Research Article

Treatment of Rats with Tannic Acid Modulates Poly(ADP-Ribose) Polymerase 1 Activity in Liver and Thymocyte Nuclei

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ABSTRACT

ADP-ribosylation of proteins by poly(ADP-ribose) polymerase 1 (PARP 1) is the earliest initiation step in DNA damage processing. The cleavage of protein-associated poly(ADP-ribose) (PAR) chains and recovery of initial conformation of proteins is accomplished by poly(ADP-ribose) glicohydrolase (PARG). The activities of PARP 1 and PARG are precisely coordinated and imbalance in dynamic equilibrium suppressed DNA repair, thereby directing cells towards different death programs. Nowadays, the interest of many scientists is focused on investigation and design of new PARP 1 and PARG inhibitors.This study is addressed to investigate the effect of in vivo treatment of rats with PARG inhibitor tannic acid (TA) on PARP 1activity and chromatin structure in rat liver and thymocyte nuclei. Herein, we demonstrated that treatment of rats with TA administered intraperitoneal impacted PARP 1 activity in thymocyte and liver nuclei in organ- and dose dependent manner. Treatment of rats with TA diminished inhibitory efficiency of benzamide (BA) on PARP 1 activity in liver nuclei. Our data show that suppression of PARP 1 in liver nuclei of rats treated with TA was paralleled with rise of chromatin accessibility to Ca^{2+}/Mg^{2+}-dependent endonuclease. Incubation of nuclei with BA led to further elevation of DNA internucleosomal fragmentation.

Keywords: Poly(ADP-ribose) polymer, tannic acid, poly(ADP-ribose)polymerase 1, DNA internucleosomal fragmentation.

INTRODUCTION

Formation of poly(ADP-ribose) polymer chains linked to lysine and glutamine residues of proteins is one of the most significant post-translational protein modifications mediated by the group of ADP-ribosyl transferases (ADPRT). The enzymes of this group cleave NAD+ and transfer ADP-ribose moieties to lysine and glutamine residues of polypeptide chains either in the form of single unite in the case of mono(ADP-ribosyl)ation, or by formation of linear or branched ADP-ribose polymers via sequential addition of ADP-ribose units to first unit attached to protein. The enzymes responsible for synthesis of poly(ADP-ribose) polymers (PAR) belong to family of poly(ADP-ribose) polymerases (PARPs) and were discovered over 50 years ago [1].

Lengthening of PAR polymers is paralleled with chain branching with up to 3%frequency. High negative charge of PAR chains bound to proteins modify their conformation and alter biological activities [2].

PARP 1(EC 2.4.2.30) is the most abundant and well investigated member of PARP family. The enzyme is localized to cell nuclei, where it catalyzes PARylation up to 10% of non-PARP proteins while approximately 90% of PAR polymers become covalently attached to automodification domain of PARP-1 [3]. The activity of PARP 1 is stimulated by DNA strand breaks and unusual DNA conformations e.g. cruciform, hairpins and loops [4]. Catalytic activity of PARP 1 is enhanced 10-500-fold within 15-30 s of DNA binding and is recognized as earliest cell reaction to DNA damage [5]. PARP-1 plays prominent role in basal nuclear functions comprising DNA repair and replication, chromatin modulation and transcription [6,7,8]. Based on the strength of DNA damage and metabolic status of the cell, PARP 1stimulation triggers biochemical pathways directing cells to death or survival [9,10,11].

Under cellular stress conditions PARP1is considered to be the greatest NAD+ consumer [12]. To circumvent energetic catastrophe and cytotoxic effects of accumulated PAR polymers their turnover in cells is strictly regulated [13]. PAR polymers in cell are rapidly (half-life 1 min) degraded mainly by the endo-exoglycosidase poly(ADP-ribose) glycohydrolase (PARG) (EC 3.2.1.143). Two additional enzymes ADP-ribosyl hydrolase and O-acyl-ADP-ribose deacylaseare implicated in complete destruction of PAR polymers and removal of the last mono(ADP-ribose) residue from PARylated molecules. In concert, aforementioned enzymes are responsible for cleavage and dissociation of PAR chains from PARylated proteins e.g. from auto-modificated PARP 1molecule. Extensively PARylated molecules of PARP1dissociate from the site of DNA damage 5. Initially formed PAR chains are trimmed off from PARP 1by PARG to form short ones. However, even this shortened PAR chains embarked on PARP prevent rejoining of enzyme molecule to sites of DNA damage and thus, suppress its activity and DNA repair [14].

In mice and humans PARG is encoded by a single gene. Due to alternative splicing in humans PARG exists at least in five variants, which differ in size and localization. Full-length hPARG111/110 is localized to nuclei, hPARG103/102 and hPARG99 localize extra-nuclearly, and hPARG55 was found in mitochondria, hPARG60 has been shown in various localizations [15].

The metabolism of (ADP-ribose) polymers (PAR) plays pivotal role in development of different pathologies

arising from disbalance in cell homeostasis e.g. inflammation, neoplasia, neurodegenerative deceases, heart failure and brain stroke.

The degradation of PAR polymers by PARG depends on the chain length and branching intensity. Affinity of PARG is much higher to long and linear polymers (KM = 0.1-0.4 µM) vs. (KM ≈ 10 µM) for short and branched polymers. Complete loss of cellular PARG activity in knock-out mice results in early embryonic lethality of homozygous mutant mice, enhances the level of PAR-modification of histones H1, H2A, and H2B, increases DNA accessibility in chromatin for MNase and acridine orange, and enhances DNA damage by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and UV radiation [16,17]. PARG inhibition slows down degradation of PAR polymers and their removal from auto-modificated PARP 1 protein.

Accumulating data suggest mutual repercussions between accumulation of PAR polymers in the cells and various epigenetic pathways e.g. regulation of PARP 1 activity after genotoxic stress. PARylated proteins are involved in basal cellular functions and their metabolism maintains variety of vital functions. Imbalance in dynamic equilibrium between PARP1 and PARG interferes with chromatin condensation and death/surviving programs determining cell fate. Given the key functions in DNA repair, replication and chromatin remodeling, PARP1 and PARG inhibition is considered a promising strategy in therapy of cancer and other diseases.

PARG can be inhibited by hydrolysable tannins, ethacridine, the ADP-ribose analogue ADP-(hydroxymethyl)-pyrrolidinediol (ADP-HPD), N-bis-(3-phenyl-propyl)9-oxofluorene-2,7-diamide (GPI 16552), and the modified PAR containing the etheno moiety [18].

Coming from the role of PARG in PAR polymer turnover, regulation of enzyme activity moved into focus of investigators interested in design of new drugs with high therapeutic outcomes.

Natural chemicals which are being tested for pharmacological purposes are preferable from the viewpoint of safer management. Tannins are naturally occurring water-soluble polyphenols widespread in plant kingdom. The curative properties of tannins are exploited for centuries. An inhibitory effect of tannin on purified PARG from human placenta was demonstrated by Tanuma et al [19]. Hitherto, the vast majority of studies were performed with gallotannin or tannic acid (TA), which demonstrated anti-

cancer properties by inducing apoptosis and controlling cancer cell proliferation [20].

In present study we examine whether *in vivo* treatment of rats with TA could have influence PARP 1 activity in liver and thymocytes nuclei and chromatin condensation in liver nuclei.

MATERIALS AND METHODS

Animals

Animals were handled in accordance with protocols approved by Committee for Bioethics of Yerevan State University. Animals were obtained from the stock of animal house of faculty of Biology, Yerevan State University. Rats were housed in laboratory conditions in polypropelene cages at standard conditions (22±2^{o}C) with a 12 light/dark cycle. Animals were fed with commercial rat feed ad libitum and were given access to water. The procedures were approved by the National Centre of Bioethics (Yerevan, Armenia) and performed according to the International Recommendations (CIOMS, 1985) guidelines.

Albino inbred male rats (6 week old) were used throughout all experiments. The animals were standardized by weight (to 100g). TA and other Reagents were purchased from Sigma-Aldrich. Vehicle (saline) and TA (25 mg/kg and 100 mg/kg weight) were injected intraperitoneal. Animals were sacrificed under light ether anesthesia by decapitation after 1, 2 and 3 h treatment with TA.

Nuclei isolation

Nuclei were isolated according to Hewish and Burgoyne[21]. Sucrose solutions utilized throughout the nuclei isolation procedure were buffered with 20 mM Tris containing 15 mM NaCl, 60 mMKCl, 0,15mM spermine and 0.5mM spermidine, pH 7,4.

PARP 1 assay

The enzymatic assay for PARP 1 activity was performed according to the original method based on estimation of residual NAD^+ concentration in PARP assay mix adapted by us to quantify NAD^+ consumed by isolated nuclei [22]. Briefly, nuclei were gently suspended in PARP assay buffer containing 20mM Tris, 6mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4. Density of nuclear suspension was normalized to 1mg DNA/ml. PARP reaction was initiated by addition of NAD^+ stock solution to 1000 µl aliquot of nuclear suspension (500 µM NAD^+ final concentration). The reaction was carried out for 10 min at 37^{0}C followed by centrifugation at 13 000g, 4^{o}C for 2 min to discard nuclear pellet. 50µl aliquot samples of supernatant were transferred to the Falcon UV-Vis transparent 96-well plate. NAD^+ quantification was performed by sequential addition of 2M KOH, acetophenone (20% in EtOH) and 88% formic acid, in accordance with the original assay. Absorbance of PARP assay mix containing 0,5mM NAD^+ was measured at 378 nm. The amount of NAD^+ was determined by using NAD^+ calibration curve and PARP 1 activity was defined as NAD^+ consumed by nuclei in 10 min per mg of DNA.

DNA fragmentation assay

100 µl aliquot samples of nuclear suspension normalized to1000 µg/ml DNA were transferred to the Eppendorf

tubes and 60mM $MgCl_2$ and 10mM $CaCl_2$ were added to yield final concentrations of 6mM $MgCl_2$ and 1mM $CaCl_2$ in aliquot probes. The ions were added to activate endogenous Mg^{+2} - and Ca^{+2} /Mg^{+2} -dependent nuclear endonucleases, which initiated internucleosomal DNA cleavage [23]. DNA isolation was performed according to standard protocol [24].

Nuclear DNA was subjected to electrophoresis in 1,8 % agarose gels (8v/cm). DNA was visualized by ethidium bromide staining and DNA fragmentation was assessed after gel densitometry using Fuji Film Image Gauge ver. 3.12 program for determination of relative content of DNA fragments.

Statistics

All results were expressed as M ± S.D. Statistical differences in the results between groups were evaluated by the Student's t-test. A probability (P) value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Poly(ADP-ribose)polymerase 1 (PARP 1) inhibition is considered as a promising strategy to improve cytotoxic effect of DNA damaging agents. From the other hand, cytotoxicity of DNA damaging impacts could be improved by imbalance in poly(ADP-ribose) polymer turnover mediated by PARG inhibition [25]. Currently, when various synthetic PARP1 inhibitors are entering clinical trials, their toxicity and side effects in cancer patients become apparent. From this viewpoint, exploitation of naturally occurring non-toxic compounds in treatment of cancer patients is preferable approach. In present study we investigated whether TA administered to rats intraperitoneal (i.p.) could influence PARP 1 activity and modulate chromatin condensation in liver and thymocyte nuclei.

It was documented earlier, that in 1 h after i.p. injection to rats, TA began to accumulate in liver and proceeded in further 2 hours. Maximal accumulation of TA in liver cell nuclei was observed 3h after injection [26]. Coming from this knowledge, we were interested to investigate the influence of i.p. injection of TA to rats in 1, 2 and 3h after animals treatment.

The data of present study come to show, that in 1 h after treatment of rats with 25mg/kg TA, PARP1 activity in liver nuclei was suppressed nearly by 12% below the basal level. The basal level was determined as PARP 1 activity displayed in liver nuclei of animals from control group. In 2 h after TA injection PARP 1 activity in rat liver nuclei did not undergo significant change. Further decrease in PARP 1 activity was estimated in 3 h after treatment of rats with 25mg/kg TA, when maximal accumulation of TA in liver nuclei occurred. As it was anticipated, changes in PARP 1activity were more significant in liver nuclei of rats treated with 100mg/kg TA. In 1 h after injection we did not estimate difference in PARP 1 activity in liver nuclei of animals treated either with 100 mg/kg or 25 mg/kg TA. In both cases PARP 1 was suppressed in liver nuclei in the same extent. However, in 2h after treatment with 100mg/kg TA, we observed PARP 1 stimulation, and enzyme activity exceeded basal level by 50%. PARP 1stimulating effect, displayed by TA in liver nuclei was not permanent, and in 3h after treatment with 100mg/kg TA we observed inhibition of the enzyme by 25% (fig. 1A). We suppose that PARP 1 stimulating effect of 100mg/kg TA, revealed herein, could be coupled with commitment of liver intoxication and necrosis induced by i.p. injection of TA, which started in 2 h after treatment according to Badawy et al in liver of rats [27].

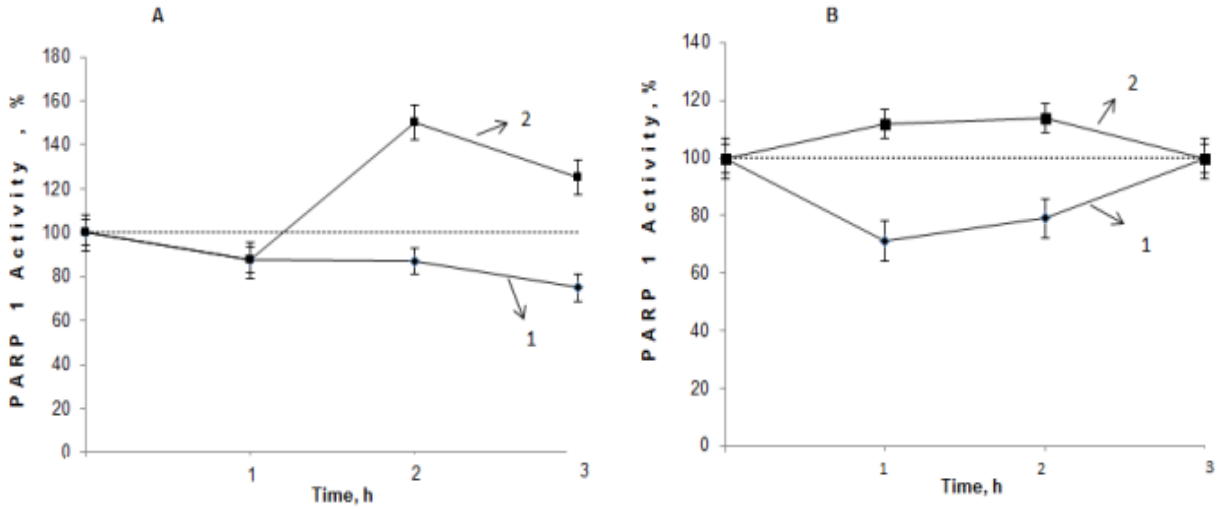

Figure 1: Time-dependent modulations in PARP 1 activity after treatment of rats with tannic acid. A) PARP 1 activity in liver nuclei, B) PARP 1 activity in thymocyte nuclei. TA administered in dose; 1- 25 mg/kg, 2- 100 mg/kg.

PARP activity was determined in nuclei isolated in 1h, 2h and3h after i.p. injection of tannic acid. Dash line presented PARP 1activity in nuclei isolated from organs of control group animals.

PARP 1 dynamics in thymocyte nuclei examined in present study was different. PARP 1 activity decreased in 1h after i.p. injection of 25 mg/kg TA to rats even more significantly than in liver nuclei (by 30%). In 2 h after TA treatment PARP 1activity in thymocyte nuclei did not undergo notable change. In 3 h after TA injection to rats the rise of PARP 1activity up to basal level was observed. In contrast to this, in 1 and 2 h after treatment of rats with 100 mg/kg TA we revealed PARP stimulation by 12-13% in thymocyte nuclei. However, in 3 h after TA injection PARP 1 activity decreased and returned back to basal level (fig. 1B).

To circumvent complexities arising from interference of different metabolic pathways engaged in TA utilization in hall organism, we design experiments which aimed to study in vitro effect of TA on PARP 1 activity in nuclei isolated from thymocytes and liver of control group animals. The results derived from this set of experiments demonstrated, that when thymocyte and liver nuclei were pre-incubated for 15 min in media containing 100 µM TA, they display elevated activity of PARP 1 (nearly by 52% in thymocyte and by 60% in liver nuclei over basal level), which could result from TA-induced prevention of NAD depletion in nuclei [13]. Benzamide (BA) is recognized as prototypic PARP 1 inhibitor. We were interested to examine whether TA could influence PARP 1 inhibition by BA, considering that enzyme suppression is accepted as a strategy in chemotherapy of cancer patients. To eliminate complications sprouting from the influence of BA on energy metabolism [28,29], we introduced BA into incubation media of thymocyte and liver nuclei after their pre-incubation in the presence of 100 µM TA. Our data come to show that pre-incubation of nuclei with TA improved efficiency of PARP 1 inhibition by BA more than by 50% vs 30% inhibition in control nuclei incubated without TA (fig 2).

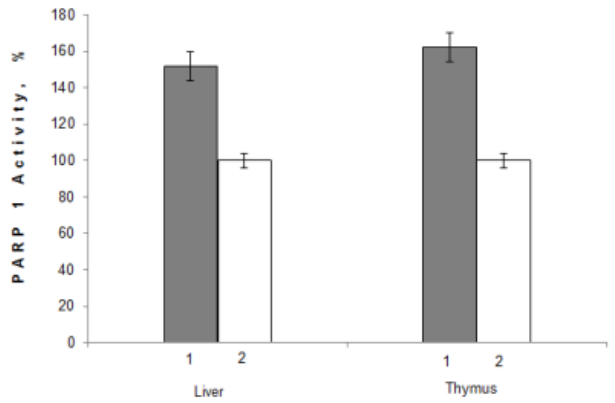

Figure 2: BA-induced inhibition of PARP 1 in nuclei isolated from liver and thymocytes of rats. PARP 1 activity in nuclei isolated from control group animals was set as 100%. 1-isolated nuclei were pre-incubated with 100 µM TA 15 min, 2-BA (20mM) was introduced into nuclei incubation media in 15 min after pre-incubation with TA.

Earlier we had demonstrated that BA-induced PARP 1 inhibition in thymocyte and liver nuclei could be modulated by cisplatin administered to rats [30]. In present study we investigated whether inhibitory potential of BA could be influenced with pretreatment of rats with TA *in vivo*.

Our data come to show, that in 1 h after treatment with 25 mg/kg TA, BA-induced inhibition of PARP 1 decreased by 15%. In 2 and 3 h after 25 mg/kg TA was administered to rats, we observed that PARP 1 inhibition by BA in liver nuclei increased and reached the basal level of inhibition (BA-induced PARP 1 inhibition in liver nuclei of control animals). In contrast, 100 mg/kg TA injection to rats suppressed BA-induced inhibition by 15% in 1 h and further decrease in inhibitory potency of BA was determined in 2 h (10%). Eventually, BA-induced PARP 1 inhibition was completely eliminated in 3h after 100 mg/kg TA injection (fig. 3). This data demonstrate, that the extent and time course in decrease of BA efficiency depended on dose of TA administered to rats.

We suppose, that PARP 1 stimulation detected in 2 h after treatment of rats with 100 mg/kg TA, could cause intensive auto(ADP-ribos)ylatation of PARP1. Though heavily PARylated enzyme molecules could dissociate from DNA, they would be “trapped” into PAR polymers due to TA-induced PARG inhibition. Eventually, the number of free PARP 1 molecules would decrease and the role of enzyme activating or inhibiting factors will diminish. The latter was manifest in suppression of inhibitory potential of BA demonstrated herein.

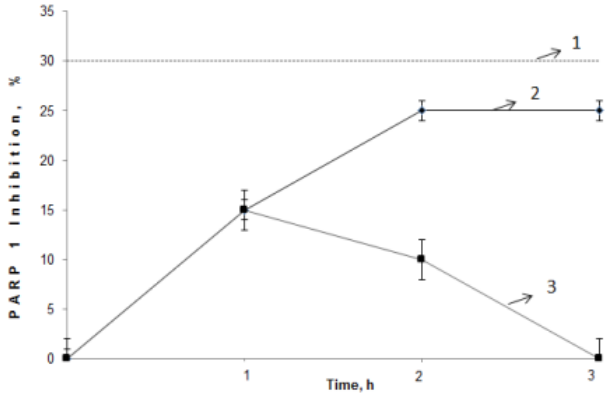

Figure 3: BA-induced PARP 1 inhibition in nuclei, isolated from liver of rats treated with TA. 1-BA-induced PARP 1 inhibition in liver nuclei isolated from control group animals (basal level of inhibition), 2-rats were treated with 25 mg/kg TA, 3-rats were treated with 100 mg/kg TA.

It is widely accepted that poly(ADP-ribos)ylation of chromatin proteins plays prominent role in maintaining chromatin structure and genome stability [11]. Recently, PARylation of DNA moved into focus of investigators and this phenomenon necessitates revalidation of wide spectrum of results [31].

From this viewpoint we were interested to investigate whether PARP 1inhibition in liver nuclei after in vivo treatment of rats with TA could affect chromatin

structure. We employed chromatin structure-dependent assay, utilizing artificial activation of endogenous nuclear Ca^{+2}/Mg^{+2}- dependent apoptotic endonuclease to perform chromatin cleavage [32,33,34].

Figure 4: Effect of in vitro treatment with BA on DNA internucleosomal fragmentation in liver nuclei isolated from rats, treated with TA 2h. Lane: 1-nuclei from liver of control group rats were incubated for 15 min with 20mM BA, prior to addition Ca^{2+}, Mg^{2+} ions into incubation media. 2- nuclei from liver of TA-treated (25 mg/kg) rats were incubated for 60 min with Ca^{2+}, Mg^{2+} ions. 3- nuclei from liver of TA-treated (25 mg/kg) rats were incubated for 15 min with 20mM BA, prior to addition Ca^{2+}, Mg^{2+} ions into incubation media.4- nuclei from liver of TA-treated (100 mg/kg) rats were incubated for 60 min with Ca^{2+}, Mg^{2+} ions. 5- nuclei from liver of TA-treated (100 mg/kg) rats were incubated for 15 min with 20mM BA, prior to addition Ca^{2+}, Mg^{2+} ions into incubation media.

Our data show, that suppression of PARP 1 in liver nuclei of rats treated with 25 mg/kg (in 2h after injection) was paralleled with the rise of chromatin accessibility to Ca^{2+}/Mg^{2+}-dependent endonuclease (fig. 4, lane 2). Incubation of these nuclei with BA led to further stimulation of DNA internucleosomal cleavage of chromatin. (fig 4 lane 3, table 1). At the time point, when injection of 100 mg/kg TA caused elevation of PARP 1 activity (2h after treatment), intensity of DNA internucleosomal fragmentation in liver nuclei did not undergo reliable changes.

However, PARP 1 inhibition by BA caused significant activation of DNA internucleosomal fragmentation. Recapitulating this data, we hypothesize, that there are two routs by which TA-induced modulation in PARP 1 activity could impact chromatin condensation in liver nuclei. First, accumulation of DNA lesions caused by suppression of DNA repair via PARP 1 inhibition, which led to elevated accessibility of chromatin to Ca^{2+}/ Mg^{2+}-dependent endonuclease in liver nuclei after injection of TA. Second, elevation of Ca^{2+}/ Mg^{2+}-dependent endonuclease activity per se, caused by release of the enzyme from inhibition by PARP 1, emerged when BA was employed for PARP 1 inhibition[35].

Table 1: Effect of in vitro treatment with BA on DNA internucleosomal fragmentation in liver nuclei isolated from rats, treated with TA 2h. Relative content (%) of DNA in different length fragments. DNA content per lane set as 100%. *-p < 0,05.

The length of DNA fragments	Control	TA treated (25mg/kg)		TA treated (100mg/kg)	
	15 min 20mM BAM + 60 min Ca^{2+}/Mg^{2+}	60 min Ca^{2+}/Mg^{2+}	15 min 20mM BAM + 60 min Ca^{2+}/Mg^{2+}	60 min Ca^{2+}/Mg^{2+}	15 min 20mM BAM + 60 min Ca^{2+}/Mg^{2+}
>1000 b.p.	5.5 ± 0.2	5.8 ± 0.29	*1.1 ± 0.01	6.7 ± 0.4	*1.0 ± 0.02
1000-200 b.p.	92.4 ± 3.6	*86.1 ± 3.4	84.3 ± 5.01	86.0 ± 3.6	82.3 ± 4.1
< 200 b.p.	2.1 ± 0.12	*8.1 ± 0.4	*14.6 ± 0.58	7.3 ± 0.36	*16.7 ± 1.1

While the roles of PARG and poly(ADP-ribose) polymerase (PARP) are closely entangled, we believe that investigation of TA-induced modulation of PARP 1 activity will support understanding pharmacological effects of therapeutic agents targeting DNA damage response.

CONCLUSION

Treatment of rats with TA administered intraperitoneal, down-regulated PARP 1 activity in thymocyte and liver nuclei. In vivo effect of TA on PARP 1 activity display organ- and dose dependent specificity. TA altered inhibitory effect of prototypic PARP 1 inhibitor BA, diminishing its inhibitory potency in liver and thymocyte nuclei. Our data show that suppression of PARP 1 in liver nuclei of rats treated with TA was paralleled with rise of chromatin accessibility to $Ca^{2+/}Mg^{2+}$-dependent endonuclease manifesting chromatin decondensation.

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