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Nitric oxide preferentially stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase compared to alcohol or lactate dehydrogenase

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Recently we demonstrated that the radical nitric oxide (NO) stimulates the auto-ADP-ribosylation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in enzyme inhibition. To further characterize this auto-ADP-ribosylation reaction we studied alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) for comparison. Whereas auto-ADP-ribosylation of ADH was stimulated to a minor extent by the NO-liberating agent 3-morpholinosydnonimine (SIN-1), LDH was unaffected. The susceptibility of dehydrogenases towards auto-ADP-ribosylation correlated with the potency of NO to decrease enzyme activity. Again, GAPDH was much more sensitive compared to ADH, whereas LDH again was unaffected. Interestingly, the efficiency of the SH-alkylating agent *N*-ethylmaleimide (NEM) to inhibit the enzymatic activity of the chosen dehydrogenases correlates with the sensitivity of dehydrogenases towards NO. These studies demonstrate the requirement of a reactive SH-group besides the NAD^+ binding site as a prerequisite for NO-stimulated auto-ADP-ribosylation reactions. Furthermore, we establish that under physiological conditions and among the dehydrognases tested, only GAPDH is a potential target for this post-translational protein modification mechanism.

Mono-ADP-ribosylation; Dehydrogenase; Nitric oxide

1. INTRODUCTION

Nitric oxide (NO) is produced in various mammalian cells by the enzymatic activity of NO-synthases, catalysing the generation of NO from the terminal guanidino nitrogen atom of L-arginine [1,2]. On one hand, NO produced by the calcium regulated constitutive NO-synthase is involved in many physiological processes, including regulation of vascular tone and platelet aggregation, furthermore functioning as a signaling pathway in the nervous system [2–4]. In this respect NO actions are mediated by binding to the iron-containing heme group of soluble guanylyl cyclase, thus increasing cGMP levels [5]. On the other hand, large amounts of NO are produced under pathophysiological conditions by the cytokine inducible NO-synthase [6]. Cytotoxic effects of NO are mediated by this long-lasting, high-output system. NO functions as a cytotoxic effector molecule by destructing several iron-containing enzymatic activities including aconitase [7], mitochondrial electron transfer proteins [8] and ribonucleotide reductase [9].

Besides interfering with heme and non-heme iron containing enzymes, we reported that NO stimulates the auto-ADP-ribosylation of the glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [10,11]. NO induces the transfer of ADP-ribose from NAD^+ to a cysteine residue of GAPDH, thereby inhibiting enzyme activity [12]. Although the exact reaction mechanism is not yet fully understood, NO first seems to interact with a cysteine residue, possibly at the active site, and in a second step promotes the ADP-ribose transfer reaction from NAD^+, which is bound close to the active cysteine group at the nearby NAD+ binding site [12].

To further characterize the auto-ADP-ribosylation reaction of GAPDH, we studied other dehydrogenases in order to determine the requirement of a reactive SH-group in combination with a nearby NAD^+ binding site as a prerequisite for this new type of NO-stimulated post-translational protein modification mechanism.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]NAD^+ (800 Ci/mmol) was purchased from Du Pont-New England Nuclear. SIN-1 was provided by Cassella-Pharma, Frankfurt, Germany, while ADH and GAPDH were from Sigma. All other chemicals were of the highest grade of purity available, mainly delivered by Boehringer Mannheim, Germany.

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; NEM, *N*-ethylmaleimide; TCA, trichloroacetate; SIN-1, 3-morpholinosydnonimine.

2.2. *Enzyme activity determination*

GAPDH activity was measured as outlined in reference [11]. Assays contained 50 mM triethylamine (pH 7.6), 50 mM arsenate, 100 μg/ml glyceraldehyde 3-phosphate and 2 mM glutathione. Incubations were carried out in the presence of 400 mU of rabbit muscle GAPDH at 37°C for 30 min with 1 μM NAD^+, as the substrate for the ADP-ribosylation reaction, before starting the enzymatic assay by the addition of 250 μM NAD^+.

ADH activity was monitored following the reduction of NAD^+ to NADH, recording the fluorescence signal above 430 nm after excitation at 313 and 366 nm, respectively. 10 mU ADH were preincubated for 30 min at 37°C in the presence of 100 mM sodium phosphate buffer (pH 8.5), 0.6 M ethanol, 6.2 mM semicarbazide and 1 μM NAD^+ as the substrate for the ADP-ribosylation reaction. The enzymatic reaction was started with 200 μM NAD^+.

LDH activity was determined by incubating 0.5 mU LDH in 0.1 M triethylammonium-buffer (pH 8.0) containing 1 mM EDTA, 1 mM lactate and 1 μM NAD^+ to allow the ADP-ribosylation reaction for 30 min at 37°C. Starting the enzymatic LDH reaction by adding 400 μM NAD^+ the reduction of NAD^+ to NADH was followed as described above.

2.3. *Pretreatment with* N-*ethylmaleimide (NEM)*

The different enzymes were preincubated as described above with increasing concentrations of NEM. After 10 min at 37°C the enzymatic reaction was started by the addition of NAD^+.

2.4. *[^{32}P]ADP-ribosylation*

ADP-ribosylation was mainly carried out as previously described [11]. The enzymes (10 μg/assay) were incubated in 100 mM HEPES (pH 7.5), 0.5 μCi [^{32}P]NAD^+, 1 μM cold NAD^+ and in the absence or presence of 250 μM SIN-1 for 30 min at 37°C. For determination of the auto-ADP-ribosylation of GADPH 1 mM DTT was added, to prevent autoxidation of the enzyme. After protein precipition (10% TCA final), resulting pellets were washed twice with cold water-saturated-ether and were resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel [13]. Radioactivity was quantified using the phosphor image system (Molecular Dynamics) [14].

3. RESULTS AND DISCUSSION

NO spontaneously released from SIN-1 has been shown to stimulate the auto-ADP-ribosylation of GAPDH in a concentration-dependent fashion [11,12]. Incubation of GAPDH with [^{32}P]NAD^+ resulted in a low basal incorporation of [^{32}P]ADP-ribose into GAPDH, whereas addition of 250 μM SIN-1 greatly enhanced the auto-ADP-ribosylation reaction of the enzyme (Fig. 1). Generally, stimulation by 250 μM SIN-1 led to a 5-fold increased incorporation of radioactivity compared to control incubation receiving no SIN-1 (Table I). Looking at GAPDH enzyme activity, known to be inhibited by the described NO-stimulated auto-ADP-ribosylation reaction [12], we detected a decreased enzymatic activity (Table I).

Crystallographic studies revealed that the coenzyme binding domain of GAPDH is structurally similar to corresponding domains of equine liver alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) [15]. Furthermore these dehydrogenases also contain SH-groups at or near their active sites [16], suggesting that NO might be able to stimulate the transfer of ADP-ribose from NAD^+ to these dehydrogenases as well.

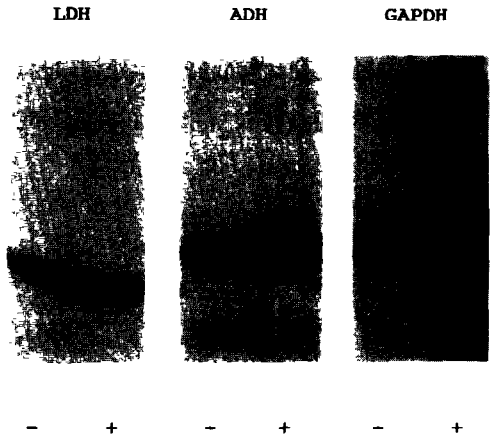

Fig. 1. Auto-ADP-ribosylation of various dehydrogenases. [^{32}P]ADP-ribosylation of different dehydrogenases (lactate dehydrogenase (LDH); alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) in the presence or absence of 250 μM SIN-1 was carried out as described in section 2. The figure is representative for three similar experiments.

To determine the ability of alcohol dehydrogenase to be ADP-ribosylated in the presence of NO, 10 μg homogeneous ADH was incubated with [^{32}P]NAD^+ as described in section 2. As shown in Fig. 1 a low basal incorporation of radioactivity was detected in control incubations (without NO-donor), whereas addition of 250 μM SIN-1 resulted in a minor, roughly 2-fold increased incorporation of [^{32}P]ADP-ribose into the protein (Table I and Fig. 1). Using rat liver cytosol instead of purified enzyme we observed an incorporation of [^{32}P]ADP-ribose in a 40 kDa protein stimulated by SIN-1. Since the molecular mass of 40 kDa is identical with the subunit molecular mass of ADH, the protein eventually becomes a target for ADP-ribosylation in the cytosolic fraction. Recently it has been reported that

Table I

Effect of SIN-1 on auto-ADP-ribosylation and enzymatic activity of different dehydrogenases.

	Radioactivity (% control)	Enzyme activity (% control)
LDH	100	100
LDH + 250 μM SIN-1	126 ± 14	97 ± 15.9
ADH	100	100
ADH + 250 μM SIN-1	172 ± 36	47.5 ± 6.2
GAPDH	100	100
GAPDH + 250 μM SIN-1	504 ± 192	37.5 ± 5.7

Auto-ADP-ribosylation and enzymatic activity of lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase Auto-ADP-ribosylation and enzymatic activity of lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined as described in section 2. Values represent mean ± S.E. of three individual experiments.

several mitochondrial dehydrogenases are affected in their activity by pathophysiological conditions [17], probably involving NO. This prompted us to investigate the effect of SIN-1 on ADH-activity as well. Measuring ADH activity after allowing ADP-ribosylation to take place by incubation with 250 μM SIN-1 we observed a 50% decreased enzymatic activity (Table I). Even higher concentrations of SIN-1 or employing another NO-donor like sodium nitroprusside did not result in enhanced inhibition under these conditions (data not shown). In comparison to GADPH, ADH is less susceptible to NO stimulated ADP-ribosylation and to a similar degree, enzymatic activity is less affected by NO. Similar experiments were carried out using lactate dehydrogenase. Interestingly, almost no stimulation of ADP-ribosylation of LDH was detected by addition of 250 μM SIN-1 (Fig. 1) and furthermore, LDH activity was completely unaffected by NO, released from 250 μM SIN-1 (Table I).

To explain the differences in the accessibility of the chosen dehydrogenases towards ADP-ribosylation associated with a variable degree of enzyme inhibition we tried to correlate the reactivity of the essential cysteine groups in the enzyme to their sensitivity towards NEM.

As shown in Fig. 2 preincubation of GAPDH with low concentrations *N*-ethylmaleimide (NEM) before starting the enzymatic reaction resulted in inhibition of GAPDH activity at low concentrations of the SH-alkylating reagent. A concentration of 50 μM NEM totally inhibited enzyme activity. Incubating ADH with the same concentration of NEM revealed only a minor decrease in ADH activity (about 20% inhibition), and no complete inhibition of ADH activity was achieved at higher concentrations of NEM (Fig. 2). Noteworthy, addition of relatively high concentrations of NEM did not affect LDH activity (Fig. 2). To sum up, LDH was neither inhibited by NEM nor by the NO donor SIN-1. Furthermore, NO-stimulated auto-ADP-ribosylation of LDH was not detectable.

Recently we reported that the NO stimulated ADP-ribosylation of GADPH is enhanced by the addition of physiological concentrations of reducing agents such as glutathione (GSH) [18]. For this reason we investigated the ADP-ribosylation of the chosen dehydrogenases also in the presence of dithiothreitol or GSH. Incubating ADH now with 250 μM SIN-1, [^{32}P]NAD$^+$ and 1 mM DTT did not result in any increased incorporation of radioactivity at all, compared to the assay when DTT was omitted (data not shown). In respect to LDH, the basal auto-ADP-ribosylation of LDH was even decreased in the presence of reducing agents (data not shown). Probably the SH-groups of the reducing agents may have a stronger affinity towards NO compared to the essential SH-group of ADH. This may result in *S*-nitrosylation of the reducing agent and therefore may prevent the NO-stimulated ADP-ribosylation of the enzyme. However, reducing conditions are necessary to

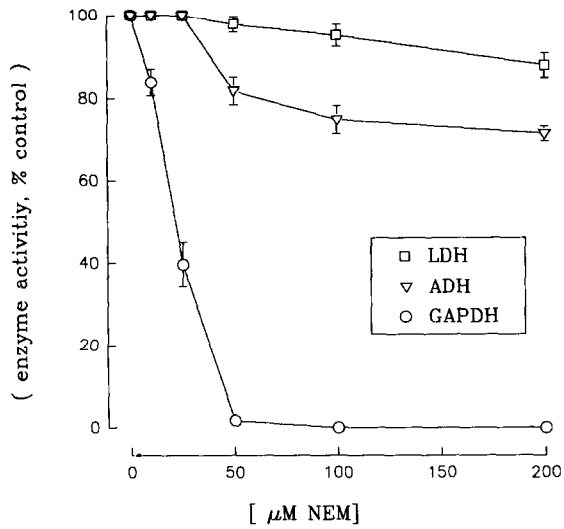

Fig. 2. Inhibition of dehydrogenase activity by *N*-ethylmaleimide (NEM). After preincubating the chosen dehydrogenases with increasing concentrations of NEM (10 min, 37°C) the enzymatic activities were determined as outlined in section 2. Data are means ± S.E. from three different experiments.

prevent oxidation of GAPDH and ADP-ribosylation of GAPDH only takes place in the presence of DTT or GSH [18]. Therefore the essential thiol group in GAPDH is much more susceptible to ADP-ribosylation and is a much more likely target under more physiological conditions compared to ADH or LDH.

Since the NAD$^+$ binding site of the different dehydrogenases is almost similar, we propose that the accessibility of the SH-group under our conditions seems to be an important restriction for the ADP-ribosylation reaction. This assumption is supported by our finding, that stimulation of the ADP-ribosylation reaction and enzyme inhibition brought about by NO correlates with the efficiency of NEM to cause similar enzyme inhibition.

The efficiency of NEM to block NO-stimulated auto-ADP-ribosylation of GAPDH has been demonstrated in the past, for the basal as well as for the stimulated ADP-ribosylation reaction [12,18]. Here we show, that the sensitivity of different dehydrogenases towards NEM correlates with the ability of NO to cause ADP-ribosylation of the chosen enzymes. Considering a reactive SH group as well as a NAD$^+$-binding site as a prerequisite for this reaction, we now clearly demonstrate that the reactivity of the accessible SH-group is another important regulatory component. The SH-group of LDH near the active site may be masked and therefore no cleavage of NAD$^+$ could be catalyzed by NO.

In conclusion, NO-stimulated auto-ADP-ribosylation is not a nonspecific reaction carried out by all dehydrogenases, only GAPDH and, to a minor extent, ADH in the absence of reducing cofactors seem to be

susceptible to NO-stimulated ADP-ribosylation and enzyme inhibition. Inhibition of GAPDH-activity by exogenous addition or endogenously generated NO recently was demonstrated using the β-cell line RINm5F [19]. These findings indicate a possible link between NO-stimulated auto-ADP-ribosylation of GAPDH, enzyme-inhibition and cytotoxic NO effects. NO mediated cytotoxicity towards pancreatic β-cells is related to inhibition of mitochondrial respiration [20] and inhibition of glycolysis [19]. Therefore, altered energy supply and cytotoxic NO effects are possibly correlated.

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