

## Neutrons affect ADP-ribosylation of proteins in human kidney T1-cells *in vitro*

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ADP-ribosylation (ADPR) of proteins has been shown to be involved with a variety of cellular responses in which chromatin organization and functions are affected. In order to look into this response, human kidney T1-cells were exposed *in vitro* to various doses up to 3 Gy of 6 MeV neutrons and compared with the effect caused by gamma photons. Whereas in case of neutrons the maximal inhibition of ADPR was reversed at 0.37 Gy, that in case of gamma-rays occurred at 1.5 Gy. For the reversal of inhibition of ADPR of proteins in T1-cells, neutrons were about 4-fold more efficient as compared to gamma rays.

ADP-ribosylation (ADPR), representing poly-ADP-ribosylation and/or mono-ADP-ribosylation, is a post-translational modification of proteins in eukaryotes wherein the ADP-ribosyl moieties from NAD<sup>+</sup> are covalently bound on the reducing ends of the target proteins<sup>1,2</sup>. Poly-ADP-ribosylation (PADPR) is catalyzed by the enzyme poly(ADP-ribose) polymerase resulting in the formation of an unbranched or a branched polymer as long as 200 monomers, sometimes larger than the target protein itself. The target proteins for PADPR in eukaryotes includes the enzyme poly(ADP-ribose) polymerase (automodification), histones, HMG proteins, DNA replicase, ligase, and RNA polymerase<sup>3</sup>. The polymer is degraded by poly(ADP-ribose) glycohydrolase and ADP-ribosyl protein lyase. Mono-ADP-ribosylation (MADPR), representing about 5% of total ADPR reactions<sup>4</sup>, is catalyzed by ADP-ribose transferase and is related to non-nuclear proteins among prokaryotes and eukaryotes<sup>3</sup> participating in signal transduction<sup>5</sup>.

Enhancement of PADPR of nuclear proteins has been shown to relax chromatin<sup>6,7</sup> indicating that PADPR of nuclear proteins, especially histones, may regulate the structural organization of

chromatin. This alters the accessibility of genes (DNA) to various enzymes<sup>1,8</sup>. Reports in the literature show that PADPR is indeed involved in multiple cellular functions<sup>9-11</sup> including DNA repair<sup>1</sup>, radiation response<sup>12,13</sup>, gene regulation<sup>6,8</sup>, signal transduction<sup>9,14</sup>, and carcinogenesis<sup>15</sup>. It has been shown that PADPR of chromosomal proteins was enhanced after exposure to DNA damaging agents as well as during DNA excision repair<sup>9</sup>.

Despite this wide spectrum of possible involvement of ADPR in eukaryotic systems, there has been no information on the neutron induced effects on ADPR of proteins. This knowledge will be necessary for the therapeutic use of neutrons in cancer treatment. Therefore, we have measured the level of total cellular ADPR and the concentration of cellular NAD<sup>+</sup> following the exposure of human kidney T1-cells *in vitro* to neutrons. For comparison, the ADPR of proteins and the NAD<sup>+</sup> pool were also monitored following gamma-irradiation.

### Materials and Methods

**Chemicals**—All chemicals were of analytical grade and were used without further purification. [<sup>32</sup>P]NAD<sup>+</sup> (0.37-1.85 TBq/mmol) was purchased from NEN, UK.

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**Cell Culture**—Human kidney T1-cells (grown in basal medium Eagle's (BME) supplemented with 10% fetal calf serum, 0.2% NaHCO<sub>3</sub>, 0.1% antibiotics, and 0.9% of glutamine in a CO<sub>2</sub>-incubator) were harvested on the fifth day after seeding. The monolayer culture was washed with PBS, trypsinized (0.25% at 37°C for 10 min) and agitated to prepare a single cell suspension. The cells were suspended in BME at 4°C until experimentation.

**Radiation sources and irradiation**—Suspensions of 3-4×10<sup>6</sup> T1-cells in BME were irradiated at 4°C by either neutron generated in the KFA Compact Cyclotron (<sup>9</sup>Be(d,n)<sup>10</sup>B; mean energy: 6 MeV) or to gamma photons from <sup>137</sup>Cs-gamma source (1.1 Gy/min). After irradiation the cells remained on ice for about 30 min before the assays.

**Assay of ADP-ribosylation**—The method published earlier<sup>11</sup> was used with slight modifications. In brief 3-4×10<sup>6</sup> T1-cells, collected by centrifugation (500×g, 2 min, 4°C), were suspended in permeation (P) buffer (10 mM tris-HCl, pH 7.8/4°C; 1 mM EDTA; 4 mM MgCl<sub>2</sub> and 30 mM 2-mercaptoethanol) at a concentration of 2×10<sup>6</sup> cells/ml for 15 min on ice. The P buffer was removed by centrifugation and the cell pellet (50 μl) was added to 25 μl of reaction (R) buffer (33 mM tris-HCl, pH 7.8/37°C; 0.67 mM EDTA; 2.67 mM MgCl<sub>2</sub>; 20 mM 2-mercaptoethanol and 0.8 μM <sup>32</sup>P-NAD<sup>+</sup> containing 222 kBq activity). It was incubated for 15 min at 37°C. The reaction was stopped by adding 6 ml of 15% trichloroacetic acid (TCA) and immersing the tubes in an ice bath for 30 min. The 15% TCA insoluble proteins were collected on GF/C filter discs (Whatman, UK) and washed with 40 ml of 15% TCA. After drying the discs, radioactivity was counted in a Beckman liquid scintillation counter using the Ultima Gold scintillation cocktail from Packard, USA.

**Assay of NAD<sup>+</sup>**—The methods described by Kato *et al*<sup>16</sup> and Jacobson & Jacobson<sup>17</sup> were followed with minor modifications. To 0.5×10<sup>6</sup> intact T1-cells in BME, 3 ml of 0.5 N HClO<sub>4</sub> was added, vortexed and left on ice for 15 min to extract NAD<sup>+</sup>. The extract was collected by a centrifugation (1,500×g; 10 min; 4°C) and pH adjusted to 7.5 by a neutralization solution (1 M KOH in 0.33 M potassium phosphate buffer, pH

7.5). The amount of NAD<sup>+</sup> of the extract was assayed at room temperature in dark for 30 min by mixing 0.5 ml of this NAD<sup>+</sup> extract with 0.6 ml of a reaction solution (200 mM bicine buffer (pH 7.8), 1 M ethanol, 0.84 mM thiazolyl blue, 3.32 mM phenazine ethosulphate, 8.32 mM EDTA, and 1.66 mg/ml BSA) and 0.1 ml (18 units) of alcohol dehydrogenase. The reaction was stopped by adding 0.5 ml of 12 mM iodoacetate. Absorption was read at 570 nm. NAD<sup>+</sup> (Sigma, USA) was used as a standard.

## Results and Discussion

Fig. 1 shows the levels of ADPR of cellular proteins in relation to different doses of 6 MeV neutrons (A) and gamma (B) rays. After exposure of T1-cells to neutrons ADPR went down to 68±35% at 0.37 Gy and went up gradually with increasing doses up to 3 Gy (115.3±26.4%). On the other hand, after gamma-irradiation ADPR decreased (35±14%) up to 1.5 Gy and increased slightly (46±15%) at 3 Gy. Fig. 2 depicts the

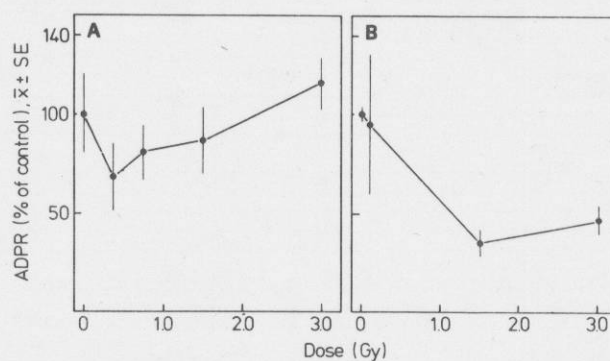


Fig. 1—ADP-ribosylation (ADPR) of proteins in human kidney T1-cells expressed as % of control following exposures to different doses of neutrons (A) and gamma rays (B) [Data represent mean±SE of 6 independent experiments]

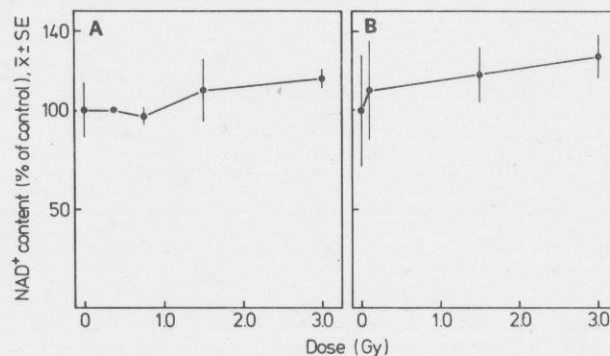


Fig. 2—Metabolic pool of NAD<sup>+</sup> in the intact human kidney T1-cells expressed as % of control following exposures to different doses of neutrons (A) and gamma rays (B) [Data represent mean±SE of 6 independent experiments]

relative metabolic pools of NAD<sup>+</sup> at corresponding doses of irradiations. After exposure of cells to neutrons the cellular pool of NAD<sup>+</sup> remained unaltered up to 1.5 Gy and later increased a little (116±9%) at 3 Gy (Fig. 2A). After gamma-irradiation the concentration of NAD<sup>+</sup> exhibited a tendency of dose dependent increase reaching the highest value (127±20%) at 3 Gy (Fig. 2B). These results show that neutrons and gamma photons affected the ADPR of cellular proteins in two different manners. Neutrons caused a relatively small reduction of ADPR of cellular proteins at a dose of 0.37 Gy while gamma-rays induced a very pronounced decrease. It is known that neutrons, by indirect reactions, (a) produce radicals at a yield higher than gamma rays at the same dose and (b) damage chromatin mainly by causing single strand breaks of DNA resulting in production of small fragments<sup>18</sup>. These lesions stimulate poly(ADP-ribose) polymerase activity to increase the ADPR level as has been shown for neurotoxic DNA damage in PC2 cells *in vitro*<sup>19</sup>. The gamma rays decreased ADPR level up to 1.5 Gy (Fig. 1B) indicating that in this range of dose gamma ray induced damages of DNA were unable to enhance ADPR. At about 0.37 Gy of neutrons and of 1.5 Gy of gamma rays the inhibitory effects of radiations on ADPR of proteins in T1-cells were reversed. Thus, the relative biological effectiveness (RBE) of neutrons was 4-fold higher for ADPR stimulation as compared to gamma rays. This is in agreement with the facts that the complexity of molecular organization of DNA and the quality of DNA lesions have direct bearing on the effectiveness of different qualities of radiations<sup>20</sup>.

The measured levels of ADPR in these experiments are the outcome of the synthesis and the degradation of ADPR on the cellular proteins<sup>1-3</sup> catalyzed by the activities of the enzymes poly(ADP-ribose)polymerase and/or ADP-ribose transferase and the poly(ADP-ribose) glycohydrolase and/or the ADP-ribosyl protein lyase, respectively (Fig. 3). The turn-over rate of ADPR is in the range of 1 min or less<sup>21</sup>. Therefore, even small changes in the levels of ADPR are likely to have significant biological consequences<sup>22</sup>. From the levels of ADPR (Fig. 1) and the corresponding levels of its substrate, NAD<sup>+</sup> (Fig. 2), it appears that initially both neutrons and gamma rays

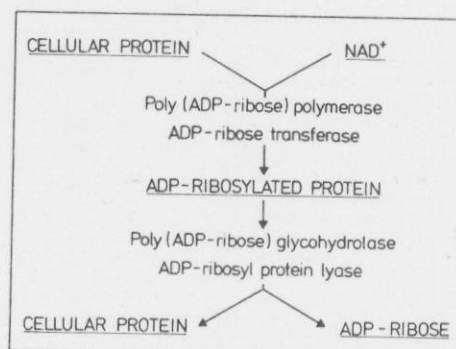


Fig. 3—Schematic pathway of ADP-ribosylation of proteins in an eukaryotic cell

stimulated the activities of the ADPR-degrading enzymes which resulted in decrease of ADPR. Above 0.37 Gy of neutrons and 1.5 Gy of gamma rays ADPR degrading enzymes were affected differently. These results (Figs 1 & 2) also indicate that the activities of ADPR-synthesizing enzymes remained unaffected for a dose up to 0.75 Gy of neutrons since the metabolic pool of NAD<sup>+</sup> did not decrease. A slight increase of the NAD<sup>+</sup> content beyond this dose of neutrons and for all doses of gamma-rays (Fig. 2) suggests that the activities of ADPR-synthesizing enzymes tended to be lowered by radiations in this dose range.

In conclusion, these data show that ADPR of cellular proteins of T1-cells respond differently to neutrons and to gamma rays. Gamma radiation primarily affected the ADPR degrading enzymes to alter the levels of cellular ADPR. Neutrons, on the other hand, influenced both synthetic and degradative enzymes of ADPR. This differential influence by neutrons on molecular components of cells such as, ADPR may be one of the causes of a higher RBE of neutrons as compared to gamma-rays.

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