The activation of neuronal NO synthase is mediated by G-protein βγ subunit and the tyrosine phosphatase SHP-2

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ABSTRACT **In CHO cells we had found that CCK positively regulated cell proliferation via the activation of a soluble guanylate cyclase. Here we demonstrate that CCK stimulated a nitric oxide synthase (NOS) activity. The production of NO was involved in the proliferative response elicited by CCK regarding the inhibitory effect of NOS inhibitors L-NAME and α-guanidinoglutaric acid. We identified the NOS activated by the peptide as the neuronal isoform: the expression of the C415A neuronal NOS mutant inhibited both CCK-induced stimulation of NOS activity and cell proliferation. These two effects were also inhibited after expression of the C459S tyrosine phosphatase SHP-2 mutant and the βARK1 (495–689) sequestrant peptide, indicating the requirement of activated SHP-2 and G-βγ subunit. Kinetic analysis (Western blot after coimmunoprecipitation and specific SHP-2 activity) revealed that in response to CCK-treatment, SHP-2 associated to G-β1 subunit, became activated, and then dephosphorylated the neuronal NOS through a direct association. These data demonstrate that the neuronal NOS is implicated in proliferative effect evoked by CCK. A novel growth signaling pathway is described, involving the activation of neuronal NOS by dephosphorylation of tyrosyl residues.—Cordelier, P., Estève, J.-P., Rivard, N., Marletta, M., Vaysse, N., Susini, C., Buscail, L. The activation of neuronal no synthase is mediated by G-protein βγ subunit and the tyrosine phosphatase SHP-2. *FASEB J.* 13, 2037–2050 (1999)**

Key Words: cholecystokinin · nitric oxide · cell growth · Gβγ complex

CHOLECYSTOKININ (CCK) IS A gastrointestinal regulatory hormone originally isolated from the porcine duodenum as a 33 amino acid peptide. CCK participates in a variety of biological processes such as contraction of the gallbladder, stimulation of the exocrine and endocrine secretions, inhibition of gastric emptying, and modulation of gut peristaltism and trophic effects, notably on pancreas (1). CCK has also been found to be expressed in brain and in nerve endings in many parts of the body, and thus acts as a neuropeptide in both the central and peripheral nervous systems. In the central nervous system, the CCK-8 form that possesses the complete biological activity of CCK is mainly represented and is involved in behavioral processes such as anxiety or satiety.

CCK is known to stimulate pancreatic growth *in vitro* and *in vivo* in the mouse, rat, or hamster (2). In rat exocrine pancreas, this effect has been shown to be mediated by the CCK_A receptor subtype (3, 4). In this latter cellular model, CCK_A receptors interact with G-proteins (guanine nucleotide binding proteins) and thereby mediate the activation of different intracellular pathways, leading to *1)* the stimulation of intracellular calcium mobilization (via phospholipase C/inositol phosphate systems), *2)* an increase of cyclic AMP, cyclic GMP (cGMP), arachidonic acid, and phosphatidic acid levels, and *3)* the stimulation of tyrosine kinase or tyrosine phosphatase activities (1, 5). However, the mechanism(s) involved in the proliferative effect induced by CCK through CCK_A receptors is not completely elucidated.

We recently demonstrated that CHO-K1 cells expressed endogenous CCK_A receptors (6). In this model we also observed that CCK-8 stimulated cell proliferation. This effect occurred through an activation of a soluble guanylate cyclase via a pertussis toxin-sensitive Gi/G0 protein, followed by activation of the protein kinase G and the MAP kinase system (6). It represented a new example of a positive effect of CCK on cell proliferation that is mediated by CCK_A receptors. It was an additional observation of the positive effect of cGMP on cell proliferation, the

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nucleotide being more frequently responsible for an inhibition of cell proliferation or migration.

The potent activator of soluble guanylate cyclase is nitric oxide (NO). NO is a ubiquitous endogenous messenger molecule that participates in a variety of physiological and pathophysiological processes. NO is produced by NO synthases (NOS), which catalyze the oxidation of L-arginine to form L-citrulline and NO (7–9). NO synthases constitute at least three distinct isoforms, which are the products of individual genes. All catalyze the formation of NO. Two of these isoforms, neuronal (nNOS) and endothelial (eNOS) NOS, are constitutively expressed (including resting cells) and regulated by Ca^{2+}/calmodulin. The third one is inducible (iNOS) in a number of cell types, including macrophages, after exposure to bacterial lipopolysaccharide in conjunction with cytokines that stimulate iNOS protein formation (9). NO is known to act as a messenger in neuronal transmission, control of vascular tone, and immune response-induced cytostasis. It is also implicated in many other processes such as cerebral blood flow and ischemia, platelets and neutrophil aggregation, coronary perfusion, lung ventilation, glomerular perfusion, endocrine and exocrine secretion of pancreas, gut peristaltism and secretion, cell proliferation, and migration (10).

To further characterize the growth-promoting effect evoked by CCK-8, we have investigated the possible involvement of NO, the production of which could activate the soluble guanylate cylase/cGMP pathways and subsequently cell proliferation. Other aims of the study were to identify the NOS isoform that could be activated by CCK treatment and the mechanism of activation of this NOS.

MATERIALS AND METHODS

Reagents

Sulfated cholecystokinin-8 (CCK-8) and quinolinequinone (LY 83583) were purchased from Bachem (Voisin le Bretonneux, France). [$^{\gamma\text{-}33}$P]ATP (3000 Ci/mmol) was purchased from Isotopchim (Ganagobie, France). AG50W-X8 resin (sodium form) and Polyprep columns were from Bio-Rad (Ivry sur Seine, France). [^{14}C] L-arginine, enhanced chemiluminescence (ECL) immunodetection system, and Hybond ECL nitrocellulose membrane were from Amersham Corp (Les Ulis, France). CHAPS was from Serva (Heidelberg, Germany). Leupeptin, tetrahydro-L-bioptherin (BH_4), β-NADPH, FMN, FAD, N^G-nitro-L-arginine (L-NAME), calmodulin, pertussis toxin, soybean trypsin inhibitor, poly(Glu, Tyr), cholesterol hemisuccinate, dithiothreitol (DTT), L-citrulline, L-arginine, Sepharose-protein A beads, and geneticin (G418) were from Sigma (Saint Quentin Fallavier, France). α-Guanidinoglutaric acid (GGA) and bovine β1γ2 recombinant protein were from Calbiochem (Meudon, France). Fugene-6 was from Boehringer Mannheim (Meylan, France).

Antibodies

Monoclonal anti-human neuronal NOS, monoclonal antiphosphotyrosine-PY20 antibodies, and monoclonal anti-human SHP-2 antibody were from Transduction Laboratories (Montluçon, France). Rabbit polyclonal anti-human neuronal NOS antibody was from Biomol (Plymouth, Pa.). Anti-β1 subunit antibody was from Gramsch Laboratories (Schwabhôusen, Germany). Polyclonal anti-SHP-2 antibody was kindly provided by Dr. F. McKenzie (CNRS-UMR134, Nice, France).

Cell culture and transfection

CHO cells (CHO K1 strain) were cultured in α-modified Eagle's medium (αMEM) containing 10% fetal calf serum (FCS), fungizone, streptomycin, and penicillin. The bovine carboxyl-terminal β-adrenergic receptor kinase 1 (βARK1) (495–689) β-globin fragment cDNA, subcloned in pRK5, was kindly provided by Drs. M. Lohse and R. Lefkovitz (HHMI, Duke University Medical Center, Durham, N.C.). Cells were concomitantly transfected with pRK5-βARK1 and pSV2neo (Clontech, Palo Alto, Calif.) vectors. Stable transfectants were selected in αMEM containing geneticin at 600 μg/ml. After selection, cells were cultured in αMEM containing 10% FCS and geneticin (200 μg/ml). pSV2neo vector alone stable transfectants were used as control clones.

The rat heme-deficient neuronal nitric oxide synthase mutant C415A nNOS was subcloned in pCMV5 vector, kindly provided by P. Rouet (INSERM U317, Toulouse, France). The C459S SHP-2 mutant (11) was subcloned in the pcDNA3 vector, a gift from C. Nahmias (ICGM Cochin, Paris). For transient transfection, CHO cells were grown in 60 mm diameter dishes for 18 h in αMEM containing 10% FCS. After αMEM was removed, cells were transfected with 2 μg of pCMV5/C415A nNOS or 1 μg of pcDNA3/C459S SHP-2 vector and 1 μg of βgal vector containing β-galactosidase cDNA (kindly provided by H. Paris, INSERM U388, Toulouse, France), used as transfection internal control, and 6 μl Fugene-6 in αMEM without FCS for 30 h. β-galactosidase activity was assayed using *o*-nitrophenolβ-D-galactopyranoside as substrate. Cells transfected with pCMV5 or pcDNA3 vectors alone were used as control. In these conditions of transient transfection, percentage of cell transfected at 30 h was 48 ± 4% (mean±SE), as measured after transient expression of green fluorescent protein (driven by the CMV promoter) and analysis under microscope (image-analysis system Viso-Lab 2000, Biocom, Paris, France).

NO synthase activity

CHO cells were plated in 60 mm diameter dishes at 50×10^3 cells/ml (5 ml of αMEM containing 10% FCS per dish) until subconfluence and, after an 18 h period of serum deprivation, were treated with CCK-8 with or without other agents tested. Cells were then homogenized using a Dounce homogenizer (60 strokes at 4°C) in 50 mM Tris buffer (pH 7.5) containing 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 20 μM leupeptin, 1 mM PMSF, 1 mM soybean trypsin inhibitor, 0.1% CHAPS. Protein was assayed using the Bio-Rad Reagent and 25 μg of cell proteins were then incubated for 15 min at 37°C in 50 mM Tris buffer (pH 7.4) containing 50 μM [^{14}C]L-arg (150,000 cpm, specific activity 58.7 Ci/mmol), 10 mM βNADPH, 1 mM DTT, 4 μM FMN, 4 μM FAD, 10 μM BH_4, 2 μg of calmodulin, and 1 mM $CaCl_2$ in a final incubation volume of 200 μl. The reaction was terminated by addition of 1 ml of quench buffer containing 20 mM HEPES, 2 mM EDTA, 0.2 mM EGTA, 1 mM L-citrulline. The samples were applied to 2 ml columns of Dowex AG50W-X8 (Na^+

form) preequilibrated with quench buffer. Columns were then eluted with 2 ml of quench buffer and radioactivity in the eluates was measured by liquid scintillation.

Cell growth assay

CHO cells were cultured in αMEM containing 10% FCS and plated in 35 mm dishes at 50×10^3 cells/ml (2 ml per dish). After an overnight attachment phase, the medium was changed to αMEM containing either 10% FCS or 0% FCS, with or without other different agents tested. Cell growth was measured after 24 h by cell counting with a Coulter counter model ZM, as described previously (12). Cell growth assays using treatment with exogenous L-arginine were performed in L-arginine-free αMEM.

SHP-2 activity assay

CHO cells were plated in 100 mm diameter dishes at 100×10^3 cells/ml for 16 h (10 ml of αMEM containing 10% FCS per dish) until subconfluence; after an 18 h period of serum deprivation, they were treated with CCK-8 or other agents. CHO and CHO/βARK1 cells were washed twice with phosphate-buffered saline (PBS) and solubilized for 10 min on ice with 50 mM Tris buffer (pH 7.6) containing 140 mM NaCl, 5 mM $MgCl_2$, 5 mM sodium orthovanadate, and 0.05% soybean trypsin (buffer A) in the presence of 1.5% CHAPS and 0.5 mg/ml cholesterol hemisuccinate. The mixture was gently agitated for 30 min at 4°C and centrifuged at 13,000 × *g* for 20 min. Soluble proteins (100 μg) were incubated for 2 h at 4°C with anti-SHP-2 or preimmune serum prebound to Sepharose-protein A beads prewashed in buffer A in the presence of 0.1% CHAPS, 60 μg/ml cholesterol hemisuccinate, and 0.3% bovine serum albumin. Immunoprecipitated proteins were then washed once with 40 mM Tris buffer (pH 7) containing 0.5 mM EDTA, 0.1% Tween 20, and 0.1% sodium dodecyl sulfate (SDS), resuspended in 380 μl of the same buffer, and duplicated for tyrosine phosphatase assay. The substrate poly-(Glu, Tyr) was phosphorylated with [$^{\gamma\text{-}33}$P]ATP as described previously (13). Dithiothreitol (5 mM) was added; the reaction was initiated by the addition of 30,000 cpm of ^{33}P-labeled poly(Glu, Tyr) and allowed to proceed for 10 min at 30°C. PTPase activity was assayed as described previously (12) and expressed in picomoles of inorganic phosphate released per minute at 30°C from radiolabeled substrate.

Immunoprecipitation and immunoblotting

CHO cells were plated in 100 mm diameter dishes at 100×10^3 cells/ml (10 ml per dish) until subconfluence, and after an 18 h period of serum deprivation were treated with CCK-8, with or without other agents tested. CHO cells were washed twice in PBS and solubilized for 10 min on ice with 50 mM Tris buffer (pH 7.6) containing 140 mM NaCl, 1 mM EDTA, 0.05% soybean trypsin inhibitor, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A) in the presence of 1.5% CHAPS and 0.5 mM sodium orthovanadate. The mixture was gently agitated for 30 min at 4°C and centrifuged at 13,000 × *g* for 20 min. Soluble proteins (500 μg to 1 mg) were incubated for 3 h at 4°C with antiphosphotyrosine or anti-nNOS, or anti-SHP-2 antibodies or preimmune serum prebound to Sepharose-protein A beads prewashed in buffer A. The beads were then washed twice with buffer A and resuspended in 50 μl of Laemmli buffer 3% SDS.

For immunoblotting, 50 μl of immunoprecipitated proteins (see above) or 50 μg of solubilized proteins were resolved through 7.5% or 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and immunoblotted with anti-human nNOS, anti-PY20, anti-β1 subunit, or anti-SHP-2 antibodies. Immunoreactive proteins were visualized by the ECL immunodetection system and quantified by image analysis using a Biocom apparatus.

For visualization of monomers and dimers of nNOS, low-temperature SDS-polyacrylamide gels were performed. Briefly, cells treated or not with CCK-8 and transfected or not with the mutant nNOS cDNA were solubilized for 30 min at 4°C in a Tris 50 mM buffer (pH 7.6) containing 140 mM NaCl, 5 mM $MgCl_2$, 0.5 mM ortho-vanadate, 0.05% soybean trypsin inhibitor, and 0.1 mM phenylmethylsulfonyl fluoride in the presence of 1% CHAPS and 10% glycerol. After addition of Laemmli buffer 3% SDS without β-mercaptoethanol or heating, 100 μg of solubilized proteins was resolved through discontinuous 5% to 15% SDS-polyacrylamide gradient gels performed at a constant current of 30 mA. Gels and buffers were equilibrated at 4°C prior to electrophoresis, which was performed at temperature below 15°C, as described previously (14). Immunoblotting was performed as described above using anti-human nNOS antibodies.

RESULTS

Nitric oxide production is involved in the growth signaling pathway of CCK

CCK-8 stimulated the proliferation of CHO-K1 cells in a dose-dependent manner, with a maximal effect observed at a concentration of 0.1 μM. This effect occurred through an activation of a soluble guanylate cyclase (6). NO is the activator of soluble guanylate cyclase. To further characterize this effect, CHO-K1 cells were treated or not with 0.1 μM CCK-8 for different times. As shown in **Fig. 1*A***, NOS activity was stimulated in a time-dependent manner, with a maximal effect after 30 s of CCK treatment, followed by a decrease until 5 min. As shown in Fig. 1*B*, an 18 h pretreatment of cells with 100 ng/ml of pertussis toxin reduced by 79 ± 4% the CCK-induced stimulation of NOS activity.

To investigate the role of NO production on cell proliferation caused by CCK, CHO cells cultured in serum-free medium were treated with the NO donor sodium nitroprusside (1 nM to 1 μM). This treatment resulted in a dose-dependent increase of cell proliferation when compared to control cells cultured in serum-free medium, maximal effect being observed at 0.1 μM sodium nitroprusside (SNP; +38±9%, ED_{50}: 1.22±0.4 nM) (data not shown from three experiments performed in triplicate). Treatment of cells with the substrate of NOS L-arginine (100 μM), resulted also in an increase of cell proliferation when compared to control cells (**Fig. 2**). We also observed that cell proliferation induced by 0.1 μM SNP was inhibited by 57 ± 1% when cells were concomitantly treated with the soluble guanylate cyclase inhibitor LY 83583 (0.1 μM) (Fig. 2).

To further characterize the possible involvement

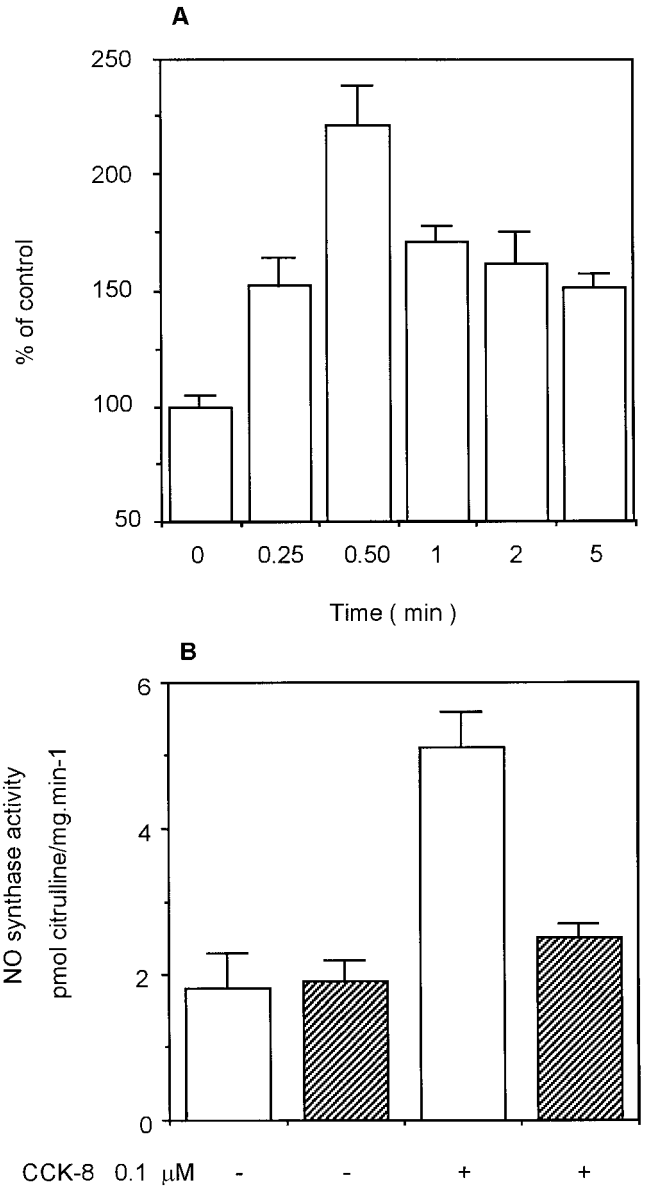


Figure 1. CCK activates NO synthase activity through a pertussis toxin-dependent mechanism in CHO-K1 cells. *A)* CHO-K1 cells were plated in αMEM containing 10% FCS until subconfluence and were treated after an 18 h period of serum deprivation with 0.1 μM CCK-8 for 5 min at 37°C. NOS activity was assayed on cell homogenates at the times indicated by measuring the conversion of L-arginine ^{14}C into L-citrulline. Results are expressed in percent of control value (1.3±0.2 pmol of citrulline produced/mg of protein per minute) obtained for untreated cells at time 0 (mean±SE of three experiments in duplicate). *B)* CHO-K1 cells were plated in αMEM containing 10% FCS until subconfluence and treated, after an 18 h period of serum deprivation, with 0.1 μM CCK-8 for 30 s at 37°C after (hatched bars) or without (open bars) a 24 h preincubation of cells with 100 ng/ml of pertussis toxin. Results are expressed in pmol of citrulline produced/mg of protein per minute and are the mean ± SE of three experiments in duplicate.

of NO production in the proliferative effect evoked by CCK, CHO cells were treated for 24 h with 0.1 μM CCK-8 in the presence or not of the inhibitors of NOS L-NAME and GGA. As shown in Fig. 2, CCK-induced CHO cell proliferation was inhibited by incubating cells with the two inhibitors. These inhibitors had no effect on the basal cell proliferation (data not shown). No additive effect could be observed on cell proliferation when cells were concomitantly treated with CCK-8 (0.1 μM) and L-arginine (100 μM), indicating that the same pathway was involved. Taken together, these results indicate that CCK stimulated CHO cell proliferation through the activation of a NOS and by a mechanism involving a pertussis-toxin-sensitive Gi/G0 protein.

The constitutive nNOS is involved in the growth signaling pathway of CCK

In CHO cells, the conditions and the kinetics of activation of NOS activity were consistent with the involvement of a constitutive NOS isoform. As described previously (15), immunoblotting revealed that CHO-K1 cells expressed endogenous constitu-

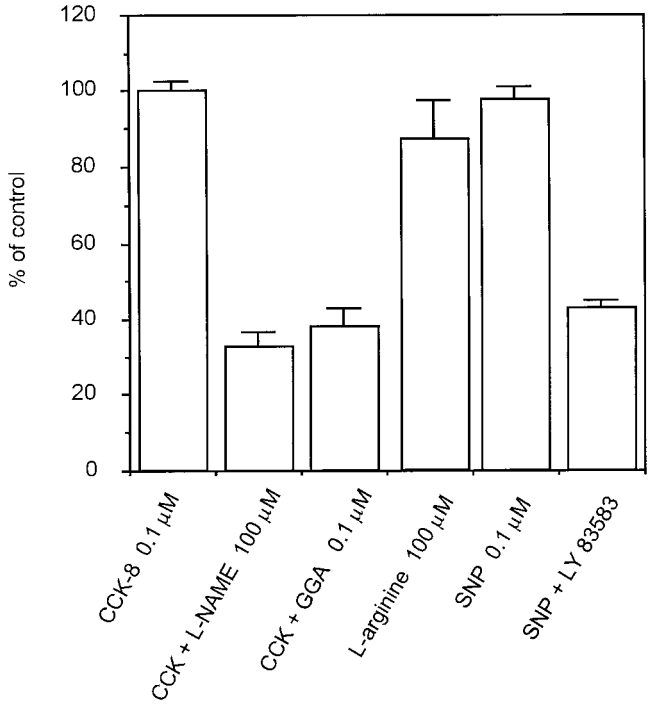


Figure 2. Nitric oxide is involved in proliferation of CHO-K1 cells. CHO cells were plated in αMEM containing 10% FCS until subconfluence; cell proliferation was induced in serum-free medium by a 24 h treatment with CCK-8. Concomitant with the CCK-8 treatment, cells were treated with the NO synthase inhibitors L-NAME and α-guanidinoglutaric acid (GGA). Cells were also treated for 24 h with the NO synthase substrate, L-arginine, or the NO donor sodium nitroprusside (SNP) with or without LY 83583 (0.1 μM). Cell proliferation was evaluated by cell counting, results are expressed as the percentage of control values obtained with CCK-treated cells. Treatment with L-arginine was performed in arginine-free medium. When given alone, LY 83583 had no effect on basal cell proliferation. Under these conditions, 0.1 μM CCK-8 induced stimulation of cell proliferation 45 ± 4% above basal values observed for cells grown in FCS-free medium (mean±SE of three experiments performed in triplicate).

tive nNOS (**Fig. 3*A***). However, we also detected the endothelial NOS isoform by Western blotting, this isoform being expressed less than nNOS (data not shown). In addition, we found that the NOS inhibitor GGA abolished the CCK-induced stimulation of both cell proliferation and NOS activity in CHO cells. This inhibitor, also known to be an anticonvulsant compound, is highly active in neuronal tissues that predominantly express nNOS. To obtain direct evidence that nNOS was activated by CCK in CHO cells and that this isoform was implicated in the positive effect of CCK on CHO cell proliferation, we transiently expressed the rat C415A nNOS mutant (Cys^{415} mutated to Ala). This mutant does not bind heme and tetrahydrobiopterin, essential cofactors required for NO synthesis (16, 17). After transient expression of the C415A nNOS mutant, immunoblotting demonstrated expression of two forms of nNOS (Fig. 3*A*) in cells transfected with the pCMV5/C415A nNOS vector when compared to cells transfected with the pCMV5 control vector alone. The 155 kDa form corresponds to the recombinant rat isoform and the 160 kDa form to the endogenous hamster nNOS form, also detected in the hamster cerebellum soluble extracts used as positive control. As shown in Fig. 3*B*, CCK-8 was unable to stimulate NOS activity in CHO cells transiently expressing the nNOS mutant when compared to cells transiently transfected with the pCMV5 vector alone. In addition, CCK-8 could not induce cell proliferation in cells expressing the nNOS mutant (Fig. 3*B*, insert). To investigate the effect of C415A nNOS mutant, solubilized cells transfected with pCMV5/C415A nNOS or pCMV5 vectors were subjected to low-temperature SDS-polyacrylamide gel electrophoresis (PAGE) in order to evaluate the amount of dimeric

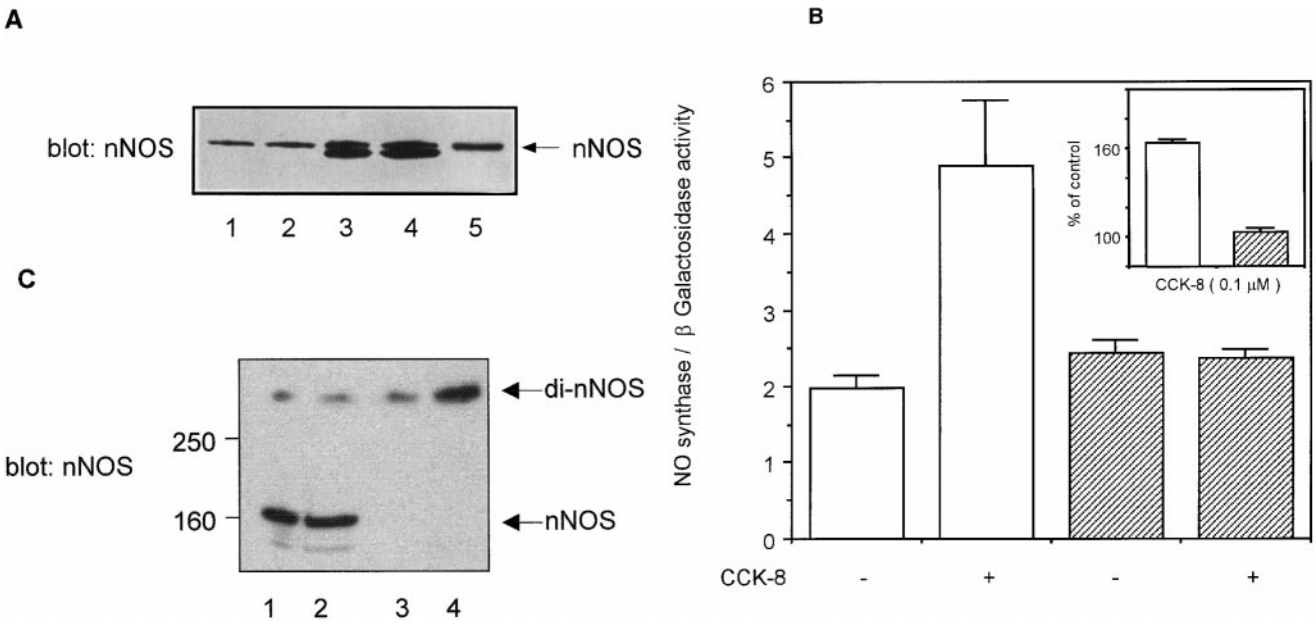


Figure 3. The constitutive nNOS is implicated in the regulation of cell proliferation evoked by CCK in CHO cells. *A)* CHO cells were transiently transfected with 2 μg of pCMV5 control vector and/or 2 μg of pCMV5/C415A nNOS vector containing the C415A rat nNOS mutant. After a 24 h expression, cells were treated or not with 0.1 μM CCK-8 at 37°C for 30 s and solubilized with 1.5% CHAPS. The lysates were then resolved by 7.5% SDS-PAGE and analyzed by immunoblotting with the anti-nNOS antibody. Lanes 1 and 2 correspond to pCMV5-expressing cells (1: untreated; 2: treated); lanes 3 and 4 correspond to pCMV5/C415A nNOS-expressing cells (3: untreated; 4: treated); lane 5 corresponds to solubilized proteins from Syrian golden hamster cerebellum, used as a positive control. Results are representative of two immunoblots each performed after different sets of cell transfection. *B)* CHO cells were transiently transfected with the pCMV5 control vector (open bars) or the pCMV5/C415A nNOS vector (hatched bars). Each vector was cotransfected with 1 μg of PGL3/βGAL vector in order to standardize results and to take into account the yield and rate of transient transfection. After 24 h expression, cells were treated or not with 0.1 μM CCK-8 at 37°C for 30 s and NOS activity was measured on cell homogenates. Results are expressed in NOS/β-galactosidase activity and are the mean ± SE of three separate experiments in duplicate. Insert: CHO cells were transiently transfected with the pCMV5 control vector (open bars) or the pCMV5/C415A nNOS mutant (hatched bars). After a 24 h expression, cells were treated or not with 0.1 μM CCK-8 for 24 h and cell proliferation was evaluated by cell counting. Results are expressed as the percentage of control values obtained in untreated cells cultured in serum-free medium and are the mean ± SE of three separate experiments performed in triplicate. *C)* CHO cells were transiently transfected with the pCMV5/C415A nNOS mutant (lanes 1, and 2) or the pCMV5 control vector (lanes 3, and 4). After 24 h expression, cells were treated or untreated with 0.1 μM CCK-8 at 37°C for 30 s and solubilized with 1% CHAPS. The lysates were then resolved by low temperature through 5–15% SDS-polyacrylamide discontinuous gradient gel and analyzed by immunoblotting with the anti-nNOS antibody (nNOS: 155–160 kDa monomeric forms of both wild-type and mutant nNOS; di-NOS: 320 kDa dimeric nNOS form). A longer exposition of films with ECL detection revealed the presence of 160 kDa bands in lanes 3 and 4 corresponding to the wild-type endogenous nNOS (data not shown). Results are representative of three immunoblots, each performed after different sets of cell transfection and CCK treatment.

nNOS (320 kDa) relative to monomeric nNOS (155–160 kDa). As shown in Fig. 3*C* (lanes 1 and 3), the amount of nNOS dimers was not increased after expression of the nNOS mutant when compared to the control cells. In addition, under CCK treatment, the amount of endogenous nNOS dimers was increased (lane 4) in control CHO cells. This increase was abolished after nNOS mutant expression (lane 2). All these results suggest that CCK can induce endogenous nNOS dimerization. This effect is prevented after transfection of inactive nNOS without apparent heterodimerization between endogenous nNOS and heterologous mutant nNOS.

SHP-2 mediates the stimulation the nNOS activity and cell proliferation induced by CCK

The activity of the constitutive NOS isoforms such as nNOS is critically controlled by an elevated level of intracellular calcium, which promotes binding of calmodulin to the enzymes. In CHO-K1 cells, we previously demonstrated that CCK can induce mobilization of intracellular calcium (12). However, this effect appeared to be pertussis toxin independent, and calcium mobilization was not implicated in the proliferative effect of CCK in these cells (6). Evidence exists that endothelial isoform of NOS could be activated by pathways other than the increase of intracellular calcium concentrations, such as by phosphorylation or dephosphorylation of proteins on tyrosine or serine-threonine residues (18). Previous reports demonstrated that CCK activated tyrosine kinase and tyrosine phosphatase activities, and these effects could be involved in the trophic effect of CCK *in vivo* (5).

We first tested the possible implication of a tyrosine phosphatase activity in the CCK-induced cell proliferation. CHO cells were concomitantly treated or not for 24 h with CCK-8 and the tyrosine phosphatase inhibitor pervanadate. At concentrations of 0.1, 0.3, and 0.5 μM, pervanadate inhibited CCK-induced cell proliferation ($-72\pm8.5\%$ at 0.3 μM) (**Fig. 4**). The basal cell proliferation of CHO-K1 was not affected at these three concentrations, but was inhibited at 1 μM of pervanadate (data not shown). In parallel, we tested the effect of okadaic acid, the inhibitor of the serine threonine phosphatases, which had no effect on CCK-induced cell proliferation at a concentration of 0.1 μM (data not shown from three experiments performed in triplicate). All these results suggested that CCK-induced cell proliferation depends on the activation of a tyrosine phosphatase.

The phosphotyrosine phosphatase SHP-2 is a positive mediator of the mitogenic signaling induced by several growth factors (19). To determine whether CCK activated the tyrosine phosphatase SHP-2 in CHO cells, cells were incubated in the presence of

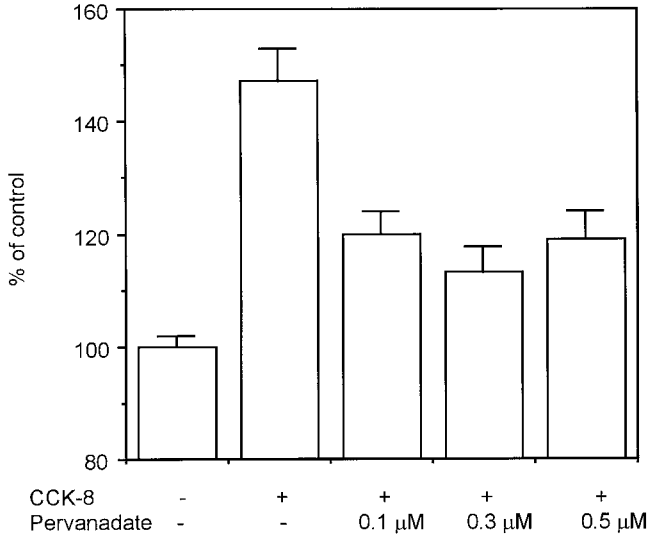


Figure 4. A tyrosine phosphatase is implicated in the CCK-induced cell proliferation of CHO cells. CHO cell proliferation was induced by a 24 h treatment with 0.1 μM CCK-8. Cells were concomitantly treated or not with the tyrosine phosphatase inhibitor pervanadate at the concentrations indicated. Cell proliferation was measured by cell counting and results are expressed as the percentage of control values obtained with control untreated cells cultured in serum-free medium (mean±SE of three experiments performed in triplicate).

CCK-8 for various times (after which they were solubilized) and SHP-2 activity was measured in SHP-2 immunoprecipitates. As shown in **Fig. 5**, treatment by CCK evoked a rapid and time-dependent stimulation of SHP-2 activity. The stimulation of SHP-2 activity was maximal after 1 min of CCK treatment and declined for up to 15 min.

To obtain direct evidence that tyrosine phosphatase SHP-2 was implicated in the positive effect of CCK on CHO cell proliferation, we transiently expressed the dominant negative mutant SHP-2 (C459S mutant: Cys^{459} mutated to serine) (11). After transient expression of the mutant, immunoblotting with anti-SHP-2 antibodies demonstrated that expression of the protein in cells (treated or untreated with CCK-8) transfected with pcDNA3/C459S SHP-2 vector was increased when compared to cells transfected with pCDNA3 vector alone (**Fig. 6*A***). This increase may correspond to the recombinant mutant transiently expressed in CHO cells. After transient expression of the SHP-2 mutant, CCK-8 was unable to stimulate cell proliferation when compared to cells transiently transfected with the pcDNA3 control vector alone (Fig. 6*B*). As shown in Fig. 6*B* (insert), the inhibition of CCK-8-induced cell proliferation depended on the concentration of the pcDNA3/C459S SHP-2 plasmid transfected, maximal effect being observed with 1.5–2 μg.

To determine whether the NOS activity stimulated by CCK depends on a tyrosine phosphatase activation, CHO cells were treated or untreated with

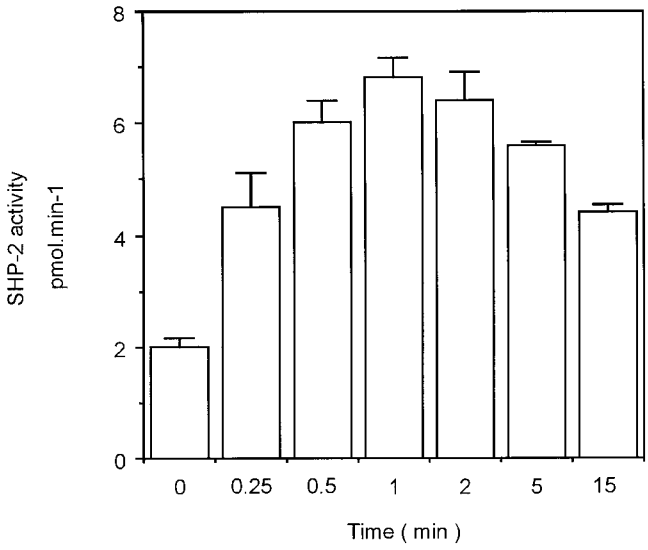
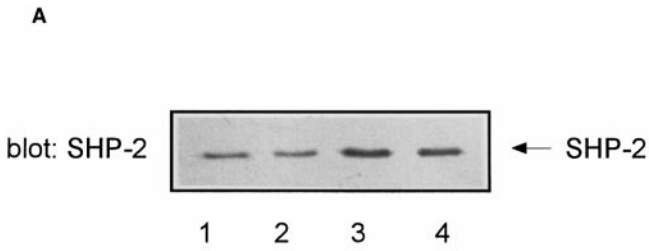


Figure 5. CCK stimulates SHP-2 activity in CHO cells. CHO cells were plated in αMEM containing 10% FCS until subconfluence and after an 18 h period of serum deprivation were treated or not with 0.1 μM CCK-8 for the indicated times prior to solubilization and immunoprecipitation with the anti-SHP-2 antibodies. Immunoprecipitates were assayed for tyrosine phosphatase activity in the presence of ^{33}P-labeled poly(Glu, Tyr). Results are expressed in pmol/min and are the mean ± SE of four experiments in duplicate.

CCK-8 for 30 s after or without a 15 min preincubation with pervanadate at a concentration of 0.3 μM. As shown in **Fig. 7*A***, pretreatment of cells with pervanadate had no effect on basal NOS activity, but inhibited by 90 ± 1% the CCK-induced stimulation of NOS activity, suggesting that activation of NOS depends on the activation of a tyrosine phosphatase.

To ask whether CCK regulates the level of tyrosine phosphorylation of nNOS, CCK-treated or untreated CHO cells were subjected to immunoprecipitation with antiphosphotyrosine antibodies. Immunoblot with anti-nNOS antibodies revealed that nNOS of untreated cells was tyrosine phosphorylated and rapidly dephosphorylated after CCK treatment, maximal dephosphorylation being observed at 15 and 30 s, which slowly decreased until 5 min. Pretreatment of cells with 0.3 μM pervanadate for 15 min inhibited nNOS tyrosine dephosphorylation induced by a 30 s CCK treatment (Fig. 7*B, D*). In addition, converse experiments revealed that a similar profile and kinetic of nNOS dephosphorylation under CCK treatment were observed after immunoprecipitation of CHO cells with anti-nNOS antibodies, followed by immunoblot with antiphosphotyrosine antibodies (Fig. 7*C*).

To obtain evidence that CCK may activate a NOS activity in CHO cells by stimulating SHP-2, we transiently expressed the inactive SHP-2 C459S mutant (immunoblotting of transfected cells with anti-SHP-2 antibodies is shown in **Fig. 8*A***). After transient expression of this mutant, CCK-8 was unable to stimulate NOS activity when compared to cells transiently transfected with the pcDNA3 control vector alone (Fig. 8*B*). As shown in Fig. 8*C*, dephosphory-



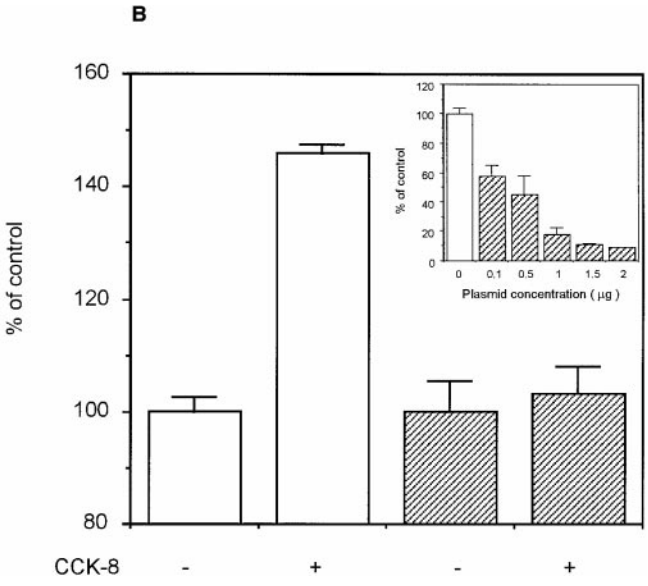


Figure 6. The tyrosine phosphatase SHP-2 is implicated in the regulation of cell proliferation evoked by CCK in CHO cells. *A)* CHO cells were transiently transfected with 1 μg of pCDNA3 control vector or 1 μg of pCDNA3/C459S SHP-2 vector containing the human C459S SHP-2 mutant. After 24 h expression, cells were treated or not with 0.1 μM CCK-8 for 30 s at 37°C, and solubilized with 1.5% CHAPS. The lysates were then resolved by 7.5% SDS-PAGE and analyzed by immunoblotting with the anti-SHP-2 antibody. Lanes 1 and 2 correspond to pCDNA3-expressing cells (1: untreated; 2: treated); lanes 3 and 4 correspond to pCDNA3/C459S SHP-2-expressing cells (3: untreated; 4: treated). Results are representative of two immunoblots, each performed after different sets of cell transfection. *B)* CHO cells were transiently transfected with 1 μg of pcDNA3 control vector (open bars) or 1 μg of pCDNA3/C459S SHP-2 vector (hatched bars). After a 24 h expression, cells were treated or not with 0.1 μM CCK-8 for 24 h and cell proliferation was assayed by cell counting. Results are expressed in percentage of control values obtained with untreated cells cultured in serum-free medium and are the mean ± SE of three separate experiments performed in triplicate. Insert: CHO cells were transiently transfected with increasing concentrations of pCDNA3/C459S SHP-2 vector (hatched bars) or 1 μg of pCDNA3 control vector (open bars), and treated with 0.1 μM CCK-8 for 24 h; cell proliferation was assayed by cell counting. Results are expressed in percentage of control values obtained with CCK-treated cells. (results are the mean±SE of three experiments performed in triplicate).

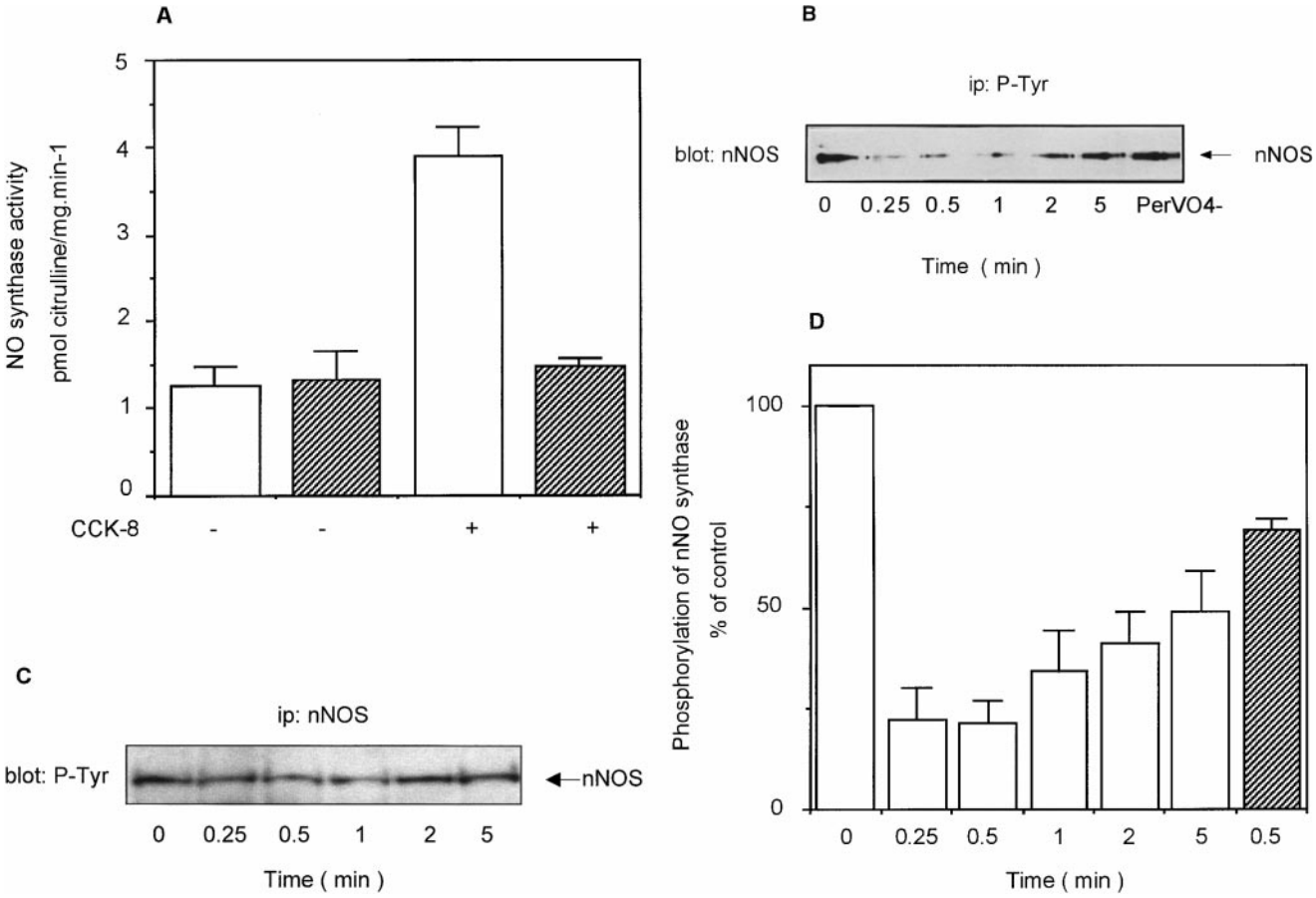


Figure 7. Activation of nNOS by CCK on CHO cells involves a tyrosine phosphatase. *A)* CHO cells were plated in αMEM containing 10% FCS until subconfluence and treated after an 18 h period of serum deprivation with 0.1 μM CCK-8 for 30 s at 37°C after (hatched bars) or without (open bars) pretreatment with 0.3 μM of pervanadate for 15 min. Results are expressed in pmol of citrulline produced/mg of protein per minute (mean±SE of three experiments in duplicate). *B)* CHO cells were plated in αMEM containing 10% FCS until subconfluence and (after an 18 h period of serum deprivation) treated or not for indicated times at 37°C with 0.1 μM CCK-8. Cells were concomitantly preincubated or not with 0.3 μM pervanadate (Per-VO_4^{2-}) for 15 min before a 30 s CCK-8 treatment, then solubilized in 1.5% CHAPS. Cell lysates were subjected to immunoprecipitation (i.p.) with antiphosphotyrosine antibodies. Immunoprecipitates were resolved by a 7.5% SDS-PAGE and analyzed by immunoblotting with anti-nNOS antibodies (blot: nNOS). Results are representative of three immunoblots performed in three separate experiments. *C)* CHO cells were plated and treated as described in panel *B* and solubilized in 1.5% CHAPS. Cell lysates were subjected to immunoprecipitation (i.p.) with anti-nNOS antibodies. Immunoprecipitates were resolved by a 7.5% SDS-PAGE and analyzed by immunoblotting with antiphosphotyrosine antibodies (blot: P-Tyr). Results are representative of two immunoblots performed in two separate experiments. *D)* Immunoblots were densitometrically analyzed and data were plotted as a percentage of control values obtained from cells at time 0 (open bars: CCK treatment/i.p. P-Tyr/blot: nNOS; hatched bars: treatment with CCK after preincubation with pervanadate/i.p. P-Tyr/blot nNOS). Data from three separate experiments are represented as mean ± SE

lation of nNOS induced by CCK-8 treatment of CHO cells (lanes 1 and 2) was abolished after transfection of inactive SHP-2 mutant, the basal phosphorylation of nNOS remaining unchanged (lanes 3 and 4). These results indicate that activation of SHP-2 is implicated in the activation of NOS activity and nNOS dephosphorylation induced by CCK in CHO cells.

The Gβγ subunit complex is involved in the coupling of CCK receptor to nNOS

Whereas it was thought that Gα was the sole or major activator of effectors, it is now recognized that Gβγ is also an important independent activator of many different effectors of G-protein actions (including Gi proteins) (20). To investigate the implication of Gβγ in the CCK-induced activation of NOS, CHO cells were first treated or not with exogenous Gβ1γ2 subunit at final concentrations of 50, 100, and 150 nM under the same conditions used for CCK-8 treatment. As shown in **Fig. 9*A***, treatment of solubilized CHO cells with the exogenous Gβ1γ2 subunit evoked a stimulation of NO synthase activity. The maximal effect was observed at a concentration of 100 nM, the stimulatory effect being similar to that observed after CCK-8 treatment of wild-type CHO cells or CHO cells stably expressing the neomycin-resistant gene alone.

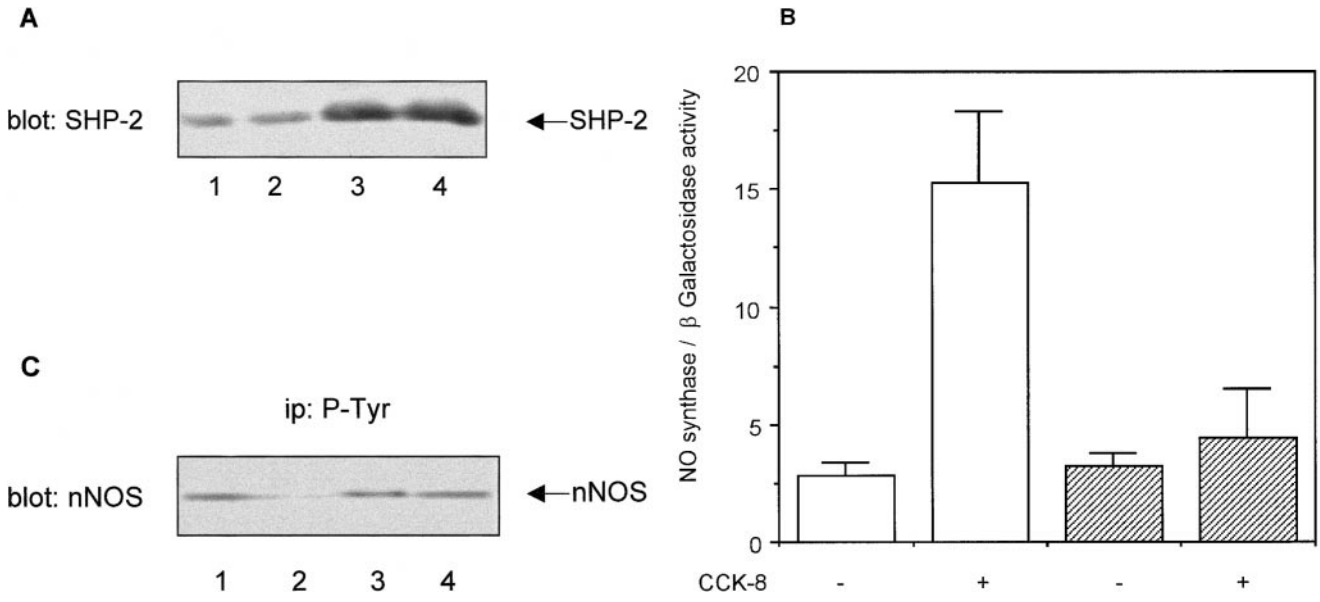


Figure 8. Activation of NOS activity by CCK involves the protein-tyrosine phosphatase SHP-2: *A)* CHO cells were transiently transfected with 1 μg of pCDNA3 control vector or 1 μg of pCDNA3/C459S SHP-2 vector containing the human C459S SHP-2 mutant. After 24 h expression, cells were treated or not with 0.1 μM CCK-8 for 30 s at 37°C and solubilized with 1.5% CHAPS. The lysates were then resolved by 7.5% SDS-PAGE and analyzed by immunoblotting with the anti-SHP-2 antibody. Lanes 1 and 2 correspond to pCDNA3-expressing cells (1: untreated; 2: treated); lanes 3 and 4 correspond to pCDNA3/C459S SHP-2-expressing cells (3: untreated; 4: treated). Results are representative of two immunoblots, each performed after different sets of cell transfection. *B)* CHO cells were transiently transfected with the pcDNA3 control vector (open bars) or with the pCDNA3/C459S vector (hatched bars) containing the C459S human SHP-2 mutant, as described in Materials and Methods. Each vector was cotransfected with 1 μg of PGL3/βGAL vector. After 24 h expression, cells were treated or not with 0.1 μM CCK-8 at 37°C for 30 s and NOS activity was measured on cell homogenates. Results are expressed in NOS/β-galactosidase activity and are the mean ± SE of four separate experiments in duplicate. *C)* CHO cells were transiently transfected with 1 μg of pCDNA3 control vector or with 1 μg of pCDNA3/C459S SHP-2 vector containing the human C459S SHP-2 mutant. After 24 h expression, cells were treated not with 0.1 μM CCK-8 for 30 s at 37°C. After CCK treatment, cells were solubilized in 1.5% CHAPS. Cell lysates were subjected to immunoprecipitation (i.p.) with antiphosphotyrosine antibodies. Immunoprecipitates were resolved by a 7.5% SDS-PAGE and analyzed by immunoblotting with anti-nNOS antibodies (blot: nNOS). Lanes 1 and 2 correspond to pCDNA3-expressing cells (1: untreated; 2: treated); lanes 3 and 4 correspond to pCDNA3/C459S SHP-2-expressing cells (3: untreated; 4: treated). Results are representative of two immunoblots performed after different sets of cell transfection.

The G-protein receptor kinases are known to phosphorylate multiple receptor serine and threonine residues. Among this kinases, the β-adrenergic receptor kinases 1 and 2 translocate to a variety of G-protein-coupled receptors by forming a complex with the Gβγ released upon activation of heterotrimeric G-proteins. A 125 amino acid domain at the carboxyl terminus of these kinases provides the binding site for Gβγ. Expression of a minigene corresponding to the carboxyl terminus amino acid sequence of βARK1 has been shown to act as a sequestrant sequence for Gβγ subunits when expressed in COS cells (21). To demonstrate the implication of Gβγ in the CCK-induced stimulation of both CHO cell proliferation and NOS activity, the cDNA encoding the carboxyl-terminal 195 amino acids of the bovine βARK1-(495–689) polypeptide were stably transfected in CHO cells. Cellular expression of the minigene product could be demonstrated by immunoblotting of solubilized cells, using a specific antibody raised against the last 221 amino acids of the carboxyl terminus domain of the rat βARK2 (kindly provided by C. Stone, Duke University Medical Center), which recognizes the isoforms βARK-1 and βARK-2, as well as the minigene βARK1 (495–689) (20). An immunoreactive protein of 28 kDa was detected in CHO cells expressing βARK1 (495–689), but not in control CHO cells expressing a neoresistance gene (data not shown). In addition, no effect was observed on the apparent expression of CCK receptor (assayed by CCK ligand binding, as described in 6) in CHO cells stably expressing the βARK1-(495–689) polypeptide when compared to wild-type CHO cells or CHO cells stably expressing the neomycin resistance gene (data not shown).

As shown in Fig. 9*A, B*, in cells expressing the sequestrant polypeptide, CCK-induced stimulation of NOS activity and cell proliferation were both abolished. In this clone, the basal NOS remained unchanged and cell proliferation could be induced by serum. Similar results were observed in another clone of CHO cells expressing βARK1-(495–689) carboxyl-terminal polypeptide (data not shown). Moreover, treatment of CHO cells stably expressing

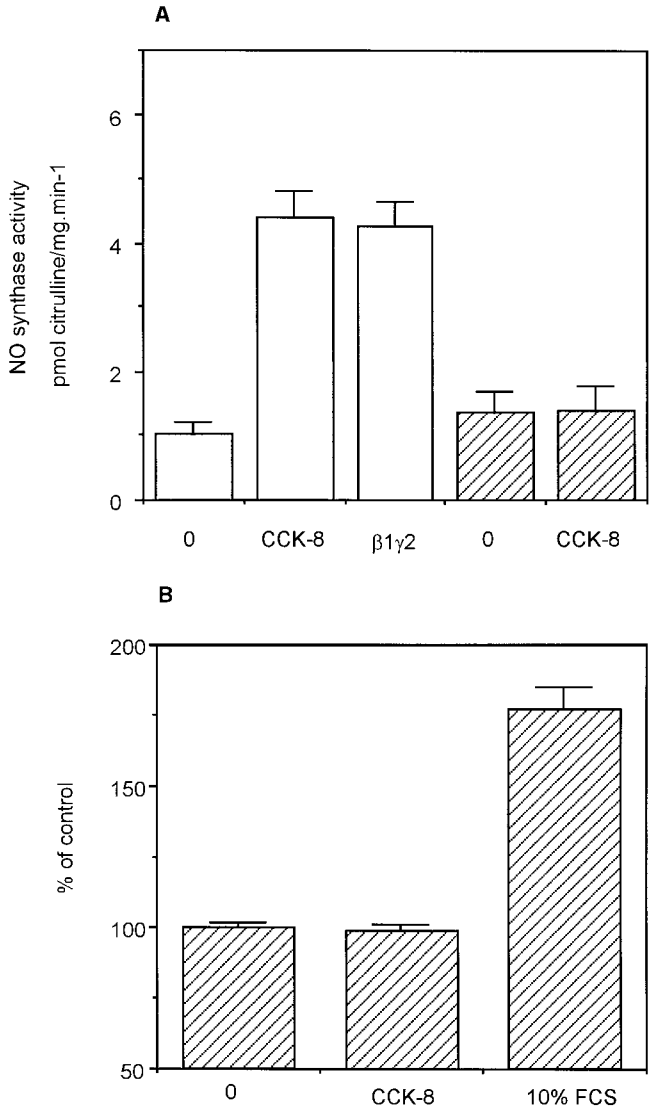


Figure 9. NO synthase activation evoked by CCK-8 required the Gβγ protein subunit. *A)* CHO-K1 cells expressing the neomycine resistance gene (open bars) were cultured in serum-free αMEM and treated or untreated (0) with 0.1 μM CCK-8 and 100 nM of the β1γ2 recombinant peptide for 30 s at 37°C. In parallel, CHO cells stably expressing the bovine βARK1-(495–689) polypeptide) (hatched bars) were also treated or untreated (0) in the same conditions with 0.1 μM CCK-8 for 30 s at 37°C. NOS activity was measured on cell homogenates. Results are expressed in pmol of citrulline produced/mg of protein per minute and are the mean ± SE of three experiments in duplicate. *B)* Proliferation of CHO cells stably expressing the bovine βARK1-(495–689) polypeptide) (hatched bars) was induced by a 24 h treatment of CCK-8 (0.1 μM) and 10% FCS. Cell proliferation was measured by cell counting. Results are expressed as the percentage of control values obtained with untreated cells grown in FCS-free medium (0) (mean±SE of three separate experiments performed in triplicate).

the βARK1-(495–689) polypeptide with CCK-8 (0.1 μM) did not induce stimulation of SHP-2 activity whenever the time of treatment (data not shown from two experiments in duplicate). These results suggest that in CHO cells, CCK stimulated cell proliferation, NOS activity, and SHP-2 activity via a Gβγ-dependent pathway.

SHP-2 associates with G-βγ subunits and nNOS in response to CCK treatment

To further characterize the implication of G-βγ, SHP-2, and nNOS in the growth signal evoked by CCK, CHO cells were either treated not with CCK for various times prior to solubilization and immunoprecipitation with anti-SHP-2 antibodies. The amounts of G-β1 subunits (always dynamically associated with the γ subunit) and nNOS were then analyzed by immunoblotting. The blots were reprobed with anti-SHP-2 antibodies to ensure that comparable amounts of SHP-2 molecules were immunoprecipitated at each time point of CCK treatment. As observed in **Fig. 10*A***, the β1 subunit was immunoprecipitated with SHP-2 antibodies in resting cells. The treatment with CCK resulted in a rapid increase of the amount of β1 subunit immunoprecipitated, which was maximal after 15 and 30 s of treatment (315±45% of control) and decreased until 5 min. In parallel, we observed that nNOS was also immunoprecipitated with SHP-2 antibodies in resting cells. In response to CCK treatment, the amount of immunoprecipitated nNOS was increased by 240%, with a maximum observed after 1 min of CCK treatment and followed by a decrease after 2 and 5 min. The kinetics of association of nNOS with SHP-2 was shifted in time when compared to the association of G-β1 with SHP-2.

DISCUSSION

CCK stimulates cell proliferation of CHO cells through the activation of neuronal NOS

We first observed that CCK induced a rapid activation of NO synthase activity. This activation occurred rapidly, with a maximal effect observed at 30 s of CCK treatment. This kinetic profile fit well with that of guanylate cyclase activation, which occurred after 2 min of CCK treatment, before subsequent activation of MAP kinase activity at 5–10 min. Moreover, the stimulation of NO synthase by CCK depended on a pertussis toxin-sensitive pathway, as previously observed for CCK-induced both cell proliferation and increase of intracellular concentrations of cGMP. We also demonstrated that both NO synthase substrate L-arginine and the NO donor sodium nitroprusside can stimulate CHO cell proliferation, indicating that endogenous and exogenous production of NO could stimulate CHO cell proliferation as did exogenous and endogenous cGMP (6). The effect of LY

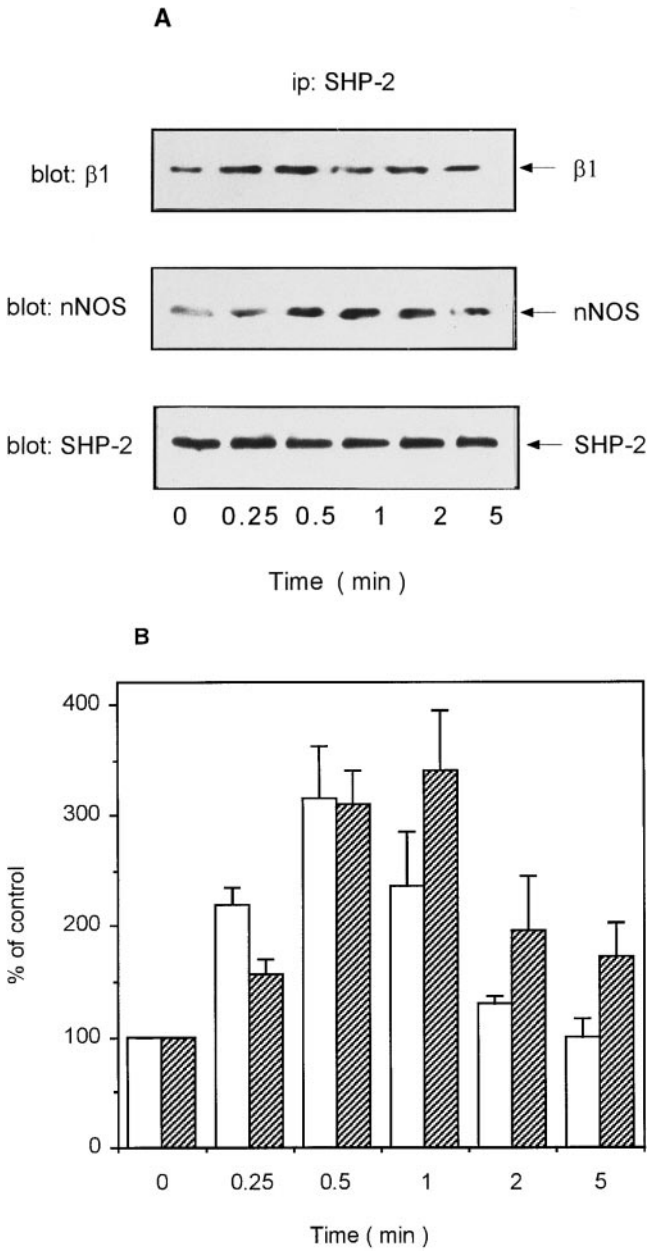


Figure 10. SHP-2 associates to G-β1 subunit and nNOS CHO-K1 cells treated with CCK. *A)* CHO cells were plated in αMEM containing 10% FCS until subconfluence and were treated or not (after an 18 h period of serum deprivation) for the indicated times with 0.1 μM CCK-8. Cell were subjected to immunoprecipitation (i.p.) with anti-SHP-2 antibody; immune complexes were fractionated by a 10% or 7.5% SDS-PAGE and subjected to sequential immunoblotting with antibodies directed against G-β1 subunit (blot: β1) or nNOS (blot: nNOS) (Fig. 8*A*). Arrows indicate the positions of the G-β1 subunit and of nNOS. Filters were reprobed with anti-SHP-2 antibodies (blot: SHP-2). After immunoprecipitation with preimmune serum, followed by immunoblotting with SHP-2 antibodies, no specific signal could be obtained (data not shown). *B)* Immunoblots were densitometrically analyzed and data were plotted as a percentage of control values from cells at time 0 (open bars: blot β1; hatched bars: blot nNOS). Data are from three separate experiments and represented as mean ± SE.

83583 suggests that NO acts through the activation of guanylate cyclase and the production of cGMP.

Besides VIP, neurotensin, angiotensin, or muscarinic receptors (14, 22), CCK_A receptor represents a novel example of G-protein-coupled receptor that can be positively coupled to a NOS activity. However, to our knowledge this is the first report demonstrating that this positive coupling is implicated in cell growth stimulation. NO has been shown to inhibit cell proliferation and cell migration and to induce apoptosis. However, it has also been shown to promote proliferation of various cell types *in vitro* and to facilitate tumor growth and neovascularization *in vivo* (23, 24). Effects of NO on cell proliferation are known to depend on concentration of the molecule—high concentrations often exerting negative effects and low concentrations evoking a positive effect (25). The results we obtained with low concentrations of sodium nitroprusside on CHO cells and a low amount of intracellular NO produced under CCK treatment correlated well with these observations.

In the present work, the rapid activation of NOS activity suggested the involvement of a constitutive NOS. Moreover, CCK treatment promoted the formation of nNOS dimers, which appear to be the active form of nNOS. The ability of the heme/BH_4-deficient nNOS mutant to inhibit CCK-induced stimulation of NOS activity and cell proliferation as well as CCK-induced nNOS dimers formation suggests that nNOS may be required to mediate the effect of CCK. As previously demonstrated, dimerization of nNOS requires heme and BH_4 (14, 26). Thus dimerization of the nNOS mutant could not be observed after its transfection in CHO cells. However, heterodimerization between mutant and wild-type nNOS did not occur. We hypothesized that the inactive nNOS could act as a competitive inhibitor of endogenous nNOS regarding the transduction pathway molecules activated by CCK such as tyrosine phosphatase SHP-2.

Neuronal NO synthase displays a widespread expression in central and peripheral nervous systems such as the gut and urogenital tract (27). This isoform is considered to be responsible for the largest proportion of tissue NO synthase activity. The enzyme has thus been implicated in the regulation of neuronal cell biology and in neuroendocrine biology as a major nonadrenergic noncholinergic neurotransmitter in enteric nerves in the contraction of skeletal muscle and relaxation of smooth muscle, among other roles (28). Many authors speculate that nNOS may be implicated in nervous system morphogenesis, neuronal differentiation, and synaptogenesis. Targeted disruption of the nNOS in mice by homologous recombination did not reveal major abnormalities of central nervous development (29). Some authors have approached the role of nNOS in cell growth or differentiation by investigating the expression of the isoform on neuronal or phaeo-

chromocytoma cells. Neuronal NOS has been induced by growth factors or expressed in surviving neurons (30). In addition, induction of different isoform of NOS in phaeochromocytoma cells has been responsible for growth arrest (31). During *Drosophila* development, high expression of NOS and the resulting NO production were responsible for cell growth arrest (32). To our knowledge, however, direct evidence of the implication of nNOS in the positive regulation of cell proliferation of eukaryotic cells has rarely been observed.

The physiological relevance of the coupling of CCK receptor with nNOS remains to be established. The mouse exocrine pancreas represents a tissue model that expresses only the CCK_A receptor subtype, as we found in our CHO-K1 strain. CCK is known to stimulate exocrine pancreatic secretion and growth in mice. NO is known to positively modulate basal and stimulated exocrine pancreatic secretion and blood flow in rodents. However, controversies still exist in the possible direct implication of the NO/cGMP system on amylase secretion by acinar cells (33). Interestingly, we found that CCK-8 evoked a time-dependent stimulation of NOS activity on mouse acinar cells and that nNOS was expressed in pancreatic acinar cells (unpublished results). Activation of nNOS could thus be implicated in the mitogenic effect of CCK on exocrine pancreas. The involvement of the coupling between CCK_A receptors and nNOS in the other biological effects of CCK that implicate NO, such as relaxation of sphincter of Oddi and lower esophagus, colonic, or gastroduodenal motility, and gastroprotection, remain to be investigated.

Activation of nNOS requires activation of the tyrosine phosphatase SHP-2 and G-βγ subunit

The SH2-containing phosphotyrosine phosphatases such as SHP-1 and SHP-2 regulate various protein tyrosine kinase signaling pathways, interacting with receptor autophosphorylation or with signaling proteins. Whereas SHP-1 appears to be a negative regulator of growth factors receptor signaling, SHP-2 is a positive mediator of the mitogenic signaling induced by several growth factors (19, 34). Many of these growth factors act through receptors that possess an intrinsic tyrosine kinase activity such as insulin, PDGF, EGF receptors, but also act though G-protein-coupled receptors such as thrombin or proteinase-activated receptor 2 receptors (11, 35).

We observed that CHO cell proliferation induced by CCK was dependent on a tyrosine phosphatase and required the stimulation of SHP-2 activity. The kinetic of activation of SHP-2 correlated well with that of stimulation of NOS activity, dephosphorylation of nNOS on tyrosine residues, and association of SHP-2 with nNOS evoked by CCK treatment. Expression of inactive SHP-2 inhibited CCK-induced cell proliferation, nNOS dephosphorylation on tyrosine residues, and NOS activity. As previously observed (11, 36), this catalytically inactive protein can bind to substrates without dephosphorylating them, thereby competitively interfering with the access of these substrates to the endogenous wild-type SHP-2. In the present work, nNOS could thus be a substrate of SHP-2. Taken together, these results suggest that CCK treatment led to the activation of SHP-2 that can dephosphorylate and then activate the nNOS.

The mechanism of association of SHP-2 with nNOS and the tyrosyl residue involved on nNOS remain to be identified. Nevertheless, this is the first evidence that nNOS activity can be regulated by tyrosine dephosphorylation. Using kinase or phosphatase inhibitors, the eNOS isoform has been found to be regulated by phosphorylation on serine threonine or tyrosine residues, but from indirect observations (9, 37). Tyrosine phosphorylation also seems to regulate association of eNOS with caveolin-1 within endothelial caveola. Tyrosine phosphorylation thus may regulate activity and subcellular trafficking of eNOS (18). It has recently been suggested that phosphorylation/dephosphorylation may alter conformation and/or protein coupling of constitutive NOS, facilitating their interactions with specific phospholipids or proteins that enhance or maintain a Ca^{2+}-independent activation (38, 39).

Expanding examples of cellular responses regulated by Gβγ have already been described. Cell growth and differentiation could implicate α or Gβγ protein subunits. In the present work, we observed that the recombinant Gβ1γ2 subunit could induce a stimulation of NOS activity. In addition, in the expression of the carboxyl terminus of β-adrenergic receptor kinase-1, a Gβγ subunit-sequestering agent inhibited the CCK-induced stimulation of SHP-2 activity, NOS activity, and cell proliferation. These results also suggested that CCK stimulated SHP-2 activity in CHO cells via a Gβγ-dependent pathway. The kinetic of G-β1 and SHP-2 associations correlated well with those of CCK-induced stimulation of SHP-2 activity, CCK-induced stimulation of NOS activity, and CCK-induced dephosphorylation of nNOS on tyrosine residues. All these observations suggest that upon CCK stimulation, the occupation of CCK_A receptors induced the recruitment of G-βγ subunit to SHP-2, which was activated and then recruited nNOS to dephosphorylate and activate it.

In conclusion, the new concept of nNOS acting as an important molecule in the signal cascade of growth factors may have some practical implication taking into account the wide distribution of this isoform both in neuronal and nonneuronal tissues. Moreover, activation of nNOS by dephos-

phorylation could be involved in other physiological roles of nNOS in epithelial, neuronal, or vascular systems. FJ

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