ADP-ribosyl–protein conjugate subclasses in various tissues

Specific influence of thyroid hormone on liver conjugates

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The amounts of endogenous mono(ADP-ribose)–protein conjugates and their hydroxylamine-sensitive and hydroxylamine-resistant subfractions in various tissues of the mouse were determined and compared with poly(ADP-ribose) conjugates. Total mono(ADP-ribose) conjugates did not correlate with poly(ADP-ribose) residues or the cellularity of a tissue, but were related to the protein content and the amounts of $\text{NAD}^+ + \text{NADH}$. The hydroxylamine-resistant subfraction, which in liver is mainly associated with the mitochondria [Adamietz, Wielckens, Bredehorst, Lengyel & Hilz (1981) *Biochem. Biophys. Res. Commun.* **101**, 96–103], did not correlate with the tissue content of cytochrome *c* oxidase. In hypothyroid mice hydroxylamine-resistant mono(ADP-ribose) conjugates of the liver were increased by a factor of two while the hydroxylamine-sensitive conjugates did not change significantly under these conditions. Upon administration of thyroxine the hydroxylamine-resistant subfraction returned to normal.

Recent data on the alteration of tissue levels of mono(ADP-ribose) and poly(ADP-ribose) protein conjugates under various metabolic and growth conditions indicated independent changes and therefore independent functions of these ADP-ribosyl–protein subclasses (Wielckens *et al.*, 1981; Garte-mann *et al.*, 1981; Hilz *et al.*, 1982). This interpretation was supported by an analysis of the subcellular distribution of (ADP-ribose)_n–protein conjugates in different tissues, which indicated that the majority of the mono(ADP-ribose)–protein conjugates is located extranuclearly, while most poly(ADP-ribose) conjugates are found in the nucleus (Adamietz *et al.*, 1981). Furthermore, the mono(ADP-ribose) conjugates could be subdivided into hydroxylamine-sensitive and hydroxylamine-resistant conjugates (Bredehorst *et al.*, 1978), the NH_2OH -resistant mono(ADP-ribose)–protein conjugates being located mainly in mitochondria (Adamietz *et al.*, 1981). It therefore seemed of interest to study the distribution of (ADP-ribose)_n–protein conjugate subclasses in various tissues to gain more information pertinent to the possible functions of these subclasses.

The present paper describes the quantification of endogenous mono(ADP-ribose)–protein conjugate subfractions from various tissues of the mouse and compares it with poly(ADP-ribose) residues in some

of these tissues. It also analyses the influence of the thyroid status on liver mono(ADP-ribose) conjugate levels.

Materials and methods

Materials

Nucleotides and reagents were obtained from E. Merck (Darmstadt) and from Boehringer (Mannheim). [^3H]Poly(ADP-ribose) of high specific radioactivity (approx. 24 Ci/mmol) was prepared according to Wielckens *et al.* (1981). 5'-[^3H]AMP (specific radioactivity 17 Ci/mmol) and [2,8- ^3H]ATP (specific radioactivity 51 Ci/mmol) were obtained from Amersham–Buchler (Braunschweig, Germany).

Animals and organs

Male Balb C mice (8 months, average weight 46 g) were killed by cervical dislocation, the organs removed and immediately frozen in liquid nitrogen. Pooled organs from four to eight animals were pulverized while frozen and extracted at 0°C with 20% (w/v) trichloroacetic acid (10 ml/g of tissue; Ultraturrax homogenizer, Jahnke and Kunkel, Freiburg, Germany). The supernatant obtained by centrifugation was freed from the acid by extraction with diethyl ether and was used for determination of

NAD. The pellet was washed three times with 20% (w/v) trichloroacetic acid, twice with ethanol and twice with diethyl ether. The dry powder was kept at -20°C .

Analytical determinations

Mono(ADP-ribose)-protein conjugates and their subfractions (NH_2OH -sensitive and NH_2OH -resistant conjugates) were determined as described previously (Bredehorst *et al.*, 1978, 1981a). Briefly, the mono(ADP-ribose) groups were released from the acceptors and concomitantly converted to 5'-AMP by treatment with alkali [total mono(ADP-ribose) residues], or released by NH_2OH treatment, separated by acid extraction and subsequently converted to 5'-AMP by alkaline treatment. The difference between total and NH_2OH -released (ADP-ribose) residues represents the NH_2OH -resistant subfraction. The procedure was verified for two of the mouse organs (heart and brain). For this purpose, (ADP-ribose) groups were released from the proteins, and purified by h.p.l.c. prior to quantification in the radioimmunoassay. As shown in Table 1, prior purification led to values nearly identical with those obtained by the 'direct' quantification.

Poly(ADP-ribose) residues were released from the proteins by alkali treatment, purified by covalent chromatography on a boronate matrix, converted to the specific derivative phosphoribosyl-AMP by phosphodiesterase I treatment, and quantified by radioimmunoassay as described previously (Wielckens *et al.*, 1981).

NAD and $\text{NAD}^+ + \text{NADH}$ were determined from the same acid extract, as previously reported (Bredehorst *et al.*, 1981a). Briefly, NAD was specifically converted to 5'-AMP with the intermediate formation of ADP-ribose by alkaline treatment and analysed by radioimmunoassay using 5'-AMP-specific antibodies. NADH was first transformed into ADP-ribose by incubation of the acid

extracts at 37°C , and subsequently, treated with alkali to effect conversion of ADP-ribose and of NAD to 5'-AMP.

DNA was quantified by the method of Burton (1968), and protein by the method of Lowry *et al.* (1951).

Results and discussion

Mono(ADP-ribose)-protein conjugates in various tissues

Quantification of total mono(ADP-ribose)-protein conjugates in various tissues of the mouse revealed marked differences (Table 2). When based on wet weight, liver, heart, kidney and spleen had values significantly higher than the remaining tissues. However, when related to the DNA content (i.e. cell number), liver and heart were the only organs with high mono(ADP-ribose)-protein conjugates.

Subdivision of tissue mono(ADP-ribose) conjugates into NH_2OH -sensitive and NH_2OH -resistant fractions showed unequal distribution among the different organs (Table 3). In most tissues, NH_2OH -

Table 2. *Mono(ADP-ribose)-protein conjugates in various organs of mice*

Mean values from two to four experiments, based on duplicate determinations from pooled organs of four to eight animals each.

Organ	Mono(ADP-ribose) residues	
	(nmol/g of tissue)	(nmol/mg of DNA)
Liver	13.06 ± 2.86	6.19 ± 1.36
Heart	11.23 ± 5.92	8.44 ± 4.93
Kidney	8.51 ± 1.60	2.66 ± 0.50
Spleen	12.12 ± 1.98	0.72 ± 0.11
Jejunum	4.68 ± 1.54	0.99 ± 0.32
Brain	2.29 ± 0.45	1.42 ± 0.28
Testes	1.76 ± 0.74	0.55 ± 0.23

Table 1. *Quantification of protein-bound mono(ADP-ribose) residues with and without prior purification by h.p.l.c.* The acid-insoluble fraction of pooled heart and brains from four animals was treated with NH_2OH as described in the Materials and methods section. Aliquots of the NH_2OH extracts were quantified in triplicate either directly (see 'Methods'), or separated by h.p.l.c. (Waters, model 6000A) on a Bondapak C18 column, using an exponential gradient of 20 mM-phosphate/tetrabutylammonium chloride (pH 6.0) to 50% (v/v) methanol in 20 mM-phosphate/tetrabutylammonium chloride (pH 6.0). The fractions corresponding to ADP-ribose were then analysed by radioimmunoassay $\pm$ prior treatment with alkali [to convert (ADP-ribose) to immunoreactive 5'-AMP] as performed in the standard test (Bredehorst *et al.*, 1981a). n.d., not determined.

Test	Tissue	ADP-ribose equivalents (pmol/100 μl)	
		Without alkaline treatment	After alkaline treatment
Radioimmunoassay	Heart	1.05 ± 0.70	14.65 ± 0.73
	Brain	0.47 ± 0.28	4.37 ± 0.60
Radioimmunoassay after h.p.l.c.	Heart	<0.10	13.04 ± 0.85
	Brain	n.d.	3.44 ± 0.66

Table 3. *Subclasses of mono(ADP-ribose)-protein conjugates in mouse organs*
For details see the legend to Fig. 1 and the Materials and methods section.

Organ	Mono(ADP-ribose) residues (nmol/mg of DNA)		Ratio (sensitive/resistant)
	NH ₂ OH-sensitive	NH ₂ OH-resistant	
Liver	2.58 ± 1.03	3.61 ± 1.19	0.7
Kidney	1.21 ± 0.52	1.45 ± 0.51	0.8
Heart	3.27 ± 2.19	5.17 ± 3.06	0.6
Brain	0.59 ± 0.24	0.83 ± 0.31	0.7
Testes	0.18 ± 0.12	0.37 ± 0.19	0.5
Jejunum	0.53 ± 0.20	0.46 ± 0.19	1.2
Spleen	0.53 ± 0.12	0.19 ± 0.08	2.8

Fig. 1. *Correlation of total mono(ADP-ribose)-protein conjugates with protein and NAD⁺ + NADH contents of mouse tissues*

Mono(ADP-ribose) values were taken from Table 2; protein and NAD⁺ + NADH values represent the mean of duplicate determinations from the pooled organs of four to eight animals. (a) $n = 9$, $P \leq 0.001$; (b) $n = 9$, $P \leq 0.01$.

resistant conjugates comprised more than half of the total mono(ADP-ribose)-proteins, while in spleen and intestine NH₂OH-sensitive conjugates were the prevailing subclass.

Relation of mono(ADP-ribose)-protein conjugates to protein content and NAD levels

Cellularity of a tissue did not appear to be a determinant of mono ADP-ribosylation of proteins, as seen in Table 2. However, there were two other parameters that exhibited a rather good correlation with the endogenous mono(ADP-ribose) conjugate levels. The first parameter is the protein content of a tissue, yielding a correlation coefficient of 0.97 (Fig. 1). The amount of mono(ADP-ribose) residues also appeared to be related to the content of the substrate

(NAD⁺ + NADH) in a tissue (correlation coefficient 0.85). The significance of these correlations is not clear. Modification by ADP-ribose is only seen with a limited number of proteins (cf. Adamietz *et al.*, 1979; Adamietz, 1982). The concentration of the substrate NAD, on the other hand, cannot be a determining factor for both subfractions, since they can change independently (cf. Wielckens *et al.*, 1979, 1980; Bredehorst *et al.*, 1980, 1981b).

Studies with rat liver had shown that the bulk of the NH₂OH-resistant mono(ADP-ribose) conjugate subfraction is located in the mitochondria (Adamietz *et al.*, 1981). In order to find out whether there is a relation of this subfraction to the mitochondrial content of the tissues, the levels were compared with the content of cytochrome oxidase, as a rough

Table 4. Cytochrome oxidase activity versus NH_2OH -sensitive and total mono(ADP-ribose)-protein conjugates in various mouse tissues

Values of cytochrome oxidase activity were taken from Greenstein *et al.* (1944), and mono(ADP-ribose) residues from Tables 2 and 3.

Organ	Cytochrome oxidase activity (units/mg of tissue)	Mono(ADP-ribose) residues (nmol/g of tissue)	
		NH_2OH -resistant	Total
Heart	18.6	6.88	11.23
Kidney	11.0	4.64	8.51
Brain	9.6	1.34	2.29
Liver	8.0	7.62	13.06
Spleen	2.0	3.20	12.12

estimate of the amount of mitochondria (or mitochondrial cristae). As shown in Table 4, however, there was no correlation between these two parameters: while liver and brain had rather similar cytochrome oxidase activities, the levels of NH_2OH -resistant mono(ADP-ribose) conjugate subfractions differed by a factor of more than five. Cytochrome oxidase activity of heart tissue was more than two times higher than that of liver, but the level of hydroxylamine-resistant conjugates was higher in liver. Negative correlation was also seen by comparing brain and spleen, or liver and kidney and it applied to total mono(ADP-ribose)conjugates as well. These data suggest that parameters other than the mitochondrial capacity for oxidative phosphorylation govern the extent of ADP-ribosylation, at least with respect to the formation of NH_2OH -resistant conjugates. Indeed, recent data suggest that part of the mitochondrial ADP-ribosyltransferase activity may be connected with peroxide-induced Ca^{2+} efflux (Hofstetter *et al.*, 1981).

Poly(ADP-ribose)-protein conjugates, a minor fraction of ADP-ribosyl proteins

When different tissues were analysed for endogenous poly(ADP-ribose) residues, it became apparent that in all the tissues studied polymeric ADP-ribose residues comprised less than 1% of the protein-bound mono(ADP-ribose) groups (Table 5). The values neither correlated with total mono(ADP-ribose) residues nor with any of the two mono(ADP-ribose) conjugate subclasses, suggesting again independent functions (cf. Hilz, 1981).

Thyroid hormone status and mono(ADP-ribose)-protein conjugates in the liver

Recently, steroid hormone-dependent changes of mono(ADP-ribose)-protein conjugate levels in a target tissue have been reported (Gartemann *et al.*, 1981). In these experiments, the hormone status had affected NH_2OH -sensitive and -resistant mono-

Table 5. Protein-bound polymeric and monomeric ADP-ribose residues in different mouse organs

Mean values are from ten determinations using pooled organs from four to eight animals. Poly-(ADP-ribose) residues are expressed as pmol of monomer residue units.

Organ	ADP-ribose residues (pmol/mg of DNA)		Ratio (poly/mo)
	Polymeric	Monomeric	
Liver	22 ± 4	6187 ± 1358	0.0036
Kidney	19 ± 3	2655 ± 504	0.0080
Heart	8 ± 2	8439 ± 4930	0.0009
Spleen	6 ± 2	724 ± 105	0.0067

Fig. 2. Induction of hypothyroidism and mono(ADP-ribose)-protein conjugate subclasses in mouse liver A, NH_2OH -sensitive conjugate; B, NH_2OH -resistant conjugates. Mice were rendered hypothyroid by propylthiouracil (PTU) treatment as described in the Materials and methods section, and subsequently injected with thyroxine or NaCl. Thyroxine serum levels in controls ($30.6 \pm 3.6 \mu\text{g/ml}$) decreased to $<5 \mu\text{g/ml}$ in PTU-treated animals, and rose to $>400 \mu\text{g/ml}$ after thyroxine replacement. In the Figure, mean values from two experiments using the pooled livers from ten animals each are listed. *, $P < 0.001$ versus control.

(ADP-ribose) conjugate subclasses to a similar extent. When we analysed the influence of thyroid hormone on liver mono(ADP-ribose) conjugates, a different response was observed. Induction of a hypothyroid

state by treatment of mice with propylthiouracil led to a significant increase of total mono(ADP-ribose)-protein conjugates of the liver (Fig. 2). This rise, however, was exclusively due to an increase in NH_2OH -resistant conjugates, which nearly doubled (Fig. 2). When the animals received thyroxine, mono(ADP-ribose) conjugate levels returned to normal.

Since in liver most of the NH_2OH -resistant conjugates are located in the mitochondria (Adamietz *et al.*, 1981), the increased formation of NH_2OH -resistant mono(ADP-ribose) conjugates in the absence of thyroid hormones may relate to a hormonal mechanism acting on this compartment. Several mitochondrial proteins can serve as acceptors for ADP-ribose groups (Kun *et al.*, 1975; C. Lindner & H. Hilz, unpublished work). However, lack of detailed information on the normal functions of these proteins precludes at present further speculations as to the metabolic consequence of their increased ADP-ribosylation.

Our data are consistent with the interpretation that covalent modification of proteins by ADP-ribosylation serves multiple functions in various compartments of differentiated cells.

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