GTP-CH I mRNA level of nontreated cells was set to 100% [$p < 0.001$ vs control (three asterisks) (100%) ($n = 6$)].

cells, and HaCat keratinocyte-like cells ($n = 3$ each, data not shown). Incubation of A673 cells with 0.5 mM db-cAMP for 24 h also enhanced nNOS protein expression (Figure 1B). Also, other cAMP analogues (100 μM 8-bromo-cAMP or 100 μM pCPT-cAMP given for 12 h each) were able to trigger nNOS mRNA expression in A673 cells, whereas the cGMP analogues db-cGMP and 8-bromo-cGMP had no effect (Table 2).

Effect of cAMP on NO and ROS Production. The level of total synthesis of NO (measured with an NO analyzer as nitrite and nitrate) increased in parallel with the level of nNOS expression upon db-cAMP treatment (Figure 2A). However, the upregulation of nNOS was associated with a decreased level of production of bioactive NO in the A673 cells (Figure 2B). ROS generation was also found to be enhanced by cAMP (Figure 2C). This increase in the level of ROS production could be prevented by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), suggesting ROS generation by nNOS itself (Figure 2C). nNOS is the only NOS isoform that can be detected in A673 cells (*18*). Incubation of A673 cells with 0.5 mM db-cAMP for 12 h resulted in a downregulation of GTP cyclohydrolase I expression [57 ± 7% ($n = 6$)] (Figure 3). Indeed, sepiapterin supplementation of db-cAMP-treated A673 cells fully restored bioactive NO production, most likely by preventing the uncoupling of nNOS (Figure 2B).

Induction of nNOS mRNA in Response to Compounds that Increase Intracellular cAMP Levels in Human A673 Cells. Stimulation of A673 cells for 12 h with 10 μM isoproterenol or 100 μM dopamine also upregulated nNOS mRNA (Figure 4A). Densitometric analyses of Western blots also demonstrated an increase in the level of nNOS protein {278 ± 32% for the cells treated with 10 μM isoproterenol for 24 h, 156 ± 21% for the cells treated with 100 μM dopamine [mean ± SEM ($n = 4$)]} (data not shown). With 10 μM isoproterenol, the level of bioactive NO increased by 38 ± 8% ($n = 3$, $p < 0.01$); with 100 μM dopamine, there was only a marginal increase of 10 ± 9% ($n = 3$, no significant difference) (Figure 4B).

The respective role of the various subtypes of dopamine receptors was investigated by studying the effects of D1- and D2-dopamine agonists and antagonists on nNOS expression in A673 cells (Figure 5). SKF-38393 (10 μM), a selective D1 agonist, strongly increased the level of nNOS expression, whereas 10 μM quinpirole, a selective D2 agonist, had no impact on the nNOS mRNA level. Furthermore, 10 μM SCH-23390, a D1-selective antagonist, could abolish the observed dopamine-induced nNOS upregulation, whereas pretreatment with 10 μM haloperidol, a D2-selective antagonist, resulted in a further increase in the level of dopamine-induced nNOS expression (Figure 5).

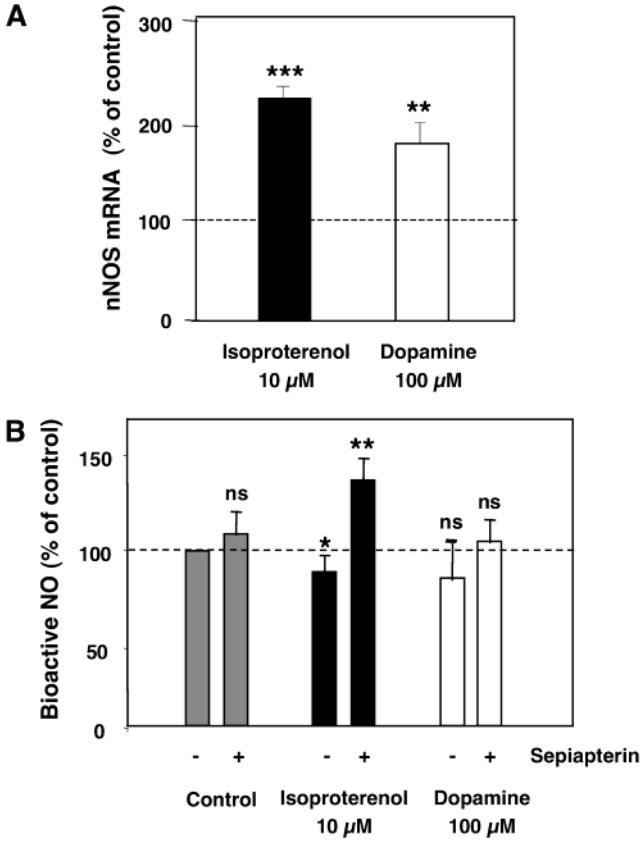

FIGURE 4: Effect of isoproterenol and dopamine on nNOS mRNA expression and the production of bioactive NO in A673 cells. (A) A673 cells were incubated with isoproterenol (10 μM) or dopamine (100 μM), and total RNA was prepared 12 h later. RNase protection assays were performed using antisense RNA probes to human nNOS and β-actin (for standardization). Columns (means ± SEM) represent relative nNOS mRNA levels as determined in densitometric analyses of six different gels. The nNOS mRNA level of untreated A673 cells was set to 100% [$p < 0.01$ (two asterisks) and $p < 0.001$ (three asterisks) vs control (100%)]. (B) Effect of a 24 h incubation with isoproterenol (10 μM) or dopamine (100 μM) on the bioactive NO production by A673 cells. The amount of bioactive NO was determined with the RFL-6 reporter cell assay. For cells treated with 100 μM sepiapterin, the compound was added 24 h before the start of the incubation. Data represent means ± SEM ($n = 4$) [$p < 0.05$ (one asterisk) and $p < 0.01$ (two asterisks) vs control cells without sepiapterin]. ns means not significant.

An upregulation of nNOS mRNA was also observed when cells were treated with 1 or10 μM forskolin (Figure 6), with the nonspecific phosphodiesterase inhibitor IBMX (100 μM) or the inhibitor of the cAMP-specific phosphodiesterase type IV Ro-20-1724 (10 μM, Figure 6).

Increased cAMP Levels Did Not Affect the Stability of the Human nNOS mRNA. When transcription of human nNOS was blocked with 10 μg/mL actinomycin D and total RNA was extracted from A673 cells after different incubation times, the nNOS mRNA levels were found to decline with

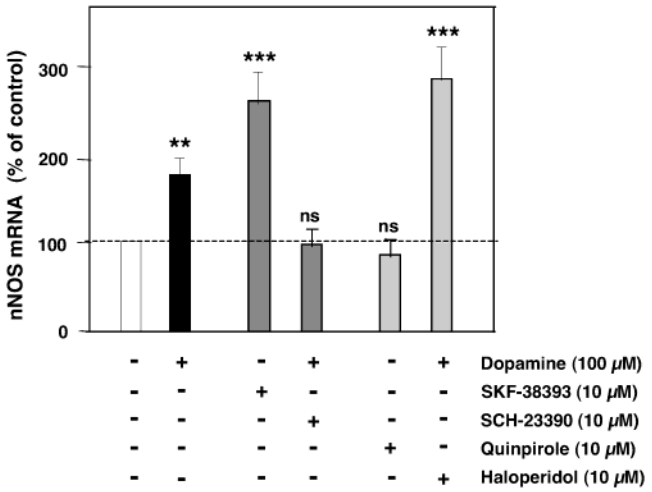

FIGURE 5: Effect of D1- and D2-dopamine receptor activation on nNOS mRNA expression in A673 cells. A673 cells were treated with dopamine (100 μM) alone or preincubated for 30 min in the presence of 10 μM D1-selective (SCH-23390) or D2-selective (haloperidol) antagonists prior to the addition of 100 μM dopamine. In other experiments, cells were also incubated in the presence of 10 μM D1-selective (SKF-38393) or D2-selective (quinpirole) agonists. Total RNA was prepared 12 h later. RNase protection assays were performed using antisense RNA probes to human nNOS and β-actin (for standardization). Columns (means ± SEM) represent relative nNOS mRNA levels as determined in densitometric analyses of six different gels. The nNOS mRNA level of untreated A673 cells was set to 100% [$p < 0.01$ (two asterisks) and $p < 0.001$ (three asterisks) vs control (100%)].

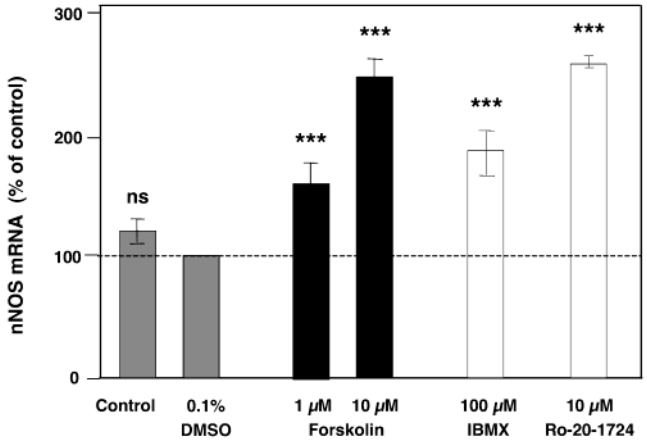

FIGURE 6: Effect of other compounds known to increase intracellular levels of cAMP on nNOS mRNA expression in A673 cells. A673 cells were incubated with DMSO (0.1%, the solvent of the subsequent compounds), forskolin (1 or 10 μM), 3-isobutyl-1-methylxanthine (IBMX, 100 μM), or compound Ro-20-1724 (10 μM). Total RNA was prepared 12 h later. RNase protection assays were performed using antisense RNA probes to human nNOS and β-actin (for standardization). Columns (means ± SEM) represent relative nNOS mRNA levels as determined in densitometric analyses of six different gels. The nNOS mRNA level of A673 cells treated with 0.1% DMSO was set to 100% [$p < 0.001$ vs DMSO control (three asterisks)].

an approximate half-life of 48 h (Figure 7). Treatment of A673 cells with 0.5 mM db-cAMP for 12 h did not change the stability of human nNOS mRNA (Figure 7).

Inhibition of CREB Phosphorylation Inhibited but Did Not Abolish the db-cAMP- or Forskolin-Induced Expression of nNOS in A673 Cells. In A673 cells, constitutive expression of high levels of messages for CREB and CREM were

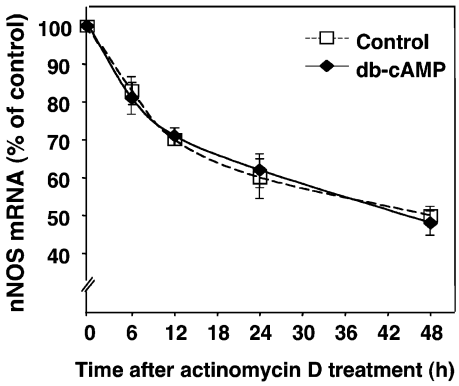

FIGURE 7: Effect of db-cAMP on nNOS mRNA stability in A673 cells. A673 cells were cultured for 12 h in medium without (control, □) or with 0.5 mM db-cAMP (◆). The inhibitor of transcription actinomycin D (10 μg/mL) was then added to the culture medium. Total RNA was prepared at various time points thereafter, and nNOS mRNA levels were determined with RNase protection assays. The nNOS mRNA levels for both experiments were set to 100% at the time of addition of actinomycin D (0 h). Values are means ± SEM of three independent sets of experiments.

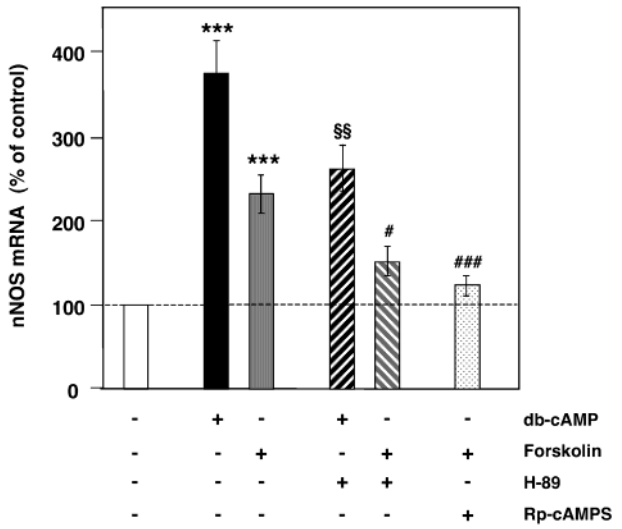

FIGURE 8: Effect of protein kinase A inhibitors on nNOS mRNA expression in A673 cells. A673 cells were incubated for 12 h with medium containing 0.1% DMSO or with medium containing 100 μM db-cAMP or 10 μM forskolin (in DMSO). An inhibitor of PKA, H-89 (1 μM), was added together with db-cAMP or forskolin. In another experiment, R_p-cAMPS (150 μM) was added together with forskolin. RNase protection assays were performed using antisense RNA probes to human nNOS and β-actin (for standardization). Columns (means ± SEM) represent relative nNOS mRNA levels as determined in densitometric analyses of four different gels. The nNOS mRNA level of A673 cells treated with 0.1% DMSO was set to 100% [$p < 0.001$ vs DMSO control (three asterisks), $p < 0.01$ vs db-cAMP alone (two section marks), $p < 0.05$ (one pound sign), and $p < 0.001$ vs forskolin alone (three pound signs)].

detected by RT-PCR and RNase protection assays ($n = 3-4$, data not shown). In the classical cAMP signal transduction cascade, CREB and CREM are activated by PKA-dependent phosphorylation at specific serine residues. The PKA inhibitors, H-89 (1 μM) and R_p-cAMPS (150 μM), inhibited the forskolin-induced expression of nNOS (Figure 8). However, the two compounds did not completely abolish nNOS mRNA induction. A similar result upon inhibition with H-89 was observed when 0.1 mM db-cAMP was used instead of forskolin ($n = 4$, Figure 8). At 30 μM, H-89 gave identical results. However, A673 cells started to detach from the

culture dishes at this concentration. Surprisingly, addition of various protein phosphatase inhibitors (okadaic acid, tautomycin, calyculin A, and cyclosporine A) did not influence basal and cAMP-induced nNOS mRNA expression (data not shown).

In addition to PKA, Ca^{2+}/calmodulin kinase and the MAP kinase pathways can lead to the activation of CREB. However, inhibitors of Ca^{2+}/calmodulin kinase II (KN-62, up to 5 μM), of MEK 1/2 (U 0126, 1 μM, and PD 98059, up to 30 μM), and of p38 MAP kinase (SB 203580, 10 μM) did not change nNOS mRNA induction ($n = 3$, each, data not shown).

An average 2.4-fold overexpression in total CREB was obtained in A673 cells stably transfected with CREB, KCREB, or $CREB_{133}$ cDNAs (Figure 9A). A673 cells stably transfected with the wild-type CREB exhibited a significant enhancement of the db-cAMP or forskolin induction of nNOS (Figure 9B). In cells overexpressing KCREB (a CREB with a mutation in the DNA binding domain), both basal and cAMP-induced nNOS expression was almost abolished (Figure 9B). Interestingly, the level of nNOS protein expression in nonstimulated cells was remarkably decreased in A673/pKCREB cells, suggesting a cAMP-dependent basal expression of nNOS in A673 cells (Figure 9A). Analysis of the 5′-flanking sequence of dual nNOS promoter exons 1f and 1g [the first exon 1f is primarily expressed in A673 cells; first exon 1g expression is upregulated upon cAMP treatment (*18*)] and of the untranslated region of exon 2 showed the presence of several CRE sites that are functionally important for basal promoter activity (J.-P. Boissel et al., unpublished data). Surprisingly, in cells expressing $CREB_{133}$ (a CREB with a mutation of the PKA-phosphorylatable serine), the overall nNOS expression level was reduced, but db-cAMP and forskolin could still trigger some degree of nNOS expression (Figure 9B).

Expression of the CREB/CREM Coactivators CBP and Four-and-a-Half-LIM-Domain Proteins (FHLs) in A673 Cells. Using RT-PCR, we found that the classic coactivator of CREB and CREM, CBP, was expressed in A673 cells (Figure 10A). Also the more recently described coactivators, four-and-a-half-LIM-domain proteins FHL1−3, were detected in A673 cells (Figure 10A). FHL1 and -2 were also found to be expressed in human cortex. In human cerebellum, FHL1 and the LIM-only protein ACT (*28*) were detected (Figure 10B).

Following treatment with forskolin or db-cAMP, FHL3 mRNA expression was induced, reaching a maximum after 3 h (Figure 10C). A time-dependent enhancement was also observed for the FHL1 message (Figure 10C). In contrast, the level of FHL2 message remained unchanged after forskolin or db-cAMP treatment (data not shown).

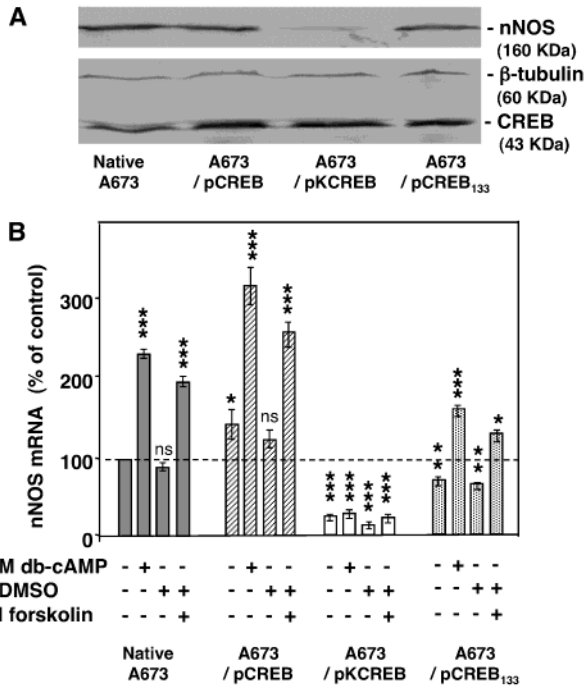

FIGURE 9: Induction by cAMP and forskolin of nNOS in native A673 cells and in A673 cells stably transfected with the wild-type murine CREB cDNA or with CREB mutants KCREB and $CREB_{133}$. Three A673-derived cell lines, stably transfected with expression plasmids containing wild-type murine CREB cDNA or two CREB mutants, KCREB and $CREB_{133}$, were created. (A) Basal expression of nNOS, total CREB, and β-tubulin in stably transfected cells. Western blots were performed with a monoclonal anti-nNOS antibody, a polyclonal anti-CREB antibody (that recognizes all the CREB variants), and a monoclonal anti-β-tubulin antibody (for normalization). Protein extracts were prepared from A673 cells or A673 cells stably transfected with CREB, KCREB, or $CREB_{133}$. Aliquots corresponding to 100 μg of total protein were loaded onto 7.5% SDS−polyacrylamide gels. Densitometric analyses of four different blots demonstrated similar increases in the amount of total CREB. The respective increases in CREB immunoreactivity were 235 ± 23% for CREB-, 243 ± 39% for KCREB-, and 227 ± 36% for $CREB_{133}$-transfected A673 cells [means ± SEM ($n = 4$ each)]. Native A673 represents nontransfected A673 cells. Basal nNOS expression was slightly enhanced in CREB-transfected cells (118 ± 12%), inhibited markedly in KCREB-transfected cells (7 ± 4%), and inhibited marginally in $CREB_{133}$-transfected cells [91 ± 8% ($n = 4$ each)]. (B) The effect of 0.5 mM db-cAMP or 10 μM forskolin on nNOS mRNA levels after a 12 h incubation was analyzed by RNase protection assays using antisense RNA probes to human nNOS and β-actin (for standardization). For the forskolin control, A673 cells were incubated for 12 h with medium containing 0.1% DMSO. Columns (means ± SEM) represent relative nNOS mRNA levels as determined in densitometric analyses of four different gels. The nNOS mRNA level of untreated and nontransfected A673 cells (native A673) was set to 100%. All cells were issued from the same A673 batch. Control cells were maintained and transportedduring the all-selection process necessary to establish the various stable CREB-transfected clones. Multiple passes and long-term culture resulted in a slight decrease in the level of cAMP and forskolin induction [$p < 0.05$ (one asterisk), $p < 0.01$ (two asterisks), and $p < 0.001$ (three asterisks) vs untreated and nontransfected A673 cells]. ns means not significant.

DISCUSSION

The current study demonstrates that several cAMP analogues, and a variety of compounds that increase the intracellular levels of cAMP (receptor agonists, forskolin, and phosphodiesterase inhibitors), stimulate the expression of human nNOS (Figures 1, 2, and 4−6). This finding was not restricted to the A673 neuroepithelioma cell line; it was also seen in human SK-N-MC neuroblastoma cells, in human NT2 testis teratocarcinoma cells, and in the human keratinocyte-like HaCat cell line. The increase in the level of nNOS mRNA (Figure 1A) was accompanied by an enhanced nNOS protein level (Figure 1B). The cAMP regulation of nNOS (promoter activity and/or mRNA/protein) has been previously reported (*18*, *29*). Likewise, in a previous study, forskolin has been shown to increase the level of nNOS protein expression in porcine ciliary processes (*30*). Furthermore,

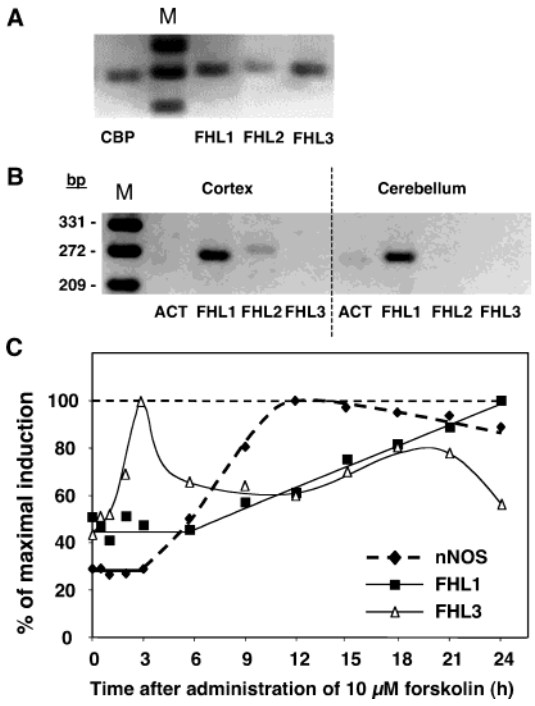

FIGURE 10: Expression of the CREB/CREM coactivators CBP and four-and-a-half-LIM-domain (FHL) proteinsin A673 cells in human brain. (A) RT-PCR analysis of the expression of CBP and FHL1−3 in A673 cells. The primers used during amplification are listed in Table 1. The PCR products were cloned into the pCRII vector. The recombinant plasmids were used to generate specific antisense RNA probes for RNase protection assays. Lane M contained molecular weight markers. (B) RT-PCR analysis of the expression of the FHLs in human cerebral cortex and cerebellum. The PCR primers are listed in Table 1. Lane M contained molecular weight markers. (C) Time course of the induction by 10 μM forskolin of human nNOS, FHL1, and FHL3 transcripts in A673 cells. Total RNA was prepared at different time points, and mRNA levels for nNOS (◆), FHL1 (■), and FHL3 (△) were determined by RNase protection assays using antisense RNA probes specific for the different human genes (Table 1) and for β-actin (for standardization). The maximal induction observed for each transcript was set to 100%. Values are means of three independent experiments. Error bars have been omitted for clarity.

the specific cAMP phosphodiesterase inhibitor cilostazol was found to stimulate NO production in the human neuroblastoma SH-SY5Y cell line (*31*). However, in the rat pheochromocytoma PC12 cells, which constitutively express nNOS, PACAP (pituitary adenylate cyclase-activating polypeptide) was shown alternatively to stimulate nNOS expression (*32*) or to inhibit NO production (*33*). Such contradictory results and the complex mechanisms involved in nNOS expressional regulation led us to conduct further investigation, using the A673 cell line as model.

In A673 cells, the cAMP-induced upregulation of nNOS was paralleled by an increase in the level of total NO production (Figure 2A). However, the level of bioactive NO was reduced (Figure 2B). This discrepancy is explained in Figure 2C, which shows that cAMP-treated cells produced more ROS. The stimulation of ROS production could be abolished by the NOS inhibitor L-NAME, suggesting that the observed increase in the level of ROS formation was due to an uncoupling of the nNOS enzyme leading to the formation of ROS (O_2^- and H_2O_2) in lieu of NO. ROS can rapidly oxidize NO, producing peroxynitrite ($ONOO^-$), thereby reducing NO bioactivity. Peroxynitrite production can lead to oxidative neuronal death as described in mice cortical cell cultures (*34*). A relative (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4) deficiency is a major cause of NOS uncoupling *in vivo*. In cAMP-treated A673 cells, the level of GTP cyclohydrolase I expression was diminished (Figure 3) and the production of bioactive NO could be restored when the cells were treated with the BH_4 precursor sepiapterin (Figure 2B). db-cAMP has been reported to inhibit cytokine-induced biosynthesis of BH_4 in human umbilical vein endothelial cells (*35*). Recent results suggested that BH_4 deficiency converts neuronal NOS into an efficient peroxynitrite synthase, which is responsible for the increase in neuronal vulnerability to hypoxia-induced mitochondrial damage and necrosis (*5*, *6*, *9*, *36*, *37*). Neurons have a high energy demand and a high level of ROS under physiological conditions. Therefore, they are subject to high risk when the level of ROS production is increased further under pathological conditions. Such conditions include neurodegenerative disorders such as Alzeimer's and Parkinson's diseases, acute traumatic and ischemic insults, chronic alcoholism, and aging. Further research may determine whether nNOS uncoupling is involved in some of those pathophysiological situations. Parallel increases in the nNOS mRNA and bioactive NO production levels in sepiapterin-treated A673 cells were also observed with the catecholamine analogue isoproterenol (Figure 4B). Upregulation of the nNOS message was also observed upon dopamine treatment. Effects of selective D1- and D2-dopamine agonists and antagonists indicated that both subtypes were present in A673 cells (Figure 5). In a variety of cell lines, it has been shown that D1- and D1-like receptors robustly stimulated cAMP accumulation. On the other hand, D2- and D2-like receptor activations were shown to inhibit adenylyl cyclase activity (for a review, see ref *38*). Our results clearly indicated that D1- and D2-dopamine receptors have a distinct effect on nNOS expression in A673 cells (Figure 5). Activation of D1 subtypes resulted in an increase in nNOS mRNA levels, whereas D2 subtypes inhibited nNOS expression. The combined activation of both receptor subtypes could explain the observed lack of a dopamine effect on bioactive NO formation. The D1 receptor is the most widespread dopamine receptor in the brain and is expressed at higher level than any other dopamine receptor. Low levels of D2 receptor expression were mainly found in cerebral cortex, hippocampus, and a restricted area of the thalamus (*38*).

Our study then aimed to characterize the mechanisms by which cAMP increases the level of nNOS gene expression. Besides its effect on gene transcription, cAMP has also been reported to stabilize the mRNA of certain genes (*39*−*41*). However, this does not seem to apply to human nNOS, because exposure of A673 cells to db-cAMP or forskolin did not change the half-life of the nNOS mRNA (Figure 7). Thus, the cAMP-induced increase in the level of nNOS expression is likely to be the consequence of enhanced gene transcription.

In eukaryotes, an established mechanism for activation of gene transcription through the adenylate cyclase signaling pathway is mediated by a family of cAMP-responsive nuclear factors, including CREB and CREM (*42*). These factors contain the basic domain/leucine zipper motifs and bind as dimers to CRE. Both CREB and CREM were found to be expressed in A673 cells. Classically, activation by CREB is

known to occur upon phosphorylation at an essential regulatory site (serine 133 in CREB) and the subsequent recruitment of CBP, a large coactivator that contacts the general transcriptional machinery (*42*). Also, CBP was expressed in A673 cells (Figure 10A). CREB phosphorylation can be brought about by PKA (and some other kinases such as MAP kinases or Ca^{2+}/calmodulin kinases) (*43*, *44*). However, inhibitors of MAP kinases or Ca^{2+}/calmodulin kinases failed to inhibit the cAMP-induced upregulation of nNOS. The absence of an effect of two inhibitors of MEK 1/2 (U0126 and PD98059) on nNOS expression also argues against an involvement of the recently discovered cAMP receptor EPAC (exchange protein directly activated by cAMP) that has been shown to modulate the MAP kinase pathway independently of PKA through activation of Rap1 and B-Raf (*45*).

Thus, the classical cAMP−PKA−CREB pathway seems to mediate a significant portion of the cAMP-induced upregulation of nNOS. On the other hand, PKA inhibitors and a 2.4-fold overexpression of a mutated CREB that lacks the DNA binding domain significantly reduced the level of basal and cAMP-induced nNOS expression (Figures 8 and 9). A 2.4-fold overexpression of wild-type CREB, on the other hand, enhanced the levels of nNOS expression (Figure 9).

Although the overall expression level of nNOS was reduced when the phosphorylation-deficient mutant $CREB_{133}$ was overexpressed in A673 cells, db-cAMP and forskolin could still double the level of expression of NOS (Figure 9). This increase could, in part, be mediated by the wild-type CREB still present in these cells. However, an alternative (or additional) explanation would be a separate signaling pathway that stimulates nNOS expression independent of PKA and CREB phosphorylation. A phosphorylation-independent pathway is also suggested by the incomplete inhibitory effect of PKA inhibitors on nNOS mRNA expression, and by the lack of an effect of various protein phosphatase inhibitors.

Recently, a family of LIM-only proteins (ACT and FHLs) has been identified. These proteins associate with CREB and CREM and produce activation independent of phosphorylation and CBP (*28*). We documented the expression of three four-and-a-half-LIM-domain proteins in A673 cells (FHL1−3, Figure 10A) and in brain (FHL1 and -2, Figure 10B). Interestingly, the expression of FHL1 and -3 was enhanced upon treatment with db-cAMP and forskolin (Figure 10C). This suggests that coactivators of CREB that act in a phosphorylation-independent manner (like FHL1 and -3) may complement the classical cAMP−PKA−CREB signaling cascade.

We, and others, have previously documented that the human nNOS gene exhibits a complex expressional regulation, with multiple nNOS mRNA species produced in a tissue-specific and developmentally regulated manner (*18*, *46*, *47*). Most of these mRNAs result from alternate splicing of 12 possible first exons to common exon 2. A673 neuroepithelioma cells express two major nNOS mRNAs (*18*). The two transcripts arise from alternate splicing of the first exons 1f and 1g to exon 2. The 1g−nNOS variant is predominantly expressed in nonstimulated A763 cells. Treatment with db-cAMP resulted in a marked upregulation of the 1f−nNOS transcript, without any noticeable effect on 1g−nNOS expression (*18*). We found that various CREB/AP1-activating agents were able to stimulate the expression of the human nNOS by acting on specific cAMP-responsive elements (CRE) located on the dual promoter complex of exons 1f and 1g and on the promoter/enhancer of exon 2. Transient cotransfections of exons 1f and 1g and of 5′-UTR exon 2 promoter−luciferase constructs with FHL-expressing vectors demonstrated that FHL1 could modulate the activities of these two genomic regions (M. Bros et al., manuscript in preparation).

Taken together, our data suggest that cAMP upregulates human nNOS expression mainly through PKA phosphorylation of CREB and CBP recruitment. However, the cAMP-mediated increase in the level of nNOS expression likely involves an additional mechanism, where CREB bypasses the classical requirement for phosphorylation and associates with other coactivators, probably the FLH proteins expressed in A673 cells. The upregulation of nNOS expression is not associated with an increase in the level of NO production. Instead, the level of production of bioactive NO decreases as the upregulated nNOS "uncoupled" and contributed to ROS production.

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