Localization and partial characterization of ADP-ribosylation products in hearts from adult and neonatal rats

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The subcellular distributions of endogenous ADP-ribosylation products in hearts from 1-day-old neonatal and adult rats were investigated. In adult rat heart a 52 kDa mono-ADP-ribosylation product was identified in the plasma membrane fraction. In contrast, in neonatal rat heart a 130 kDa poly-ADP-ribosylation product was present in the nuclear fraction. The monomeric and polymeric nature of the two ADP-ribosylation products was determined by their sensitivity to thymidine and by analysis of their snake venom phosphodiesterase products. NADP⁺ enhanced both the mono- and polymeric reactions. The ADP-ribose–protein linkage of the adult 52 kDa product was stable to 1 h of treatment with hydroxylamine (0.5 M) and mercury ions, but was sensitive to alkali and a 12 h treatment with hydroxylamine (1 M). This is suggestive of an arginine linkage. The 130 kDa poly-ADP-ribosylation product from the neonatal rat heart was alkali-labile but stable to both hydroxylamine and HgCl₂. This implies the presence of an unusual linkage in the 130 kDa product. The presence of these different ADP-ribosylation products in adult and neonatal rat hearts suggests the possible importance of these proteins and their ADP-ribosylation during cardiac development.

INTRODUCTION

ADP-ribosylation is a post-translational covalent modification of proteins, whereby the ADP-ribose moiety of NAD⁺ is transferred to a cellular protein. Proteins can be poly- or mono-ADP-ribosylated. Mono-ADP-ribosylations generally occur outside the nucleus and their function is not clearly established. This may be the mechanism of regulation of certain enzymes and regulatory proteins [1]. The most clearly defined example of mono-ADP-ribosylation of an enzyme as a regulatory mechanism is the reversible ADP-ribosylation of the enzyme nitrogenase in the photosynthetic bacterium *Rhodospirillum rubrum* [2]. Several bacterial toxins also possess ADP-ribosyltransferase activity, and this activity is responsible for their major toxic effects [3]. Poly-ADP-ribosylations occur mainly intranuclearly. These reactions seem to be involved in DNA repair, replication and translation as well as in cellular differentiation and malignant transformation [4]. Several nuclear acceptor proteins have been identified, but it is still not completely clear how they are linked to these functions [5]. The activity of poly(ADP-ribose) polymerase (EC 2.4.2.30) is dependent upon DNA and is stimulated by a variety of DNA-damaging agents. There is a direct correlation between the activity of the enzyme and the extent and number of DNA strand breaks in most tissues [6].

In rat heart, the mono-ADP-ribosylation of a 39 kDa cytosolic protein increases upon treatment with nitrous oxide-generating agents [7]. Several authors have also demonstrated the presence of poly(ADP-ribose) polymerase activity in rat cardiac muscle nuclei [8,9]. Several hormones, as well as circulatory stress (i.e. aortic coarctation), decrease the activity of this enzyme in cardiomyocytes significantly, whereas they increase it in the non-muscle cells of the heart [10–12]. The activity of the poly(ADP-ribose) polymerase is ten times higher in cardiocyte nuclei of 5-day-old rats than in nuclei of adult rats [13]. Not only are there known differences between neonatal and adult hearts with respect to poly-ADP-ribosylation, but at birth the rat heart and its regulation have not yet reached a mature state. The cardiomyocytes are not completely differentiated and continue to divide for

several days after birth [14]. After the third week of postnatal development, the growth of the heart is primarily due to muscle cell enlargement and to some extent to the accumulation of non-muscle cells [8]. The extracellular matrix composition [15] and the isoform of myosin heavy chain [16] are different in neonatal compared with adult heart. The parasympathetic innervation becomes functional at day 15 after birth [17]. Thus whereas the anatomy of adult and neonatal rat hearts is very similar, and their ultimate function as a blood-pumping organ is the same, their biochemistry is very different. Our objective was to study the ADP-ribosylation patterns in subcellular fractions of adult and neonatal rat hearts and to start the characterization of these products.

MATERIALS AND METHODS

Subcellular fractionation

The subcellular fractionation method was modified from that of Hosey & Fields [18]. Hearts were removed from 1-day-old or adult Sprague–Dawley (Holtzman, Madison, WI, U.S.A.) or Wistar (house-bred) rats and placed on ice into precooled buffer A (0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.4, 0.1 mM-EDTA). Hearts were trimmed, minced and homogenized in 10 vol. of buffer A at 4 °C using a Brinkman Polytron homogenizer at setting 6 for three 5 s periods, with 30 s rest periods in between. The homogenates were centrifuged for 10 min at 600 *g*. The pellet was resuspended in 5 vol. of buffer A and centrifuged again for 10 min at 600 *g*. The resultant pellet (P600) was resuspended in 5 vol. of buffer A and stored. The supernatant from the original 600 *g* spin was centrifuged at 100 000 *g* for 60 min. The resultant supernatant (S33 000) was stored. The pellet (P33 000) was resuspended in 13 ml of buffer A, applied to five-step sucrose gradients [15%, 28%, 32%, 36% and 40% sucrose (w/v in 20 mM-Tris/HCl, pH 7.4, 0.1 mM-EDTA: buffer B)] and centrifuged at 140 000 *g* in a SW 40 rotor for 2 h. Subsequently, 2 ml fractions were collected, diluted with buffer B and centrifuged at 20 000 *g* for 90 min. The final pellets were resuspended in a minimal volume of buffer B and stored. Fractions were designated

Abbreviations used: Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; G-protein, guanine-nucleotide-binding protein; α -G_s, α -subunit of the guanine-nucleotide-binding protein G_s; PEI, poly(ethyleneimine).

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as follows: F1 (8.6 % sucrose layer), F2 (15 %), F3 (28 %), F4 (32 %), F5 (36 %), F6 (40 %) and F7 (pellet). All stored samples were kept at -70°C until used. All protein assays were conducted by the method of Lowry *et al.* [19].

For use with cholera toxin labelling, crude membrane preparations from adult rat hearts were prepared according to the method of McMahon [20].

ADP-ribosylation assay

Endogenous and pertussis toxin (List Biological Laboratories, Campbell, CA, U.S.A.)-catalysed [^{32}P]ADP-ribosylation reactions were by the method of Burns *et al.* [21], as modified by McMahon [20]. The mixture was incubated for 30 min at 30°C and the reaction was terminated by the addition of either SDS-sample-buffer or trichloroacetic acid (final concentration 20 %). Trichloroacetic acid-soluble samples were processed by t.l.c., and trichloroacetic acid-insoluble and SDS-sample-buffer samples were processed by SDS/PAGE or processed for the hydrolysis studies described below.

Cholera toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed by the method of Gill & Woolkalis [22]. In brief, cholera toxin (List Biological Laboratories) was activated by incubating 0.2 mg of cholera toxin/ml in 5 mM-dithiothreitol, 0.5 % SDS, 130 mM-NaCl and 10 mM-Hepes (pH 7.3) for 10 min at 37°C . Membranes were preloaded with guanine nucleotide by incubating 100 mg of crude membranes/ml with 1 mM-guanosine 5'-[$\beta\gamma$ -imido]triphosphate (Gpp[NH]p) for 10 min at 37°C . The activated toxin (final concentration 0.02 mg/ml) and guanine nucleotide-loaded membrane (150 μg) were then incubated at room temperature for 60 min with 10 mM-thymidine, 10 mM-[^{32}P]NAD $^{+}$ (5 $\mu\text{Ci}/\text{tube}$), 10 mM-dithiothreitol, 0.1 % Triton X-100 and 0.5 mM-Gpp[NH]p in 100 mM-NaCl/10 mM-Hepes, pH 7.3. The reactions were terminated by the addition of trichloroacetic acid (10 % final) and centrifuged at 12000 g for 5 min.

T.l.c.

Poly(ethyleneimine) (PEI)-treated cellulose plates (Brinkman Instruments, Westbury, NY, U.S.A.) were spotted with 2 μl of sample (trichloroacetic acid supernatant) and an internal standard (10 nmol of NAD $^{+}$, 40 nmol of ADP-ribose and/or 40 nmol of AMP). The plates were developed in 0.1 M-LiCl/0.5 M-formic acid [23]. Untreated cellulose plates (Eastman, Rochester, NY, U.S.A.) were spotted as above and developed in 60 % (v/v) ethanol/0.3 M-ammonium acetate (pH 5.0) [22].

Snake venom phosphodiesterase digestion of ADP-ribosylated samples

This method was modified from those of Tanuma *et al.* [24] and Lee & Iglewski [25]. Samples were prelabelled with [^{32}P]NAD $^{+}$ (New England Nuclear-Dupont, Boston, MA, U.S.A.) and the reactions were terminated by addition of trichloroacetic acid (final concentration 20 %) with subsequent centrifugation (2000 g , 10 min, 4°C). The pellets were washed four times by resuspension with cold 20 % trichloroacetic acid, twice with ethanol and twice with diethyl ether. The pellets were resuspended in 50 mM-Tris/HCl (pH 9.0)/10 mM-MgCl $_2$ and incubated with snake venom phosphodiesterase (1 mg/ml final concentration) (Worthington, Freehold, NJ, U.S.A.) at 37°C for 1 h. The reactions were terminated by the addition of trichloroacetic acid (final concentration 20 %). The supernatants were ether-extracted and spotted on cellulose plates. The pellets were washed with ether, suspended in SDS sample buffer and subjected to electrophoresis with subsequent autoradiography.

Determination of the stability of the ADP-ribose-protein bond

These methods were modified from those of Hsia *et al.* [26], Bredehorst *et al.* [27], Payne *et al.* [28] and Meyer *et al.* [29]. Samples were prelabelled and washed as described for the snake venom phosphodiesterase experiments. For assessment of the stability to alkali, the samples were suspended by sonication in 50 μl of water, and subsequently 50 μl of 1 M-NaOH or 1 M-NaCl was added to the samples, which were incubated at 56°C for 1 h. The reaction was terminated by the addition of 50 μl of 2 M-HCl and trichloroacetic acid (final concentration 20 %).

For assessment of the stability to hydroxylamine and HgCl $_2$, samples were suspended by sonication in 50 μl of 100 mM-Hepes (pH 7.5)/2 % SDS. An equal volume of either 1 M-NH $_2$ OH or 1 M-NaCl (control), 2 M-NH $_2$ OH or 2 M-NaCl (control), or 2 mM-HgCl $_2$ or 2 mM-NaCl (control) was added, and samples were incubated for 1 h (1 M-NH $_2$ OH, 2 mM-HgCl $_2$) or 12 h (2 M-NH $_2$ OH). After the indicated time at 37°C , cold trichloroacetic acid (final concentration 20 %) was added. The supernatants were ether-extracted and spotted on t.l.c. plates. The pellets were washed with ether, suspended in SDS sample buffer and subjected to electrophoresis and autoradiography.

RESULTS

Demonstration of subcellular distribution of endogenous ADP-ribosylation products in adult and neonatal rat hearts

The distribution of the endogenous ADP-ribosylation products in the different subcellular fractions was determined. The ADP-ribosylation assay was performed in the presence and absence of pertussis toxin (as a control and a marker), followed by electrophoresis and autoradiography. In adult heart (Fig. 1), an endogenous 52 kDa band was observed in fractions F2, F3 and F4. Pertussis toxin did not affect the labelling of this protein. The 40 kDa pertussis toxin substrate was used as a marker for the plasma membrane (Fig. 1) and was present in the highest relative concentration in fractions F2, F3 and F4. This was confirmed by using the muscarinic receptor as an additional plasma membrane marker (results not shown). This suggested that the endogenous ADP-ribosylation products were localized in the plasma membrane.

In neonatal hearts (Fig. 2), the 52 kDa ADP-ribosylation product was not observed in any of the fractions. Instead, a 130 kDa product was present in fractions H, S600 and to the largest extent in the nuclear fraction P600. P600 was defined as the nuclear pellet by its centrifugational properties and by using DNA as a nuclear marker (results not shown). This suggested that the major acceptor protein in the neonatal rat heart was localized in the nucleus. Further experiments were performed with fractions F2-F4 for the adult and fractions S600 and P600 for the neonatal animal.

Characterization of the ADP-ribosylations as monomeric or polymeric

Our original assay contained 10 mM-thymidine, a known inhibitor of poly-ADP-ribosylation, and to a much lesser extent of mono-ADP-ribosylation [30]. Since the ADP-ribosylation in the neonatal heart appeared to be a nuclear event, and most nuclear ADP-ribosylations are polymeric in nature, it was hypothesized that this ADP-ribosylation product was polymeric. Likewise, the adult product was hypothesized to be monomeric, since it was localized in the plasma membrane. Two experiments were performed to test these hypotheses. Initially, assays were performed in both the absence and presence of 10 mM-thymidine (Fig. 3). In neonatal rat heart, the absence of thymidine increased the degree of ADP-ribosylation. In addition, ADP-ribosylation of proteins in the molecular mass range above

Fig. 1. Subcellular distribution of ADP-ribosylation products in adult rat heart

The ADP-ribosylation assays were performed on subcellular fractions as defined in the Materials and methods section in the absence (–) and presence (+) of pertussis toxin (PT), followed by SDS/PAGE and autoradiography. All lanes contained 25 µg of protein, except F1 in which the protein concentration was too low to be determined. The positions of the 52 kDa endogenous product and the 40 kDa pertussis toxin substrate are indicated on the left hand side. In addition, minor bands with a molecular mass between 30 and 40 kDa were present in fractions S600, P600, S33000 and P33000 of this particular preparation, but these were not consistently detected. The subcellular fractions are marked above the respective lanes [H, homogenate; S600, supernatant of 600 g spin; P600, precipitate of 600 g spin; S33000, supernatant of 33000 rev./min (100000 g) spin, P33000, pellet of 33000 rev./min spin; F1–F7, sucrose gradient fractions (F1, 8.6% sucrose, sample layer; F2, 15%; F3, 28%; F4, 32%; F5, 36%; F6, 40%; F7, pellet)].

130 kDa occurred. This may have been due to the attachment of ADP-ribose polymers of variable length to the neonatal protein, which would make it move at various slower mobilities on electrophoresis. Preliminary data suggest that the IC_{50} (concn. causing 50% inhibition) for thymidine in the neonatal heart is about 0.2 mM ($n = 1$; results not shown). In contrast, in adult heart, thymidine did not exert any effect. For subsequent experiments with neonatal fractions, thymidine was omitted from the reaction mixture.

To further test these hypotheses, snake venom phosphodiesterase digestion of the radiolabelled substrates was conducted. This enzyme will cleave the PP_i bond. In the case of poly-ADP-ribosylation, one AMP molecule will be cleaved per chain or branch, originating from the terminal residue. The internal

Fig. 2. Subcellular distribution of ADP-ribosylation products in neonatal rat heart

The ADP-ribosylation assays were performed on subcellular fractions in the presence (+) and absence (–) of pertussis toxin (PT), followed by SDS/PAGE and autoradiography. All lanes contained 25 µg of protein, except F1 in which the protein concentration was too low to be determined. The positions of the 130 kDa endogenous product and the 40 kDa pertussis toxin substrate are indicated on the left-hand side. The subcellular fractions are marked above the respective lanes as in Fig. 1 (M = molecular mass markers). No ADP-ribosylated bands were detected in any subcellular fraction outside the molecular mass range shown.

Fig. 3. Effect of thymidine on the ADP-ribosylation reaction in adult and neonatal heart

Adult (F2–4) and neonatal (S600) fractions were ADP-ribosylated in the presence (+) and absence (–) of 10 mM-thymidine. The reaction mixture also contained 5 mM-NADP⁺. The reactions were terminated by the addition of SDS sample buffer, followed by electrophoresis and autoradiography.

residues will appear as phosphoribosyl-AMP. AMP will be the only released product after digestion of mono-ADP-ribosylated proteins [31].

Prelabelled samples were digested with snake venom phosphodiesterase as described in the Materials and methods section. Fig. 4 shows the digestion products after t.l.c. In the adult, all of the radiolabelled material co-migrated with authentic AMP. In

Fig. 4. Snake venom phosphodiesterase products from radiolabelled adult and neonatal rat heart fractions

Adult (F2-4) and neonatal (S600) fractions were prelabelled in the presence of NADP⁺, washed and then incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of snake venom phosphodiesterase as described in the Materials and methods section. The ether-extracted supernatants were analysed by t.l.c. on cellulose plates. The positions of the AMP and phosphoribosyl-AMP (prAMP) internal standards are shown on the right-hand side.

contrast, the digestion products from the neonatal hearts co-migrated with both AMP and phosphoribosyl-AMP. GMP was used as the marker for phosphoribosyl-AMP, since it behaves similarly in most chromatography systems [32]. This confirmed that the 52 kDa band in the adult heart was a mono-ADP-ribosylation product, whereas the 130 kDa band in the neonatal heart was the result of a poly-ADP-ribosylation.

Establishment of the requirements for the ADP-ribosylation reaction

To investigate whether [³²P]NAD⁺ was being consumed and therefore was rate-limiting in the reactions, the trichloroacetic acid-soluble material of incubations with adult (F4) or neonatal (S600) rat hearts was subjected to t.l.c. The majority of the NAD⁺ was degraded by the end of the incubation period in both adult and neonatal rat hearts (Fig. 5a, -). The major NAD⁺ breakdown product was identified as ADP-ribose in both adult and neonatal hearts. The assay was also performed in the presence of 5 mM-NADP⁺ (Fig. 5a, +), a known inhibitor of NAD⁺ breakdown [33]. Under the assay conditions used, NADP⁺ inhibited NAD⁺ degradation in both adult and neonatal rat hearts. Since NADP⁺ can also compete with NAD⁺ as a substrate for ADP-ribosylation [34], we investigated whether NADP⁺ affected the ADP-ribosylations in either age group. Fig. 5(b) shows that the presence of NADP⁺ increased the degree of ADP-ribosylation in both adult and neonatal animals. For all subsequent experiments, NADP⁺ was included in the reactions.

In subsequent experiments (results not shown), we established that several of the original ingredients of our assay were not required for the ADP-ribosylation reactions. The deletion of the individual components GTP (as well as 10 mM-Gpp[NH]p),

Fig. 5. Effects of NADP⁺ on the ADP-ribosylation reaction

(a) Inhibition of NAD⁺ breakdown by NADP⁺ in adult and neonatal rat fractions. The ADP-ribosylation assays of adult (F4) and neonatal (S600) fractions were performed in the absence (-) and presence (+) of 5 mM-NADP⁺. Both adult and neonatal fractions were labelled in the presence of thymidine. The reactions were terminated by trichloroacetic acid precipitation and 2 μ l portions of the supernatants were analysed by t.l.c. on PEI-treated cellulose plates. ADPR, ADP-ribose. (b) Effect of NADP⁺ on the ADP-ribosylation reaction. The ADP-ribosylation assays of adult (F4) and neonatal (S600) fractions were conducted in the presence (+) and absence (-) of NADP⁺, followed by electrophoresis and autoradiography. Each lane contains 25 μ g of protein. The positions of the molecular mass markers are indicated on the left hand side.

Fig. 6. Time course of ADP-ribosylations in rat heart preparations

Adult (F4, ●) and neonatal (P600, ○) fractions were incubated with reaction mixture for the amount of time indicated. After electrophoresis and autoradiography, the bands were excised and counted in a liquid scintillation counter. Incorporations from [³²P]NAD⁺ (pmol/mg of protein) is expressed on the left y-axis for the adult and the right y-axis for the neonatal rat.

Fig. 7. Stability of the ADP-ribosylated proteins to dilute alkali

After prelabelling the adult (F3, *a*) and neonatal (P600, *b*) fractions, (both in the presence of thymidine), samples were treated as described in the Materials and methods section and incubated with 0.5 M-NaOH or NaCl (control). After trichloroacetic acid precipitation, the pellets were washed and resuspended, and electrophoresis followed by autoradiography was performed.

dithiothreitol, digitonin, cholate and ATP had no effect either on the adult monomeric or on the neonatal polymeric reactions. Deletion of all ingredients except NAD⁺ decreased the reaction rates of both preparations substantially (results not shown).

Time courses of ADP-ribosylation were determined for both the adult and neonatal preparations (Fig. 6). Both time courses were linear during the first 10 min of the reaction. After that time, the level of ADP-ribosylation reached a plateau in the adult and was stable for at least 60 min. In the neonatal preparation, the ADP-ribosylation reached a peak at 15 min and gradually declined up to 60 min.

Stability of the ADP-ribose-protein bond to alkali, hydroxylamine and HgCl₂

The ADP-ribosylation of proteins occurs through a number of ADP-ribose-protein linkages [35]. Polymeric ADP-ribosylations are frequently linked to the γ-carboxyl group of glutamic acid or the α-carboxyl group of terminal lysine residues. The most

Fig. 8. Stability of the ADP-ribosylated proteins to hydroxylamine

After radiolabelling of the adult (F2-4) and neonatal (S600) fractions and cholera toxin labelling of an adult crude membrane preparation, the fractions were treated as described in the Materials and methods section and incubated with (a) 0.5 M-NH₂OH, pH 7.5 (+) or 0.5 M-NaCl (-) for 1 h or (b) 1 M-NH₂OH, pH 7.0 (+) or 1 M-NaCl (-) for 12 h. After trichloroacetic acid precipitation, the pellets were washed and resuspended, and electrophoresis followed by autoradiography were performed.

common mono-ADP-ribosylations are on arginine residues, but linkages through cysteine, dipthamide (modified histidine) and asparagine residues [36] have also been described. The various ADP-ribose-amino acid linkages described can be discriminated on the basis of their different sensitivities to chemical treatment. All known ADP-ribose-amino acid bonds, except with dipthamide, are alkali-labile [35,36]. In both adult and neonatal rat hearts, treatment with 0.5 M-NaOH removed the label from both the adult and neonatal ADP-ribosylated proteins, thus excluding the possibility that the modified amino acid was a dipthamide residue (Fig. 7).

The thio-glycosidic bond formed between a cysteine residue and the ADP-ribose moiety can be identified by its unique sensitivity to Hg²⁺ [29]. Both adult and neonatal ADP-ribosylated proteins maintained their label in the presence of 1 mM-HgCl₂ (results not shown). This demonstrated that neither of the proteins was modified at a cysteine residue.

The different ADP-ribose-protein linkages have different sensitivities to hydroxylamine [28]. The ADP-ribose-glutamate and other carboxylate ester bonds are extremely labile to hydroxylamine. The ADP-ribose-arginine bond is relatively

stable to 0.5 M-NH₂OH for 1 h, but is labile to 1 M-NH₂OH for 12 h. The cysteine and diphthamide bonds are completely stable under these conditions, whereas the recently described asparagine bond is stable in 0.5 M-NH₂OH for at least 5 h. The adult ADP-ribosylated protein was stable in 0.5 M-NH₂OH for 1 h (Fig. 8a), but released its label when treated with 1 M-NH₂OH for 12 h (Fig. 8b). The cholera toxin-labelled α -subunit of G_s was an internal standard, since it is known to be ADP-ribosylated at an arginine residue [37]. The sensitivity of prelabelled α -G_s to hydroxylamine was identical with that of the adult protein under the conditions used. These results are suggestive of the presence of an arginine-ADP-ribose bond in the adult. Interestingly, the poly-ADP-ribose bond of the neonatal preparation was stable under both sets of hydroxylamine conditions (Fig. 8). Since the most commonly described carboxylate ester linkages are extremely sensitive under these conditions, it can be inferred that a different bond may be involved in the neonatal rat heart nuclear protein.

DISCUSSION

The results of this study demonstrate the presence of a 52 kDa mono-ADP-ribosylation product in the plasma membrane fraction of adult rat hearts and a 130 kDa poly-ADP-ribosylation product in the nuclear fraction of hearts from 1-day-old rats. Interestingly, the 52 kDa product was not detected in the neonatal rat, nor was the 130 kDa product detected in the adult. The 52 kDa protein that was observed in adult heart may well be a protein that is not yet present or able to be ADP-ribosylated in 1-day-old rat hearts. Alternatively, the protein may be present but the mono-ADP-ribosyltransferase may not yet be present or functional. The reverse may be true for the 130 kDa protein, which may either be absent in adult rat heart or not be ADP-ribosylated due to a decrease in poly(ADP-ribose) polymerase activity. Previous workers have reported both complementary and contradictory results concerning poly(ADP-ribose) polymerase activity. Claycomb [8] demonstrated that the activity of poly(ADP-ribose) polymerase in rat heart nuclei increased by about 2-fold between day 1 and day 16 of postnatal development and is even higher in the adult. In contrast, Jackowski & Kun [13] showed that the poly(ADP-ribose) polymerase activity of neonatal (5-day-old) cardiocyte nuclei is about 10 times greater than in nuclei of adult rats. These latter authors discriminated between cardiocyte and non-cardiocyte nuclei. In our preparation, which was more similar to Claycomb's procedure, no discrimination was made between different cell types. Thus, although the present data do not resolve the apparent discrepancies, they are more consistent with the results of Jackowski & Kun [13].

The results demonstrated that the presence of NADP⁺ enhanced the endogenous ADP-ribosylation reactions. This could be due to an increase in the availability of the substrate (NAD⁺) or to a direct stimulatory action on the enzyme. Other work in heart has also shown that NADP⁺ prevented NAD⁺ breakdown. In dog cardiac sarcolemma, NADP⁺ inhibits NAD⁺ degradation and increases the efficiency of cholera toxin labelling [33]. In platelets and erythrocytes, NADP⁺ also acts as an inhibitor of NAD⁺ degradation, but in contrast it inhibits endogenous ADP-ribosylation [23].

The studies on the stability of the ADP-ribose-protein bond are suggestive of an arginine linkage in adult heart. One has to be careful, however, since the stability of the asparagine bond has not yet been completely established. Nevertheless, the only amino acids that are known to be modified by endogenous ADP-ribosylation are arginine [35] and cysteine [38], and the possibility that the modification is at a cysteine residue has been excluded.

In contrast, the neonatal ADP-ribosylation product appears to have an uncommon linkage. The presence of an alkali-labile hydroxylamine-stable poly-ADP-ribose-protein bond is unusual. Most described poly-ADP-ribose-protein bonds are of the carboxylic ester type and are highly labile under these conditions. This suggests the presence of a new ADP-ribose-protein bond. Further investigation should clarify the exact nature of this bond.

In summary, a 52 kDa mono-ADP-ribosylation product is modified at an arginine residue in the plasma membrane of adult rat heart. In contrast, a 130 kDa poly-ADP-ribosylation product in the nucleus of the 1-day-old rat heart contains an unusual ADP-ribose-protein linkage.

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