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Effect of the carboxylesterase inhibitor bis-(4-nitrophenyl)phosphate *in vivo* on aspirin hydrolase and carboxylesterase activities at first-pass sites of metabolism in the guinea pig

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The therapeutic activity of aspirin (acetylsalicylic acid) as an anti-inflammatory, analgesic and antipyretic agent is thought to be mediated by its ability to inhibit the biosynthesis of prostaglandins and related autocoids by an irreversible acetylation of the enzyme cyclooxygenase [1]. Salicylic acid, the hydrolysis product of aspirin, cannot acetylate proteins and is almost inactive against cyclooxygenase *in vitro*, but it has been shown to reduce production of prostaglandins *in vivo* [2] and may mediate its actions by a mechanism different from that of aspirin. Although aspirin and salicylic acid are regarded as being equipotent, for example, in the treatment of rheumatoid arthritis [3], other data show a difference in therapeutic action; for example, only aspirin has an analgesic effect when used to treat post-operative dental pain [4]. It was thought of interest, therefore, to develop a means of comparing the pharmacologies of aspirin and salicylic acid *in vivo*. Such a comparison is made difficult under normal circumstances because aspirin is rapidly hydrolysed to salicylic acid once in the circulation—the half-life is about 15 min [5]. To overcome this problem, the esterases that hydrolyse aspirin would have to be inhibited *in vivo* prior to administration of aspirin. Biochemical studies have identified aspirin hydrolases as belonging to a group of serine esterases known as carboxylesterases (EC 3.1.1.1) [6–8]. Bis-(4-nitrophenyl)phosphate (BNPP) irreversibly inhibits carboxylesterases and is comparatively non-toxic [9]. This study describes the use of BNPP to inhibit aspirin hydrolases in the guinea pig *in vivo*, at the three major sites of hydrolysis after oral administration of aspirin, revealed by pharmacokinetic studies to be liver stomach and blood [10–12].

Materials and methods

Carboxylesterase activity, measured with 1-naphthylacetate, and hydrolysis of aspirin were assayed spectrophotometrically [8].

Treatment of animals. Male guinea pigs of mass 350–650 g received an injection of BNPP (Sigma Chemical Co., Poole, U.K.) of 60 mg/kg, i.p., as a solution of 6 mg/ml, made isotonic with NaCl. Control animals received saline only. In each experiment a pair of animals, control and treated with BNPP, were killed, 4 hr after injection, a period sufficient for BNPP to inactivate esterases in rat liver *in vivo* [7].

Preparation of tissues. Livers were weighed and homogenised as described before [13] in 0.3 M sucrose, 1 mM EDTA and 1 mM dithiothreitol. Mucosae were scraped off freshly excised stomachs, weighed, and homogenised following the same procedure as for liver. Tissue homogenates were centrifuged at 100,000 g for 30 min. Supernatant fractions were assayed as cytosolic fractions, and the sediments were resuspended by homogenisation in 25 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol, 1.0% (v/v) in Triton X-100, pH 7.5, to the volume of the initial homogenate. The solubilised membranes were centrifuged at 100,000 g for 30 min, and the supernatant fractions were retained for assay. Blood was collected by cardiac puncture into heparinised syringes and spun at 1300 g for 20 min. The plasma was used for assay, and the erythrocytes were discarded in view of their low amount of aspirin-hydrolysing activity [14].

Results and discussion

In each set of experiments the carboxylesterase and aspirin-hydrolysing activities were compared in the liver, gastric mucosa and plasma obtained from a pair of guinea pigs, one treated with BNPP and the control with saline only. Data in Table 1 give the hydrolytic activities measured in each preparation of cytosol and membranes, and in plasma. Of the three sites of metabolism studied, liver contained by far the greatest amount of aspirin hydrolase activity—13.4 ± 1.5 units/g (equivalent to 308 ± 54 units total activity in the tissue) compared with 2.65 ± 0.50 units/g (6.2 ± 1.5 units total tissue activity) for gastric mucosa and 0.003 ± 0.001 units/ml in plasma. Most of the aspirin hydrolase activity in liver is due to microsomal enzymes sensitive to inhibition by BNPP *in vitro* ([13] and K. N. White, unpublished results). In this study, virtually all (99%) of the aspirin hydrolase activity attributable to microsomal enzymes was abolished by treating animals with BNPP. In contrast, the uniquely cytosolic aspirin hydrolase of liver is much less sensitive to inhibition by BNPP *in vitro* [15] and was consequently inhibited only 41% by BNPP *in vivo*. Cytosolic carboxylesterases were similarly resistant to inhibition by BNPP, being inhibited by only 29%. In total, 97% of the aspirin-hydrolysing activity of liver was inhibited by treatment with BNPP.

In the gastric mucosa, both membranous and cytosolic aspirin hydrolases were almost completely inhibited by BNPP, 97% in both cases, compared with about 50% inhibition of carboxylesterases. In general, aspirin hydrolases were more sensitive to inhibition by BNPP than were carboxylesterases. In the liver and gastric mucosa, the total aspirin hydrolase activity of the tissue was reduced by 97% in both cases, compared with a reduction in total carboxylesterase activity of 88% and 51%, respectively. The difference in sensitivities between the two esteratic activities may be accounted for by the existence of a larger number of distinct protein forms of carboxylesterases, compared with aspirin hydrolases, which vary in their sensitivities to inhibition by BNPP [16]. Only the cytosolic aspirin hydrolase of liver has been found to be partially inhibited by BNPP; all other aspirin hydrolases studied thus far in liver and other tissues are inhibited completely by micromolar concentrations of BNPP. The low activity of aspirin hydrolase in plasma (about 0.003 units/ml) was strongly inhbited (87%) by BNPP as was the much higher (0.515 ± 0.213 units/ml) carboxylesterase activity (90% inhibition).

Data from pharmacokinetic studies on rats [10] suggest that the contribution of a tissue to the hydrolysis of aspirin *in vivo* is not correlated to the absolute amount of aspirin hydrolase activity measurable in the tissue *in vitro*. In the rat, the liver and stomach contribute approximately the same to the first-pass hydrolysis of aspirin [10] despite the greater, by fifteen times, specific activity of aspirin hydrolase in liver [17]. Results from this study with guinea pigs show that the total tissue activity remaining in liver after treatment of animals with BNPP (9.6 ± 2.5 units) is greater than that found in the gastric mucosa of untreated animals (6.2 ± 1.5 units). It remains to be seen from pharmacokinetic studies whether the aspirin hydrolase activity left in liver after treatment of animals with BNPP can contribute significantly to the hydrolysis of aspirin *in vivo*. In this respect, it is noteworthy that the cytosolic aspirin hydrolase of liver, which accounts for most of the activity resistant to BNPP, had a much lower K_m, (about 1 μM) compared with the microsomal aspirin hydrolase (450 μM) (K. N. White, unpublished results).

In conclusion, the carboxylesterase inhibitor BNPP administered *in vivo* comprehensively abolished the total aspirin-hydrolysing activity at important sites of first-pass metabolism in the guinea pig and showed greater selectivity

Table 1. Effect of i.p. BNPP on carboxylesterase and aspirin hydrolase activities in liver, gastric mucosa and plasma

	Aspirin hydrolase (units/g)	Aspirin hydrolase (units/ml × 1000)	Carboxylesterase (units/g)	Carboxylesterase (units/ml)
Liver				
Membranes				
Control	12.8 ± 1.4		309 ± 115	
BNPP	0.11 ± 0.02 (99)		29.7 ± 15.5 (90)	
Cytosol				
Control	0.53 ± 0.07		10.3 ± 2.1	
BNPP	0.31 ± 0.07 (41)		7.3 ± 4.2* (29)	
Total activity in tissue				
Control	13.4 ± 1.5		319 ± 116	
BNPP	0.41 ± 0.09 (97)		36.6 ± 13.2 (88)	
Gastric mucosa				
Membranes				
Control	1.38 ± 0.33		10.9 ± 2.94	
BNPP	0.05 ± 0.03 (97)		4.98 ± 3.19 (54)	
Cytosol				
Control	1.26 ± 0.21		8.73 ± 3.20	
BNPP	0.04 ± 0.02 (97)		4.64 ± 1.29* (47)	
Total activity in tissue				
Control	2.65 ± 0.50		19.6 ± 5.80	
BNPP	0.08 ± 0.05 (97)		9.62 ± 3.39 (51)	
Plasma				
Control		2.62 ± 1.08		0.515 ± 0.213
BNPP		0.35 ± 0.22 (87)		0.050 ± 0.016 (90)

Hydrolytic activities are expressed in units of 1 μmol of substrate hydrolysed per min per g of wet weight tissue or per ml. Data are mean ± SD for six animals. Data in parentheses are percent decrease in activity in animals treated with BNPP compared with control animals. All data from animals treated with BNPP were statistically significantly different from controls within confidence limits of 95% or greater (Student's *t*-test) except where indicated by an asterisk.

towards aspirin hydrolases than to carboxylesterases. However, a small amount of activity in the liver which is attributable to an esterase with a high affinity for aspirin could not be inhibited completely by BNPP, although greater inhibition may result from longer exposure to, or higher doses of, BNPP. Pharmacokinetic studies are required to confirm that hydrolysis of aspirin by guinea pigs *in vivo* can indeed be abrogated by prior administration of BNPP. A means whereby significant levels of intact aspirin can be maintained in the circulation would allow a better comparison of the pharmacological properties of aspirin and salicylic acid than is presently possible.

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