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Nitric Oxide Causes ADP-ribosylation and Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase*

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Nitric oxide and nitric oxide-generating agents like 3-morpholinosydnonimine (SIN-1) stimulate the mono-ADP-ribosylation of a cytosolic, 39-kDa protein in various tissues. This protein was purified from human platelet cytosol by conventional and fast protein liquid chromatography techniques. N-terminal sequence analysis identified the isolated protein as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Nitric oxide stimulates the auto-ADP-ribosylation of GAPDH in a time and concentration-dependent manner with maximal effects after about 60 min. Associated with ADP-ribosylation is a loss of enzymatic activity. NAD^+-free enzyme is not inhibited by SIN-1, indicating the absolute requirement of NAD^+ as the substrate of the ADP-ribosylation reaction. Inhibition of the glycolytic enzyme GAPDH may be relevant as a cytotoxic effect of NO complementary to its inhibitory actions on iron-sulfur enzymes like aconitase and electron transport proteins of the respiratory chain.

Adenosine diphosphate ribosyltransferases (EC 2.4.2.30) are known to catalyze the transfer of ADP-ribose from NAD^+ to proteins. Such reactions represent versatile mechanisms for the post-translational modification of proteins and have been identified in phages, bacteria, and eukaryotic cells (1, 2). Poly(ADP-ribosyl)transferases catalyze the transfer of many ADP-ribose residues resulting in the modification of nuclear proteins with ADP-ribose polymers (3, 4). In contrast, mono-ADP-ribosyltransferases transfer only one single ADP-ribose group to the acceptor protein and are most clearly defined in the action of certain bacterial toxins on animal cells (5–8). Cellular endogenous mono-ADP-ribosyltransferases are currently divided into three different types. One group of enzymes modifies elongation factor 2 (9), which also is known to be auto-ADP-ribosylated. A second group ADP-ribosylates arginine residues (10), while a third group transfers ADP-ribose to cysteine residues (11, 12). The physiological role of endogenous ADP-ribosylation largely remains unknown.

The conversion of L-arginine to the short-lived free radical nitric oxide and citrulline is an ubiquitous biochemical pathway that has been identified in various tissues and cell types (13). Activation of nitric oxide synthase (14) in its various isoforms (15) produces NO, initially characterized as the endothelium-derived relaxing factor (16, 17). NO with its physiological actions maintains the vascular tone, regulates blood pressure, inhibits platelet activation, and functions as a signaling pathway in the nervous system (18–20). These effects are mediated by binding NO to the heme subunit of soluble guanylate cyclase, thus increasing cellular cGMP (21).

On the other hand, as a macrophage-derived molecule, NO is linked to pathophysiological and cytotoxic effects (22), such as tumor (23) and pancreatic islet cell killing (24) and possibly mediating the neurotoxicity of glutamate (19, 25). Cytotoxic effects of NO are thought to be mediated by intracellular iron loss (22) and the destruction of iron-containing enzymes, including mitochondrial electron transfer proteins and aconitase.

Recently we demonstrated new effects of NO, independent of the known action on guanylate cyclase and cGMP (26, 27). NO-generating agents like sodium nitroprusside or 3-morpholinosydnonimine (SIN-1)[1] were shown to stimulate the endogenous mono-ADP-ribosylation of a cytosolic 39-kDa protein in various tissues. ADP-ribosylation was also observed in rat cerebellum and HL-60 cells, when NO was released from its physiological source L-arginine by the enzymatic activity of the NO synthase (28), indicating that it can proceed under physiological conditions. NO-stimulated ADP-ribosylation was recently confirmed by Duman *et al.* (29) using cerebral cortex homogenates. All previous studies used cytosolic fractions to investigate the NO effect, not allowing a discrimination between the enzyme itself and the substrate of the reaction. However, all experiments suggested a stimulation of an endogenous enzymatic ADP-ribosylation reaction can occur. To further characterize the ADP-ribosylation reaction and to explore the identity of the 39-kDa protein, we purified the cytosolic protein to homogeneity. Sequence analysis of the N-terminal portion revealed its identity as GAPDH.

EXPERIMENTAL PROCEDURES

Purification of the 39-kDa Protein—The 39-kDa protein was purified using platelet-rich plasma from outdated blood samples provided by the German Red Cross. After centrifugation at 800 × *g* for 20 min, thrombocytes were resuspended in Tyrode-Hepes buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM NaH_2PO_4, 1 mM $MgCl_2$, 25 mM Hepes, 1 mM phenylmethylsulfonyl fluoride) (pH 7.4) and sonified with a Branson sonifier (10 times for 30 s; 60-s cooling intervals; output 3). After 100,000 × *g* centrifugation and ADP-ribosylation of the protein in order to follow purification (detection of radioactivity), the 50–90% (NH_4SO_4) precipitation was collected, resuspended in 10 mM sodium phosphate buffer (pH 9.0), and desalted with a Sephadex G-25 column, at a flow rate of 2 ml/min. Subsequently, the protein was applied to a Q-Sepharose (fast flow) column and eluted with 10 mM sodium phosphate buffer, pH 9.0. The pH of the collected protein was adjusted to pH 7.0, and the protein was applied to the cation

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[1] The abbreviations used are: SIN-1, 3-morpholinosydnonimine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

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exchange column S-Sepharose (fast flow) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The 39-kDa protein was eluted using the same buffer, with 100 mM NaCl added. The protein was desalted using a Sephadex G-25 column and loaded to a blue Sepharose CL-4B column, equilibrated with 10 mM sodium phosphate buffer (pH 7.0), and eluted with 1 M NaCl. The purified protein was stored at −80 °C.

The purity of the purified protein was determined by 10% SDS-polyacrylamide gel electrophoresis (30) and silver staining.

ADP-ribosylation—ADP-ribosylation of the unlabeled protein was carried out essentially as outlined in Ref. 26, but the system was optimized for enzymatic GAPDH activity. Therefore the assays (30 min, 37 °C) contained 30 μg of GAPDH, 50 mM triethylammonium (pH 7.6), 50 mM arsenate, 100 μg/ml glyceraldehyde 3-phosphate, 2.4 mM glutathione, 1 μM NAD^+, and 0.5 μCi of [^{32}P]NAD^+/assay (40).

Studies on the stability of the ADP-ribose-protein bond were accomplished according to Aktories *et al.* (31). Briefly, the trichloroacetic acid precipitate of the ADP-ribosylation reaction was washed with water-saturated ether, resuspended in 100 mM Hepes buffer pH 7.5, and incubated for 30–60 min in the presence of 2 mM $HgCl_2$ or 2 mM NaCl as a control, respectively. After precipitation, proteins were resolved on SDS gels.

Gels were either subjected to autoradiography or radioactivity was quantified using the phosphor image system (Molecular Dynamics) (32). Protein was determined by the Bradford assay (40).

GAPDH Activity Determination—GAPDH activity was monitored by the reduction of NAD^+ to NADH, recording the fluorescence signal above 430 nm after excitation at 313 and 366 nm, respectively. Assays contained triethylammonium, arsenate, glutathione, and glyceraldehyde 3-phosphate as indicated for ADP-ribosylation. Incubation was carried out in the presence of 7.5 μg of GAPDH (rabbit muscle enzyme) at 37 °C for the times indicated with 1 μM NAD^+ as the substrate for ADP-ribosylation added, before starting the enzymatic assay by adding 250 μM NAD^+.

The back reaction of GAPDH was measured in the presence of 50 mM triethylammonium buffer (pH 7.6), 3.3 mM $MgCl_2$, 2.4 mM reduced glutathione, 1.5 mM ATP, 7 mM glycerol 3-phosphate, 4.5 units of phosphoglycerate kinase, and 0.8 units of GAPDH (10 μg/ml) in a final volume of 1 ml. After a preincubation period of 30 min at 37 °C with 1 μM NAD^+ added, the reaction was started with 100 μM NADH.

In order to prepare a NAD^+-free enzyme, we followed the protocol of Taylor *et al.* (33).

Materials—GAPDH from human erythrocytes and glyceraldehyde 3-phosphate were purchased from Sigma. SIN-1 was provided by Cassella-Pharma, Frankfurt, Germany, while [^{32}P]NAD (800 Ci/mmol) was ordered from Du Pont-New England Nuclear. All column material was bought from Pharmacia LKB Biotechnology Inc. Other chemicals were of the highest grade of purity available, mainly delivered by Boehringer Mannheim or other local commercial sources.

RESULTS AND DISCUSSION

In previous work (26), we measured protein ADP-ribosylation by incorporation of [^{32}P]ADP-ribose from [^{32}P]NAD^+ into a 39-kDa cytosolic protein under the influence of NO-liberating agents in various tissues.

For the isolation and identification of the 39-kDa protein, human platelet cytosol was incubated with [^{32}P]NAD^+, and the labeled protein was separated by conventional and fast protein liquid chromatography techniques as outlined under "Experimental Procedures." Starting with 1 g of human platelet cytosolic protein we succeeded in obtaining 450 μg of homogeneous protein as shown by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 1, *lanes A* and *B*). Additionally, two-dimensional gel electrophoresis confirmed no impurity of the isolated 39-kDa protein.

The purified unlabeled protein when incubated with [^{32}P] NAD^+ incorporated radioactivity at a low basal rate (Fig. 1, *lane C*), but the compound SIN-1, known to release NO spontaneously, greatly increased this labeling (Fig. 1, *lane D*).

Sequencing (477A gas phase sequenator, Applied Biosystems) of the isolated, homogeneous protein shown in Fig. 1, immobilized on siliconized glass fiber (Glasibond Biometra) in order to desalt the preparation, established the identity of the first 29 residues (Gly-Lys-Val-Lys-Val-Gly-Val-Asn-Gly-Phe-Gly-Arg-Ile-Gly-Arg-Leu-Val-Thr-Arg-Ala-Ala-Phe-Asn-Ser-Gly-[Lys]-Val-Asp-Ile-) with the exception of uncertainty at Lys-26. For sequencing, about 10 μg of homogeneous protein per lane, judged by Coomassie Blue staining, was subjected to electrophoresis on a 10% SDS gel, followed by blotting the protein band onto a glass fiber membrane. Sequencing was performed with an initial yield of 50% and a repetitive yield of 96.5%. Based on comparisons with known sequences (NBRF data base, Washington, D.C.), a match with the N terminus of human GAPDH (EC 1.2.1.12) was observed. The identity of the 39-kDa protein with GAPDH (36 kDa) was confirmed by measuring NADH formation from NAD^+ and glyceraldehyde 3-phosphate with the isolated platelet protein. The specific activity was 76 μmol × mg^{-1} protein × min^{-1} at 37 °C, compared with a specific activity of 40–80 units/mg at 25 °C using human erythrocyte enzyme. Furthermore, the identity of the 39-kDa protein with GAPDH was proven by immunoprecipitation of the radiolabeled protein with polyclonal antibodies raised against GAPDH.

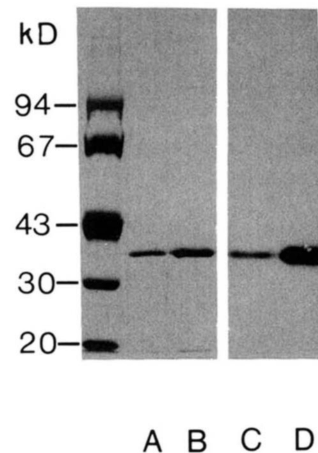

FIG. 1. **Purification and auto-ADP-ribosylation of the 39-kDa protein.** *Lanes A* and *B* show a silver stain of the purified 39-kDa protein (*A*, 0.25 μg of protein; *B*, 0.5 μg of protein) separated by 10% SDS-polyacrylamide gel electrophoresis. *Lanes C* and *D* show an autoradiography of the auto-ADP-ribosylated protein (*C*, basal activity; *D*, stimulated activity in the presence of 200 μM SIN-1).

Commercially available GAPDH from rabbit muscle as well as from human erythrocytes showed an identical incorporation behavior of ^{32}P label from [^{32}P]NAD^+ in the presence of SIN-1 indicating an auto-ADP-ribosylation mechanism. The NO donor stimulated the ADP-ribosylation of the purified protein in a dose-dependent manner with concentrations of 400–500 μM proving to be optimal. A representative time course of this reaction with rabbit muscle GAPDH is shown in Fig. 2.

SIN-1 led to observable incorporations of radioactivity after about 10 min; the reaction was completed within 60 min. In the absence of SIN-1, a slow basal incorporation of radioactivity was noticeable in line with the observations of the 39-kDa platelet protein in Fig. 1. Generally, there is a 5–7-fold differential between controls and experimental samples with respect to ADP-ribosylation after 45–60 min. Replacing the NO donor SIN-1 by sodium nitroprusside, another compound known to release NO spontaneously, resulted in the same stimulatory efficiency concerning the auto-ADP-ribosylation reaction of GAPDH (data not shown). Furthermore, authentic NO solutions stimulated the auto-ADP-ribosylation of GAPDH. In this case, half of the incubation volume was replaced by previously degassed and then NO-saturated water.

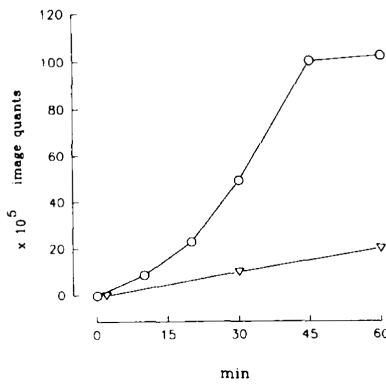

FIG. 2. **Auto-ADP-ribosylation of GAPDH.** Auto-ADP-ribosylation of GAPDH was carried out as described under "Experimental Procedures." *Triangle*, basal ADP-ribosylation; *circle*, auto-ADP-ribosylation in the presence of 500 μM SIN-1. Similar results were obtained in three other experiments.

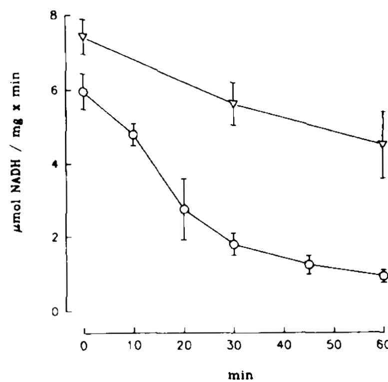

FIG. 3. **Inhibition of GAPDH activity by the NO donor SIN-1.** GAPDH activity was monitored following the reduction of NAD^+ to NADH as outlined under "Experimental Procedures." *Triangle*, basal activity; *circle*, enzyme activity in the presence of 500 μM SIN-1. Values represent means ± S.D. of three to five individual experiments.

It was essential to study the effect of ADP-ribosylation on GAPDH activity which is complicated by the fact that NAD^+ also is the substrate for the enzyme. Since the time course of the NAD^+-dependent ADP-ribosylation is slow, however, compared with the duration of the optical test for NADH formation, it was possible to correlate the NO-catalyzed modification of the enzyme with its enzyme activity (Fig. 3).

GAPDH activity slowly decreased in the absence of SIN-1. In the presence of NO, enzyme inhibition was greatly accelerated approximating 80% inhibition after 60 min. Under these conditions, an IC_{50} value for GAPDH inhibition around 100–200 μM SIN-1 was determined with 400–500 μM SIN-1 being optimal. Obviously, addition of SIN-1 caused only a minor immediate inhibition of GAPDH as seen in Fig. 3. Generally, there is a 4–6-fold difference between controls and experimental samples with respect to specific GAPDH activity after 45–60 min. Again employing sodium nitroprusside under the same assay conditions showed a similar inhibitory effect on enzymatic GAPDH activity (data not shown). When measuring the back reaction with NADH, ATP, glycerol 3-phosphate, and phosphoglycerate kinase by GAPDH no inhibition by NO was observed, and also no ADP-ribosylation of the enzyme was detectable. High ATP and NADH concentrations, necessary for the reverse enzymatic reaction, were shown to inhibit the transfer of ADP-ribose.

Since commercially available GAPDH preparations still contain high amounts of NAD^+ (33), it was necessary to prepare an NAD^+-free form of the enzyme. To further link ADP-ribosylation and enzyme inhibition, we studied GAPDH activity using the NAD^+-free enzyme compared with the enzyme with bound NAD^+. As expected, the NAD^+-free enzyme was not inhibited at all (Table I) when preincubated in the presence of 250 μM SIN-1 for 30 min, compared with the NAD^+-containing form of the enzyme.

If the NAD^+-free form of GAPDH is supplemented with NAD^+ during the preincubation period, the ability of SIN-1 to inhibit GAPDH is restored almost completely. Therefore, enzyme inhibition by NO requires the presence of NAD^+.

A cysteine residue of GAPDH is the probable target for ADP-ribosylation, because *N*-ethylmaleimide prevented labeling of the protein by ADP-ribosylation (27). Cysteine-149 is close to the NAD^+ binding site and is also responsible for GAPDH sensitivity against –SH modification (34). In order to test the hypothesis of a cysteine residue being involved, we took advantage of the recent finding that the ADP-ribose-cysteine bond is sensitive to mercury ions (31). Treatment of ADP-ribosylated GAPDH with 2 mM $HgCl_2$ for 1 h (as outlined under "Experimental Procedures") largely removed the ADP-ribose residue compared with control incubations carried out in the presence of 2 mM NaCl. $HgCl_2$ treatment removed 90 ± 4.4% (mean ± S.D., $n = 4$) of the label compared with NaCl treatment (100% control reference value), consistent with a cysteine as the target of ADP-ribosylation.

Our results provide evidence for a novel NO-catalyzed auto-ADP-ribosylation of the important glycolytic enzyme GAPDH. Although this reaction leads to inactivation of the enzyme, a direct and unequivocal proof for a physiological, cytotoxic action is difficult to obtain. Previously (26) addressing this reaction as stimulation of an endogenous ADP-ribosyltransferase we now identify this process as an auto-ADP-ribosylation reaction stimulated by NO. This reaction results in a nonenzymatic posttranslational protein modification. Further experiments will explore whether *S*-nitrosylation as previously reported for protein SH groups (35) is involved in the NO-stimulated ADP-ribosylation. NO as a ligand to heme and non-heme proteins is involved in several important biological events. NO functions as an activator of soluble guanylate cyclase, a signal-transducing enzyme, and as an effector molecule in cytotoxicity. Enhancing auto-ADP-ribosylation of GAPDH in a probably nonenzymatic fashion may contribute to the role of NO as a cytotoxic effector

TABLE I

Effect of SIN-1 on GAPDH activity using a complete and NAD^+-free form of the enzyme

Preparation of the NAD^+-free form of GAPDH and GAPDH activity were measured as outlined under "Experimental Procedures." The enzyme (complete enzyme, NAD^+-free form of GADPH, and the NAD^+-free enzyme with NAD^+ added) was preincubated for 30 min at 37 °C in the presence of the NO donor SIN-1 and 1 μM NAD^+ as indicated before 250 μM NAD^+ was supplemented to start the enzymatic reaction. Results (mean ± S.D., $n = 4$) are compared with control incubations incubated for 30 min without further additions.

	GAPDH activity
	% of control
Control	100
GAPDH (complete) + 250 μM SIN-1 + 1 μM NAD^+	37.5 ± 5.7
GAPDH (NAD^+-free) + 250 μM SIN-1	96.5 ± 6.4
GAPDH (NAD^+-free) + 250 μM SIN-1 + 1 μM NAD^+	56.9 ± 7.1

molecule. To explain a potential cytotoxic NO effect, other investigators have suggested that NO effectively interferes with iron enzymes such as aconitase or the iron-sulfur proteins of the respiratory chain (36, 37). However, in the case of NO-mediated inhibition of hepatocyte protein synthesis, a blocking of mitochondrial respiration could be excluded (38). The autoribosylation of GAPDH would be an alternative mechanism to explain such NO effects. Indeed, by inhibiting glycolysis in parallel to the respiratory chain and aconitase in the citric acid cycle, the cellular energy production would be efficiently reduced. Currently, we are trying to establish a link between glucose oxidation and ADP-ribosylation in β-cells which have been reported to be destroyed in the presence of NO (24).

The question of how to separate beneficial and regulatory roles of NO from its cytotoxic actions arises. One possible explanation lies in the different NO synthases from which only the high capacity, cytokine-inducible enzyme may be responsible for cytotoxicity, whereas the constitutive enzyme mediates cGMP-dependent actions.

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Addendum—While this manuscript was under revision a similar finding was reported by Kots *et al.* (39).

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