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Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase

(nitroarginine/glutamate neurotoxicity/cGMP/biotinylated NAD)

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ABSTRACT **Nitric oxide generation in brain cytosolic fractions markedly enhances ADP-ribosylation of a single 37-kDa protein. By utilizing a biotinylated NAD and avidin affinity chromatography, we purified this protein. Partial amino acid sequencing establishes its identity as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is further confirmed by detection of GAPDH enzymatic activity in the purified 37-kDa protein. GAPDH is ADP-ribosylated in the absence of brain extract. This auto-ADP-ribosylation is enhanced by nitric oxide generation. ADP-ribosylation appears to involve the cysteine where NAD interacts with GAPDH so that ADP-ribosylation likely inhibits enzymatic activity. Such inhibition may play a role in nitric oxide-mediated neurotoxicity.**

Nitric oxide (NO) is a major biological messenger molecule accounting for endothelium-derived relaxing factor activity in blood vessels (1, 2) and the tumoricidal and bactericidal actions of macrophages (3) and serving a neurotransmitter-like role in the brain (4). NO is synthesized from arginine by NO synthase (NOS). Distinct forms of NOS have been molecularly cloned from brain (5), macrophages (6–8), and endothelium (9). NO exerts some of its actions by stimulating guanylyl cyclase, which is not likely the sole physiologic target of NO, however. Thus, the localization of guanylyl cyclase protein and mRNA throughout the brain differs markedly from that of NOS (4, 10). NO mediates the neurotoxicity of glutamate acting through *N*-methyl-D-aspartate receptors, as removal of arginine from the incubation medium for brain cultures or treatment with NOS inhibitors prevents *N*-methyl-D-aspartate toxicity (11). NO mediation of neurotoxicity does not involve guanylyl cyclase, as inhibitors of the enzyme fail to influence neurotoxicity, and 8-bromo-cGMP does not elicit neurotoxicity (12).

In a search for alternative targets of NO, Brune and Lapetina (13) observed that NO can enhance ADP-ribosylation of a 37-kDa cytosolic protein. In the present study we have developed a biotinylated derivative of NAD, the substrate for ADP-ribosylation, and have isolated an NO-enhanced ADP-ribosylated protein (NAP) from brain tissue. We demonstrate that NAP is glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

MATERIALS AND METHODS

Preparation of Brain Extract. Adult rat brain was homogenized in ice-cold buffer (8 ml of buffer per g of brain) consisting of 20 mM Tris·HCl (pH 7.4), 5 mM EDTA, 5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, chymostatin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and trypsin inhibitor (1 μg/ml; all from Sigma). The homogenate was centrifuged at 100,000 × *g* for 40 min. The supernatant was used as cytosolic fraction for *in vitro* ADP-ribosylation.

We followed the published procedure for purification of NOS from rat cerebella (14).

***In Vitro* ADP-Ribosylation of NAP.** A typical ADP-ribosylation reaction mixture contained 10 μl of brain extract and a freshly prepared solution containing 0.5 mM sodium nitroprusside (SNP) and 0.5 μM [*adenylate*-^{32}P]NAD (800 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN) or 60 μM biotinylated NAD added to a final volume of 20 μl. In one experiment, SNP was replaced by 1 μg of purified cerebellar NOS, 0.1 mM arginine, 1 mM NADPH, 0.1 μM calmodulin, and 20 μM $CaCl_2$ with or without 1 μM nitroarginine. For NAP purification, 20 ml of the biotinylated ADP-ribosylation mixture was incubated at 37°C for 1 hr and either terminated with 2× SDS gel loading buffer [0.25 M Tris·HCl, pH 6.8/4% (wt/vol) SDS/20% (wt/vol) glycerol/10% (wt/vol) 2-mercaptoethanol] or used immediately for purification of NAP.

^{32}P-labeled NAP was detected by autoradiography after SDS/PAGE. To detect biotinylated NAP, proteins were electrophoretically transferred from SDS gel to nitrocellulose. The nitrocellulose filter was blotted with horseradish peroxidase-avidin D (0.5 μg/ml; Vector Laboratories) plus 2% (wt/vol) bovine serum albumin in buffer A consisting of 10 mM sodium phosphate (pH 7.4), 140 mM NaCl, and 0.5% Triton X-100 for 1 hr, washed for four 10-min periods with buffer A, and developed by using the Enhanced Chemiluminescence (ECL) method according to manufacturer's protocol (Amersham).

Use of Biotinylated NAD to Label NAP for Purification. The detailed procedures for synthesizing biotinylated NAD and purification of NAP will be published elsewhere. Briefly, biotinylated NAD, N^6-[*N*-(*N*-biotinyl-ε-aminocapronyl-6-aminohexylcarbamoylmethyl]NAD, was synthesized from N^6-[6-(aminohexyl)carbamoylmethyl]NAD (Sigma) and biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide (Pierce). Biotinylated NAD was purified by reverse-phase chromatography on a C_{18} column.

Purification of *in vitro* biotinyl-ADP-ribosylated NAP employed 65% saturation ammonium sulfate fractionation, followed by phenol-Superose chromatography of NAP-containing supernatant. The phenol-Superose NAP fraction was further purified by Superose 12 gel filtration and then affinity chromatography on avidin D-agarose (Vector Laboratories). After binding and extensive washing with 0.5 M NaCl, NAP was eluted by 10 mM mercuric chloride.

Protein Sequencing. Purified NAP (0.1 nmol) was sequenced from the N terminus on an Applied Biosystems model 475A sequencer. Phenylthiohydantoin amino acid

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; NAP, nitric oxide enhanced ADP-ribosylated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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derivatives were detected by a model 120A amino acid analyzer.

Enzymatic Assay for GAPDH. The assay mixture (1 ml) contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium phosphate, 0.25 mM NAD, 3 μM dithiothreitol, and either 1 μg of purified GAPDH (Sigma) or purified NAP (5 μg of protein from a Superose 12 fraction). After incubating in a spectrophotometer at 25°C for 5 min to achieve temperature equilibrium and establish blank values, the reaction was initiated with the addition of glyceraldehyde 3-phosphate to 0.4 μM. A_{340} from 0 to 5 min was recorded.

ADP-Ribosylation of Dehydrogenases. Dehydrogenases (1 μg) were incubated at 37°C for 1 hr with 0.3 μM [^{32}P]NAD (800 Ci/mmol) in 20 μl of 100 mM Tris·HCl, pH 8.0/1 mM EDTA/10 mM thymidine/2.5 mM $MgCl_2$, in the presence or absence of 1 mM SNP. Dehydrogenases examined included GAPDH (from chicken muscle or *Bacillus stearothermophilus*), alcohol dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, and malate dehydrogenase (all from Sigma). After 1 hr at 37°C, the reactions were terminated by ice-cold 20% (wt/vol) trichloroacetic acid. Protein pellets were washed with acetone and solubilized in SDS-gel loading buffer for SDS/PAGE.

RESULTS

We examined ADP-ribosylation in cytosolic preparations of rat brain incubated with [^{32}P]NAD (Fig. 1). In control samples, radiolabel was incorporated most prominently into a 37-kDa band. Addition of SNP, which generates NO, substantially intensified this band, resembling results of others (13, 15, 16). To ascertain whether generation of NO from endogenous sources can influence ADP-ribosylation, we added purified brain NOS to the specimens to increase labeling of the 37-kDa band. Nitroarginine, a potent NOS inhibitor, abolished the enhanced ADP-ribosylation produced by NOS. Indeed, nitroarginine-treated samples demonstrated negligible labeling of NAP even compared to control samples. In other experiments, nitroarginine added to brain samples without additional NOS also abolished labeling of NAP (data not shown). This establishes that endogenous NO can mediate ADP-ribosylation of NAP.

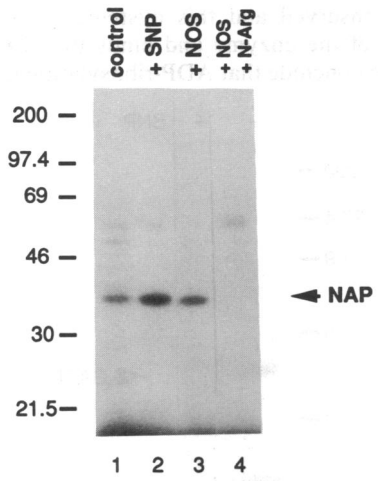

FIG. 1. NOS stimulates ADP-ribosylation of NAP in brain. Rat brain cytosolic fraction was incubated with [^{32}P]NAD in the absence (lane 1) or presence of NO (lanes 2 and 3). The arrow at right points to NAP as revealed by autoradiography after SDS/PAGE on a 12.5% gel. Molecular masses in kDa are indicated on the left. NO was derived from SNP (lane 2) or the actions of purified cerebellar NOS (lane 3). In lane 4, NO production was blocked by N^G-nitroarginine (N-Arg), an inhibitor of NOS.

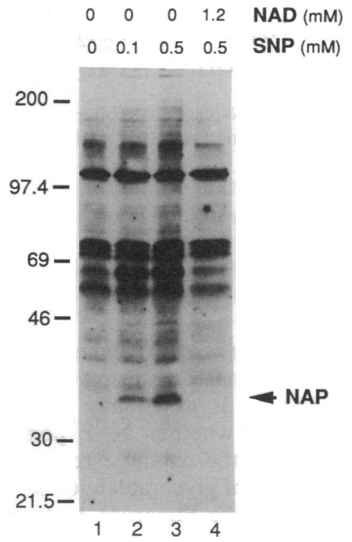

FIG. 2. Labeling NAP with biotin. Biotinylated NAD was used for *in vitro* ADP-ribosylation of NAP. After SDS/PAGE on a 12.5% gel and electrophoretic transfer of proteins to nitrocellulose, biotinylated ADP-ribosylated NAP was detected by horseradish peroxidase-avidin coupled to an ECL system. As indicated by the arrow, ADP-ribosylation of NAP was stimulated with increasing concentration of SNP (lanes 2 and 3). NAD competitively abolished the biotin labeling (lane 4), demonstrating the specificity of ADP-ribosylation. Molecular masses in kDa are indicated at left.

As a potential reagent for purification of ADP-ribosylated proteins, we synthesized NAD with biotin attached to the ADP moiety at the sixth position. We utilized this biotinylated NAD as a substrate for ADP-ribosylation, visualizing ADP-ribosylated substrates with avidin-conjugated horseradish peroxidase (Fig. 2). In the presence of SNP, we detected selective enhancement of ADP-ribosylation of a 37-kDa band whose labeling was abolished by exogenous NAD.

We purified NAP to homogeneity, taking advantage of the high solubility of NAP in ammonium sulfate so that it was recovered in the supernatant fraction after 65% ammonium sulfate precipitation (Fig. 3). Fractionation on a phenyl-Superose column separated unincorporated biotinylated NAD. The final purification step involved elution of NAP from an avidin affinity column with mercuric chloride, which abolished the ADP-ribosylation of NAP. Hydroxylamine did not interfere with the ADP-ribosylation of NAP. Mono-ADP-

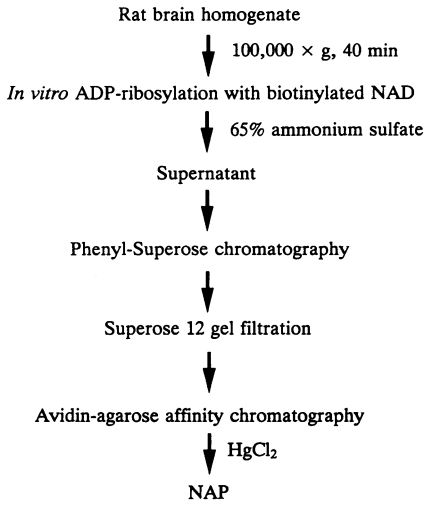

FIG. 3. NAP purification.

	1										11										21								
NAP:	NH_2-	V	K	V	X	V	N	G	F	G	R	I	G	R	L	V	T	R	A	A	F	S	X	D	K	V	D	I	V	A-
GAPDH:	NH_2-	V	K	V	G	V	N	G	F	G	R	I	G	R	L	V	T	R	A	A	F	S	C	D	K	V	D	I	V	A-

FIG. 4. Comparison of N-terminal sequences of NAP and GAPDH. The N-terminal amino acid sequence of NAP was determined by Edman protein sequencing and aligned with the rat GAPDH sequence (19). X denotes an amino acid that could not be determined during sequencing.

ribosylation involves linkages predominantly to arginine, lysine, asparagine, or cysteine. Hydroxylamine selectively reverses interactions with arginine in some proteins (17), whereas mercuric chloride mainly abolishes interactions with cysteine (18). Thus, ADP-ribosylation of NAP presumably occurs on cysteine.

We obtained the amino acid sequence of the N-terminal 29 amino acids by an automated Edman sequencing technique (Fig. 4). The sequence is essentially identical to that of GAPDH. To ascertain whether NAP possesses GAPDH activity, we monitored the catalytic reduction of NAD to NADH upon oxidation of glyceraldehyde 3-phosphate (Fig. 5). Purified NAP from the Superose 12 fraction displayed GAPDH activity with a specific activity for NAP protein identical to that for homogeneous GAPDH. Incubation of this fraction with avidin-agarose did not diminish GAPDH activity. This suggests that the biotinylated, and hence ADP-ribosylated, portion of isolated NAP does not contribute to GAPDH activity.

To confirm that GAPDH itself can be ADP-ribosylated, we utilized a preparation of chicken muscle GAPDH (Fig. 6). We detected ADP-ribosylation of GAPDH in the absence of brain extract and in the absence of SNP. SNP produced a 15-fold enhanced labeling of GAPDH. Deletion of thymidine or Mg^{2+} markedly diminished both basal and SNP-stimulated ADP-ribosylation. We wondered whether chicken GAPDH preparation might be contaminated with an ADP-ribosyltransferase. Accordingly, we repeated these experiments with GAPDH from *B. stearothermophilus* purified by a different procedure than that used for chicken GAPDH. The same amount of ADP-ribosylation was observed with bacterial GAPDH and chicken GAPDH (data not shown). This establishes that GAPDH can auto-ADP-ribosylate and the process is enhanced by NO.

GAPDH is an NAD-dependent enzyme, suggesting that ADP-ribosylation occurs at the NAD recognition site. To determine whether ADP-ribosylation is a general feature of NAD-dependent dehydrogenases, we evaluated four other NAD-dependent dehydrogenases (Table 1). Some ADP-ribosylation of alcohol dehydrogenase was observed in the absence of SNP, but the level of labeling was less than 10% of levels obtained with GAPDH. ADP-ribosylation of alcohol dehydrogenase was markedly enhanced by SNP. No ADP-ribosylation was evident in the absence or presence of SNP for glutamate dehydrogenase, lactate dehydrogenase, or malate dehydrogenase. This comparison demonstrates that GAPDH is selectively targeted for NO-enhanced ADP-ribosylation.

DISCUSSION

The major finding of this study is that NO-enhanced ADP-ribosylation in the brain involves GAPDH. GAPDH appears to be the only protein in the brain whose ADP-ribosylation is stimulated by NO. In peripheral tissues, Brune and Lapetina (13) have also found only a single 37-kDa protein whose ADP-ribosylation is enhanced by NO, and while this work was in preparation, Kots *et al.* (16) observed that SNP stimulates ADP-ribosylation of GAPDH in human erythrocytes. However, we employed a different technique for monitoring ADP-ribosylation that utilizes biotinylated NAD as a substrate and may greatly facilitate the identification and isolation of protein targets of ADP-ribosylation.

A particularly striking finding was that GAPDH is auto-ADP-ribosylated. Other known mammalian proteins that are ADP-ribosylated, such as guanine nucleotide binding proteins and elongation factor 2, do not auto-ADP-ribosylate (20). Further, NO enhances the auto-ADP-ribosylation by acting either upon GAPDH or upon NAD to facilitate cleavage to ADP-ribose. In addition, Tanaka *et al.* (21) reported that ADP-ribose itself can directly attach to skeletal muscle GAPDH.

The reversal of GAPDH ADP-ribosylation by mercuric chloride indicates that ADP-ribosylation involves a cysteine in GAPDH. In the family of GAPDH proteins, only a single cysteine is conserved and this cysteine is located at the catalytic site of the enzyme and binds the NAD substrate (22). Thus, we conclude that ADP-ribosylation takes place at

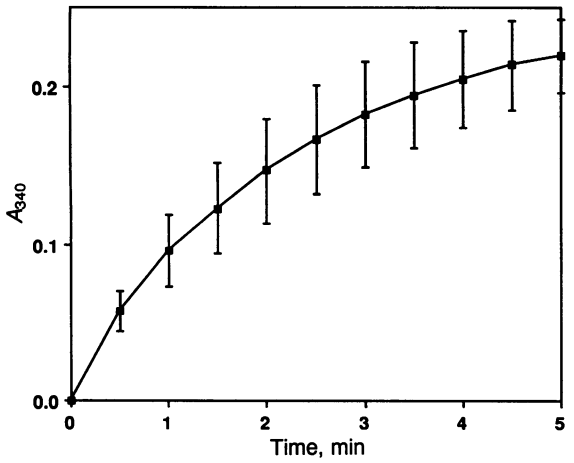

FIG. 5. Assaying NAP for GAPDH enzymatic activity. Purified NAP was incubated with glyceraldehyde 3-phosphate and NAD. The catalytic reduction of NAD to NADH was monitored by the increasing absorbance at 340 nm. The enzymatic activity was plotted as A_{340} vs. time. Each data point is an average of three experiments and the standard deviation is shown with an error bar.

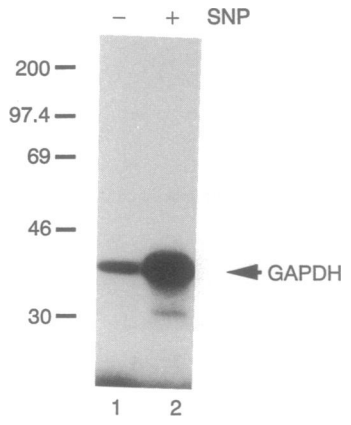

FIG. 6. Auto-ADP-ribosylation of GAPDH. The ability of GAPDH to auto-ADP-ribosylate was tested by incubating pure chicken muscle GAPDH with [^{32}P]NAD in the absence (lane 1) and presence (lane 2) of SNP. ^{32}P-labeled GAPDH was resolved by SDS/PAGE on a 12.5% gel. Shown here is the autoradiograph where NAP (GAPDH) is indicated with an arrow.

Table 1. NO-enhanced ADP-ribosylation of selected dehydrogenases

Dehydrogenase	ADP-ribosylation, %	
	− SNP	+ SNP
GAPDH	0.3	5
ADH	0.01	0.05
GDH	Nondetectable	Nondetectable
LDH	Nondetectable	Nondetectable
MDH	Nondetectable	Nondetectable

To evaluate the specificity of NO-enhanced ADP-ribosylation, five NAD-dependent dehydrogenases were tested. GAPDH, ADH (alcohol dehydrogenase), LDH (lactate dehydrogenase), GDH (glutamate dehydrogenase), and MDH (malate dehydrogenase) were incubated with [^{32}P]NAD in the presence or absence of SNP. ^{32}P-labeled dehydrogenases were resolved by SDS/PAGE. The stimulation of NO on ^{32}P-labeling of each dehydrogenase was quantified by scintillation counting of the ^{32}P-labeled band that was excised by using an autoradiograph of the SDS gel as a template.

the same cysteine where the substrate NAD binds. Accordingly, ADP-ribosylation should terminate enzyme activity.

Does ADP-ribosylation physiologically regulate GAPDH? In our experiments, ≈5% of GAPDH is ADP-ribosylated. This is likely an underestimate, because NAD is known to bind tightly to GAPDH (23) and would compete with the labeled NAD that we add for ADP-ribosylation assays.

What might be physiologic functions of NO-mediated GAPDH ADP-ribosylation? The regional brain localization of guanylyl cyclase, one physiologic target of NO, differs markedly from NOS (10) and closely matches that of heme oxygenase 2, the enzyme that generates carbon monoxide (24). Potent and selective inhibitors of heme oxygenase deplete endogenous cGMP in brain extracts, suggesting that carbon monoxide, rather than NO, is the principal endogenous regulator of cGMP levels in most parts of the brain. If GAPDH is ADP-ribosylated *in vivo*, then its isoelectric point (pI) should display heterogeneity, which is consistent with the observation that human brain GAPDH has subspecies with various pI values (25).

GAPDH might mediate neurotoxicity associated with *N*-methyl-D-aspartate receptor activation and NO (11). cGMP is not likely involved in this process, as 8-bromo-cGMP does not elicit neurotoxicity and inhibitors of guanylyl cyclase fail to block it (12). Inhibition of GAPDH would diminish the amount of ATP formed from glycolysis and decrease the flow of substrates to the electron transport chain. These effects might produce a greater depletion of ATP than the NO-mediated inhibition of iron-sulfur proteins of the citric acid cycle and respiratory chain (3).

Although ADP-ribosylation by bacterial toxins has been extensively elucidated, mammalian ADP-ribosyltransferases have only recently been characterized (20) and, heretofore, the best studied substrates have been elongation factor 2, guanine nucleotide binding proteins, and structural proteins. GAPDH may provide a target whereby ADP-ribosylation could influence energy metabolism.

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