

Tannic Acid-induced Modulation of Poly(ADP-Ribose) Glycohydrolase and NAD⁺ Content in Rat Liver and Thymocyte Nuclei

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Abstract

Objective: The goal of the present study was to determine whether the treatment of intact rats with tannic acid (TA) could alter activities of the main players in poly(ADP-ribose) (PAR) polymers turnover. **Materials and Methods:** Animals were treated according to the regulations of the National Center of Bioethics (Armenia). Cell nuclei were isolated according to standard procedure. PAR polymerase 1 (PARP 1) activity was evaluated by NAD⁺ consumption. PAR glycohydrolase (PARG) protein was estimated by sandwich ELISA method. Data are expressed as mean \pm standard deviation (SD). Statistical differences in the results between groups were evaluated by the Student's *t*-test. $P < 0.05$ was considered statistically significant. **Results:** The results demonstrate relatively stable stimulation of PARP 1 activity in liver and thymocyte nuclei of rats treated with TA. TA stimulated PARP 1 activation in thymocyte and liver nuclei is paralleled with drop in NAD⁺ content for more than 30% below basal level. The concentration of PARG in liver nuclei exceeded the basal value of PARG content in thymocyte nuclei nearly 3.5-fold. In 24 h after administration of TA to animals the content of PARG protein in thymocyte nuclei decreased by 30%. Decrease of PARG content in liver nuclei was more substantial and proceeded for 48 h after the treatment of rats with TA. **Conclusion:** TA-induced stimulation of PARP 1 in liver and thymocyte nuclei paralleled with decrease in NAD⁺ and PARG content could alter PAR polymer turnover and mediate genotoxic effect of TA revealed by light microscopy examination of liver.

Key words: Hepatotoxicity, NAD⁺ content, nuclei, poly(ADP-ribose) glycohydrolase, polymerase 1, tannic acid

INTRODUCTION

Modulation of poly(ADP-ribose) (PAR) polymer content in cell could trigger or suppress cell death and thus is accepted as a promising strategy for the development of new therapeutic approaches in the treatment of various diseases.^[1] The main enzymes involved in PAR polymer turnover are members of PAR polymerase (PARP) family that accomplishes PAR synthesis and an axial enzyme involved in PAR cleavage PARG. The members of PARP family are encoded by 18 different genes^[2] and are located in different cell compartments. Although they have different domain organizations, all of them share homology in conservative catalytic domain, which accomplishes binding, cleavage of substrate NAD⁺, and transfer of ADP-ribose moieties

to acceptor sites. The most abundant member of the family PARP 1 is localized to cell nuclei. C-terminal end of the enzyme molecule comprises catalytic pocket which consists of helical subdomains implicated in allosteric regulation of enzymatic activity, NAD⁺-binding subdomain, where NAD⁺ is cleaved to nicotine amide and ADP-ribose moiety, and subdomain which are responsible for ADP-ribose transferase activity.^[3] PARP 1 is responsible for the synthesis of 99% of

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PAR in cell and is activated in response to DNA damage and under various genotoxic stress conditions.^[4] PAR polymer synthesis is initiated by the formation of an ester bond between the amino acid acceptors and the first ADP-ribose. Stepwise elongation occurs at the 2'-OH of the ribose moiety, whereas branching occurs at the 2''-OH position resulting a multibranched polyanion poly(ADPR)/(PAR). The diverse spectra of target proteins attached to linear and branched chains of PAR polymer include histones, DNA repair proteins, transcription factors, and chromatin modulators.^[5] PARP 1 possesses automodification domain (AD) containing the residues targeted for PARylation. AD is located in proximity to the catalytic site of the enzyme, thus PARP 1 molecule itself is the most preferable target protein for PARylation.^[6] Regulation of auto-PARylation of PARP 1 plays crucial role in regulation of the enzyme activity.^[7]

The initial conformation of PARylated proteins is restored by PARG which has endo- and exo-glycosidic activities and is responsible for the degradation of PAR polymer chains.^[8] Branched and short PAR molecules are degraded slowly and with lower affinities by PARG than long and linear polymers.^[9] PARG is encoded by a single gene, but in mammals, several PARG isoforms located in different cell compartments are identified.^[10] PARG isoforms resulted from alternative splice variants from PARG RNA transcripts. The full-length and enzymatic most active PARG protein (111-kDa) localize to the nucleus.^[11,12]

PAR metabolism plays an important role in the development of stress responses, viral infections, and cancer. Although it is recognized that PARG and PARP 1 play an integral role in PAR metabolism, the mechanisms coordinating their activities are poorly understood.

As early as in 1989, Tanuma *et al.* reported that tannic acid (TA) could inhibit the activity of PARG purified from human placenta.^[13] Later, it was demonstrated that TA could suppress basal PARG expression in cells.^[14]

TA (penta-m-digalloyl glucose) is hydrolysable tannin of plant origin, which has numerous food applications and is employed in medicinal purposes for centuries. It is documented that TA exhibits antimutagenic, antioxidant, and anticancer effects.^[15] Several PARG inhibitors exhibit cross-inhibition of PARP 1.^[16]

Although TA-induced PARG inhibition is established in *in vitro* experimental settings, the data obtained in cell culture experiments and animal models should be interpreted consciously. In some cases, treatment with TA caused downregulation of PARP 1 cleavage,^[17-19] which prompted possibility that high activity of PARP 1 could be responsible for PAR accumulation rather than PARG inhibition. In the present paper, we were interested to study whether treatment of rats with TA could modulate PARP 1 activity, PARG cleavage, and NAD⁺ content in liver and thymocytes

nuclei. The second goal of the study was histopathological examination of the liver of intact rats treated with TA.

MATERIALS AND METHODS

Animals

Albino outbred male rats were treated according to the regulations of the committee for bioethics of Yerevan State University.

Animals were obtained from stock of Yerevan State University Biology Faculty of Animal House. Rats were housed in laboratory conditions (20 ± 2°C) with a 12 h light/dark cycle, fed with commercial rat feed *ad libitum* and were given free access to water. Animals were standardized by weight (100 g) and divided into two different groups: Group 1: Control animals – saline was administered intraperitoneal (i.p.) and Group 2 – 100 mg/kg TA administered i.p. In different time intervals after TA treatment (2, 24, and 48 h), rats were sacrificed under light ether anesthesia by decapitation. The procedures were approved by the National Center of Bioethics (Armenia) and performed according to the International Recommendations (CIOMS, 1985) guidelines. All reagents were purchased from Sigma-Aldrich.

Nuclei Isolation

Livers and thymuses were collected and nuclei were isolated according to Hewish and Burgoyne.^[20] Sucrose solutions utilized throughout nuclei isolation procedure were buffered with 20 mM Tris pH 7.4, containing 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine.

Determination of NAD⁺ Content in Nuclei

Purified nuclei from liver cells and thymocytes were suspended in nuclei isolation media and suspension was normalized by DNA (1 mg/ml DNA). DNA was estimated by ultraviolet (UV) absorption in 1 N NaOH. Nuclei from liver and thymus were pelleted from 5 ml of nuclear suspension by centrifugation at 9000 g for 15 min. Supernatant was discarded and ice-cold 0.5 N HClO₄ was added to nuclear pellet. Acid-soluble materials were extracted by vortexing and insoluble acidic pellet, containing proteins and other acid-insoluble substances were discarded (centrifugation 15 min 9000 g). Supernatant was moved to fresh tubes and equal volume of 1 N KOH was added to neutralize acid. Neutralized supernatant was clarified by centrifugation at 9000 g 15 min and NAD⁺ was quantified according to Putt and Hergenrother.^[21]

The enzymatic assay for PARP 1 activity was performed according to original method based on chemical determination of NAD⁺.^[21] The original method was adapted in purpose to

quantify NAD⁺ consumed by nuclei in 10 min from PARP assay buffer. Briefly, nuclei were gently suspended in PARP assay buffer which contains 20 mM Tris, 6 mM MgCl₂, 1 mM CaCl₂, and pH 7.4. Density of nuclear suspension was normalized to 1 mg DNA/ml. PARP reaction was initiated by addition of NAD⁺ stock solution into nuclear suspension to 0.5 mM NAD⁺ final concentration. The reaction proceeded for 10 min (37°) and was stopped by nuclei withdrawal from reaction mixture by centrifugation at 13,000 g, 1°C for 2 min. The supernatant was transferred to the wells of Falcon UV-visible transparent 96-well plate. NAD⁺ quantification was performed in supernatant aliquots after sequential addition of 2 M KOH and 20% acetophenone (in EtOH) and formic acid in accordance with original assay. The absorbance of PARP assay buffer containing 0.5 mM NAD⁺ was determined at 378 nm alongside with the samples derived from nuclear suspensions and was set as standard. NAD⁺ content in samples of nuclear suspension was determined by subtraction of test sample absorbance from the standard. PARP 1 activity was defined as NAD⁺ consumed by nuclei in 10 min.

Statistical Analysis

Data are expressed as mean ± SD. Statistical differences in the results between groups were evaluated by the Student's *t*-test. *P* < 0.05 was considered statistically significant.

Estimation of PARG Protein Content in Isolated Nuclei

PARG content in isolated liver and thymus nuclei was estimated according to the recommendations of manufacturer (MyBioSource, Inc. Rat PARG, ELISA kit).

Light Microscopy

Liver samples were collected from examined groups of animals immediately after decapitation. The samples were fixed in 10% formalin in saline, dehydrated in ascending grades of alcohol, and embedded in paraffin. Paraffin-embedded

5 μm (5 mkm) sections were dewaxed with xylenes, stained with hematoxylin and eosin, and examined under light microscope.^[22]

RESULTS

Previously, we had shown that in 2 h after i.p. injection of TA (100 mg/kg) into rats PARP 1 activity increased nearly by 40%.^[23] Herein, we study whether PARP 1 stimulation in rat liver nuclei reflected short-term fluctuation induced by intervention with biologically active substance or PARP 1 stimulating effect of TA is stable for relatively long time after the treatment of animals. In this purpose, we examine PARP 1 activity in liver and thymocyte nuclei in 24 and 48 h after the treatment of rats with TA. Our results come to show that in 24 h and 48 h after administration of TA to animals PARP 1 activity in rat liver nuclei increased nearly by 2.5-fold and more. Stimulation of enzyme activity in thymocyte nuclei was less dramatic and did not exceed the basal level for more than 22% [Figure 1].

More than three decades ago, it was reported that PARP 1 activity in hepatocytes is linked to intracellular NAD⁺ content.^[24] In the present study, we intended to examine whether stimulation of PARP 1 activity in liver cell and thymocyte nuclei of rats treated with TA could be coupled with modulation in NAD⁺ content.

The results obtained in this set of experiments demonstrated that liver nuclei content nearly by 35% more NAD⁺ than their counterparts from thymocytes. As early as in 2 h after the treatment of rats with TA, we estimated a drop in NAD⁺ level both in liver and thymocyte nuclei nearly for the same extent (10–12% below basal level). The decrease in NAD⁺ content proceeded and in 24 h and 48 h after TA administration to rat NAD⁺ content dropped by 32% and 34% in thymocyte and liver nuclei correspondingly [Table 1].

Coming from the knowledge that treatment of rats with TA affected protein and nuclear RNA synthesis in liver cells^[25]

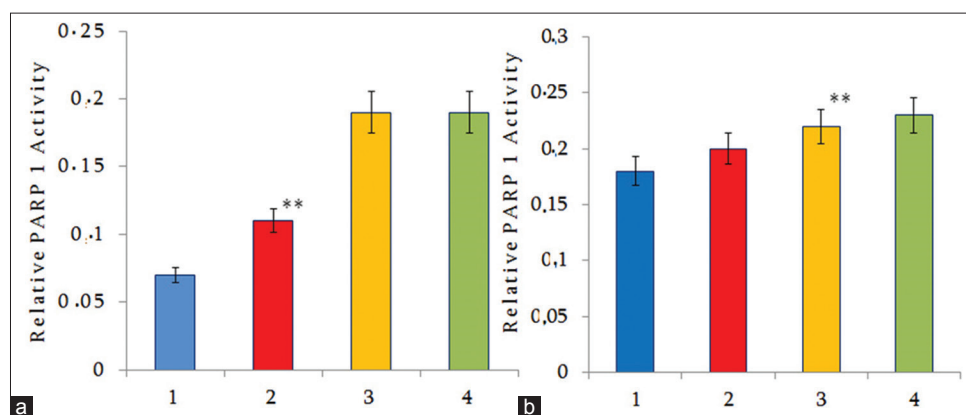


Figure 1: Modulation of poly(ADP-ribose) polymerase 1 activity in: (a) Liver nuclei, (b) thymocyte nuclei. 1 – Control group, 2, 3, and 4 – 2, 24, and 48 h after treatment with tannic acid, ***P* < 0.05

and could reduce the elevation of PARG proteins in murine liver,^[18] we were interested to investigate whether TA could affect PARG protein content in liver nuclei of rats.

We estimated endogenous PARG protein concentration in liver cell and thymocyte nuclei in 2 h, 24 h, and 48 h after i.p. injection of TA into rats. The data come to show that liver nuclei possess larger amount of PARG protein than their counterparts from thymocytes [Figure 2].

PARG concentration in the liver nuclei exceeded more than 3.5-fold the basal value of PARG content in thymocytes nuclei. Initially, high PARP 1 activity in thymocyte nuclei is paralleled with low basal level of PARG protein concentration. In 24 h after administration of TA to rats, we observed that PARG content in thymocytes nuclei dropped by 30%; however, it returned to basal level in 48 h after treatment with TA. In contrast, more significant decrease of PARG content in liver nuclei of rats treated with TA for 24 h (50% below the basal level) was stable and in 48 h after TA administration to animals, it was still by 60% less than basal level.

To investigate whether treatment with TA may influence the viability of hepatocytes, we implemented histological examination of liver collected from rats pre-treated with TA.

Light microscope observations revealed that the treatment of rats with TA caused dissolution of liver lobules, degeneration of hepatic cords, and massive periportal fibrosis in 24 h after injection. These changes indicate on massive hepatotoxicity and became more pronounced in 48 h after administration of TA. At that time, the cytoplasm of the hepatocytes contained

transparent vacuole relevant to clustered lipids and pronounced steatosis emerged. In 48 h after treatment with TA, many hepatocytes with nuclei abnormalities were identified [Figure 3]. Liver imprints revealed nuclear protrusions, vacuolization, and karyolysis. The results of micronucleus test demonstrate that TA display marked hepatotoxicity and genotoxicity as early as in 24 h after administration of TA to rats [Figure 4].

DISCUSSION

It is well documented that when the cells underwent genotoxic stress, PARP 1 is overactivated and PAR polymer turnover is modulated. These events are causative in necrosis/apoptosis or in triggering of PARP-regulated death pathway.^[26-28] Here, we were interested to study whether the treatment of rats with TA could impact PARP 1 activity and cause morphological changes in hepatocytes of rats, considering liver as the main organ responsible for detoxification of xenobiotics in mammals.

Hepatotoxicity of TA in man was discovered in 1925–1945 when it was widely used in the treatment of burns. In 1969, Badawy *et al.* described an inhibitory effect of TA on protein and RNA synthesis in rat liver.^[25] Later, it was revealed that TA could inhibit the activity of purified PARG from human placenta.^[13]

These observations come to show that TA could affect PAR turnover in cells by modulating the activity of enzymes which are involved in metabolic pathway and play a pivotal role in regulation of cell life or die decisions.^[29]

We hypothesized that in addition to direct effect on PARG activity in cells, TA could indirectly affect PAR turnover through modulation of PARP 1 activity.^[30,31]

The results obtained in the present study demonstrate that in 24 h and 48 h after administration of TA to animals PARP 1 activity increased nearly 2.5-fold in rat liver and by 22% in thymocyte nuclei over basal level. In concert with our data reported earlier,^[22] this result comes to show that stimulation of PARP 1 activity in rat liver and thymocyte nuclei by TA is relatively

Table 1: NAD⁺ content (mM) in liver and thymocytes nuclei of rats treated with TA (100 mg/kg)

TA (h)	Liver	Thymocyte
0	0.045±0.006	0.033±0.003
2	0.04±0.003**	0.029±0.001**
24	0.03±0.002**	0.022±0.001**
48	0.03±0.002**	0.022±0.002**

***P*<0.05

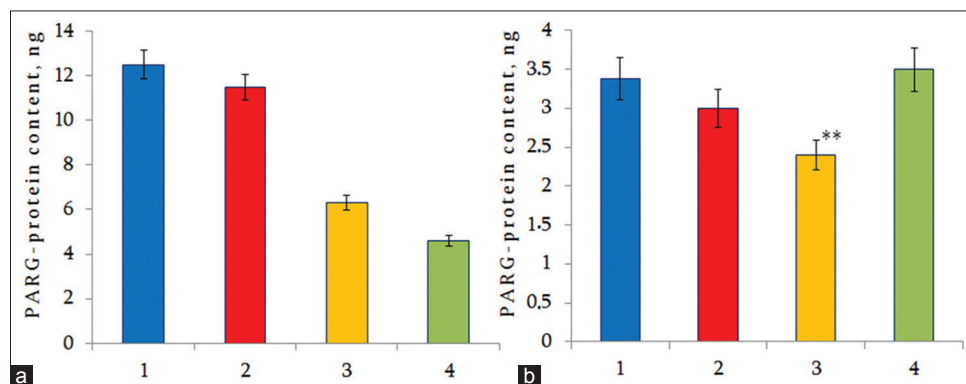


Figure 2: Content of poly(ADP-ribose) glycohydrolase protein in: (a) Liver, (b) thymocyte nuclei. 1 – Control group, 2, 3, and 4 – 2, 24, and 48 h after treatment with tannic acid, ***P* < 0.05

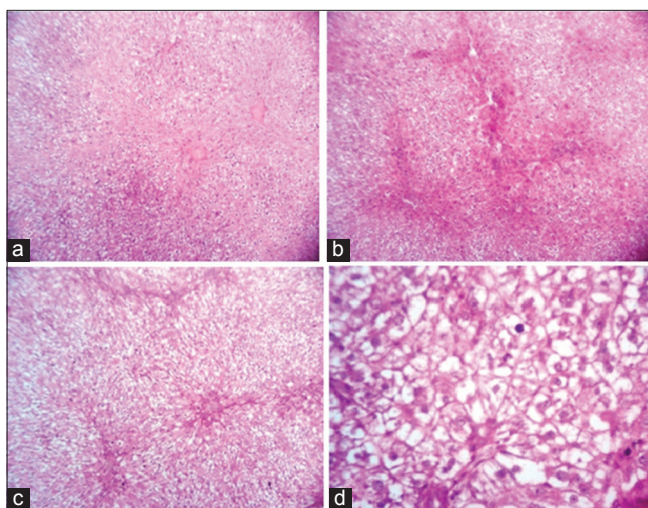


Figure 3: (a) Rat liver from control group; (b) liver in 24 h after treatment with tannic acid (TA); (c) liver in 48 h after treatment with TA, $\times 100$; (d) liver in 48 h after treatment with TA, $\times 400$

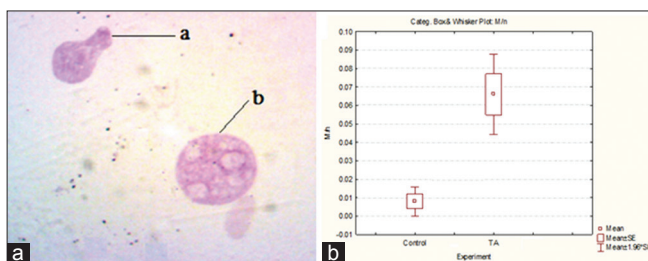


Figure 4: (a) Liver nuclei protrusion a; b – nuclei vacuolization, $\times 1000$; (b) mean values of micronucleus test; control -0.008 ± 0.004 ; tannic acid -0.066 ± 0.011

stable phenomenon and is not a short-term fluctuation, induced by intervention with biologically active substance.

It has been suggested earlier that the level of PARP 1 activation depended on the concentration of NAD⁺ in cells and nuclei.^[32,33] In recent years, the mechanism that linked PARP 1 activity with NAD⁺ content was investigated from the viewpoint of its role in triggering cell death programs. It is well documented that overactivation of PARP 1 results in severe NAD⁺ depletion in cell under stress conditions.^[26,34]

Coming from this knowledge, we anticipated that stimulation of PARP 1 activity in liver and thymocyte nuclei of rats treated with TA might increase NAD⁺ consumption, and here, we examined whether the treatment of rats with TA could impact NAD⁺ content in liver and thymocyte nuclei. The results obtained in this set of experiments demonstrated that liver nuclei content nearly by 35% more NAD⁺ than their counterparts from thymocytes. We found that as early as in 2 h after the treatment of rats with TA NAD⁺ content in nuclei decreased. The drop in NAD⁺ in 24 h and 48 h after TA administration to rats reached up to 32% and 34% below basal level in thymocyte and liver nuclei correspondingly [Table 1]. These data are in good accordance with the results reported by other investigators.^[35,36]

In the present study, we demonstrated that thymocyte nuclei exhibit higher PARP 1 activity than liver nuclei, whereas the latter possess larger amount of PARG protein than their counterparts from thymocytes. Thus, coming from our data, low basal PARP1 activity in liver cell nuclei is correlated with high concentration of PARG protein, in contrast, high basal PARP 1 activity in thymocyte nuclei is paralleled with low PARG protein concentration. It was revealed that administration of TA to rats caused decrease of PARG content in thymocyte and liver nuclei. We suppose that the drop in PARG content in thymocyte and liver nuclei of TA-treated rats could result from downregulation of protein and RNA synthesis in nuclei by TA reported earlier.^[24] We suppose that low levels of PARG content could cause impairment of NAD⁺ recycling due to its “locking” in PAR chains in result of decreased PARG activity. However, the drop in NAD⁺ content could result from TA-induced stimulation of PARP 1 activity in liver and thymocytes nuclei documented herein. Increased PAR levels, resulted from to TA-induced PARP 1 activation, in concert with decreased PARG and the drop in NAD⁺ content could trigger PAR-mediated cell death.^[37,28] We hypothesize that this chain of biochemical events could underlay genotoxic effect of TA revealed by micronucleus test and histopathology of liver collected from TA-treated rats.

CONCLUSION

Activation of PARP 1 in rat liver and thymocyte nuclei by TA is relatively stable phenomenon induced by intervention with biologically active substance. TA stimulated PARP 1 activation in thymocyte and liver nuclei is paralleled with drop in NAD⁺ content. Concentration of poly(ADP-ribose) glycohydrolase (PARG) in liver nuclei exceeded the basal value of PARG content in thymocyte nuclei nearly 3,5 fold. In 24 h after administration of TA to animals content of PARG protein in thymocyte nuclei decreased. Decrease of PARG content in liver nuclei was more substantial and proceeded for 48 h after treatment of rats with TA. TA-induced stimulation of PARP 1 in liver and thymocyte nuclei which was paralleled with decrease in NAD⁺ and PARG content, could alter poly(ADP-ribose) polymer (PAR) turnover and mediate genotoxic effect of TA revealed by light microscopy examination of liver.

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