NUCLEAR POLY (ADPR) AND MONO (ADPR) RESIDUES IN TISSUES WITH DIFFERENT GROWTH RATES

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1. Introduction

Poly(ADPR) is formed in eukaryotes by a nuclear enzyme with NAD as a substrate [1–3]. Its localization, its association with nucelar proteins [4], its influence on DNA polymerase activity [5–7], the stimulation of poly(ADPR) formation in vitro by exogenous DNA [1,8], and the inhibition of poly(ADPR) degradation in vitro by DNA [9,10] are consistent with the postulate that the polymer might be involved in the regulation of DNA synthesis and cell proliferation [5,6]. This interpretation has been questioned on the basis of similar enzymic activities per unit DNA for the formation and degradation of poly (ADPR) [7,11], and the inability of poly(ADPR) to suppress endogenous DNA polymerase activity in lymphoid cells [12]. On the other hand, in stimulated lymphocytes [13], as well as in regenerating rat liver [11], poly(ADPR) synthetase activity per unit DNA increased significantly, which, however, did not correlate with DNA synthesis. When poly(ADPR) levels were determined by an isotope dilution method, rather similar values were obtained for adult and neonatal rat liver tissues [14,9]. This method was suited to detect ADPR residues from poly(ADPR) down to the dimer only. Therefore, the possibility of a shift from (relatively few) polymer chains to (many) oligomeric or monomeric chains as a regulatory mechanism related to cell proliferation has not been excluded. Such an explanation was first suggested by H. Grunicke at the poly(ADPR) workshop in Hamburg (1972).

In addition, Dietrich et al. [15] recently reported on the determination of mono (ADPR) residues formed in adult rat liver nuclei which can be rendered acid soluble by NH_2OH as described first by Hayaishi and coworkers [16]. Dietrich et al. interpreted their data as indicating that most of the radioactivity bound to nuclear material after incubation with [^{14}C]NAD is not poly(ADPR) but protein-bound monomeric ADPR. They were also not able to detect any poly(ADPR) in rat liver nuclei isolated after in vivo injection of $^{32}P_i$ [17]. This paper demonstrates that in isolated rat liver nuclei considerable degradation of poly(ADPR) takes place even at 0°C, and during incubation with 0.4 M NH_2OH. It is also shown, that after exclusion of enzymic degradation subsequent to incubation, mono(ADPR) residues were nearly absent in Ehrlich ascites carcinoma nuclei, low in neonatal, and only moderate (1/5 of total ADPR residues) in adult rat liver.

2. Material and methods

[^{3}H]NAD labeled in the adenine moiety was prepared as described previously [7]. Alkaline phosphatase and snake venom phosphodiesterase were products from E. Merck (Darmstadt). The latter was freed of slight phosphatase contamination by chromatography on Whatman DE-cellulose (G.R. Philipps, personal communication). NMN, NAD, NAD-pyrophosphorylase and pyrophosphatase were obtained from Boehringer (Mannheim), [^{3}H]ATP from Buchler–Amersham (Braunschweig).

Preparation of nuclei: The nuclei of rat liver (adult: 220 g male rats; neonatal; 1 day old) were prepared according to Blobel and Potter [18]. Nuclei of Ehrlich ascites carcinoma cells (6 days after transplantation into Balb/c mice) were prepared from washed cells as described previously [7].

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Incubation of nuclei: Nuclei (3-5 mg protein) were incubated in a total volume of 1.0 ml containing 0.05 M Tris–HCL buffer pH 8.2; 0.014 M $MgCl_2$; 0.037 M KCl; 5×10^{-4} M [^{3}H]NAD (1.3×10^7 dpm), at 25°C for 10 min, and immediately precipitated with cold perchloric acid (3% final concentration) unless otherwise stated. They were washed three times with cold 3% perchloric acid. Aliquots of the acid insoluble residue were hydrolyzed in 3% perchloric acid at 95°C for 30 min, and analyzed for dpm in a BF Betascint 5000 connected to a Diehl Algotronic computer (Berthold and Friesecke, Karlsruhe).

NH_2OH-treatment: An aliquot of the acid insoluble nuclear residue (1–2 mg protein) was suspended in 0.5 ml 0.4 M NH_2OH pH 7.5 (freshly prepared) without significant change of the pH value, and incubated for 10 min at 25°C. Kinetic analyses of the NH_2OH reaction revealed completion within 5 min. Cold perchloric acid was added to a final concentration of 3%. After standing in ice for 10 min, the precipitate was removed by centrifugation, hydrolyzed in 3% perchloric acid and analyzed for radioactivity as described above. The supernatant was carefully adjusted to a final pH of 4–6 by the addition of KOH, and centrifuged.

Chromatographic analysis of NH_2OH-sensitive (ADPR) residues was performed by putting an aliquot of the supernatant onto chromatography paper (2043b, Schleicher and Schüll, Dassel, Germany), together with 100 nmoles each of ADPR, AMP and adenosine, and developed in the isobutyric acid/NH_3 system [20]. Five mm strips of the dried chromatogram were cut out, hydrolyzed with 0.5 ml 0.1 N HCl for 60 min at 100°C in a counting vial, and finally analyzed for radioactivity.

DNA was determined according to Burton [21].

3. Results and discussion

3.1. *In washed liver nuclei, poly(ADPR) is degraded during treatment with NH_2OH*

Rat liver nuclei were incubated with labeled NAD and freed from the incubation mixture by centrifugation. When resuspended in a solution containing 50 mM Tris buffer pH 7.4 and 1 M NaCl, one third of the total acid insoluble radioactivity (= (ADPR) residues) was transformed into an acid soluble form over a period of 10 hr at 0°C (table 1). Further incubation for 30 min at 30°C led to a loss of nearly 60% of the labeled acid insoluble material. These observations point to an enzymic degradation of poly(ADPR) and oligo(ADPR) to acid soluble split products even at 0°C and even in the presence of high salt concentrations. This was confirmed by experiments in which nuclei after incubation with labeled NAD either were precipitated and washed with perchloric acid in order to inactivate nuclear enzymes (experiment A), or were washed successively with the incubation medium at 25°C as described by Dietrich et al. [15] (experiment B). In experiment C, the washed nuclei of B were incubated for an additional 60 min at 25°C. The nuclei of the three experiments were subsequently subjected to 0.4 M NH_2OH treatment for 30 min at 37°C [15], cooled, and mixed with perchloric acid. When the supernatant was analyzed for the labeled material rendered acid soluble by NH_2OH (fig. 1A–C), it became apparent that during incubation with NH_2OH, extensive enzymic degradation of oligo(ADPR) units to AMP and adenosine (or ribosyl adenosine) had taken place, which was especially pronounced in experiment C. It should be pointed out, that NH_2OH treatment of acid-inactivated nuclei liberated little AMP. It therefore appears, that AMP and adenosine in the fraction rendered acid soluble by NH_2OH treatment are indicative of enzymic degradation of poly and oligo(ADPR) residues.

Table 1
Loss of acid insoluble ADPR residues in non-inactivated nuclei

Treatment after incubation	Acid insoluble ADPR residues (cpm/nucleus)	(1%)
None	3.95 ± 0.66	100
10 hr at 0°C in 1 M NaCl	2.57 ± 0.13	65
Additional 30 min at 30°C	1.65 ± 0.03	43

Liver nuclei from adults rats were incubated for 45 min as described in methods, centrifuged, resuspended in 1 M NaCl–50 mM Tris acetate pH 7.4, and kept for 10 hr at 0°C. An aliquot was incubated for an additional 30 min at 30°C. Aliquots of the nuclear suspension were put on filter paper, processed in TAC-alcohol–ether, and counted for (acid insoluble) radioactivity.

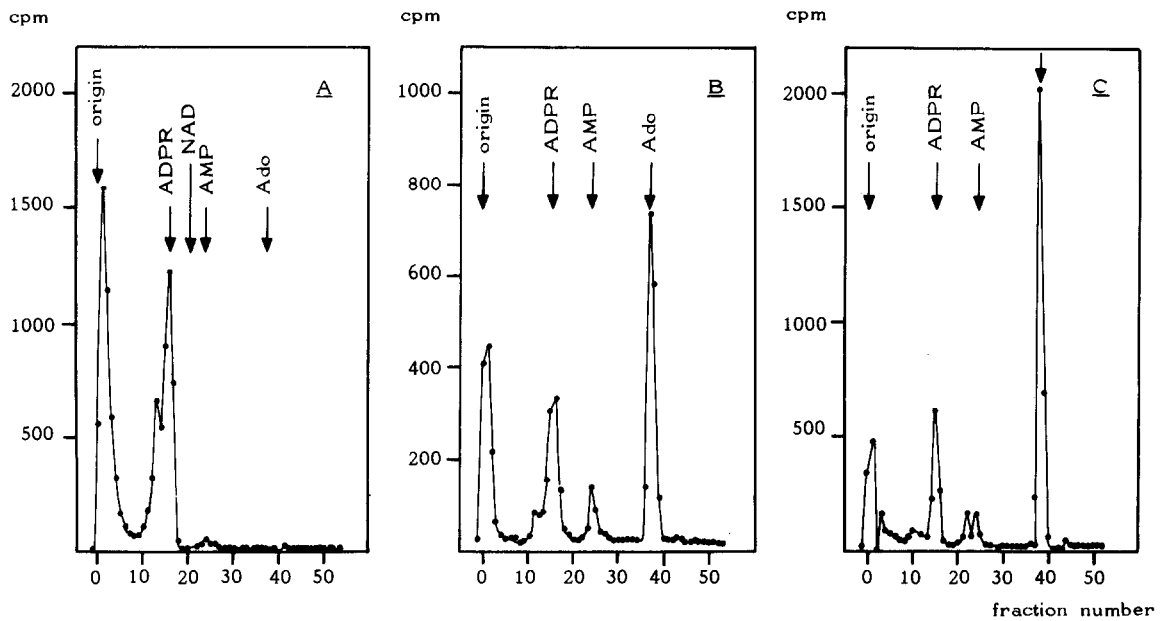

Fig. 1. Degradation of oligo(ADPR) residues in non-inactivated nuclei. Adult rat liver nuclei were incubated with [^{3}H]NAD for 10 min as described in methods. An aliquot was inactivated and washed with perchloric acid (A), another aliquot washed 5 times acc. to [15] with incubation medium at 25°C (B), or treated as in (B) plus further incubation for 60 min at 30°C (C). Nuclei were centrifuged down, and treated with NH_2OH. Chromatographic analysis of the fraction rendered acid soluble by this treatment was performed as described in methods.

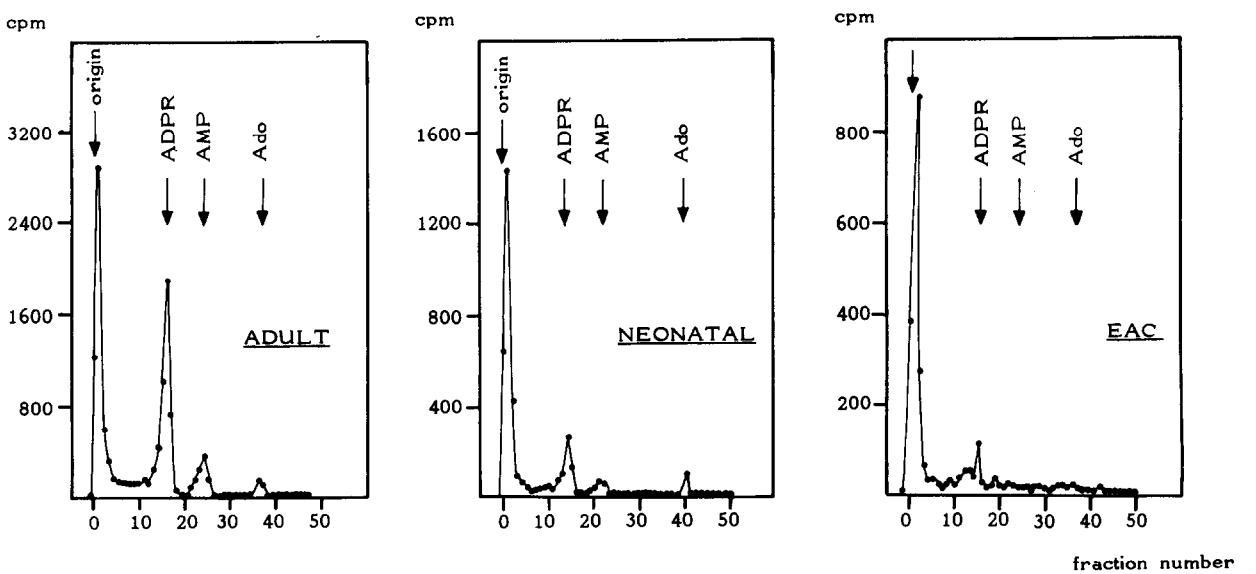

Fig. 2. NH_2OH-released mono(ADPR) residues in different tissues. Nuclei of the different tissues were incubated with [^{3}H]NAD for 10 min, precipitated and washed with perchloric acid. Chromatographic analysis of the NH_2OH-released material was performed as described in Material and methods.

3.2. *The ratio of NH_2OH-sensitive oligo(ADPR) to mono(ADPR) residues differs in proliferating versus non-proliferating tissues.*

When nuclei from adult and neonatal rat livers, and from Ehrlich ascites carcinoma (EAC) cells were analyzed for (ADPR) residues labile to NH_2OH, remarkable differences emerged (fig. 2): whereas in adult liver nuclei a considerable amount of the material rendered acid soluble by NH_2OH was in the form of single ADPR residues, the identity of which was confirmed by enzymic analysis with snake venom phosphodiesterase, and alkaline phosphatase, respectively, (Stone et al. [19]), this fraction was strongly reduced in neonatal liver nuclei in favor of oligo (ADPR) residues which moved only slightly from the starting point of the chromatogram. In the nuclei of fast growing EAC cells, very little mono(ADPR) was found, oligo(ADPR) being the only major labeled species rendered acid soluble by NH_2OH. A relationship similar to the mono(ADPR) applies also to AMP, which was highest in adult liver, and lowest in EAC nuclei.

Quantification of these differences was accomplished by extraction of the labeled spots according to the UV absorption of carrier substances. When the data were based on the DNA content of the nuclear preparations and compared to the total acid insoluble (ADPR) residues, it became apparent (table 2) that total (ADPR) residues did not differ significantly in adult versus neonatal liver thus confirming our previous observations [7]. Also, oligo (ADPR) released on NH_2OH treatment exhibited identical levels in both tissues. Drastic differences, however, were found in mono(ADPR) moieties rendered acid soluble by incubation with NH_2OH. An inverse relationship existed with the rate of cellular proliferation. In non-proliferating adult liver, mono(ADPR) residues comprised one fifth of the total acid soluble material. Its proportion was reduced by a factor of 3 in proliferating neonatal liver nuclei. In tumor tissues (EAC), mono(ADPR) residues could barely be detected ($< 2\%$ of total).

The data presented above were obtained under conditions preventing enzymic degradation after the ten minute incubation period of the nuclei with labeled NAD. With adult liver nuclei, it resulted in substantially lower mono(ADPR) values than those obtained by Dietrich et al. with non-inactivated nuclei. Rather rapid degradation of poly(ADPR) in non-inactivated nuclei is also indicated by the strongly elevated formation of AMP and adenosine (or ribosyl adenosine) under the conditions of NH_2OH incubation when compared to perchloric acid-inactivated nuclei. The time-dependent formation of these metabolites of poly(ADPR) appears to be indicative for enzymic degradation during analysis. It cannot be excluded, therefore, that the relatively high levels of mono(ADPR) in adult rat liver are the result of an artefactual degradation of newly synthesized poly(ADPR) during the 10 min incubation period. Any method allowing enzymic attack after killing the cell or the animal (cf. [17]), then, must be considered inadequate for the determination of poly(ADPR), and especially its monomeric form, the existence of which is significant amounts in vivo has yet to be proven.

Table 2
Distribution of labeled (ADPR) residues in nuclei of different tissues after incubation with [^{3}H]NAD

Tissue	Σ (ADPR) residues (dpm/μg DNA)	NH_2OH-released oligo (ADPR) residues (dpm/μg DNA)	(% of Σ)	NH_2OH-released mono(ADPR) residues (dpm/μg DNA)	(% of Σ)
Adult liver	1970 ± 400	650 ± 120	35	430 ± 40	22
neonatal liver	1750 ± 270	656 ± 24	37	124 ± 4	7
EAC	820 ± 250	186 ± 13	23	14 ± 3	2

Nuclei were prepared and incubated with [^{3}H]NAD, as described in methods. In the case of EAC nuclei, final [^{3}H]NAD concentration was 1 mM instead of 0.5 mM. The perchloric acid inactivated nuclei were treated with NH_2OH, and the fraction rendered acid soluble was chromatographed. Oligo(ADPR) remained at the origin, while mono(ADPR) had an R_f-value identical with authentic ADPR (cf. fig. [1] or [2]. For further details see Material and methods. EAC = Ehrlich ascites carcinoma.

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