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Preventive Effect of 3-Aminobenzamide on the Reduction of NAD Levels in Rat Liver Following Administration of Diethylnitrosamine

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Nicotinamide adenine dinucleotide is utilized as the substrate of a chromatin-bound enzyme, poly(ADP-ribose) polymerase. The effects of diethylnitrosamine and/or 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose) polymerase, on the cellular NAD levels in rat liver were investigated. 3-Aminobenzamide (600 mg/kg) administered intraperitoneally was not detectable in the liver within 12 hr after administration; the inhibitor had a calculated half life of 90 min. Diethylnitrosamine reduced the NAD levels in rat liver in a dose-dependent way. The NAD content reached a minimum level at 8 hr, returning to 78% of the control value after 48 hr. The reduction of the NAD levels caused by diethylnitrosamine was completely prevented when 3-aminobenzamide was administered either simultaneously with diethylnitrosamine or 4 hr after diethylnitrosamine treatment. Furthermore, an immunohistochemical study showed that nuclear poly(ADP-ribose) decreased 1 hr after the administration of 3-aminobenzamide. These results suggest that inhibition of poly(ADP-ribosyl)ation is involved in the initiation of liver carcinogenesis by diethylnitrosamine and 3-aminobenzamide.

Key words: NAD levels — 3-Aminobenzamide — Rat liver

Poly(ADP-ribose) is a biopolymer which is synthesized enzymatically from nicotinamide adenine dinucleotide (NAD^+).[1-5] Accumulated data suggest an involvement of poly(ADP-ribose) in DNA repair,[6] cell differentiation,[5, 6] sister chromatid exchanges[7] and cell proliferation.[8]

Previously, we have reported the enhanced initiation of liver carcinogenesis by inhibitors of poly(ADP-ribose) polymerase in rats.[9-11] In these studies, 3-aminobenzamide (ABA)[*7] was used as a representative inhibitor of poly(ADP-ribose) polymerase to investigate its effect on the early stage of liver carcinogenesis induced by diethylnitrosamine (DEN). Estimation of the number and size of γ-glutamyltranspeptidase (γ-GTP)-positive foci, assayed as a marker representing populations of initiated cells,[12] showed that ABA enhanced the number of such foci in rat liver initiated by DEN in a dose-dependent way.

Alkylating agents and irradiation reduce the cellular NAD level,[6, 13-15] and cause a transient accumulation of poly(ADP-ribose)[6, 16, 17] in various cell lines. NAD is the substrate for poly(ADP-ribose) synthesis. The demonstration of changes in NAD levels *in vivo* caused by inhibitors of poly (ADP-ribose) polymerase would provide evidence for the involvement of the poly(ADP-ribosyl)ation reaction in liver carcinogenesis.

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[*7] Abbreviations: ABA, 3-aminobenzamide; DEN, diethylnitrosamine; NAD, nicotinamide adenine dinucleotide; γ-GTP, γ-glutamyltranspeptidase; poly (ADP-ribose), poly (adenosine diphosphate ribose); DMSO, dimethylsulfoxide; PES, 5-ethylphenazinium ethyl sulfate; MTT, 3-(4,5-dimethylthiazolyl - 2) - 2,5 - diphenyltetrazolium bromide, PBS; phosphate-buffered saline.

In this study we have observed a reduction of NAD levels in rat liver following DEN treatment and a protective effect of an inhibitor of poly(ADP-ribose) polymerase. In addition, we demonstrate by immunohistochemistry that ABA decreases nuclear poly(ADP-ribose) in rat liver. These data are evidence of the possible involvement of poly(ADP-ribosyl)ation reactions in the initiation of liver carcinogenesis by DEN and ABA in rats.

MATERIALS AND METHODS

Animals Male Fischer 344 rats (Shizuoka Laboratory Animal Center, Shizuoka) weighing approximately 200 g each were used throughout the experiments. Rats were given a commercial stock diet (Oriental MF, Oriental Yeast Ind., Tokyo) and water *ad libitum* in an air-conditioned room at 24°. Rats were sacrificed by decapitation after starvation for 14 hr and the livers were immediately removed.

Chemicals DEN (Wako Pure Chemicals Ind., Osaka) was dissolved in 0.9% (w/v) NaCl at a concentration of 50 mg/ml and given intraperitoneally (ip) at doses of 20, 100 and 200 mg/kg body weight. ABA (Tokyo Kasei Kogyo, Co. Ltd., Tokyo) was dissolved in dimethylsulfoxide (DMSO; Sigma Chemicals Co., USA) at a concentration of 300 mg/ml and given ip at doses of 100, 300 and 600 mg/kg body weight. An authentic sample of β-NAD^+ was purchased from Sigma Chemicals Co. Nicotinamide was purchased from Nakarai Chemicals Ind., Kyoto. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and alcohol dehydrogenase were from Sigma Chemicals Co. All other reagents were of the highest grade available from commercial sources.

Determination of ABA Content in Rat Liver We developed a method for the determination of ABA content of rat liver. ABA at a dose of 600 mg/kg body weight was given ip and the rats were sacrificed by decapitation at 5 min, 30 min, 1 hr, 4 hr, 12 hr and 24 hr after administration. The livers were immediately removed and a 1 g sample was taken from each of the three different lobes. The tissue samples were homogenized in 5 ml of 10% (w/v) TCA and centrifuged at 3000*g* for 10 min. The centrifugation was repeated 3 times and the supernatants were collected and mixed with a solution containing 1 ml of 0.1% (w/v) $NaNO_2$. Then 1 ml of 0.5% (w/v) ammonium sulfaminate and 1 ml of 0.05% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in ethanol were added. The samples were then incubated at 30° for 30 min. The absorbance of the ABA derivative was measured at 550 nm on a spectrophotometer (Model 124, Hitachi Ltd., Tokyo). For calibration, ABA was dissolved in DMSO, diluted with 10% (w/v) TCA and measured as described above.

NAD Determination Cellular NAD levels were measured using the method described by Bernofsky and Swan[18] and modified slightly by us. One gram of liver from an individual was homogenized in 9 ml of ice-cold 50m*M* potassium phosphate buffer (pH 6.0) and immersed in boiling water for 5 min. The samples were cooled on ice and centrifuged at 3000*g* for 10 min at 4°. The supernatant was then preincubated with 1.2*M* sodium-bicine buffer (pH 7.8) containing 5m*M* PES, 5m*M* MTT, 6*M* ethanol and 50m*M* EDTA in a dark room at 30° to establish thermal equilibrium. The assay was initiated by adding alcohol dehydrogenase (0.125 mg) which was reconstituted in 40m*M* Tris, 40m*M*, $KH_2P_2O_7$ (final pH 8.1) immediately before use. Changes in absorption were measured at 570 nm on a spectrophotometer (Model 124, Hitachi Ltd., Tokyo). Calibration standards of β-NAD^+ in the range of 1 to 5 μg/ml were used.

Immunohistochemical Studies of Poly(ADP-ribose) Polyclonal antibody against poly(ADP-ribose) was prepared as previously described.[19] A portion of rat liver was removed and immediately frozen in a dry ice/acetone mixture. The tissue was sectioned at 2 μm in a cryostat, then the sections were thawed on glass slides, dried with a fan at 4° and fixed with 95% ethanol for 5–10 min at 0°. The sections were incubated for 30 min at 37° with rabbit anti-poly(ADP-ribose) antiserum dilution. Controls were incubated with preimmune rabbit IgG. Slides were washed by immersing and agitating in PBS. Sections were then incubated for 30 min at 37° with FITC-conjugated swine anti-rabbit IgG and washed as above. Slides were observed with a Nikon fluorescence microscope equipped with an epi-illumination system.

RESULTS

Kinetics of ABA Disappearance from Rat Liver A linear relationship was obtained between the amount of ABA and absorbance, using authentic ABA, over the range of 0.5 to 1 mg/ml.

The kinetics of ABA disappearance in rat liver was as follows. Values of ABA content in rat liver were 0.68, 0.49, 0.39 and 0.20 mg/g liver at 5 min, 30 min, 1 hr and 4 hr, respectively; 5.5% of administered ABA was present in the liver at 5 min after ABA administration (600 mg/kg). Within 12 hr ABA content was below 0.1 mg/g liver weight, corresponding to a calculated half-life of 90 min.

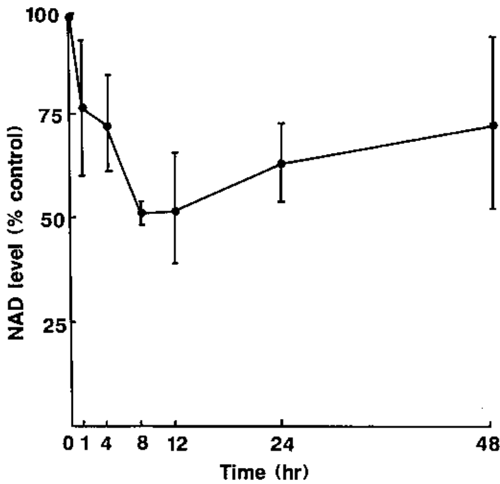

Fig. 1. NAD levels in rat liver as a function of time after DEN administration. DEN was given at 200 mg/kg body weight. Each point represents the mean±SD (n=5).

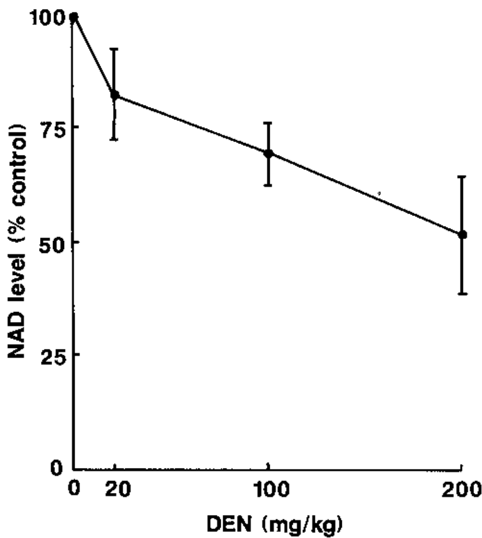

Fig. 2. Dose-response effect of DEN on the NAD levels in rat liver 12 hr after administration. Each point represents the mean±SD (n=5).

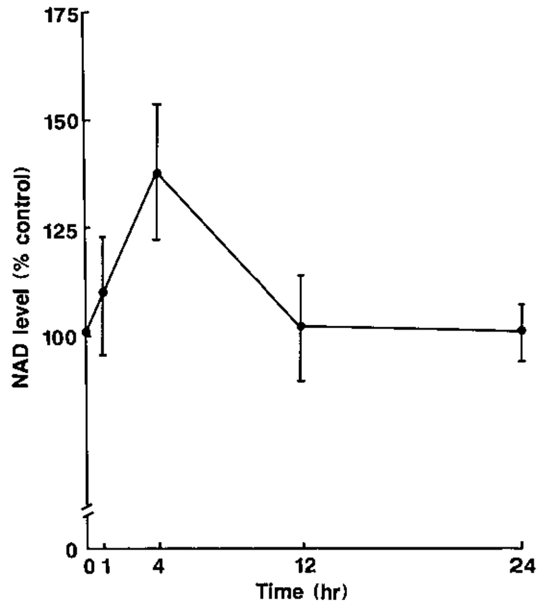

Fig. 3. NAD level as a function of time after ABA treatment in rat liver. ABA at a dose of 600 mg/kg body weight was given. Mean values±SD (n=4).

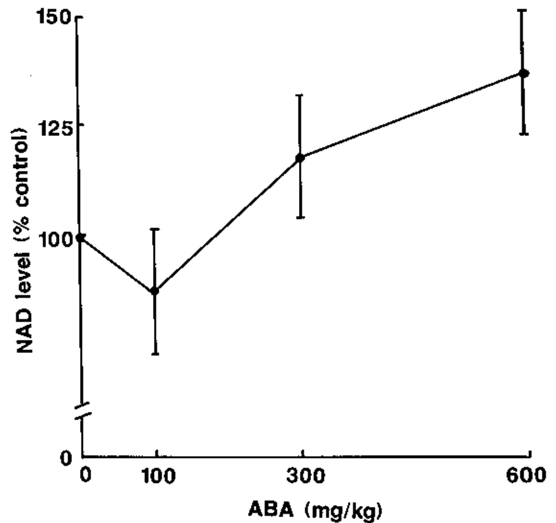

Fig. 4. The effect of ABA on the NAD level in rat liver 4 hr after administration. Mean values±SD (n=5).

Effects of DEN or ABA on the NAD Content in Rat Liver Figure 1 shows the NAD level as a function of time after DEN treatment at 200 mg/kg body weight. The NAD level reached a minimum at 8 hr and had returned to 78% of the control level at 48 hr after DEN administration. The dose-response effect of DEN on the NAD levels assayed at 12 hr after administration is shown in Fig. 2. A dose-dependent lowering of cellular NAD content 12 hr after administration was observed following DEN

Table I. ABA Protects Liver from Loss of NAD due to DEN Administration

Group	Treatments[a]	Dose of DEN (mg/kg)	No. of rats	Time after administration			
				4 hr		12 hr	
				NAD content[b] (μg/g liver)	Ratio[c]	NAD content[b] (μg/g liver)	Ratio[c]
1	0.9% NaCl		13	364±27.0	1.00	388±38.2	1.00
2	DEN	20	9	319±45.0	0.82	320±39.5	0.81
3	DEN+ABA	20	6	387±58.7[d]	0.98	392±30.6[e]	1.00
4	DEN	200	9	285±48.7	0.72	204±53.4	0.52
5	DEN+ABA	200	9	525±59.6[f]	1.34	490±23.3[g]	1.52

a) ABA at a dose of 600 mg/kg body weight was given ip.
b) Mean±SD.
c) Ratio of NAD content in rat liver treated with DEN and/or ABA was calculated from the value of NAD content in rat liver treated with 0.9% NaCl.
d) $P<0.05$ compared with Group 2.
e) $P<0.01$ compared with Group 2.
f) $P<0.001$ compared with Group 4.
g) $P<0.001$ compared with Group 4.

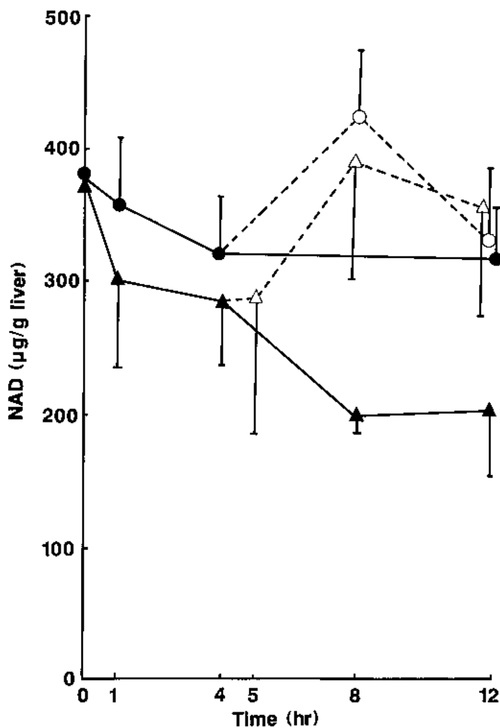

Fig. 5. Protective effect of ABA on the reduction of NAD level in rat liver caused by DEN treatment. Each point represents the mean±SD (n=5). ●, 20 mg/kg DEN; ▲, 200 mg/kg DEN; ○, 600 mg/kg ABA 4 hr after 20 mg/kg DEN; △, 600 mg/kg ABA 4 hr after 200 mg/kg DEN.

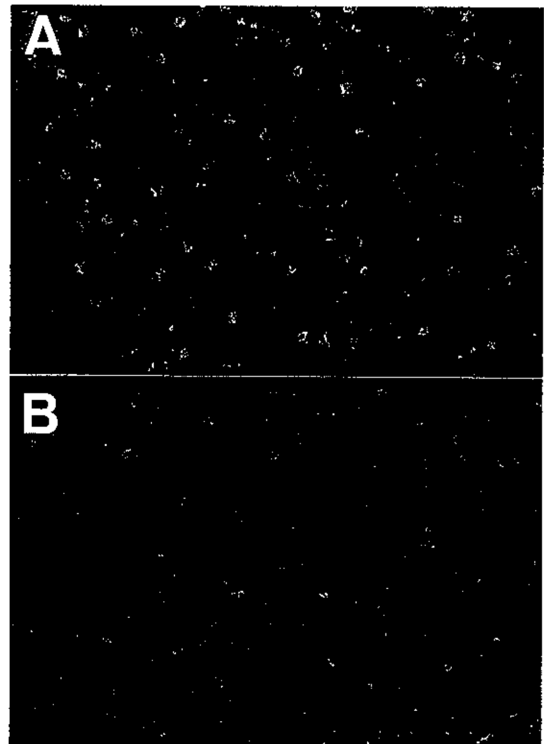

Fig. 6. Histochemical demonstration of poly(ADP-ribose) stained by the indirect immunofluorescence method. Poly(ADP-ribose) in the liver of a control rat sacrificed 1 hr after DMSO treatment (A). The liver of a rat sacrificed 1 hr after administration of ABA at 600 mg/kg body weight (B).

treatment. The effect of ABA (600 mg/kg) on the NAD level as a function of time after administration is shown in Fig. 3. ABA induced a small transient increase in liver NAD level with a peak at 4 hr, after ABA treatment, then the level returned to the control value by 12 hr, and remained constant thereafter. Cellular NAD level 4 hr after administration was increased in a dose-dependent way by 300 and 600 mg/kg body weight of DEN (Fig. 4).

Prevention of the Loss of NAD by ABA Following DEN Treatment Table I shows the effects of ABA on cellular NAD content in rat liver after the administration of DEN. DEN alone decreased the NAD levels at both 4 hr and 12 hr in a dose-dependent way, while ABA (600 mg/kg) completely prevented this decrease. The protective effect of post-treatment with ABA on the reduction of NAD level caused by DEN is shown in Fig. 5. ABA (600 mg/kg) administered at 4 hr after DEN treatment (20 or 200 mg/kg), clearly prevented the reduction of cellular NAD level.

Effect of ABA on Poly(ADP-ribose) In an indirect immunohistochemical investigation, conspicuous nuclear immunofluorescence due to poly(ADP-ribose) was observed in the liver of control rats treated with DMSO (Fig. 6A), while it was decreased 1 hr after the administration of ABA (600 mg/kg) (Fig. 6B).

Discussion

NAD is the substrate of poly(ADP-ribose) polymerase, which polymerizes the ADP-ribose moiety of NAD^+ to form homopolymers covalently attached to various nuclear proteins. The present experiments demonstrate that the administration of DEN to rats reduced the cellular NAD content in liver in a dose-dependent way and that ABA, an inhibitor of poly(ADP-ribose) polymerase, prevented this reduction. This demonstrates protection by ABA *in vivo*.

The reduction of the NAD level in rat liver by DEN suggests that NAD was rapidly converted to poly(ADP-ribose), since the changes in NAD content and poly(ADP-ribose) polymerase activity are related *in vitro*. The present findings suggest that the increase in cellular NAD level observed after ABA administration is due to the inhibition of poly(ADP-ribosyl)ation since it correlated with a decrease in the nuclear immunofluorescence of poly(ADP-ribose). However, further dose-response and time-course studies on the changes in poly(ADP-ribose), poly(ADP-ribose) polymerase activity and NAD concentration *in vivo* are needed for this relationship to be clarified. The effects of DEN at various doses and of combined administration of DEN and ABA on poly(ADP-ribose) levels in rat liver are under investigation and preliminary data showed that DEN treatment increased the immunofluorescence due to poly(ADP-ribose) in rat liver and ABA inhibited its increase (unpublished data).

ABA administered simultaneously with DEN or even 4 hr after DEN treatment prevented the reduction of NAD levels caused by DEN. It is perhaps significant that in our previous studies,[9-11] such simultaneous or even subsequent administration of ABA was effective in enhancing the induction of DEN-initiated γ-GTP-positive foci in rat liver. Administered ABA in rat liver was removed rapidly with a calculated half life of 90 min. Although ABA disappeared rapidly from the liver, the remaining amount of ABA in rat liver after 4 hr is equivalent to a concentration of 0.7–1.4mM, which is known to inhibit poly(ADP-ribose) polymerase activity *in vitro*. The amount of ABA retained in a rat liver might be effective for the prevention of NAD reduction by DEN.

ABA is reported to have side effects when it is used at high concentration in cell lines.[20] We have checked the toxicity of ABA in rat liver histologically and found no necrotic cell at a dose of 600 mg/kg (data not shown).

There have been several reports concerning a role of poly(ADP-ribosyl)ation in cellular transformation and carcinogenesis, though some showed different results. *In vitro*, inhibitors of poly(ADP-ribose) polymerase prevented the transformation following exposure to carcinogens and this protective effect of inhibitors on transformation had no relationship with DNA repair processes.[21] But there is no clear explanation of the role of poly(ADP-ribosyl)ation in transformation *in vitro*. In animal experiments, inhibitors of poly(ADP-ribosyl)ation enhanced oncogenic processes during renal cell carcinogenesis in rats.[22] Our previous studies demonstrated the enhancing effect of an inhibitor of poly(ADP-

ribosyl)ation, ABA, on rat hepatocarcinogenesis.[9–11] Other workers found no effect or a protective effect of ABA on carcinogenesis in rats.[23,24] However, the experimental protocols, carcinogens and strains of rats used in these experiments were different. To clarify the mechanisms involved in the enhancing effect of ABA during carcinogenesis, we focused on NAD metabolism and poly(ADP-ribose) at the initiation step of rat hepatocarcinogenesis.

It is known that poly(ADP-ribose) polymerase of the cell nucleus is dependent on nicked DNA for its enzymatic activity[25,26] and that treatment with DNA damaging agents causes a rapid conversion of NAD^+ to poly(ADP-ribose), resulting from increased poly(ADP-ribose) polymerase activity, and leads to decreased cellular NAD levels *in vitro.*[14] DEN induces DNA fragmentation and produces N^7-ethylguanine as a major product.[27] Shall[6] has shown that poly(ADP-ribose) polymerase activity is required for efficient DNA strand rejoining. Cleaver and Morgan[28] reported that poly(ADP-ribose) did not regulate the ligation step of repair but might regulate nuclease and other enzymes and change chromatin structure. The present documentation of the prevention by ABA of a DEN-induced reduction of cellular NAD content indicates that a decrease of poly-(ADP-ribosyl)ation activity is perhaps associated with the inhibition of some processes in the repair of DNA damage, thus contributing to the enhanced initiation of rat liver carcinogenesis by DEN. Such protective effects of ABA on the reduction of NAD levels probably correlate with phenotypical changes of the initiated cells. Miwa *et al.*[29] have demonstrated that ABA increased the hepatocarcinogenesis by methylazoxymethanol acetate (MAM acetate) in the small fish "Medaka" (*Oryzias latipes*), with a decrease of NAD level after MAM acetate treatment. Some discrepancy in the kinetics of NAD concentration after the carcinogen treatments may be due to a different route of administration and the use of different carcinogens. However, these data further indicate the importance of involvement of poly (ADP-ribosyl)ation in carcinogenesis.

Evidence for the involvement of poly ADP-ribosylation reactions in liver carcinogenesis initiated with DEN provides support for the view that DNA lesions are necessary for the initiation of liver carcinogenesis and that processes of repair of DNA damage, including those involving changes in the levels of NAD and poly(ADP-ribose), are involved in neoplasia in the rat liver.

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