CISPLATIN MODULATES POLY(ADP-RIBOS)YL POLYMERASE 1 INHIBITION AND DNA INTERNUCLEOSOMAL FRAGMENTATION IN RAT LIVER NUCLEI

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ABSTRACT

In last decade poly(ADP-ribos)yl polymerase 1 (PARP1) inhibitors enter into clinical trials for combination cancer chemotherapy improving curative potential of DNA-damaging agents. Information about the role of age and sex-dependent differences in development of drug toxicity during co-treatment with cisplatin and PARP 1 inhibitors is limited.In present study we investigated the role of sex-and age-dependent variables on PARP 1 activity in rat liver nuclei. Taking into consideration that the vast majority of pharmacological PARP 1 inhibitors are designed as benzamide (Bam) analogues and different ATP mimetics are used in chemotherapy, we study effect of Bam and ATP on PARP 1 in rat liver nuclei. The data come to show that PARP1 activity in liver nuclei decreased in the course of sexual maturation of rats (6-10 weeks). No sex-dependent differences in PARP 1 inhibition in rat liver nuclei by Bam and ATP were revealed, whereas reliable age-dependent differences in PARP 1 inhibition were apparent.The in vivo treatment of rats with cisplatinwas found to cause 2,5-3 fold PARP1 suppression in 6 week old rat liver nuclei, activation of the enzyme in nuclei of 10 week old rats by 100-150%% andmodulated inhibitory efficacy of Bam and ATP. Chromatin structure-dependent assay revealed that PARP 1 inhibitors affected intensity of DNA internucleosomal fragmentation in rat liver nuclei. PARP1 inhibition by 5mM ATP coincided with complete suppression of DNA internucleosomal fragmentation in liver nuclei. Treatment of 10 week old rats with cisplatin led to increased accessibility of chromatin to endonucleolitic attacks and changed the effect of inhibitors on DNA fragmentation. The data of present study come to show that complicated interplay between cisplatin and PARP 1 inhibitors can influence chromatin structure in liver cells.

Keywords-cisplatin, PARP 1 inhibition, nuclei, DNA internucleosomal fragmentation

INTRODUCTION

Cisplatin [cis-diammine-1,1-cyclobutanedicarboxylate platinum(II)] is one of the most effective and commonly used antitumor drugs which kills cancer cells by forming inter- and intra-chain cross –links in DNA. However, to maintain genome stability cells evolve DNA repair mechanisms which display different activities in normal and cancer cells[1,2]. Chromatin-associated enzyme poly(ADP-ribose)polymerase 1 (PARP 1) plays central role in DNA repair by different pathways e.g. BER, NER, MMR[3]. DNA-lesions caused by cisplatinare readily recognized by PARP1 which is identified as platinum DNA damage response protein. Upon binding to damaged DNA PARP1 undergoes activation and facilitates DNA repair thus, attenuating cytotoxic potential of cisplatin and other DNA-alkylating drugs[4,5]. Mounting data come to show that PARP1 inhibition sensitize cancer cells to cytotoxic insults induced by temozolomide and cisplatinthereby improving therapeutic responses[6-8]. Currently PARP1 inhibitors are entering the phase of clinical testing and are used as monotherapeutic agents in treatment of BRCA-deficient cancers. Though drug-drug interaction in the course of combination chemotherapy is widely recognized phenomenon which challenges salutary effect of pharmacotherapy, the mechanisms standing behind this are poorly investigated. Coming from the data indicating on sexually dimorphic response to therapies with PARP 1 inhibitors, in present study we examine whether cisplatin can influence PARP1 inhibition by competing inhibitor benzamide (Bam) and allosteric inhibitor ATP in rat liver nuclei in sex- and age-dependent manner[9].

The link between PARP 1 and chromatin structure and functional activity is well established. In present study we intend to reveal whether cisplatin and PARP1 activitycan affect the ability of chromatin to undergo DNA internucleosomal cleavage, which is widely recognized hallmark of apoptosis and, from the other hand, reflect structural features of chromatin[10,11].

MATERIALS AND METHODS

Animals

Animals were treated according to regulations of Committee for Bioethicsof Yerevan State University.

Albino inbred male and female rats (6 and 10 week old) were used throughout all experiments. The animals were standardized by weight in either age group (to 100g and 150g in 6 and 10 week old correspondingly). Cisplatin, Bam, ATP and other reagents were purchased from Sigma-Auldrich. Vehicle (saline) and cisplatin (10mg/kg weight), were injected intraperitoneal. Animals were sacrificed under light ether anesthesia by decapitation after 48 h treatment with cisplatin.

Liver nuclei isolation

Liver nuclei were isolated according to Hewish and Burgoyne[12]. Sucrose solutions utilized throughout the nuclei isolation procedure were buffered with 20 mMTris containing 15 mMNaCl, 60 mMKCl, 0,15mM spermine and 0.5mM spermidine, pH 7,4.

PARP1 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[13]. 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 (0.5 mM NAD^+final concentration). The reaction was carried out for 10 min at 37^0C followed by centrifugation at 13 000g, 4^oC for 2 min to discard nuclear pellet. 50µl aliquote samples of supernatant were transferred to the Falcon UV-Vis transparent 96-well plate. NAD^+quantitation 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.

PARP 1 protein identification

PARP-1 protein was identified by Western-blot analysis. Nuclear extract was denatured for 5 min at 95^oC and electrophoresed through 7.5% SDS polyacrylamide gel. The proteins were transferred on to Hybond™ C-Extra nitrocellulose membrane (Amersham).The membrane was blocked in 3% bovine serum albumin (BSA) overnight at 4^oC and was probed with monoclonal anti-PARP-1 antibody (Sigma, clone# C-2-10) diluted 1:1000 in standard incubation buffer (150mM NaCl, Tris 10mM, pH 7,4, Tween-20 0,05%) for 2 h at room temperature. Alkaline-phosphatase- linked anti-rabbit IgG (1:30000 dilution, 1 h at room temperature) was used and PARP 1 was detected by NBT/BCIP solution.

DNA fragmentation assay

100 µlaliquote 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 aliquote probes. The ions were added to activate endogenous Mg^{+2} - and Ca^{+2} /Mg^{+2} -

dependent nuclear endonucleases, which initiated internucleosomal DNA cleavage[11]. DNA isolation was performed according to standard protocol[14].

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 two-tailed Student's t-test. A probability (p) value of < 0.05 was considered significant.

RESULTS

Estimation of PARP 1 activity

According to our data the baseline activity of PARP 1 in 6 week old male rat liver nuclei exceeded enzyme activity of female rat nuclei by 25%. However, nuclei of 10 week old rats exhibited significant decrease in PARP 1 activity in both sexes. Age-dependent PARP 1 down-regulation was paralleled with elimination of sex-dependent differences (fig 1, a).

To circumvent physiological complications and side effects sprouted from the in vivo treatment of rats with Bam and ATP, we examined enzyme inhibition in the in vitro system where inhibitors were introduced into nuclei incubation media in 15 minutes prior to addition of constituents of PARP 1 reaction mixture.
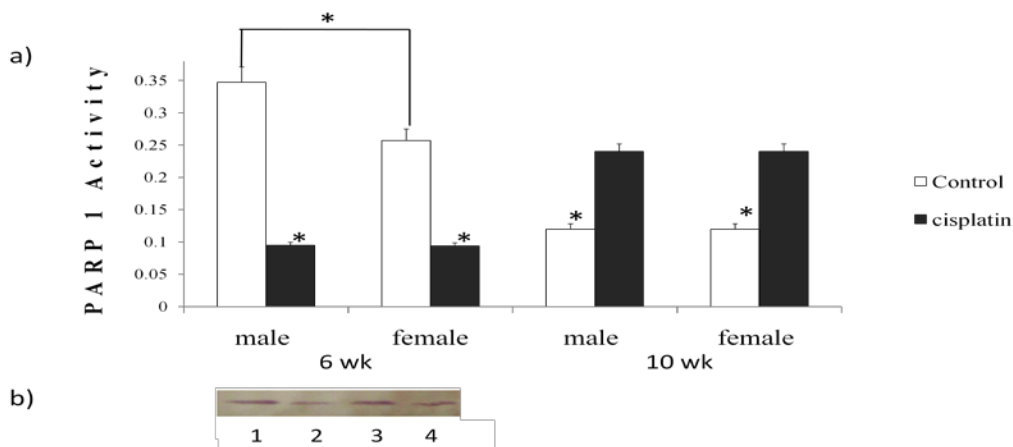

Fig 1. The effect of cisplatin on age-dependent modulation of PARP 1 activity in rat liver nuclei (a); PARP 1 protein immunodetection in liver nuclei (b); 1- PARP 1 human expressed in E.Coli, 2- PARP 1 in nuclei of 6 week old control animal, 3-6 week old male rat treated with cisplatin, 4-6 week old cisplatin treated female. p *<0,05
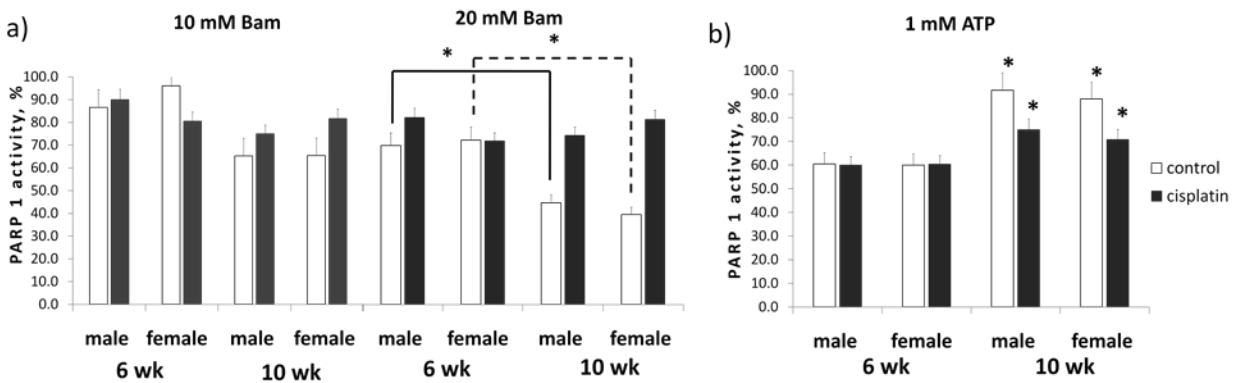
Fig 2. Inhibition of PARP 1 by Bam (a) and ATP (b) in liver nuclei of rats treated with cisplatin. p *<0,05

Our data (fig. 2) show that 10mM Bam didn't affect PARP 1 activity in nuclei of 6 week old rats. When Bam concentration in incubation media was increased up to 20 mM, enzyme activity was suppressed nearly equally in female and male nuclei (by 30%). In nuclei of 10 week old rats Bam exhibited higher inhibitory efficacy, more pronounced when 20 mM of Bam was applied. 1mM ATP added into incubation media inhibited PARP 1 activity in nuclei of 6week old females and males by 40% and did not affect PARP 1 in nuclei of 10 week old rats. In the presence of high physiologic concentration of ATP (5mM) in incubation medium PARP 1 activity was completely inhibited in nuclei of all examined groups. In general, these data demonstrated that there were no sex dependent differences in PARP 1 inhibition in rat liver nuclei by Bam and ATP, whereas reliable age-dependent differences in PARP 1 inhibition were apparent.

Modulations of PARP 1 activity in liver nuclei after administration of cisplatin to intact animals

Administration of cisplatin to intact animals (fig. 1, a) elicited opposite effects on PARP 1 activity in liver nuclei of 6 and 10 week old rats. Enzyme activity in liver nuclei of 6 week old rats treated with cisplatin decreased nearly for 3,5 and 2,5 fold in male and female nuclei respectively and this diminution was not paralleled with enzyme protein cleavage (fig. 1,b). In contrast, PARP 1 activity of cisplatin treated 10 week old rat nuclei increased more than twofold regardless sex.PARP 1 activation in nuclei of cisplatin treated 10 week old rats coincided with sensitization to inhibition by ATP, whilst inhibitory potential of Bam decreased nearly two fold (fig.2)

DNA electraphoresis

As it was shown previously PARP 1 inhibition by ATP was paralleled with suppression of DNA internucleosomal cleavage in the in vitro systems comprising artificial chromatin assemblies or different DNA constructs[15]. In present study we examined whether PARP 1 inhibition by ATP is capable to suppress

internucleosomalcleavage of chromatin in liver nuclei isolated from animals of different age and sex. The cleavage of chromatin was accomplished by artificially activated intra-nuclear Mg^{2+} and Ca^{2+} /Mg^{2+} -dependent apoptotic endonucleases by addition of Ca^{2+} and Mg^{2+} ions into nuclei incubation media.

The data show that intensity of DNA fragmentation in rat liver nuclei was age-independent and had no sex bias. Further examination of DNA internucleosomal cleavage was performed with male rat nuclei. When nuclei were preincubated for 15 min with 20 mM Bam, which was added to nuclei incubation medium prior to divalent ions DNA fragmentation intensity decreased in nuclei of 10 week old rats nearly two fold. It was reflected by increased relative content of fragments exceeding 1000 b.p. concomitantly with diminution of olygonucleosomal fragments (1000 b.p.-200 b.p.) content. In 6 week old rat nuclei we did not find reliable changes (fig. 3, table 1). In contrast to Bam, 1mM ATP did not significantly suppress DNA internucleosomal fragmentation in nuclei of 6 and 10 week old rats. However, when nuclei were preincubated for 15 min in the presence of 5mM ATP, DNA internucleosomal fragmentation was completely inhibited in nuclei isolated from rats of both age groups (fig. 4).
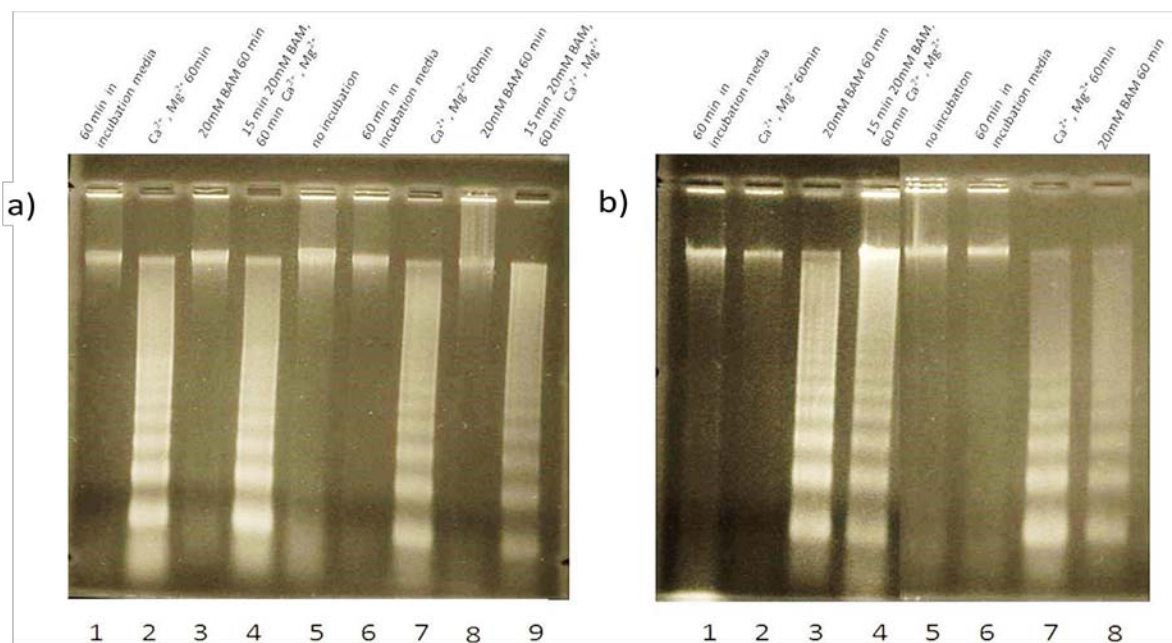

Fig 3. The effect of Bam on DNA internucleosomal cleavage in liver nuclei of (a) -6 and (b) -10 week old rats in control (lane 1-4) and after cisplatin administration (lane 5-9). Nuclei were incubated in isolation media. Bam was added into incubation media in 15 minutes prior to addition of Ca^{2+} and Mg^{2+} ions.

Cisplatin administration to rats led to elevation of high molecular weight DNA fragments content more than for 28% in nuclei of 6 week old rats, in contrast to nuclei of 10 week old rats, where their content decreased two fold.These changes indicated on distinct age-dependent modulations of chromatin structure in liver nuclei induced by the in vivo treatment of rats with cisplatin.They prompt us that treatment of rats with cisplatin

caused condensation of liver chromatin in 6 week old rats, whilst induced decondensation of 10 week old rat liver chromatin. We intend to determine whether cisplatin can influence Bam and ATP effects on liver chromatin structure. The in vivo treatment of rats with cisplatin did not lead to significant suppression of DNA internucleosomal fragmentation by Bam in nuclei of 6 week old rats.In contrast, in 10 week old rat nuclei Bam caused nearly two fold increase in content of high molecular weight DNA fragments, thereby indicating on inhibitor induced chromatin condensation. Treatment with cisplatin modulated effect of 1mM ATP on chromatin accessibility to endonucleolytic attacks. In 6 week old rat nuclei we revealed 40% increase in content of high molecular weight DNA fragments. The effect of cisplatin administration to rats was greater in 10 week old rat nuclei reflected in three fold increase in content of large DNA fragments (table 1).
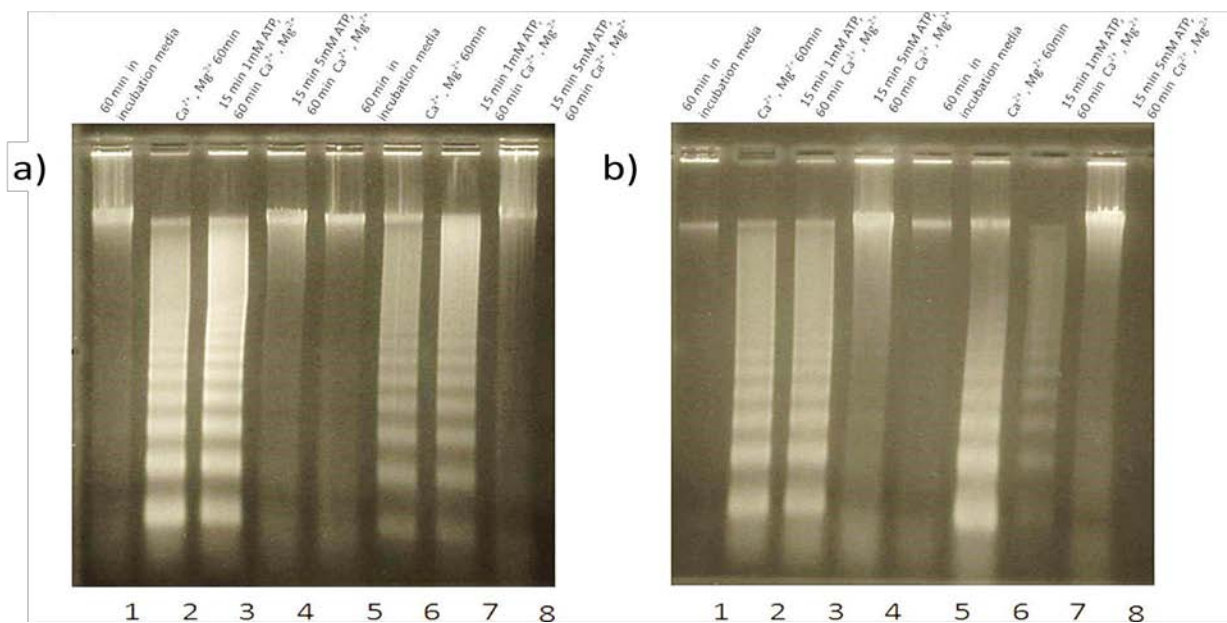

Fig 4. The influence of ATP on DNA internucleosomal cleavage in liver nuclei of (A) -6 and (B) -10 wk old rats in control (lane 1-4) and after cisplatin administration (lane 5-8). ATP was added into incubation media in 15 minutes prior to addition of Ca^{+2} and Mg^{+2} ions.

Table 1. Relative content (%) of DNA fragments of different length in nuclei preincubated with Bam or ATP for 15minutes before addition of divalent ions. DNA content per lane was set as 100%. p*< 0,05

age week	Length of DNA fragments base pair	control			treatment with cisplatin		
		60 min Ca^{+2}/Mg^{+2}	60 min Ca^{+2}/Mg^{+2} + 20mM Bam	60 min Ca^{+2}/Mg^{+2} + 1mM ATP	60 min Ca^{+2}/Mg^{+2}	60 min Ca^{+2}/Mg^{+2} + 20mM Bam	60 min Ca^{+2}/Mg^{+2} + 1mM ATP
6 week old rats	> 1000	25,3±1,51	20,5±1,63	30,2±1,8	32,5±1,62*	38,1±2,28	45,2±3,16*
	1000-200	62,3 ±4,75	56,2±3,93	63,1±3,8	65,1±4,94	54,7±2,18	53,5±4,81
	<200	12,4±0,62	23,3±1,16	6,7±0,53	2,4±0,12	7,2±0,21	1,3±0,06
10 week old rats	> 1000	30,3±2,45	60,4±4,80*	31,4±2,19	13,8±1,10*	28,7±2,01*	40,4±2,4*
	1000-200	61,0±4,90	31,1±1,86	56,9±2,84	71,4±6,42	62,5±5.62	54,6±3,28*
	<200	8,7±0,90	8,6±0,60	11,8±0,70	14,8±1,01	8,8±0,26	4,96±0,34

DISCUSSION

PARP1 plays prominent role virtually in all chromatin-associated nuclear functions and is the most abundant member of PARP enzyme family localized to nuclei[16]. Pharmacologic inhibition of the enzyme increases cytotoxic potential of cisplatin and benefits therapeutic responses of cancer patient treated with DNA-damaging agents. Though efficacy of PARP 1 inhibition depends on the enzyme baselineactivity[17] and curative potential of pharmacological PARP 1 inhibitors demonstrate sex-bias, the role of age-and sex-dependent factors in maintenance of PARP 1 activity is poorly investigated. In present study we examined the link between the PARP 1 basline activity and efficacy of its inhibition before and after the in vivo treatment of rats with cisplatin. Taking into consideration that hall nuclei mimics natural occurring system more closelythe experimental system employed here (naked nuclei) minimized disparities between kinetics of inhibition of purified PARP 1 and its cellular forms,and from the other handdiscriminated non-specific effect of PARP 1 inhibitors on cellular bioenergetic pathways[18-20].

Our data come to showage-dependent differences of PARP1 activity in liver nuclei and these results are consistent with data reported earlier for aged animals[21-24]. Here we show for the first time that PARP1 down-regulation is not featured to process of aging and can start earlier in the course of sexual maturation of rats from 6-10 weeks in sex-independent manner.

PARP1 inhibition can be released via two different routes: disturbance of mechanisms responsible for the enzyme protein binding to coenzymic DNA and second, suppression of catalytic domain function. Bam is wellknown competing PARP1 inhibitor of first generation which represents a family of structural analogues of NAD+ employed in clinical trials. NAD+ analogues block the binding of NAD^+ to PARP1catalitic domain, thereby inhibiting the enzyme[3].The data presented in this paper revealed that there were no sex-dependent differences in PARP 1 inhibition by Bam which suggests, that sex- bias in curative potential of PARP 1 inhibitors can be determined by sex-dependent differences in drug pharmacokinetics or pharmacodynamics [25-27].However, inhibitory potency of Bam was higher in 10week old rat nuclei, indicating on age-dependent sensitization of PARP 1 regarding inhibition by Bam.

It is widely recognized that PARP1 comprises two different activities: auto- and trans- poly(ADP-ribos)ylation. In 2006 Kun et al suggested bioenergetic model for PARP 1 regulation in cells by ATP[28]. This model emphasized the role of ATP as specific inhibitor of auto-ADP-polyribosylating capacity of PARP 1 in the in vitro system. The results of present study indicate on biological significance of PARP1 regulation by ATP in cell nuclei.Our data come to show that efficacy of PARP1 inhibition by ATP was greater in nuclei of 6 week old rats. Age-dependent down-regulation of basal PARP 1 activity in liver nuclei of 10 week old rats was paralleled with diminution of ATP inhibitory potency. It was reported earlier that trans-poly(ADP-ribos)ylating activity, which is not inhibited by ATP, prevails in less actively proliferating cells[29]. We suppose, that elevated resistance to ATP in liver nuclei of 10 week old rats could be determined by prevalence of trans-poly(ADP-ribos)ylationin less actively growing liver of elder animals. In concert, these data prompt us that sex-independent suppression of PARP1 in liver of 10 week old rats could be linked to switching of auto- poly(ADP-ribos)ylatingto trans- poly(ADP-ribos)ylating capabilities of PARP1.

Mounting evidence demonstrate that drug-drug interactions can modify pharmacologic effects and attenuate therapeutic outcomes. We were interested to investigate whether the in vivo treatment of hall animals with cisplatin could influence PARP1 inhibition in liver nuclei by Bam and ATP. Our results show that treatment of rats with cisplatin led to significant changes in PARP 1 activity in liver nuclei. Cisplatin administration to 6 week old rats suppressed PARP1, whilst stimulated enzyme activity in 10 week old young adults' liver regardless sex. It was reported that cisplatin dramatically suppressed glucose and NAD+ metabolism in cells[19,30]. Coming from this, we suppose that PARP1 suppression in 6 week old rats liver nuclei which was not paralleled with enzyme protein cleavage (fig 1, b), could be determined by decrease in NAD^+ content in liver cells. Greater detoxifying potency of 10 week old rat liver could be responsible for less pronounced effect of cisplatin on energy metabolism and NAD^+ content, thereby diminishing the rate limiting role of substrate in PARP1 activity[31].

Cisplatin induced PARP1 activation in nuclei of 10 week old rats could reflect enzyme activation due to formation of DNA-cisplatin adducts in accordance with data reported earlier[4].

It was demonstrated that inhibition of auto-poly(ADP-ribos)ylpolymerizing activity of PARP 1 by ATP led to "trapping"of the enzyme molecule to DNA[15]. To study whether PARP1 trapping to DNA via DNA-cisplatin adducts can interfere with liver chromatin accessibility we employed chromatin structure-dependent assay, using artificially activated intra-nuclear apoptotic Mg^{+2} and Ca^{+2}/Mg^{+2}-dependent endonucleases which are responsible for DNA internucleosomal fragmentation (laddering) in apoptosis[32]. It was demonstrated earlier that Mg^{+2}-dependent endonucleases are involved in initial DNA cleavage in nuclei where they perform large scale DNA fragmentation (DNA fragments exceeding 1000 bp in length), whilst Ca^{+2}/Mg^{+2}-dependent endonuclease is responsible mainly for olygonucleosomal DNA fragmentation[10] (1000-200 bp in length).After addition of divalent ions into nuclei incubation media we did not reveal age-and sex-dependent differences in the pattern or intensity of DNA internucleosomal cleavage incontrol. Our data show that the in vivo treatment of rats with cisplatin, which dramatically diminished PARP 1 activity in liver nuclei of 6 week old rats affected large scale and olygonucleosomal DNA fragmentation not markedly. In contrast, intensity of DNA fragmentation in nuclei of 10 week old rats treated with cisplatin increased, indicating on elevated accessibility of chromatin to endonucleolytic attacks and chromatin loosening. We consider that PARP1 activation could be responsible for enhanced poly(ADP-ribose)polymerization of chromatin proteins. Thus, chromatin decondensation could result not only from DNA unwindingvia DNA-cisplatin adducts formation reported earlier by other authors, but could be determined by poly(ADP-ribose)polymerization of chromatin proteins[33]. This hypothesis was supported by the data which show that more effective PARP 1 inhibition by Bam and ATP in nuclei of 10 week old rats coincided with greater suppression of DNA fragmentation. Results of our study come to show that 5mM ATP, which completely inhibited PARP 1 activity suppressed DNA internucleosomal fragmentation as well. We suppose that firm association of PARP1 with chromatin due to inhibition of auto-poly(ADP-ribosyl)polymerisation by ATP led to shielding of linker internucleosomal regions from cleavage with endonucleases and eventually to suppression of DNA-laddering[15]. Chromatin decondensation in nuclei of cisplatin treated 10 week old rats could be consequential to at least two biochemical events: poly(ADP-ribosylation) of chromatin associated proteins by activated PARP 1, and DNA unwinding capabilities of DNA-cisplatin adducts.

CONCLUSION

Here we show that PARP1 down-regulation is not featured to process of aging and can start earlier in the course of sexual maturation of rats from 6-10 weeks in sex-independent manner.Treatment of rats with cisplatin led to age-dependent modulations in PARP 1 activity and inhibition in rat liver nuclei.

Activation of PARP 1 in liver nuclei of cisplatin treated 10 week old rats was paralleled with chromatin decondensation evidenced by enhanced susceptibility of chromatin to endonucleolyticattacs. PARP1 inhibitors can affect DNA internucleosomal cleavage in nuclei of cells,therebyinfluencing accomplishment of apoptotic death program. To avoid futile therapeutic approaches sex- and age-dependent factors should be considered when ATP-mimetics or competing PARP 1 inhibitors are employed in combination therapy with cisplatin in the treatment of cancer patients.

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