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Unexpected stimulation of mitochondrial ADP-ribosylation by cyanide

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Cyanide, the classical inhibitor of the mitochondrial respiratory chain at site III, stimulates ADP-ribosylation of a number of mitochondrial proteins, the major protein being the 50–55 kDa band. Sodium azide, sharing the same inhibitory site, does not have the same effect. Rotenone or antimycin A have no influence on mitochondrial ADP-ribosylation. Data suggest that no apparent correlation exists between oxidoreductase function and protein ADP-ribosylation. Purified nuclear poly(ADP-ribose) polymerase activity was not affected by cyanide. The cyanide effect on mitochondrial ADP-ribosylation seems intriguing and may be attributed to NAD^+-CN complex formation, since NAD reacts with cyanide at pH >8 with *N*-substituted nicotinamide which may prevent inhibition of ADP-ribosylation.

ADP-ribosylation; ADP-ribosyltransferase; Mitochondria; Cyanide; Cu-Zn superoxide dismutase

1. INTRODUCTION

Recently, cellular ADP-ribosylation has received considerable attention because it is agreed that it constitutes one of the pathways of post-translational protein modification [1–3]. In this process adenosine diphosphate ribosyltransferase (EC 2.4.2.30) catalyzes the cleavage of NAD^+, thereby transferring the ADP-ribose moiety to proteins. Poly(ADP-ribosylation) appears to be involved in DNA repair and in cell differentiation [4] while mono(ADP-ribosylation) occurs in eukaryotes and bears some resemblances to the action of bacterial toxins. Thus, one of the pathways of signal transduction is believed to be operated via ADP-ribosylation, although its precise molecular mechanism remains unclear [6]. There is general agreement regarding nuclear ADP-ribosylation while the nature of ADP-ribosylation in mitochondria has been questioned [7,8]. In view of the fact that mitochondria have their own distinct genetic machinery [9], their bacterial origin continues to be debated [10] and the presence of an ADP-ribosyltransferase system in mitochondria appears a logical corrollary. Moreover, when NAD^+ glycohydrolase activity was inhibited, the transfer of ADP-ribose from NAD^+ to mitochondrial proteins still occurred [11,24].

Here, mitochondrial ADP-ribosylation is shown to be stimulated by potassium cyanide. It is intriguing that this cyanide effect is not shared by other mitochondrial respiratory chain inhibitors (azide included). Cyanide does not affect nuclear or cytoplasmic ADP-ribosylation.

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2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria and mitoplasts from rat liver*

Mitochondria were isolated from male Wistar rat liver. All animals were fasted overnight prior to decapitation. Livers were removed, cut into small pieces and washed with isolation medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. Livers were homogenized in the same medium, with a motor-driven Potter Elvehjem homogenizer with 10 up-down strokes. The homogenate was diluted to 10% (w/v) with isolation medium and centrifuged twice at $1000 \times g$ for 10 min to remove debris and the nuclear fraction. The resulting supernatant was centrifuged at $10000 \times g$ for 10 min. The pellet was suspended

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in isolation medium and recentrifuged at 10000 × *g* for 10 min. This was repeated twice. The final mitochondrial pellet was suspended in the homogenizing medium to give approx. 50 mg/ml protein.

Mitochondria were incubated with digitonin for 15 min at 4°C according to Schnaitman and Greenwalt [12]; the suspension was diluted with 3 vols isolation medium and centrifuged at 15000 × *g* for 15 min. The resulting pellet was resuspended in 3 vols isolation medium and centrifuged again. The final pellet contained all the inner membrane and matrix and was considered as mitoplasts.

2.2. *Preparation of mitochondria free of lysosomes*

Male rats (200–250 g, Wistar strain) were injected intraperitoneally with 85 mg Triton WR-1339 per 100 g body wt for 3–4 days prior to decapitation. The isolation of mitochondria was as described above. Mitochondria were layered onto a linear density gradient of 25–45% sucrose containing 5% dextran T-10, 20 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The tubes were centrifuged in a Beckman SW41 rotor for 112 min at 36000 rpm [13]. Mitochondria constituted the middle band in the gradient. Lysosomes were found in the upper band while peroxisomes sedimented to the bottom of the centrifuge tube. Mitochondria were carefully removed with a Pasteur pipette, finally washed twice and suspended in isolation medium to a protein concentration of approx. 50 mg/ml. The isolation of mitoplasts from this mitochondrial preparation was carried out as above.

2.3. *ADP-ribosylation assay*

ADP-ribosyltransferase activity was determined by the incorporation of [^{32}P]ADP-ribose from [^{32}P]NAD into protein precipitable by cold trichloroacetic acid. The reaction mixture contained: 10 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 0.4 mM dithiothreitol and 100 μM [^{32}P]NAD (100 cpm/pmol) in a volume of 125 μl. The reaction was carried out for 10 min at 37°C and was terminated by the addition of 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The precipate was collected and washed and radio-activity determined as in [14].

One enzyme unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol ADP-ribose into acid-insoluble material per 10 min at 37°C.

2.4. *Preparation of radiolabelled ADP-ribose*

[^{32}P]ADP-ribose was prepared according to Payne et al. [15], the ADP-ribosylation assay being performed as described above.

2.5. *Polyacrylamide gel electrophoresis*

Lithium dodecyl sulfate gel electrophoresis was performed according to Jones et al. [16], using 10% acrylamide at a constant current of 15 mA for 16 h. SDS gel electrophoresis was performed essentially according to Laemmli [17], employing 10% acrylamide plus 0.1% SDS. Mitochondria or mitoplasts were incubated with [^{32}P]NAD for 60 min at 37°C and proteins were precipitated with trichloroacetic acid. The prepcipitate was centrifuged. The pellet was washed twice with diethyl ether, dried and supended in the respective solubilization buffer for 2 h at room temperature before electrophoresis [16,17]. The gels were stained with 0.05% Coomassie brilliant blue R-250 in 40% methanol and 5% acetic acid. For autoradiography, gels were exposed to Kodak XAR 5 film at −70°C.

3. RESULTS

Treatment of mitochondria or mitoplasts from rat liver with rotenone, antimycin A or sodium azide did not influence ADP-ribosylation activity. Potassium cyanide, however, markedly stimulated ADP-ribosylation in both mitochondria and mitoplasts (table 1). The lack of effect of sodium azide and stimulation with cyanide on mitochondrial ADP-ribosylation is intriguing since both azide and cyanide share the common inhibitory site III in the mitochondrial respiratory chain. This led us to investigate possible cyanide inhibitory centres in mitochondria. In view of the fact that Cu-Zn superoxide dismutase [18] prevents ADP-ribosylation stimulated by active oxygen species and since liver mitochondrial preparations not free of lysosomes are likely to contain Cu-Zn superoxide dismutase [13], the first step was to remove lysosomes from mitochondria using sucrose-dextran T-10 gradient centrifugation. These data are presented in table 2. The stimulatory influence of cyanide on ADP-ribosylation was further enhanced after lysosome removal from mitochondria and with the mitoplasts prepared from them.

Fig.1 illustrates various proteins ADP-ribosylated in mitochondria and in mitplasts with or without cyanide. In the absence of cyanide

Table 1

Influence of respiratory chain inhibitors on ADP-ribosylation in mitochondria or mitoplasts

Inhibitors	pmol ADPR incorporated from [^{32}P]NAD per 10 min at 37°C	
	Mitochondria	Mitoplasts
None	35.1	35.2
Rotenone	28.7	37.4
Antimycin A	31.2	30.6
Sodium azide	30.4	29.2
Potassium cyanide	135.4	172.5

Mitochondria or mitoplasts were used at 500 μg protein per assay medium. Rotenone (4 μM), antimycin A (5 μM) sodium azide (1 mM), potassium cyanide (5 mM) were added. The reaction was started with [^{32}P]NAD and terminated by adding 10% chilled trichloroacetic acid. Values are means of three separate experiments with standard deviations ranging between 5 and 10%

Table 2

Cyanide stimulation of ADP-ribosylation in mitochondria free of lysosomes

	pmol ADPR incorporated from [^{32}P]NAD per 10 min at 37°C		% increase compared with mitochondria not freed from lysosomes
	−KCN	+KCN	
Mitochondria	41.85	177.4(431)	42
Mitoplasts	46.4	242.2(521)	33

Mitochondria were freed of lysosomes as described in section 2. Mitoplasts were prepared using 0.12 mg digitonin per mg mitochondrial protein. Other experimental conditions were the same as in table 1 including cyanide concentration. The values in parentheses show the percentage increase due to the cyanide effect.

significant protein ADP-ribosylated is observed at 50–55 kDa (fig.1B, lanes 1,3). When cyanide was added to the assay medium, either containing mitochondria or mitoplasts, a number of minor proteins were ADP-ribosylated (fig.1B, lanes 2,4). However, the extent of ADP-ribosylation of the 50–55 kDa protein band was distinctly enhanced by cyanide.

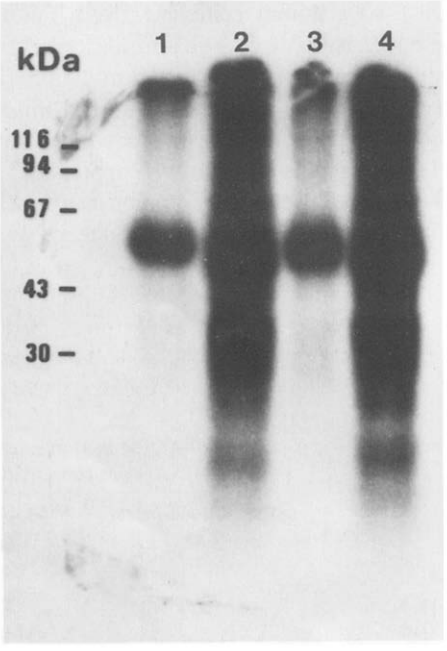

Fig.1 LiDS-polyacrylamide gel electrophoresis of mitochondria or mitoplasts demonstrating protein ADP-ribosylation. Polyacrylamide gel was run for 16 h at 15 mA. Autoradiogram of the gel exposed to Kodak XAR5 film. Lanes: 1, mitochondria; 2, mitochondria + 5 mM potassium cyanide; 3, mitoplast, 4, mitoplast + 5 mM potassium cyanide.

In lysosome-free mitochondria an almost equal effect of cyanide on ADP-ribosylation was seen (fig.2A,B). This effect was further examined on both mitochondria and mitoplasts at two concentrations of NAD (10 and 100 μM). Cyanide increased ADP-ribosylation irrespective of the amount of NAD employed in the assay medium. Fig.2B illustrates protein ADP-ribosylation in mitochondria (lane 1, 10 μM NAD; lane 2, 100 μM NAD) and mitoplasts (lane 3, 10 μM NAD; lane 4, 100 μM NAD). The effect of cyanide (under similar conditions) is shown in lanes 5 and 6 (as in lanes 1

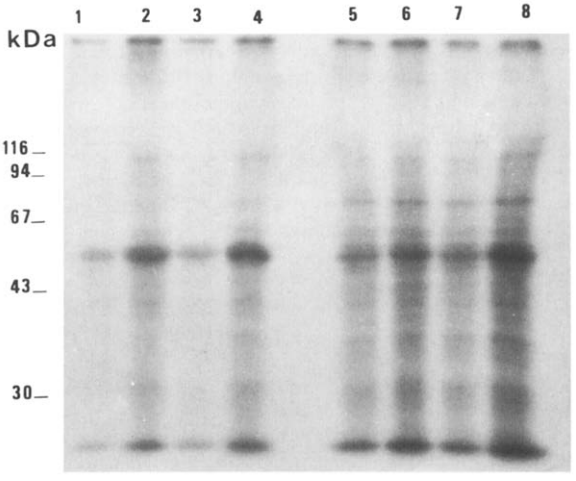

Fig.2 SDS-polyacrylamide gel electrophoresis of mitochondrial preprations freed of lysosomes [12] illustrating protein ADP-ribosylation. Autoradiogram of the gel. Lanes: 1, mitochondria + 10 μM NAD; 2, mitochondria + 100 μM NAD; 3, mitoplasts + 10 μM NAD; 4, mitoplasts + 100 μM NAD; 5,6, 5 mM potassium cyanide as in 1,2, respectively; 7,8 contained 5 mM potassium cyanide as in 3,4 respectively.

and 2, respectively) and in lanes 7 and 8 (as in lanes 3 and 4, respectively).

It has been reported that ADP-ribosylation can occur in mitochondria by non-enzymatic transfer of ADP-ribose to protein acceptors [8]. When mitochondria were incubated with [^{32}P]ADP-ribose in the presence of cyanide, there was no cyanide stimulation of ADP-ribosylation (not shown).

4. DISCUSSION

In an attempt to elucidate the controversy concerning ADP-ribosylation in mitochondria [7,8] data are presented here demonstrating that cyanide stimulates ADP-ribosylation in mitochondria or mitoplasts (inner mitochondrial membrane preparation stripped of outer membrane). Cyanide is a potent irreversible inhibitor of cytochrome *c* oxidase, i.e. site III of the mitochondrial respiratory chain. Another site III inhibitor, sodium azide, was observed to exercise no influence on mitochondrial ADP-ribosylation. Similarly, rotenone which specifically inhibits site I (i.e. NADH and flavoprotein) or antimycin A, the classical site II inhibitor (i.e. at the cytochrome *b* level), showed no effect whatsoever on ADP-ribosylation of mitochondrial protein (table 1). The major protein ADP-ribosylated in the absence of cyanide was at 50–55 kDa while the cyanide stimulated ADP-ribosylation of a number of minor proteins (see fig.2). These minor bands, however, were not apparently ADP-ribosylated in the absence of cyanide.

Furthermore, the lack of cyanide stimulation of mitochondrial ADP-ribosylation with free ADP-ribose suggests that this effect is specific and not due to chemical ADP-ribosylation in mitochondria. This observation furter supports the view that the major pathway of ADP-ribosylation in mitochondria is catalyzed enzymatically [11,24].

Since it is known that cyanide reacts with NAD [18], the validity of the observed cyanide effect may be questioned. The possibility that the nicotinamide-cyanide complex formed as a result of the NAD^+-CN^- complex after ADP-ribosylation reaction may not be able to inhibit ADP-ribosylation and hence the observed stimulation remains open.

It is pertinent to discuss the role of Cu-Zn superoxide dismutase in mitochondrial ADP-ribosylation, since it is well known that if mitochondria have not been freed of contaminating lysosomes, Cu-Zn superoxide dismutase is present in mitochondrial preparation [13] and this enzyme is sensitive to cyanide [20]. In view of reports that active oxygen species stimulate nuclear ADP-ribosylation which is reversible by Cu-Zn superoxide dismutase [19], it is logical to question if cyanide stimulation of ADP-ribosylation is due to inhibition of Cu-Zn superoxide dismutase by cyanide. To address this question mitochondria were freed of contaminating lysosomes employing sucrose-dextran T-10 gradient centrifugation [13]. Mitoplasts were derived from lysosome-free mitochondria and the influence of cyanide was examined on ADP-ribosylation in both mitochondria and mitoplasts. Cyanide further increased by 42 and 33% ADP-ribosylation in mitochondria and mitoplasts devoid of lysosomes, compared with lysosome-contaminated preparations (table 2). It has been shown [21] that superoxide anion causes nuclear DNA strand breakage and consequent enhancement of ADP-ribosylation in nuclei. Whether the same mechanism operates in mitochondria remains to be elucidated.

It has also been reported that pentobarbital, another inhibitor of the mitochondrial respiratory chain at site I, induces neobiogenesis of mitochondria with enhancement of mitochondrial DNA synthesis [22]. Barbiturate has also been shown to increase nuclear (ADP-ribosyl) transferase with covalent modification of histones, particularly histone H1 [23]. The increase in ADP-ribosylation is involved in cell proliferation and DNA replication. It is possible that the inhibition of the mitochondrial respiratory chain by cyanide stimulates the initiation of mitochondrial neobiogenesis – in parallel with an enhancement of ADP-ribosylation – as has been shown for pentobarbital [22].

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