Ubiquitination of Neuronal Nitric-oxide Synthase in Vitro and in Vivo

Received for publication, January 10, 2000, and in revised form, March 3, 2000
Published, JBC Papers in Press, April 5, 2000, DOI 10.1074/jbc.M000155200

Andrew T. Bender‡, Damon R. Demady, and Yoichi Osawa§

From the Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0632

It is established that suicide inactivation of neuronal nitric-oxide synthase (nNOS) with guanidine compounds, or inhibition of the hsp90-based chaperone system with geldanamycin, leads to the enhanced proteolytic degradation of nNOS. This regulated proteolysis is mediated, in part, by the proteasome. We show here with the use of human embryonic kidney 293 cells transfected with nNOS that inhibition of the proteasome with lactacystin leads to the accumulation of immunodetectable higher molecular mass forms of nNOS. Some of these higher molecular mass forms were immunoprecipitated by an anti-ubiquitin antibody, indicating that they are nNOS-polyubiquitin conjugates. Moreover, the predominant nNOS-ubiquitin conjugate detected in human embryonic kidney 293 cells, as well as in rat brain cytosol, migrates on SDS-polyacrylamide gels with a mobility near that for the native monomer of nNOS and likely represents a conjugate containing a few or perhaps one ubiquitin. Studies in vitro with the use of [125I]-ubiquitin and reticulocyte extracts could mimic this ubiquitination reaction, which was dependent on ATP. The heme-deficient monomeric form of nNOS is preferentially ubiquitinated over that of the heme-sufficient functionally active homodimer. Thus, we have shown for the first time that ubiquitination of nNOS occurs and is likely involved in the regulated proteolytic removal of non-functional enzyme.

Nitric-oxide synthases (NOS) are cytochrome P450-like heme protein enzymes that catalyze the conversion of L-arginine to citrulline and nitric oxide by a process that requires NADPH and molecular oxygen (1–4). The enzymes also require bound FMN, FAD, (6R)-5,6,7,8-tetrahydro-L-biopterin (BH$_4$), and Ca$^{2+}$/calmodulin for activity. Three main isoforms of NOS have been identified: isoform I or neuronal NOS (nNOS), which is constitutively expressed in a variety of neuronal cells as well as other cells; isoform II or inducible NOS, which is usually not constitutively expressed but can be induced by bacterial lipopolysaccharide and/or cytokines in macrophages and other cells; and isoform III or endothelial NOS, which is expressed in endothelial cells (5, 6).

Both nNOS and endothelial NOS are hsp90-associated proteins and inhibition of hsp90 by geldanamycin causes the loss of NOS protein in cells (7, 8). Based on studies with other hsp90-associated proteins, this loss of NOS is likely due to enhanced proteasomal degradation, presumably as a mechanism for the removal of misfolded proteins (9–11). It has been shown for some proteins, including p53 (11) and tyrosine kinase p185$^{erbB-2}$ (12), that geldanamycin treatment leads to the accumulation of ubiquitinated forms of the target protein. More recently, our laboratory has shown that suicide inactivation of nNOS enhances the proteolytic removal of the enzyme that is mediated, in part, by the proteasome (13). Based on these studies, we asked if nNOS could be regulated by ubiquitination prior to proteolytic removal by the proteasome in HEK 293 cells transfected with nNOS. This cellular model was chosen since it is the same model used in studies on the suicide inactivation and hsp90 regulation of nNOS (7, 13).

In the current study, we have found that inhibition of the proteasome leads to the accumulation of higher molecular mass forms of NOS, which are in part due to conjugation with ubiquitin (Ub). The major nNOS-Ub conjugate did not greatly change the relative mobility of the protein on SDS-PAGE gels, suggesting that the conjugate contains a few or perhaps one Ub. This limited ubiquitination of nNOS could be reproduced with an in vitro system containing purified nNOS, reticulocyte extracts, ATP, and Ub. Studies with [125I]-Ub indicate that the heme-deficient monomeric form of NOS is preferentially ubiquitinated over the dimeric active form of NOS. Thus, these studies establish for the first time that ubiquitination of nNOS occurs and likely plays a regulatory role in the removal of misfolded or non-functional protein.

EXPERIMENTAL PROCEDURES

Materials

The affinity-purified rabbit IgG used for Western blotting of nNOS was from Transduction Laboratories (Lexington, KY) or from RBI (Natick, MA) where indicated. The immunogen used for production of the antibody from Transduction Laboratories was a C-terminal peptide (residues 1095–1289) of nNOS, whereas that from RBI was an N-terminal peptide (residues 251–270). The affinity-purified IgG used for Western blotting of Ub was from Dako Corp. (Carpinteria, CA). The rabbit antiserum used to immunoprecipitate nNOS was raised against rat neuronal NOS and was the generous gift of Dr. Lance Pohl (NHLBI, National Institutes of Health, Bethesda, MD). The antibody was affinity purified prior to use. N$^6$-Nitro-L-arginine (NNA) and the rabbit antiserum used to immunoprecipitate Ub were purchased from Sigma. Peroxidase conjugated anti-rabbit IgG antibody was from Roche Molecular Biochemicals. [125I]-Labeled Ub was purchased from Amersham Pharmacia Biotech. Lactacystin was purchased from BIOMOL (Plymouth Meeting, PA). BH$_4$ was purchased from Dr. Schirks Laboratory (Jona, Switzerland). The CDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Balti-
more, MD). Male Wistar rats (150–250 g) were purchased from Charles River Laboratories (Wilmington, MA).

Methods

Cell Culture and Cytosol Preparation—Human embryonic kidney (HEK) 293 cells stably transfected with rat neuronal NOS by Bredt et al. (14) were obtained from Dr. Bettie Sue Masters (University of Texas Health Science Center, San Antonio, TX). HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and G418 as described previously (3). When treated with lactacystin, cells were seeded at a density of 0.8 × 10^6 cells/ml and grown for 48 h. After 48 h, the cell medium was aspirated and replaced with Dulbecco’s modified Eagle’s medium containing 0.1 mM arginine and supplemented with either 10 μM lactacystin or H2O as a vehicle control. HEK cells were harvested, washed with ice-cold phosphate-buffered saline, and homogenized with a Tenbroek ground glass homogenizer in three volumes of HE lysis buffer (10 mM HEPES, pH 7.4, 0.32 M sucrose, 2 mM EDTA, 6.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 10 mM Na3VO4, 1% Nonidet P-40, 5 mM N-ethylmaleimide (NEM)). Homogenates were centrifuged at 16,000 × g for 10 min with the supernatant taken as the cytosolic fraction. Cytosol from rat brains was prepared as above in three volumes of HE lysis buffer.

Immunoprecipitation and Western Blotting—nNOS was immunoadsorbed from 50 μl of HEK 293 cytosol or 100 μl of rat brain cytosol with 20 μl of anti-nNOS IgG and 10 μl of protein A-Sepharose in a total volume of 300 μl of HE lysis buffer for 2 h at 4 °C. In studies where Ub was immunoadsorbed, 25 μl of anti-Ub IgG replaced anti-nNOS IgG. Immune pellets were washed three times with 1 ml of ice-cold HE lysis buffer. Immune pellets were boiled in SDS sample buffer containing dithiothreitol (6.0 mg/ml), and the proteins were resolved on 6% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for 6 h at 850 mA. The membranes were probed with either a 0.1% anti-nNOS polyclonal antibody from Transduction Labs or 0.1% anti-nNOS antibody from RBI or 0.1% anti-Ub polyclonal antibody (Dako Corp.). Prior to probing with the anti-Ub antibody, the nitrocellulose membranes were autoclaved in distilled H2O for 20 min. An anti-rabbit IgG conjugated to peroxidase (Roche Molecular Biochemicals) was used as a secondary antibody. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and X-Omat film (Eastman Kodak Co.).

Expression and Purification of nNOS—nNOS was expressed in Sf9 cells using a recombinant baculovirus as described previously (7). Sf9 cells were grown in SPM 900 II serum-free medium (Life Technologies, Inc.) supplemented with Cytomax (Kemp Biotechnology, Rockville, MD) in suspension cultures maintained at 27 °C with continuous shaking (150 rpm). Cultures were infected in log phase of growth with recombinant baculovirus at a multiplicity of infection of 1.0. After 48 h, oxyhemoglobin (25 μM) was added as a source of heme. Cells were harvested and lysates prepared as described previously (7). Lysates from Sf9 cells (5 × 10^5) were centrifuged at 100,000 × g for 30 min. The supernatant fraction was loaded onto a 2.5-ADP Sepharose column, and the nNOS was affinity-puriﬁed as described previously (15), except that 10 mM 2-AMP in high salt buffer was used to elute the protein. The nNOS containing fractions were loaded onto a Sephacryl S-300 HR gel ﬁltration column (2.6 × 100 cm, Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10 mM BH4. The proteins were eluted at a flow rate of 1.3 ml/min, and 1.5-ml fractions were collected and analyzed for protein content and NOS activity. The fractions containing NOS activity were pooled and supplemented with 10 mM FAD, 10 mM FMN, and 10 mM BH4 before concentration with the use of a Centriplus concentrator (Amicon, Beverly, MA). The concentrated enzyme was aliquoted and stored at −80 °C. For preparation of puriﬁed heme-deﬁcient apo-nNOS, the same procedure was followed except that oxyhemoglobin was not added to the insect cells during expression.

In Vitro Ubiquitination of nNOS—To conjugate Ub to nNOS, puriﬁed nNOS (10 μg) was incubated for 1 h at 37 °C in a total volume of 60 μl of HKD buffer (10 mM HEPES, 100 mM KCl, 2 mM dithiothreitol), containing either 10 μM Ub, an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM MgOAc, and 100 units/ml creatine phosphokinase), and 2 mg/ml DE52-retained fraction of rabbit reticulocyte lysate (RET), which was prepared as described previously (7). After the incubation, reactions were quenched with SDS sample buffer containing dithiothreitol (6 mg/ml) and Western blotted with a 0.1% polyclonal antibody from Dako Corp.

RESULTS

Inhibition of the Proteasome Leads to Accumulation of Higher Molecular Mass Forms of nNOS in Vivo—As shown in Fig. 1A, treatment of nNOS-transfected HEK 293 cells with 10 μM lactacystin leads to the appearance of nNOS bands, which were detected by probing with an antibody directed against the C-terminal region of nNOS, that were of higher molecular mass than the native enzyme (cf. lane 2 with lane 1). HEK 293 cells that have not been transfected with nNOS do not give these intensely staining higher molecular mass bands (lane 4), indicating specificity of the antibody. As shown in Fig. 1B, higher molecular mass bands were also detected with an antibody directed against the N-terminal region of nNOS (lane 2). The pattern of higher molecular mass forms of nNOS differed from that found with the use of the antibody directed against the C terminus, suggesting that some of the epitopes may be masked. Moreover, these bands were only found when the lysis buffer contained NEM, suggesting an instability of these higher molecular mass nNOS forms due to a thiol-sensitive degradative pathway (cf. lane 2 with lane 1).

Ubiquitination of nNOS in Vivo—As expected from previous studies (12, 18, 19), lactacystin treatment of HEK 293 cells was found to cause a large increase in the level of immuno-detectable Ub-protein conjugates (data not shown). Moreover, Ub-protein conjugates are known to be ubiquitinated by an NEM-sensitive pathway (20). Thus, we suspected that the higher molecular mass forms of nNOS were due to conjugation with Ub. As shown in Fig. 2A, immunoprecipitation of Ub-conjugates from cytosol that was prepared from lactacystin-

FIG. 1. Accumulation of higher molecular weight immunode- tectable nNOS proteins in lactacystin-treated HEK 293 cells. A. HEK 293 cells stably transfected with nNOS (lanes 1 and 2) or non- transfected HEK 293 cells (lanes 3 and 4) were treated for 20 h with 10 μM lactacystin (lanes 2 and 4) or H2O as a vehicle control (lanes 1 and 3). Cells were harvested, lysed in HE lysis buffer, and cytosols prepared as described in Experimental Procedures. nNOS in the cytosol (14 μl) was measured by SDS-PAGE and Western blotting with the use of polyclonal antibody from Transduction Labs. B. HEK 293 cells transfected with nNOS were treated for 20 h with 10 μM lactacystin and harvested in HE lysis buffer (lane 2) or HE lysis buffer that did not contain NEM (lane 1). The cell cytosols were prepared and incubated at 37 °C for 30 min. An aliquot (17 μl) was taken for immunodetection of nNOS with the use of polyclonal antibody from RBI. 125I-Ub was conjugated to nNOS using a procedure modified from Ref. 16 by incubating 5 μg of nNOS for 2 h at 37 °C in a total volume of 25 μl of HKD buffer containing 0.2 pmol of 125I-Ub (9 × 10^6 cpm), ATP-regenerating system, and 0.8 mg/ml RET. The reaction was halted by the addition of SDS sample buffer, and aliquots were resolved on 6% SDS-polyacrylamide gels. To separate nNOS monomers and dimers by SDS-PAGE, 100 μM BH4 and 100 μM L-arginine were included in the SDS sample buffer and samples were kept ice-cold prior to loading on gels. This method has previously been described by Klatt et al. (17) to prevent the dissociation of nNOS dimers prior to and during electrophoresis. Gels were stained with Coomassie Blue and dried using a Bio-Rad model 583 gel dryer. Dried gels were then exposed to X-Omat film overnight at −80 °C. The radioactivity was quantified with the use of a PhosphorImager (model 445 SI, Molecular Dynamics, Sunnyvale, CA).
treated nNOS-transfected HEK 293 cells revealed higher molecular mass bands that were recognized by anti-nNOS IgG in the immune (I), but not non-immune pellet (N), indicating the formation of nNOS-Ub conjugates. Unexpectedly, we observed a very dark band corresponding to the nNOS monomer that could not be entirely explained by nonspecific immunoprecipitation of nNOS (cf. lanes N with lane I). As will be described below, this is due to ubiquitination of nNOS in a manner that did not greatly alter the relative mobility of the protein on SDS-PAGE.

As shown in Fig. 2B, anti-nNOS IgG was able to immunoprecipitate large amounts of the native nNOS but not the higher molecular mass forms of the enzyme (left panel). This may be due to masking of nNOS epitopes under the non-denaturing conditions of the immunoprecipitation. Even though we could not address the nature of the higher molecular mass species by immunoprecipitation with anti-nNOS IgG, we found that Western blotting and probing of the nNOS-immunoprecipitate with an anti-Ub IgG gave an immune-specific signal corresponding approximately to the mobility of the nNOS monomer (right panel, cf. lane I with lane N). This signal was increased in lactacystin-treated cells (LC) over that found for control cells (CT), strongly suggesting that the signal was not due to cross-reactivity of the antibody and that ubiquitination of nNOS occurred. Furthermore, the ubiquitination did not greatly alter the relative mobility of the conjugate on SDS-PAGE gels, suggesting that the conjugate contained only one or a few Ub molecules. To further confirm these observations, an in vitro system was investigated as described below.

**Ubiquitination of nNOS in Vitro**—As shown in Fig. 3A, ubiquitination of purified nNOS was also found to occur in vitro when RET and ATP were present during the incubation (cf. lane 3 with lane 1). Again, the nNOS conjugate appeared as a band with the relative mobility on SDS-PAGE gels near that of the monomer of nNOS. The changes in intensity of this nNOS-Ub band were quantified by laser densitometry as shown in Fig. 3B. The ubiquitination was greatly increased when exogenous Ub was added (lane 4). The smear of higher molecular mass proteins recognized by the Ub antibody were not due to nNOS conjugates, as they were not recognized by anti-nNOS IgG (data not shown), and likely represent various ubiquitinated proteins from RET. The immunoreactivity to the Ub antibody found here was not due to cross-reactivity as the same amount of nNOS was present in all samples. To further support that ubiquitination of nNOS occurs, we utilized $^{125}$I-Ub in the in vitro system. As shown in Fig. 4, the radiolabeled Ub was conjugated to nNOS only when incubated with RET and ATP (cf. lane 3 with lane 1). There were three higher molecular mass bands that were radiolabeled, but these were found in reaction mixtures not containing nNOS and are due to ubiquitination of proteins found in RET (cf. lane 3 with lane 4). Thus, taken together, nNOS was ubiquitinated in a manner that had little effect on the relative mobility of nNOS. Higher molecular mass nNOS-Ub conjugates were not observed in these in vitro studies.

Under conditions where $^{125}$I-Ub could be conjugated to nNOS in vitro, we asked whether the monomeric or dimeric forms of nNOS were ubiquitinated. The dimeric or catalytically active form of nNOS is resistant to SDS and can be detected as a dimer if the gel and sample are kept cold (17). We utilized this procedure to separate monomer from dimer after ubiquitination with $^{125}$I-Ub and subsequently analyzed the samples by Coomassie Blue (CB) staining and PhosphorImager analysis for radioisotope ($^{125}$I-Ub) (Fig. 5). As shown in Fig. 5A, our nNOS preparation was a mixture of monomer and dimer as determined by Coomassie Blue staining (lane 9). The nNOS was converted to the monomer during incubation with RET and ATP (lane 2) and reflects the instability of the enzyme during the incubation. The nNOS could be stabilized as a dimer if 10 μM NNA was added to the ubiquitination reaction mixture (lane 4). Conversely, we have a purified preparation of inactive heme-deficient apo-nNOS that exists only as a monomer (lane 3). We have previously shown that this apo-nNOS preparation can be functionally reconstituted to give the active dimer (7).

As shown in Fig. 5B, treatment of nNOS gave $^{125}$I-Ub conjugated to the nNOS monomer (lane 2) as well as to other proteins found in RET (lane 1). The intensity of the $^{125}$I-Ub-nNOS monomer band increased when heme-deficient apo-NOS was used instead of native nNOS (lane 3), whereas the intensity decreased when the dimeric nNOS was stabilized with 10 μM NNA (lane 4). An $^{125}$I-Ub-labeled band corresponding to the dimer of nNOS was not observed, even though a substantial amount of dimer was found by Coomassie Blue staining (cf. panel A, lane 4, with panel B, lane 4). As shown in Fig. 5C, the amount of radioiodine was quantified in samples that were
Ubiquitination of nNOS

A

B

C

125I-Ub

nNOS -

nNOS + + + +

RET - + + +

ATP - + + +

217-

lanes 1–4, 9

lanes 5–8

FIG. 4. In vitro conjugation of 125I-Ub to nNOS. nNOS was treated with a DE52-retained fraction of rabbit reticulocyte lysate (RET), an ATP-regenerating system (ATP), and 125I-Ub (9 × 10^6 cpm) as described under "Experimental Procedures." Where indicated RET, ATP or nNOS were omitted from the reaction mixtures. The reaction mixtures were resolved by SDS-PAGE and the radioactivity detected by exposure of the gel to x-ray film.

IP: anti-nNOS

nNOS -

nNOS + + + +

Ub - - - -

lanes 1–4, 9

lanes 5–8

FIG. 6. Immunoprecipitation of nNOS-Ub conjugates from rat brain cytosol. Cytosol from male Wistar rats was prepared as described in Experimental Procedures. nNOS was immunoadsorbed from rat brain cytosol with an anti-nNOS antibody (1) or non-immune IgG (NI). Immune pellets were washed, analyzed by SDS-PAGE, and Western blotted with a polyclonal antibody to nNOS from RBI (left panel) or a polyclonal antibody to Ub from Dako (right panel).

DISCUSSION

Suicide inactivation of nNOS enhances the proteolytic degradation of the enzyme, in part, due to the proteasome (13). We show here that inhibition of the proteasome caused the accumulation of ubiquitinated forms of nNOS, strongly suggesting that ubiquitination regulates nNOS degradation. That ubiquitination is a mechanism for proteasomal removal of proteins is well recognized (19, 21, 22), although this is the first report of the ubiquitination of nNOS. These observations are consistent with the finding that liver microsomal P450 cytochromes, which are heme-thiolate enzymes similar to NOS, are ubiquitinated and proteasomally degraded after suicide inactivation (23, 24).

In the case of suicide inactivation of liver P450 cytochromes, structural changes and not the functional inactivation per se appears to be the "trigger" for proteolysis (25, 26). In particular, the cross-linking of heme to the protein, which occurs during suicide inactivation of cytochrome P450, plays a prominent role in the enhanced turnover of cytochrome P450 (25–27). This process is selective in that other structural changes that occur during suicide inactivation, including the N-alkylation of the heme (25), the formation of apoprotein (28, 29), and the conformational change of the protein (30), all fail to enhance the proteolysis of the affected P450 cytochromes.

In the case of nNOS, it is not known if heme-protein cross-links can lead to enhanced proteolytic degradation, and further studies...
are needed to define this pathway of nNOS heme alteration. We have found, however, that the functionally inactive monomeric form of the protein was preferentially ubiquitinated over the enzymatically active homodimer. Moreover, the slowly reversible inhibitor, NNA, stabilized the dimeric state of nNOS and decreased the extent of ubiquitination. This is consistent with the observation that NNA causes a slight increase in the amount of immunodetectable nNOS in vivo (31) and slows the rate of proteolytic degradation of nNOS in HEK 293 cells (13). That functionally inactive monomeric nNOS would be processed for removal is consistent with the recent postulate that ubiquitination and proteasomal degradation as well as chaperone-based folding and unfolding are mechanisms for cellular “quality control” of proteins (32). In this regard, our laboratory has also shown that inhibition of the hsp90-based chaperone system leads to the enhanced degradation of nNOS (7). Thus, it is possible that under conditions where heme is not available for assembly of newly synthesized nNOS to functionally active homodimers in vivo that the excess monomeric nNOS would be ubiquitinated and proteolytically removed. Alternatively, the monomeric form of nNOS may form during autoinactivation of the holoenzyme (Fig. 5). Thus, ubiquitination may be critical in regulating the level of monomeric nNOS in vivo. The amount of monomeric NOS may be important as a reserve pool of protein that could be rapidly assembled in the absence of protein synthesis to give the functional homodimer (33).

The major ubiquitinated form of nNOS found in vitro and in vivo was a conjugate that did not greatly alter the relative mobility of nNOS, suggesting that only one or a few Ub are attached. It is likely that the limited ubiquitination of nNOS gives a more stable conjugate whereas the polyubiquitinated forms, which were only observed in cells treated with lactacystin, are rapidly proteolyzed. It is noteworthy that the major conjugate observed in rat brains was that containing only limited amounts of Ub. The lability of the polyubiquitinated forms may be a possible explanation for not detecting the polyubiquitinated conjugates in our in vitro studies. The mono-ubiquitinated forms of proteins are thought to be involved in subcellular targeting (see Ref. 34 for review), and it would be important to further define if limited ubiquitination of nNOS is biologically important in this regard as a certain portion of nNOS exists in the membrane particulate fraction (35).

The sites on nNOS that serve to conjugate Ub are not known. It is likely that some structural change, which is related to inactivation and monomerization, serves to expose a lysine residue for Ub conjugation. For some proteins such as 1xBc and the large subunit of RNA polymerase II, phosphorylation serves to initiate structural changes that lead to ubiquitination (36, 37). In this regard, the phosphorylation of nNOS has been shown to occur on a serine residue and lead to the inhibition of the enzyme (38, 39). Moreover, suicide-inactivated liver microsomal P450 cytochromes are phosphorylated prior to ubiquitination and degradation (23). Thus, the elucidation of the structural features that predispose nNOS to ubiquitination will aid in understanding the post-translational events that govern the steady state levels of nNOS. The current report describes the ubiquitination of nNOS and the initial studies to define these structural features.

Acknowledgments—We thank Bettie Sue Masters, Solomon Snyder, William Pratt, and Lance Pohl for providing cell lines, cDNAs, reticulocyte extracts, and antisera used in this work.

REFERENCES

1. White, K. A., and Marletta, M. A. (1992) Biochemistry 31, 6627–6633
2. Studer, D., and Bada-Geisler, M. (1992) J. Biol. Chem. 267, 20547–20550
3. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11141–11145
4. Marletta, M. A. (1995) J. Biol. Chem. 268, 12231–12234
5. Lancaster, J. H., Jr., and Hibi, J. B., Jr. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1223–1227
6. Forstermann, U., Closs, E. I., Pollock, J. S., Nakane, M., Schwarz, P., Gath, I., and Kleinert, H. (1994) Hypertension 23, 1121–1131
7. Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., and Osawa, Y. (1999) J. Biol. Chem. 274, 1472–1478
8. Garcia-Cardenas, G., Fan, R., Shah, Y., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sees, W. C. (1998) Nature 392, 821–824
9. Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouwerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F. U. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14536–14541
10. Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998) EMBO J. 17, 6879–6887
11. Whitesell, L., Sutphin, P. A., Ans, W. G., Schulte, T., Blagosklonny, M. V., and Neckers, L. (1997) Oncogene 14, 2809–2816
12. Minn Aqua, E. G., Chavany, C., and Neckers, L. (1996) J. Biol. Chem. 271, 22796–22801
13. Noguchi, S., Jianmongkol, S., Bender, A. T., Kamada, Y., Demady, D. R., and Osawa, Y. (2000) J. Biol. Chem. 275, 2376–2380
14. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
15. Roman, L. J., Shetz, E. A., Martasek, P., Gross, S. S., Liu, Q., and Masters, B. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8428–8432
16. Sokolik, C. W., and Cohen, R. E. (1991) J. Biol. Chem. 266, 9100–9107
17. Kluft, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., and Mayer, B. (1995) EMBO J. 14, 3687–3695
18. Chen, E., Hrilickova, R., Nebyha, J., Lonto, D. L., Rose, H. R. Jr, and Li, C. C. (1998) J. Biol. Chem. 273, 35201–35207
19. Lee, D. H., and Goldberg, A. L. (1996) Trends Cell Biol. 6, 397–403
20. Minn Aqua, E. G., Bonvini, P., and Neckers, L. (1999) Electrophoresis 20, 418–428
21. Ciechanover, A. (1994) Cell 78, 13–21
22. Peters, J.-M., Harris, J. R., and Finley, D. (1998) Ubiquitin and the Biology of the Cell, Plenum Press, New York
23. Karsmeyer, K. K., Davoll, S., Figuroedo-Pereira, M. E., and Correia, M. A. (1999) Arch. Biochem. Biophys. 365, 51–44
24. Wang, H. F., Figuroedo Pereira, M. E., and Correia, M. A. (1999) Arch. Biochem. Biophys. 365, 45–53
25. Terney, D. J., Haas, A. L., and Koop, D. R. (1992) Arch. Biochem. Biophys.
26. Correia, M. A., Decker, C., Sugiyama, K., Caldera, P., Bornheim, L., Wrighton, S. A., Rettie, A. E., and Trager, W. F. (1987) Arch. Biochem. Biophys. 258, 436–451
27. Yao, K., Falic, A. M., Patel, N., and Correia, M. A. (1993) J. Biol. Chem. 268, 59–65
28. Bornheim, L. M., Underwood, M. C., Caldera, P., Rettie, A. E., Trager, W. F., Wrighton, S. A., and Correia, M. A. (1987) Mol. Pharmacol. 32, 299–308
29. Correia, M. A., Davoll, S. H., Wrighton, S. A., and Thomas, P. E. (1992) Arch. Biochem. Biophys. 297, 228–238
30. Lunetta, J. M., Sugiyama, K., and Correia, M. A. (1989) Mol. Pharmacol. 35, 10–17
31. Nakatsuiva, M., Nakatsuka, K., and Osawa, Y. (1998) Drug Metab. Dispos. 26, 497–501
32. Wickner, S., Maurizi, M. R., and Gettesmann, S. (1999) Science 286, 1888–1893
33. Wolf, D. J., Lubeskie, A., Gaud, D. S., and Neulander, M. J. (1998) Eur. J. Pharmacol. 350, 325–334