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Human phenol sulfotransferases hP-PST and hM-PST activate propane 2-nitronate to a genotoxicant

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The industrial solvent 2-nitropropane (2-NP) is a genotoxic hepatocarcinogen in rats. The genotoxicity of the compound in rats has been attributed to sulfotransferase-mediated formation of DNA-reactive nitrenium ions from the anionic form of 2-NP, propane 2-nitronate (P2N). Whether human sulfotransferases are capable of activating P2N is unknown. In the present study we have addressed this question by investigating the genotoxicity of P2N in various V79-derived cell lines engineered for expression of individual forms of human sulfotransferases, the phenol-sulfating and the monoamine-sulfating phenol sulfotransferases (hP-PST and hM-PST) and the human hydroxysteroid sulfotransferase (hHST). Genotoxicity was assessed by measuring the induction of DNA repair synthesis and by analyzing the formation of DNA modifications. P2N induced repair synthesis in V79-hP-PST and V79-hM-PST cells, whereas induction of repair synthesis in V79-hHST cells was negligible. P2N also resulted in the formation of 8-aminodeoxyguanosine and increased the level of 8-oxodeoxyguanosine in V79-hP-PST cells, but not in the parental V79-MZ cells, which do not show any sulfotransferase activity. Acetone oxime, the tautomeric form of the first reduction product of 2-NP, 2-nitrosopropane, was inactive in all cell lines. The results show that the human phenol sulfotransferases P-PST and M-PST are capable of metabolically activating P2N (P-PST >> M-PST) and that the underlying mechanism is apparently identical to that resulting in the activation of P2N in rat liver, where 2-NP causes carcinomas. These results support the notion that 2-NP should be regarded as a potential human carcinogen.

Introduction

2-Nitropropane (2-NP) is an industrial chemical which causes hepatocellular carcinomas in rats when administered by inhalation (1) or by gavage (2). It has been suggested that the carcinogenicity of the compound in rats results from enzymatic conversion of the anionic form of 2-NP, propane 2-nitronate (P2N), into a DNA-damaging, unsubstituted nitrenium ion through the intermediate formation of acetoxime-*O*-sulfonate and hydroxylamine-*O*-sulfonate (3) (Figure 1). 8-Oxoguanine and aminated guanines (8-aminoguanine and 2-hydrazinohypoxanthine) were identified as products of the reaction of the aminating species with hepatic DNA and RNA *in vivo* (4–6) and 3-aminotyrosine was detected in liver proteins (7).

In rats, the metabolic activation of P2N appears to be a liver-specific process (8,9) which is catalyzed by hepatic cytosolic sulfotransferases (3). Using V79-derived cell lines engineered for expression of individual forms of rat hepatic sulfotransferases, we have recently identified two distinct sulfotransferases, SULT1A1 and SULT1C1, which are capable of activating P2N in the rat (10). Nothing is known, however, about the ability of human sulfotransferases to activate 2-NP or P2N to a genotoxicant. Initial studies in cultured human cells showed that 2-NP did not induce DNA damage, as detected by induction of DNA repair synthesis, in the cell lines A549 and NCI-H322 (derived from adenocarcinomas of the lung), WI38 (derived from embryonic lung fibroblasts) and HEp2 (derived from an epidermoid larynx carcinoma) (8). No phenol sulfotransferase activity, measured as sulfation of 3-hydroxybenzo[*a*]pyrene, could be detected in A549 cells (11) and only a very low activity was observed in NCI-H322 cells (12). No information is available on sulfotransferase activity of WI38 and HEp2 cells.

The question of whether 2-NP is genotoxic in human hepatocytes has been addressed in a study by Davies *et al.* (13), who compared the effects of 2-NP and P2N on unscheduled DNA synthesis (UDS) and DNA modifications in primary cultures of hepatocytes from rat, mouse and man. All three species showed UDS after exposure to 2-NP, but the effects observed in human hepatocytes were much lower than those observed in rat hepatocytes. In addition, there was a striking interindividual variability with regard to the response of the human hepatocyte preparations to 2-NP and P2N, as only three and four of six preparations showed induction of DNA repair by 2-NP and P2N, respectively. All preparations efficiently activated 2-acetylaminofluorene, which served as a positive control chemical. An analysis of the DNA from 2-NP-treated hepatocytes by high performance liquid chromatography with electrochemical detection (HPLC-EC) revealed the presence of a major, electrochemically active modified deoxynucleoside in the DNA from rats and, at a lower level, from mice. This DNA modification, which most likely represented a DNA modification previously called 'DX1' and now known to be 2-hydrazinohypoxanthine (6) was not detected in human hepatocytes. The level of 8-oxodeoxyguanosine (8-oxodGuo), which is increased in the hepatic DNA of rats treated with 2-NP, was not elevated in the hepatocytes of any of the species. Taken together, the observations indicate that 2-NP/P2N is clearly genotoxic in hepatocytes from rat and mouse, whereas the observed effects in human hepatocytes are weaker. It remains unclear whether the genotoxicity of 2-NP/P2N in rat and man is due to the same mechanism. In particular, it is

Abbreviations: 8-aminodGuo, 8-amino deoxyguanosine; AO, acetone oxime; HMP, 1-hydroxymethylpyrene; HPLC-EC, high performance liquid chromatography with electrochemical detection; 2-NP, 2-nitropropane; 8-oxodGuo, 8-oxodeoxyguanosine; P2N, propane 2-nitronate; UDS, unscheduled DNA synthesis.

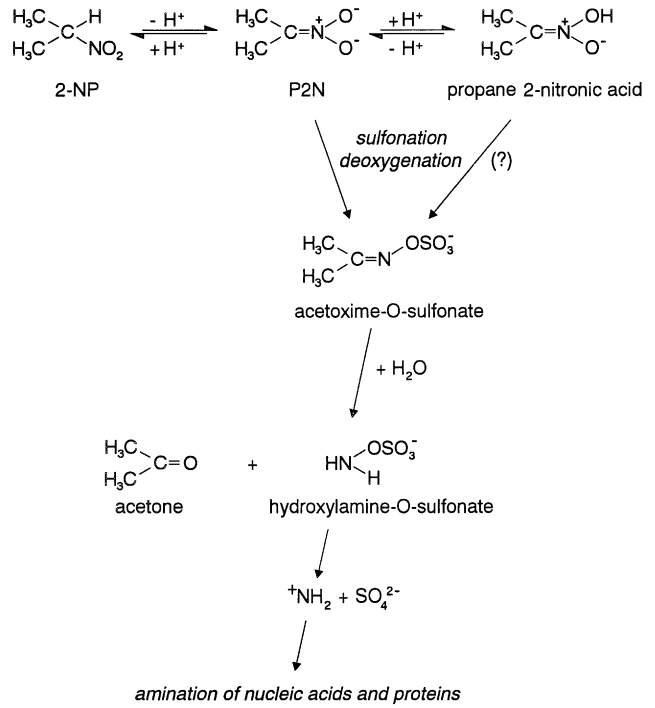

Fig. 1. Mechanism of the activation of 2-NP as proposed by Sodum *et al*. (3).

unknown whether a human sulfotransferase(s) is capable of sulfonating 2-NP/P2N.

Therefore, we have assessed the genotoxicity of P2N in various V79-derived cell lines engineered for expression of individual human sulfotransferases, the phenol-sulfating phenol sulfotransferase (hP-PST, also termed P-PST, TS PST, TS PST 1, ST1A3, human SULT1A1, HAST1 and HAST2), the monoamine-sulfating phenol sulfotransferase (hM-PST, also termed M-PST, TL PST, ST1A5, human SULT1A3 and HAST3) and the hydroxysteroid sulfotransferase (hHST, also termed human DHEA ST, ST2A3 and human SULT2A1). Genotoxicity was determined by measuring the capacity of P2N to induce DNA repair synthesis. In parental V79 cells, which do not show any xenobiotic-metabolizing sulfotransferase activities (14), 2-NP/P2N does not induce repair (8,10,15). Thus, the induction of DNA repair synthesis by P2N in a V79 line engineered to express a sulfotransferase would clearly indicate the ability of this enzyme to activate 2-NP/P2N. In addition, we determined whether the DNA modifications 8-aminodeoxyguanosine (8-aminodGuo) and 8-oxodGuo, which are formed in rat liver following exposure of the animals to 2-NP, could also be detected in the DNA of the V79 cells.

Materials and methods

Chemicals

2-NP (CAS no. 79-46-9, purity >99%) was donated by Angus-Chemie (Ibbenbüren-Uffeln, Germany). P2N was prepared by mixing equal amounts of 2-NP and NaOH in water and allowing the mixture to stand at room temperature for 30 min. Acetone oxime (AO) (98%) was purchased from Riedel-de Haen (Seelze) and 1-hydroxymethylpyrene (HMP) (98%) from Aldrich (Steinheim). All other chemicals were of the highest purity available.

Cells

The V79 cell line used for the construction of recombinant cell lines and as the control cell line has been characterized previously (16). It is now termed V79-MZ. The construction of cell lines V79-hHST (17) and V79-hM-PST (18) has been described previously. The same methods were used to prepare a new V79-hP-PST cell line. For construction of this cell line, the wild-type sequence of hP-PST, designated allele SULT1A1*1 by Raftogianis *et al*. (19), was used. Expression of the sulfotransferases was analyzed by immunoblotting and enzyme activity measurements. The expression levels in the cell lines, determined by immunoblotting, were somewhat higher (2–3 times) than those in the human tissues showing the highest expression levels, i.e. liver for hP-PST, duodenum for hM-PST and liver and adrenals for hHST. Levels of expression of the recombinant proteins in the three cell lines were approximately equal. Specific activities in cytosol preparations were determined as described previously (18,20). They amounted to 30, 100 and 330 pmol product/min/mg protein for V79-hHST (substrate dehydroepiandosterone), V79-hP-PST (substrate *p*-nitrophenol) and V79-hM-PST (substrate dopamine) cells, respectively.

Cells were grown as monolayer cultures in Dulbecco's modification of Eagle's medium (from PAA) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom) at 37°C in a humidified atmosphere of 93% air/7% CO_2.

DNA repair synthesis

DNA repair synthesis in V79 cells was determined using the 5-bromo-2′-deoxyuridine (BrdUrd) density shift method (21) with the modifications described (8,22). Cells were preincubated in 5 ml basal Earle's medium (Biochrom) supplemented with 20 mM HEPES, 2 µM fluorodeoxyuridine (Sigma) and 10 µM BrdUrd (Sigma) for 1 h. After preincubation the medium was removed and the cells were provided with fresh medium additionally containing 10 µCi/ml [5-^{3}H]deoxycytidine (25.6 Ci/mmol; NEN), 2 mM hydroxyurea (Sigma) (23) and the test compound. P2N and AO were dissolved in medium, 1-HMP in dimethylsulfoxide (DMSO). The final DMSO concentration in the medium was 0.5%. For UV irradiation, cells were rinsed with phosphate-buffered saline, drained and irradiated with UV light (254 nm, 1 J/m^2/s). After 5 h incubation, the DNA was isolated and unreplicated ('light') DNA strands were separated from replicated, density labeled ('heavy') strands by equilibrium centrifugation in alkaline $CsCl/Cs_2SO_4$ gradients. Repair synthesis was determined by measuring the incorporation of radioactivity into unreplicated DNA strands and expressed as c.p.m./µg DNA.

Analysis of DNA modifications

The ability of P2N to induce the DNA modifications 8-aminodGuo and 8-oxodGuo in V79-hP-PST and V79-MZ cells was investigated by exposing the cells to P2N (0.3–10 mM) or medium only for 4 h, isolating and hydrolyzing the DNA and analyzing the DNA hydrolysates using HPLC-EC.

For the isolation of DNA, cell pellets were suspended in ~5 vol of extraction buffer (10 mM Tris–HCl, 100 mM EDTA, 0.5% SDS, pH 8.0). DNA was isolated by a modification (24) of the method described by Gupta (25) using RNases A and T1 (Sigma) and proteinase K (Boehringer) treatment followed by extraction with phenol and chloroform/isoamyl alcohol (24:1). DNA was precipitated by addition of 0.1 vol NaCl solution (5 M in H_2O) and 1 vol absolute ethanol. The precipitated DNA was washed twice with 70% ethanol.

The isolated DNA was hydrolyzed to nucleosides by sequential incubations with nuclease P1 (Sigma) and alkaline phosphatase (Sigma) (4). The hydrolysates were purified by filtration (Microcon-3 microconcentrators; Amicon) and analyzed by reverse phase HPLC-EC within a maximum of 5 h after hydrolysis (4), essentially as described by Sodum *et al*. (5). Analysis was performed on a System Gold HPLC system (Beckman) using two 0.46×25 cm Ultrasphere ODS columns (particle size 5 µm) in series with a 0.46×4.6 cm Ultrasphere ODS guard column. Elution with buffer containing 12.5 mM citric acid, 25 mM sodium acetate and 7.5% methanol (pH 5.1) was performed at a flow rate of 1 ml/min. Separations were monitored with a photodiode array detector (model 168; Beckman) at 254 nm and an amperometric electrochemical detector (model 41000; Chromsystems). The potential of the detector was +700 mV versus the Ag/AgCl/3 M KCl reference electrode. Data obtained were processed using special HPLC software (System Gold; Beckman). For quantification of 8-oxodGuo, calibration curves were constructed relating the concentration of 8-oxodGuo to the integrated response of the EC detector and the concentration of deoxyguanosine (dGuo) to its integrated UV absorbance. The DNA modification 8-aminodGuo was identified by co-chromatography with a mixture of 8-aminodGuo and 8-oxodGuo obtained by reacting dGuo with hydroxylamine-*O*-sulfonic acid as described (5). 8-AminodGuo was quantified by relating the integrated response of the EC detector to the integrated UV absorbance of dGuo and expressed as relative area units.

Results

The results on the capacity of P2N to induce DNA repair synthesis in V79-hP-PST, V79-hM-PST and V79-h-HST cells are shown in Table I. Incubation of V79-hP-PST cells with P2N for 5 h resulted in a very strong increase in repair synthesis. Even at 0.1 mM P2N, the lowest concentration

Table I. DNA repair synthesis in V79-hP-PST, V79-hM-PST and V79-hHST cells exposed to P2N, 1-HMP, UV light and AO[a]

Treatment[a]	Repair synthesis (% of control)[b]		
	V79-hP-PST	V79-hM-PST	V79-hHST
Control (medium)	100[c]	100[d]	100[e]
P2N (0.1 mM)	359 ± 78[f]	n.d.[g]	n.d.
P2N (0.3 mM)	920 ± 18[h]	125 ± 7	84 ± 7
P2N (1 mM)	1679 ± 32[h]	181 ± 14[i]	112 ± 3
P2N (3 mM)	2419 ± 31[h]	286 ± 22[f]	115 ± 10
P2N (10 mM)	2451 ± 58[h]	453 ± 61[h]	149 ± 31
1-HMP (10 μM)	2768 ± 276[h]	794 ± 101[f]	436 ± 184
1-HMP (30 μM)	2864 ± 317[h]	1618 ± 148[f]	497 ± 205
UV (20 J/m^2)	2672 ± 726[f]	2596 ± 382[f]	1780 ± 152[f]
AO (1 mM)	n.d.	99 ± 2	n.d.
AO (3 mM)	n.d.	94 ± 11	n.d.
AO (10 mM)	89 ± 12	116 ± 15	115 ± 20

[a]Cells were exposed to the test compound for 5 h.
[b]Results are the means ± SEM of three independent experiments.
[c]88 ± 11 c.p.m./μg DNA.
[d]57 ± 5 c.p.m./μg DNA.
[e]63 ± 7 c.p.m./μg DNA.
[f]Significantly different from the value of the control, $P < 0.01$ (two-sided Student's *t*-test).
[g]Not determined.
[h]Significantly different from the value of the control, $P < 0.001$ (two-sided Student's *t*-test).
[i]Significantly different from the value of the control, $P < 0.05$ (two-sided Student's *t*-test).

tested, repair was increased 3- to 4-fold above the control level and at 3 mM the increase was ~24-fold. No further stimulation of repair occurred when the P2N concentration was raised from 3 to 10 mM, suggesting that these concentrations saturated the capacity of the cells either to metabolize P2N to the DNA-damaging species or to repair the P2N-induced DNA lesions.

P2N was also effective in inducing DNA repair in V79-hM-PST cells, although to a much lesser extent than in V79-hP-PST cells. Repair synthesis increased continuously up to the highest concentration tested (10 mM), where a 4.5-fold increase over control levels was obtained. In contrast, no significant effect of P2N on DNA repair was observed in V79-hHST cells. At 10 mM P2N, there was a slight elevation of repair (150% of the control value), but this increase was not statistically significant.

All three cell lines were capable of activating 1-HMP to a DNA-damaging metabolite, although to different extents (Table I). V79-hP-PST cells were again the most efficient. The two concentrations of 1-HMP tested (10 and 30 μM) led to nearly identical levels of DNA repair, which were similar to those induced by irradiation with 20 J/m^2 UV light and by 3 and 10 mM P2N. These observations suggest that all these treatments saturated the capacity of V79-hP-PST cells for excision repair. 1-HMP caused a concentration-dependent increase in repair in V79-hM-PST cells, but the effects were markedly lower than those observed with V79-hP-PST cells. As UV irradiation was similarly effective in inducing repair in V79-hM-PST and V79-hP-PST cells, it appears likely that the lower effect of 1-HMP in V79-hM-PST reflects a lower capacity of the cells for metabolic activation of 1-HMP. The lowest genotoxicity of 1-HMP was observed in V79-hHST cells, where both concentrations of the compound induced a 4- to 5-fold increase in repair synthesis above the control level. In these cells, UV irradiation resulted in a repair response that was ~30% lower than in the other two cell lines, but the difference was not statistically significant.

Table II. 8-AminodGuo and 8-oxodGuo in the DNA of V79-hP-PST and V79-MZ cells exposed to P2N

Treatment[a]	8-aminodGuo (relative area units)[b]		8-oxodGuo (8-oxodGuo/10^5 dGuo)[b]	
	V79-hP-PST	V79-MZ	V79-hP-PST	V79-MZ
Control (medium)	<0.5[c]	<0.5[c]	0.49 ± 0.06	0.34 ± 0.16
P2N (0.3 mM)	1.06 ± 0.42	<0.5	0.52 ± 0.12	0.57 ± 0.22
P2N (1.0 mM)	4.41 ± 0.53	<0.5	0.84 ± 0.24	0.44 ± 0.10
P2N (3.0 mM)	9.44 ± 1.15	<0.5	1.17 ± 0.24[d]	0.37 ± 0.05
P2N (10.0 mM)	15.97 ± 1.31	<0.5	1.94 ± 0.71[d]	0.40 ± 0.21

[a]Cells were incubated with P2N or medium for 4 h.
[b]Results are the means ± SEM of three independent experiments.
[c]Limit of detection.
[d]Significantly different from the value of the control, $P < 0.05$ (two-sided Student's *t*-test).

AO, the tautomeric form of the first reduction product of 2-NP, 2-nitrosopropane, did not induce DNA repair in any of the V79 cell lines (Table I). This observation supports the notion that the compound, which is formally the direct precursor of the reactive intermediate acetoxime-*O*-sulfonate (Figure 1), is not a substrate of sulfotransferases capable of activating P2N (10).

We determined whether exposure of V79-hP-PST cells, the cell line with the highest capacity to activate P2N, also resulted in formation of DNA modifications characteristic of 2-NP and P2N. Table II shows that the same P2N concentrations that elicited DNA repair also resulted in concentration-dependent formation of both 8-aminodGuo and 8-oxodGuo in the DNA of the cells. In contrast to the amount of repair induced, the levels of both modifications continued to increase between 3 and 10 mM P2N, indicating that the metabolism of P2N was not saturated at 3 mM. Exposure of the parental V79-MZ cells, which do not express sulfotransferase activity nor exhibit increased repair following exposure to P2N (10), did not result in the formation of detectable amounts of 8-aminodGuo and did not affect the level of 8-oxodGuo in the DNA of the cells (Table II).

Discussion

The data presented show that the human phenol sulfotransferases hP-PST and hM-PST are capable of metabolizing P2N to a DNA-damaging species. Treatment of V79 cell lines engineered to express these enzymes with P2N resulted in the induction of DNA repair synthesis, an indicator of the presence of DNA modifications subject to excision repair. An analysis of the DNA modifications in P2N-exposed V79-hP-PST cells by HPLC-EC revealed the presence of 8-aminodGuo and increased levels of 8-oxodGuo. These DNA base modifications have been previously observed in the hepatic DNA of rats treated with 2-NP (4,5) and in the DNA of cultured ovine seminal vesical cells (26) exposed to P2N. Whereas 8-oxodGuo may be formed by a variety of mechanisms, the presence of the characteristic DNA modification 8-aminodGuo in all these systems strongly suggests that the mechanisms of activation are very similar in rat liver and in the recombinant cell lines

used. The reactions involved in activation, as proposed by Sodum *et al*. (4,5), are shown in Figure 1.

Comparisons of the species, sex and organ dependence of tumor formation by 2-NP with the corresponding genotoxicity of 2-NP in animals *in vivo* have indicated that DNA damage plays a pivotal role in the induction of hepatocarcinomas by 2-NP in rats (8,9,27). Since the induction of DNA damage in rat liver is mediated by sulfotransferases and since at least two human sulfotransferases are capable of performing the same activation step, it appears likely that the same DNA lesions that give rise to liver tumors in 2-NP-exposed rats are also produced in 2-NP-exposed humans. However, in contrast to rat cytosolic sulfotransferases, which are expressed primarily in the liver (28), human sulfotransferases show a much wider organ distribution (29–33). Human P-PST is predominantly expressed in the liver, but significant activity has also been found in colon, small intestine, lung, endometrium, platelets, skin and brain. In contrast, human M-PST has its highest activity in the colon and the small intestine, some activity occurs in the lung, platelets and the brain, but hepatic expression is negligible. Thus, it is likely that organs other than the liver are potential targets for 2-NP genotoxicity in humans.

In addition to metabolism by sulfotransferases, oxidative denitrification, probably mainly catalyzed by cytochromes P450 (34), represents another route for the metabolism of 2-NP. This pathway, which has been demonstrated to occur in rabbits (35) and rats (36,37) *in vivo*, is believed to result in detoxification of 2-NP (37). It is possible that this pathway also contributes to the metabolism of 2-NP in humans and therefore the relative contributions of sulfonation and oxidative denitrification to the metabolism of 2-NP in specific organs would be a major determinant of possible species differences in response and for the risk of tumor initiation in humans exposed to 2-NP.

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