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In vivo effect of L-ascorbic acid on benzo(α)pyrene metabolite-DNA adduct formation in rat liver

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Abstract. Pretreatment of male Wistar rats with L-ascorbic acid results in a decrease in the *in vivo* covalent binding of benzo(a)pyrene to hepatic nuclear DNA. *In vitro* formation of this adduct is also found to be low in liver slices and in liver nuclei of pretreated rats. No inhibition of the adduct formation is, however, observed when benzo (a) pyrene and exogenous DNA are incubated with liver microsomes isolated from ascorbic acid treated rats.It appears that the presence of ascorbate in the cellular or subcellular environment is essential for its inhibitory action.

Keywords. L-ascorbic acid, benzo(a) pyrene, DNA, adduct formation, carcinogenesis.

Introduction

The environmental carcinogen benzo (a) pyrene, a polycyclic aromatic hydrocarbon, requires metabolic activation by microsomal enzymes to exert its toxic, mutagenic and carcinogenic action (Gelboin *et al.,* 1972; Sims and Grover, 1974). When activated thus, the compound undergoes covalent interaction with cellular DNA (Gelboin, 1969). The formation of an adduct of DNA with the activated metabolite of benzo(a)pyrene is regarded as a critical step in the process of carcinogenesis (Sims and Grover, 1974; Jerina and Daly, 1974). We have recently shown that such an adduct formation *in vitro* as well as metabolic activation of benzo (a) pyrene by isolated microsomes are partially inhibited in the presence of L-ascorbic acid (Shah and Bhattacharya, 1980). Since ascorbic acid is known to afford protection against experimental carcinogenesis induced by chemicals (Schlegal *et al.,* 1970; Shamberger, 1972; Slaga and Bracken, 1977) including that by benzo (a) pyrene (Kallistratos and Fasske, 1980), it is desirable to study the effect of administration of ascorbic acid on the formation of the carcinogen-DNA adduct in the liver of rats. We report here results of our investigation with benzo (a) pyrene.

Materials and methods

Benzo(a)pyrene was obtained from Calbiochem, La Jolla, California, USA. [^{3}H]-Benzo(a)pyrene (Sp. act. 3.7 Ci/m mol) was a product of Isotope Division, Bhabha

Atomic Research Centre, Bombay. [^{3}H]-Benzo(a)pyrene was diluted suitably with non-radioactive material prior to use. L-Ascorbic acid was from E.Merck, Darmstad, W. Germany and calf thymus DNA was from Sigma Chemical Co., St. Louis, Missouri, USA.

Male Wistar rats (100 -150 g) were kept in standard cages and fed a laboratory stock diet and water *ad lib.* Treatment consisted of a single intraperitonial injection of L-ascorbic acid (100 or 300 mg/kg body weight). For *in vivo* experiments, further treatment involved intraperitonial administration of 20 mg/kg [^{3}H]-benzo(a)pyrene (64.2 mCi/m mol) 2 h after ascorbic acid injection. 16 h after administration of [3H] -benzo (a)pyrene the animals were sacrificed by decapitation and the livers were quickly removed, washed free of blood and used for isolation of nuclear DNA. For *in vitro* experiments, the livers were removed from rats 2 h after ascorbic acid treatment and used for the preparation of slices, nuclei and microsomes. All subsequent operations were carried out at 4°C unless otherwise mentioned.

Liver slices were prepared by cutting sections of 0.3-0.4 mm thickness using a Stadie-Riggs tissue slicer. The slices were suspended in Krebs-Ringer solution-phosphate buffer, pH 7.4. The nuclear fraction was purified from the liver by incorporating certain modifications in the method employed by Farber *et al.* (1974). Whole liver or liver slices were homogenized in 3 volumes of 0.32 M sucrose-3 mM $MgCl_2$ using a glass teflon homogenizer. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was saved for the isolation of microsomes. The nuclear pellet was suspended in 1.7 M sucrose and layered over a cushion of 2.1 M sucrose.. Intact and pure nuclei were obtained by centrifugation at 42,500 *g* for 1 h. The purified nuclear fraction was suspended in 0.25 M sucrose-1 mM $MgCl_2$ at a concentration of 8 g liver equivalent/ml. The supernatant was centrifuged at 24,000 *g* for 10 min to remove mitochondria, and the resultant supernatant from this step was further centrifuged at 110,000 g for 1.5 h to obtain pure microsomal fraction. This was Suspended in 0.25 M sucrose-1 mM $MgCl_2$ to give a concentration of 0.5 g liver equivalent/ml. Liver slices, nuclei and microsomes were incubated independently with [^{3}H]-benzo(a)pyrene under appropriate conditions. Exogenous DNA (calf thymus) was added in the case of microsomes.

The isolation of nuclear DNA was performed according to the following procedure. The nuclear fraction was washed with 0.14 M NaCl-0.02 M EDTA, and the nuclei were reisolated by centrifugation at 800 *g* for 10 min. The final nuclear pellet was lysed in 4 volumes of 1 M NaCl-0.1M EDTAA2% Na-dodecyl sulphate at 60°C for 10 min. After cooling in ice the sample was extracted with an equal volume of chloroform: isoamyl alcohol: phenol (24:1:25) followed by two further extractions with chloroform: isoamyl alcohol (24:1). The DNA was precipitated from the aqueous layer by 2.5 volumes of cold ethanol, washed in ethanol and dissolved in 0.14 M NaCl-0.014 M Na-citrate, pH 7.0. Ribonuclease (200 µg), previously heated at 85°C for 10 min, was added and the mixture was incubated at 37°C for 15 min. The mixture was cooled and extracted once with water saturated phenol:*m* cresol (4:1) and twice with chloroform: isoamyl alcohol (24:1) The

DNA was finally precipitated from the aqueous phase with 2 volume of cold ethanol, washed and dissolved in 0.14 M NaCl-0.014 M Na-citrate, pH 7.0. An aliquot was used to measure $A_{260\ nm}$' and a second aliquot containing about 2 mg DNA was hydrolysed with an equal volume of 1 M perchloric acid for 15 min at 70°C. The sample was cooled, neutralized with 1 M NaOH and centrifuged. The hydrolysed sample was counted for radioactivity in a Beckman model LS-100 liquid scintillation spectrometer using a Triton X-100-toluene cocktail (7 g PPO, 200 mg POPOP, 350 ml Triton X-100 and 650 ml toluene). The DNA from the microsomal mixture was isolated, purified and the bound carcinogen measured according to a procedure reported earlier (Shah and Bhattacharya, 1980).

Results

The result of the *in vivo* experiment is shown in figure 1. Subsequent to administration of [^{3}H] -benzo(a) pyrene, the DNA bound carcinogen was measured to be 1.89 µmol/mol DNA-P in the hepatic nuclei of normal rats. Pretreatment of rats with L-ascorbic acid reduced the *in vivo* binding of the carcinogen. The respective inhibition of adduct formation was 60% at 100 mg/kg^{-1}and 76% at 300 mg/kg body weight dose levels. This inhibition by ascorbic acid is considered highly significant, inspite of the fact that the extent of binding was low.

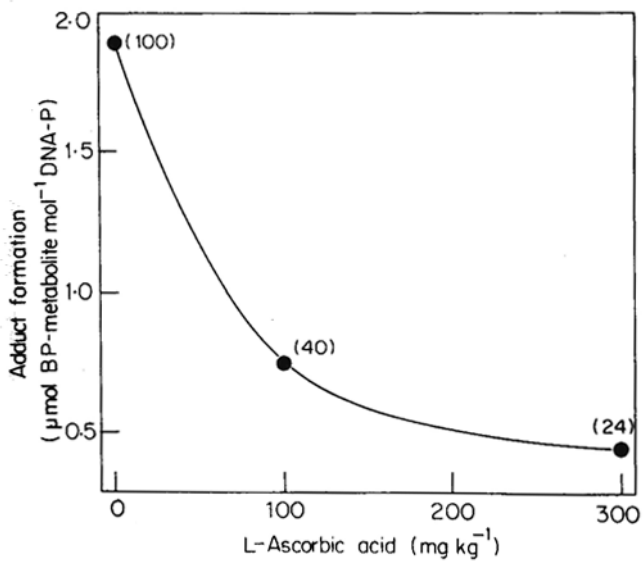

Figure 1. *In vivo* adduct formation in hepatic nuclei of rats pretreated with L-ascorbic acid

Adduct formation was calculated from the amount of radioactive benzo(a)pyrene metabolite covalently bound to DNA (millimolar extinction coefficient of DNA-P at 260nm was taken as 6.6). Each value represents average from 4 rats. Figures in parenthesis are per cent of control value.

The extent of *in vitro* binding of [^{3}H] -benzo (a) pyrene to DNA in three different liver preprations obtained from ascorbic acid treated rats is presented in table 1.

Table 1. *In vitro* adduct formation in liver subcellular preparations of rats pretreated with L-ascorbic acid.

L-Ascorbic acid treatment	Adduct formation, µmol benzo(a)pyrene-metabolite mol^{-1}		
	Slices	Nuclei	Microsome
None (control)	2.80±0.15 (100)	2.32±0.20 (100)	28.12±3.1 (100)
100 mg/kg	2.31±0.20 (82)	2.30±0.24 (99)	34.92±4.5 (124)
300 mg/kg	1.67±0.22 (60)	1.50±0.25 (64)	26.52±3.9 (94)

Liver slices, nuclei and microsome were prepared from normal and ascorbic acid treated rats as described in the text. *In vitro* reaction conditions were as follows:

Liver slices —5 g fresh weight of slices were suspended in 15 ml Krebs-Ringer solution—phosphate buffer, pH 7.4 and incubated at 37°C with 0.4 µmol [^{3}H]-benzo(a)pyrene (322.5 mCi/m mol) for 2 h with constant shaking. Nuclear fraction was then prepared.

Liver nuclei—each reaction mixture (2 ml) contained Na-phosphate buffer, pH 7.4, 30 µmol, EDTA,50 µmol,NADPH, 1 mg; [^{3}H]-benzo(a)pyrene,0.2 µmol (322.5mCi/m mol), control microsome, equivalent of 100 mg fresh liver (about 1 mg protein) and nuclei, equivalent of 2 g fresh liver. Incubation was at 37°C for 30 min. Nuclear DNA was isolated and purified as described in the text.

Liver microsome — reaction condition was same as in nuclei except that sp. act. of [^{3}H]–benzo(a)pyrene was 129 mCi/m mol and nuclei were omitted, but calf thymus DNA (1 mg) was added. DNA was reisolated and purified according to earlier method (Shah and Bhattacharya, 1980). Adduct formation was calculated from the amount of covalently bound radioactive metabolite to DNA (millimolar extinction coefficient of DNA-P at 260 nm was 6.6). Each value represents average from 4 rats with standard deviation, and figures in parenthesis are per cent of control of a respective group.

When [^{3}H] -benzo (a) pyrene was incubated with liver slice preparations from ascorbic acid treated rats, covalent binding to nuclear DNA observed to be low as compared to the binding observed in liver slices of normal animals. The degree of inhibition was somewhat dose related, being 18% and 40% with 100 mg/kg and 300 mg/kg body weight respectively. With liver nuclei, inhibition in adduct formation was observed only in preparations from rats treated with ascorbic acid at 300 mg/kg. The magnitude of ascorbic acid inhibition was, therefore, more pronounced in liver slice preparations than in isolated nuclei.

The binding of [^{3}H]-benzo(a)pyrene to exogenous DNA mediated by isolated microsome was found to be markedly higher. This was mainly due to more efficient utilization of substrate by the microsomal enzyme and easy accessibility of the metabolite to native DNA which was exogenously added. This was in contrast to the *in situ* situation in liver slices and nuclei where the metabolite had limited access to DNA in regions of chromatin (Jahn and Litman, 1979). The microsomal activity to bring about the binding of benzo(a)pyrene metabolite to exogenous DNA, however, was not found to be inhibited following ascorbic acid treatment of rats. The slightly higher activity observed at 100 mg/kg body weight level was perhaps non-specific with respect to ascorbic acid, in as much as no further stimulation was noted with a higher dose of ascorbic acid.

Discussion

Liver microsomal enzymes metabolise benzo(a)pyrene subsequent to its administration to rats. It has been established that benzo (a)pyrene-7,8-dihydro-diol, a metabolite among others which are produced, is more potent as a carcinogen than benzo (a)pyrene (Kapitulnik *et al.,* 1977). When activated by microsomal preparation, this product also binds to DNA (Borgen *et al.,* 1974). The reactive metabolite involved in the binding to DNA has been identified as a diol epoxide of benzo (a) pyrene (Sims *et al.,* 1974). One stereoisomer (±)-7β, 8α-dihydroxy-9α, l0α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, termed benzo(a) pyrene-diol epoxide I, is the predominant species generated enzymatically from benzo (a) pyrene-7,8-dihydrodiol (Huberman *et al.,* 1976; Yang *et al.,* 1976), and is the ultimate carcinogenic metabolite of benzo(a)pyrene. The benzo (a) pyrene diol epoxide I is also carcinogenically most potent (Kapitulnik *et al.,*1977). In the present study we have measured the formation of the adduct DNA-benzo(a)pyrene diol epoxide I in rat liver using [^{3}Hl-benzo(a)pyrene. We have also demonstrated that in the ascorbic acid treated rats this adduct formation is inhibited at the cellular and nuclear levels but not at the microsomal level. We had shown earlier that addition ofL-ascorbate inhibited the adduct formation partially but significantly *in vitro* in a microsome mediated reaction using [^{3}H]-benzo(a)pyrene or [^{3}H] -benzo(a)pyrene-7,8-dihydrodiol, and in a non-enzymatic reaction between [^{3}H]-benzo(a)pyrene diol epoxide I and DNA (Shah and Bhattacharya, 1980). These studies utilized microsome from normal rats and exogenous DNA.

The absence of any inhibition of microsomal activity while there is inhibition at the whole liver cell level due to *in vivo* ascorbic acid treatment indicates that L ascorbate does not have any action at the organelle level. At the cellular level, it is predominantly the microsomal enzymes which convert benzo (a) pyrene to benzo (a) pyrene-diol epoxide I, but ascorbic acid treatment does not seem to inhibit the enzymatic functions of the microsome. It appears, therefore, that the presence of ascorbate in the cellular environment is essential to bring about an inhibitory response. Purified microsomal fractions are not expected to contain any ascorbate, while the whole cells (in slices) and nuclear fractions can have a substantial amount of ascorbate, especially after administration of high doses (Burch, 1961).

Regarding the different inhibitory responses to ascorbate under *in vivo* and *in vitro* binding conditions two points are to be noted. First, at 100 mg/kg dose level ascorbate inhibits adduct formation in liver to the extent of 60% *in vivo* but 18% *in vitro.* Thus the inhibition is far more efficient *in vivo.* This is probably because of ready replenishment of ascorbate in liver through the blood and lymphatic systems assuring its continued presence. Ascorbate is also known to induce fragmentation in DNA (Yamafuji *et al.,* 1971; Stitch *et al.,* 1977) which may result in possible repair synthesis. Sixteen hours after benzo (a) pyrene treatment bound benzo (a) pyrene will thus be considerably reduced. Secondly, on increasing the ascorbate dose from 100 mg/kg to 300 mg/kg, the inhibition of *in vivo* adduct formation increases from 60% to only 76%, while inhibition under *in vitro* binding

conditions increases from 18% to 40%. Thus the *in vitro* binding system responds better to increase in the dose of ascorbate. Since these livers are obtained only 2 h after ascorbic acid treatment, the marked inhibition is probably due to the increased concentration of ascorbate present in liver slices.

Under *in vitro* binding conditions with the nuclear preparation, the inhibition of adduct formation is not apparent at 100 mg/kg body weight dose level, while at 300 mg/kg dose, it is as much as that in liver slices. Liver nuclei from rats treated with a low level of ascorbate may not have enough ascorbate to bring about reduction in the availability of benzo(a)pyrene-diol epoxide I generated at the outer nuclear envelope and by exogenously added microsome for its subsequent reaction with DNA. In the case of liver slices, on the other hand, effective inhibition is brought about by the presence of ascorbate both in cytosol and in the nuclei. Significant inhibition in nuclear preparation at 300 mg kg^{-1} dose level, therefore, seems to be a reflection of adequate ascorbate in the nuclei. It is evident furthermore that there is a limit to the inhibitory action of ascorbate in as much as the inhibition on adduct formation is neither linear with dose both *in vivo* and *in vitro,* nor complete as shown in the present study and in earlier studies (Shah and Bhattacharya, 1980).

The mode of action of ascorbic acid in inhibiting the formation of DNA-benzo(a)pyrene diol epoxide I complex is not clearly understood. One explanation is that ascorbic acid, owing to its existence as ascorbate anion at physiological pH (Edgar, 1974), can be considered as a nucleophile which can react with the electrophilic benzo(a)pyrene diol epoxide I. We presume that the *in vivo* effect of L-ascorbic acid with regard to carcinogenesis induced by benzo(a)pyrene or other chemicals can be explained on this basis. Further investigation is clearly needed to verify this hypothesis.

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