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CONJUGATION OF 1-NAPHTHOL BY HUMAN BRONCHUS AND BRONCHOSCOPY SAMPLES

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Abstract—Human bronchus, in short-term explant culture, metabolized 1-naphthol to both its sulphate ester and glucuronic acid conjugates. At low concentrations of 1-naphthol (20 μM), more 1-naphthyl sulphate than 1-naphthyl-β-D-glucuronide was formed. At similar substrate concentrations, short-term explant cultures of both bronchus without cartilage and bronchoscopy samples, which consisted largely of bronchial epithelium, formed predominantly 1-naphthyl-β-D-glucuronide. Thus cartilage, which alone did not significantly metabolize 1-naphthol, may in some way influence the metabolism of 1-naphthol by intact bronchus. A large inter-individual variation was observed in the conjugation of 1-naphthol by cultured human bronchus. Such variations in the conjugation of chemicals may be of importance in determining individual susceptibility to chemically induced damage.

Lung cancer has produced many fatalities in Western Society [1]. By identifying major differences in metabolic pathways between normal and tumour tissues, it may be possible to design drugs which are selectively toxic to the cancerous tissue. Recent studies, using short-term organ cultures of human peripheral lung and tumour tissue from the same patients, have shown striking qualitative differences in the routes of conjugation of 1-naphthol, a model phenolic substrate. Whereas normal lung tissue formed primarily 1-naphthyl sulphate [2, 3], certain types of human lung tumours, in particular squamous cell carcinomas, formed almost exclusively the glucuronic acid conjugate, 1-naphthyl-β-D-glucuronide [3]. Other types of lung tumours demonstrated a greater variability in the routes of conjugation. However, there appears to be a major biochemical difference in the routes of conjugation of 1-naphthol between normal peripheral lung and squamous tumour tissue which may be exploitable in chemotherapy.

Squamous carcinoma of the lung is thought to arise from the bronchial epithelium [4]. Thus, it is important to determine if the conjugation pathways present in the tumour are the same or different from the bronchial epithelium from which the tumours have originated. In the present study, we have examined the metabolism of 1-naphthol by using short-term explant cultures of surgically derived bronchial specimens and bronchoscopy samples. Intact whole bronchus formed both 1-naphthyl sulphate and 1-naphthyl-β-D-glucuronide. At low concentrations of 1-naphthol (20 μM), sulphate ester conjugates predominated.

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MATERIALS AND METHODS

Short-term explant culture. Samples obtained either from surgery or bronchoscopy were collected from hospitals and cultured within a period of 3 hr of removal. The tissue was transported to the laboratory in Leibovitz L-15 medium (Flow, Irvine, Ayrshire, U.K.) containing the antibiotics: gentamicin sulphate (50 μg/ml; Sigma Chemical Co., Poole, Dorset, U.K.), fungizone (25 μg/ml; Gibco-Biocult, Paisley, U.K.), penicillin (100 units/ml) and streptomycin (100 μg/ml; Gibco-Biocult). The human bronchus and bronchoscopy samples were cultured as explants, essentially as described by Barrett *et al.* [5] and Stoner *et al.* [6], on gelatin sponge (UpJohn, Crawley Down, Sussex, U.K.) in a supplemented CMRL-1066 medium (Gibco-Biocult). Samples were placed in plastic tissue culture dishes (Nunc, Denmark or Sterilin, Teddington, Middlesex, U.K.), maintained at 37° in an atmosphere of 50% oxygen, 45% nitrogen and 5% carbon dioxide, and rocked at 10 cycles/min for 24 hr so that the explants were in the medium for 50% of each cycle. In addition to culturing the intact bronchus from surgery, in some cases this was split into two, i.e. the cartilage and the surface layer containing bronchial epithelium.

1-Naphthol conjugation in short-term explant culture. After culture at 37° for a period of 18 or 24 hr, the medium was decanted and replaced with one containing [1-^{14}C]-1-naphthol (sp. act. 19.2 mCi/mmole; Amersham International Ltd., Bucks., U.K.). The tissue was then cultured for a further 24 hr, after which the medium was removed and stored at −20°, prior to the analysis of 1-naphthol metabolites. The tissue was also removed and stored at −20° for protein determination. The tissue was dissolved in 100 μl 1 M sodium hydroxide in a sealed tube at 37° and aliquots of the resulting solution were employed for a protein assay [7] using bovine serum

albumin as a standard. In some cases, the tissue was further cultured by replacing the media containing [1-^{14}C]-1-naphthol with fresh culture media. Appropriate controls for the metabolism studies were obtained by culturing media containing [1-^{14}C]-1-naphthol for 24 hr in the absence of tissue, and the media were removed, stored and analysed.

Chromatography of 1-naphthol conjugates. The conjugates in the culture media were analysed by thin-layer chromatography (TLC) essentially as described by Mehta *et al.* [8]. Aliquots (50 μl) of the culture medium from one incubation together with 50 μl of authentic standards of 1-naphthol, 1-naphthyl-β-D-glucuronide (Koch-Light Laboratories Ltd., Colnbrook, U.K.) and 1-naphthyl sulphate (Sigma Chemical Co., Poole, Dorset, U.K.) were applied to silica gel TLC plates (200 mm × 50 mm; Kieselgel 60F$_{254}$, 0.2 mm thickness, aluminium-backed plates) and the metabolites separated using a solvent system of toluene–glacial acetic acid–acetone (2:1:2 by vol.) at room temperature. The metabolites associated with radioactivity were detected by radiochromatogram scanning, while the quantitation of the conjugates present in each medium was carried out by cutting the chromatogram at the position of the UV-absorbing standards on the fluorescent background, when examined under UV light. This was followed by liquid scintillation counting, the amount of radioactivity detected at similar R_f positions as the conjugates, in the appropriate controls, being subtracted from the corresponding values obtained in the experimental determinations. The R_f values for 1-naphthol, 1-naphthyl sulphate and 1-naphthyl-β-D-glucuronide were 0.85, 0.47 and 0.19, respectively. In order to assess the degree of reproducibility and variability of the method, medium containing metabolites was analysed eight times as described above and the results were averaged. The percentages of glucuronic acid and sulphate ester conjugates were found to be 41.1 ± 2.1% and 31.9 ± 1.3%, respectively (mean ± S.D.).

Hydrolysis of conjugates for further identification of metabolites. Aliquots of culture media were incubated with either Ketodase (General Diagnostics, Warner Lambert U.K. Ltd., Eastleigh, Hants., U.K.) or β-glucuronidase (Sigma, 5000 units/ml) in 0.1 M acetate buffer, pH 5.0; or with arylsulphatase (Sigma, 700 units/ml) and D-saccharic acid 1,4-lactone (Sigma, 40 mM) in 0.1 M acetate buffer, pH 5.0; or with 0.1 M acetate buffer, pH 5.0, alone. Saccharic acid 1,4-lactone was included with arylsulphatase to inhibit β-glucuronidase, a known contaminant of arylsulphatase. The hydrolyses were carried out in sealed tubes in a 37° incubator for periods between 20 and 24 hr. In order to quantitate the conjugates formed, the hydrolysates were then analysed by TLC. The TLC procedure was carried out as already explained, but without radiochromatogram scanning. A metabolite was identified as a glucuronide if it was hydrolysed by β-glucuronidase and as a sulphate ester if it was hydrolysed by arylsulphatase.

RESULTS

Metabolism of 1-naphthol by bronchus samples

Short-term organ cultures of intact bronchus metabolized 1-naphthol to both 1-naphthyl sulphate and 1-naphthyl-β-D-glucuronide (Table 1). However, considerable inter-individual variation was observed both in the total extent of conjugate formation and in the relative amounts of the different conjugates formed. In some cultures of intact bronchus, sulphation predominated (patients F, P and U), in others glucuronidation predominated (patients N, O and R), whereas in others (patients M and S) more 1-naphthyl sulphate than 1-naphthyl-β-D-glucuronide was formed at lower concentrations, but the latter predominated at higher concentrations. Specimen K formed equal quantities of both conjugates (Table 1). Even though these statements are quantitatively true, some of the absolute differences between the two pathways are small, possibly being within the limits of experimental error. However, when all the data with the intact whole bronchus at low 1-naphthol concentrations ($\leq$20 μM) are examined, sulphate ester conjugation was significantly higher than glucuronic acid conjugation ($P = 0.05$), using the Wilcoxon matched pairs signed ranks test.

When sufficient sample was available, in addition to being cultured intact, it was also split into underlying cartilage and bronchial epithelium and cultured. In three samples (P, R and U), the cartilage metabolized 1-naphthol very slowly and in all cases forming 1-naphthyl sulphate with little, if any, 1-naphthyl-β-D-glucuronide. In contrast to the results with the cartilage, the tissue containing the bronchial epithelium metabolized 1-naphthol more rapidly and produced both conjugates, but formed more 1-naphthyl-β-D-glucuronide (Table 1).

Metabolism of 1-naphthol by bronchoscopy samples

To extend the data obtained with the bronchial samples, tissue from bronchoscopy was examined. This tissue was either taken from the bronchus wall and thus contained some epithelial cells, or it was removed from the surface of the tumour. The results of the incubation of [1-^{14}C]-1-naphthol with tissue samples from bronchoscopy are shown in Table 2. Most samples were again able to metabolize 1-naphthol to both its sulphate ester and glucuronic acid conjugates. A large inter-individual variation in the formation of both conjugates was again observed. With the samples from bronchoscopy, significantly more 1-naphthyl-β-D-glucuronide than 1-naphthyl sulphate was formed ($P = 0.005$, using Wilcoxon matched pairs signed ranks test). No major differences were observed in the metabolism of 1-naphthol by bronchial mucosa from normal or bronchitic patients (Table 2).

When intact bronchus and bronchoscopy samples were maintained in culture for more than 48 hr, the metabolism of 1-naphthol to its two conjugates was still observed (Tables 1 and 2). This illustrated that viable tissue, capable of metabolizing this substrate, was maintained over several days. The pattern of metabolites from 1-naphthol was very similar when these tissues were cultured for 24 hr or several days suggesting that the tissues were well-maintained in culture.

The samples of tumour from bronchoscopy showed little metabolism in two cases (specimens T3

Table 1. Conjugation of [1-^{14}C]-1-naphthol by short-term organ cultures of normal human bronchus: intact and divided

Patient	Tissue	1-Naphthol concn (μM)	% of total radioact. recovered in medium as conjugates		Conjugates formed (nmoles/24 hr per mg protein)	
			1-NS	1-NG	1-NS	1-NG
F	Intact	10	53.2 (2)	13.2 (2)	6.3	1.6
		100	32.8 (2)	9.5 (2)	25.0	7.5
K	Intact	10	28.0 (2)	24.6 (2)	6.0	5.1
		10*	32.1 (2)	35.7 (2)	10.9	10.0
M	Intact	5	14.4 (3)	2.1 (3)	2.8	0.4
		10	3.6 (3)	1.9 (3)	1.5	0.8
		20	5.3 (3)	1.9 (3)	4.5	1.6
		50	1.2 (3)	2.6 (3)	2.7	5.7
		100	0.7 (3)	2.0 (3)	3.5	9.7
N	Intact	20	10.8 (3)	14.7 (3)	4.7	6.5
O	Intact	20	16.7 (3)	21.6 (3)	1.9	2.6
P	Intact	5	21.0 (2)	13.5 (2)	1.9	1.3
		10	15.0 (2)	7.0 (2)	2.8	1.3
		20	20.0 (2)	16.1 (2)	42.9	34.1
	Cartilage	5	8.7 (2)	2.0 (2)	1.5	0.4
		10	7.5 (2)	1.0 (2)	1.9	0.2
		20	1.9 (2)	1.2 (2)	0.7	0.5
	Epithelial layer	5	13.0 (2)	14.9 (2)	6.1	6.3
		10	18.7 (2)	23.5 (2)	11.5	15.2
		20	10.6 (2)	25.7 (2)	15.0	36.2
R	Intact	20	21.3 (3)	21.8 (3)	11.4	16.1
		100	8.8 (2)	26.0 (2)	8.0	35.6
	Cartilage	20	5.6 (3)	0.6 (3)	1.5	0.2
		100	2.0 (2)	0.5 (2)	4.2	1.2
	Epithelial layer	20	8.0 (3)	38.0 (3)	9.1	37.7
		100	3.8 (2)	17.2 (2)	14.8	68.4
S	Intact	10	17.7 (2)	5.1 (2)	1.2	0.4
		20	28.7 (2)	36.1 (2)	4.7	5.9
		100	7.3 (2)	9.7 (2)	4.9	6.3
U	Intact	20	8.7 (2)	1.1 (2)	2.3	0.2
	Cartilage	20	0.4 (2)	ND (2)	0.3	ND
	Epithelial layer	20	3.9 (2)	9.0 (2)	6.4	16.0

Bronchial samples from patients with lung cancer were cultured, within approximately 3 hr of surgical removal, for either 18 or 24 hr at 37°. After this time, the culture medium was changed for one containing [1-^{14}C]-1-naphthol and incubated for a further 24 hr. The amount of radioactivity in the medium at the end of the culture was 70–90% of the total. Aliquots of the media were analysed for 1-naphthol conjugates by TLC. The results are generally expressed as the mean values from a number of incubations, this being added in parentheses.

'Intact' refers to whole intact bronchus, while 'cartilage' and 'epithelial layer' refer to the two layers obtained when the latter was stripped from the underlying cartilage.

ND, Not detectable; 1-NS, 1-naphthyl sulphate; 1-NG, 1-naphthyl-β-D-glucuronide.

* In this experiment the bronchus was cultured for 8 days prior to incubation with [1-^{14}C]-1-naphthol.

and T4). However, in the two samples (T1 and T2) where significant metabolism occurred more 1-naphthyl-β-D-glucuronide than 1-naphthyl sulphate was formed. This was in agreement with our earlier results using short-term explant cultures of human squamous carcinomas of the lung [3].

DISCUSSION

Since most lung cancers arise from the bronchial epithelium, it was decided to examine the metabolism of 1-naphthol by human bronchus. A very marked inter-individual variation in metabolism was observed (Table 1). A similar variation has also been observed in other studies of human drug metabolism both *in vivo* [9] and *in vitro* [10]. These variations may be due to both genetic and environmental factors. Previous reports, with cultured human bronchus [11] or lung [12] and also with human lung microsomes [13, 14], have observed a large inter-individual variation in Phase I oxidative metabolism and in some cases covalent binding of xenobiotics.

Table 2. Conjugation of [1-^{14}C]-1-naphthol by short-term organ cultures of bronchial epithelium and lung tumours from bronchoscopy

Patient	Tissue	1-Naphthol concn (μM)	% of total radioact. recovered in medium as conjugates		Conjugates formed (nmoles/24 hr per mg protein)	
			1-NS	1-NG	1-NS	1-NG
B1	Normal	20	8.8	24.4	1.8*	4.9*
B2	Bronchitic	5	13.7 (2)	24.8 (2)	11.9	22.1
		10	11.0 (2)	31.5 (2)	0.5*	1.4*
		20	6.6	27.2	44.0	181.3
		20†	2.9	22.7	0.1*	0.8*
B3	Normal	10	10.7	28.2	1.1*	2.8*
B4	Bronchitic	20	0.6	ND	0.2*	ND*
B5	Normal	20	ND	0.7	ND*	0.7*
B6	Normal	20	1.3	8.5	5.2*	34.5*
B7	Bronchitic	5	4.3 (2)	10.0 (2)	7.2	16.6
		10	3.6 (2)	16.2 (2)	7.2	34.2
		20	3.3 (3)	14.4 (3)	16.3	72.2
		20	0.9	7.2	1.8*	14.4*
B8	Bronchitic	5	1.1 (2)	6.9 (2)	0.4	2.5
		10	2.4 (2)	30.2 (2)	2.6	31.9
		20	0.4 (2)	5.2 (2)	1.2	13.9
		20†	1.1 (2)	3.2 (2)	3.7	10.5
B9	Bronchitic	20	0.4	0.8	2.0	4.0
B10	Bronchitic	20	2.9 (3)	8.7 (3)	14.8	38.1
		20	4.4	10.5	1.5*	3.5*
B11	Normal	20	13.0	16.7	1.5	2.0
B12	Bronchitic	20	2.4 (2)	12.1 (2)	12.0	60.3
B13	Normal	20	3.2 (3)	23.0 (3)	28.7	196.9
B14	Normal	20	1.4 (2)	5.1 (2)	8.8	26.5
T1	Tumour	20	1.5	7.9	6.0	31.6
T2	Tumour	5	0.6 (2)	ND (2)	0.2	ND
		10	1.4 (2)	1.9 (2)	1.5	2.0
		20	ND (2)	5.2 (2)	ND	20.6
T3	Tumour	20	ND (3)	0.6 (3)	ND	3.0
T4	Tumour	20	0.2 (2)	ND (2)	1.0	ND

Tissue from bronchoscopy was cultured within approximately 3 hr of removal. Each sample was very small and few explants, if any, could be made. Thus, the tissue was cut in half and placed straight on the gelfoam and cultured for 24 hr at 37° as explained in Materials and Methods. After this time period, the culture medium was changed for one containing [1-^{14}C]-1-naphthol and incubated for a further 24 hr. The amount of radioactivity in the medium at the end of the culture was 70–90% of the total. Aliquots of the media were analysed for 1-naphthol conjugates by TLC as described in Materials and Methods. Where more than two explants from a sample were cultured, the result is given as a mean value, with the number of incubations added in parentheses. Some tissue was used for histopathological examination and so was unavailable for protein determination. These results were then expressed per mg tissue (wet wt).

ND, Not detectable; 1-NS, 1-naphthyl sulphate; 1-NG, 1-naphthyl-β-D-glucuronide.

* nmoles product/24 hr per mg tissue (wet wt).

† Cultured for 5 days not 24 hr before metabolism studies.

This study is to our knowledge the first report of such a large inter-individual variation in Phase II conjugation reactions in cultured human bronchus. These variations occur both in sulphate ester and glucuronic acid conjugation (Table 1). Such large variations may be responsible, at least in part, for inter-individual variation in susceptibility to chemically induced carcinogenicity and/or toxicity in the respiratory tract.

Short-term organ culture of bronchus metabolized 1-naphthol ($\leq$20 μM) predominantly to its sulphate ester conjugate (Table 1). This is in agreement with the results of Autrup *et al.* [15], who found that sulphate ester conjugation was quantitatively more important than glucuronic acid conjugation for cultured human bronchus using benzo[a]pyrene (1.5 μM) as substrate. As the concentration of 1-naphthol was increased, the percentage of glucuronic acid

conjugate increased (Table 1), most probably due to the limited availability of sulphate required for the synthesis of the sulphate donor, adenosine 3′-phosphate 5′-sulphatophosphate (PAPS). Sulphation is thought to be a saturable process [16, 17]. In several studies primarily using the liver, a preference for sulphation with phenolic substrates was observed at low substrate concentrations, whereas at higher concentrations glucuronidation was the major pathway of conjugation [18–20]. This seems to be related to the lower K_m for sulphotransferase and a higher V_{max} for UDP-glucuronosyltransferase [18].

Thus, with intact bronchus at low concentrations of 1-naphthol ($\leq 20\ \mu M$) the production of 1-naphthyl sulphate predominated (Table 1). At similar substrate concentrations, bronchus without the cartilage and bronchoscopy samples, which usually consisted of bronchial epithelium with some underlying tissue of the bronchus wall, both formed predominantly 1-naphthyl-β-D-glucuronide (Tables 1 and 2). In addition, a primary cell culture of human bronchial epithelial cells [21] metabolized 1-naphthol almost exclusively to the glucuronic acid conjugate with little, if any, 1-naphthyl sulphate being formed (E. M. Gibby, G. M. Cohen, C. C. Harris and R. C. Grafstrom, unpublished results). Thus cartilage, which alone did not significantly metabolize 1-naphthol, may influence the metabolism of the intact bronchus. The differences in conjugation between the intact bronchus and the tissue from which cartilage had been removed may have been due to cellular damage incurred by stripping off the upper layer or to artefacts of the culture system, e.g. leakage of sulphate from the cut ends of the cartilage followed by uptake into the upper epithelial layer. However, this latter possibility would require the presence of a carrier to transport the sulphate anion across biological membranes, as has been reported for erythrocytes [22]. Uptake of inorganic sulphate by hepatocytes [23] and by ascites tumour cells has also been demonstrated [24].

In summary, short-term organ cultures of intact bronchus, with low concentrations ($\leq 20\ \mu M$) of 1-naphthol, produced more 1-naphthyl sulphate than 1-naphthyl-β-D-glucuronide. Thus the intact bronchus conjugates 1-naphthol in a similar manner to human peripheral lung [2, 3]. These culture systems probably represent the situation *in vivo* more closely than other methods. In contrast, bronchoscopy samples and bronchus without the underlying cartilage both formed mainly the glucuronic acid conjugate at similar substrate concentrations. Thus the conjugation pathway appears to change when the bronchus wall is disrupted. A marked inter-individual variation in the ability of cultured human bronchus to conjugate 1-naphthol was observed.

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