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ASPIRIN, PROTEIN TRANSACETYLATION AND INHIBITION OF PROSTAGLANDIN SYNTHETASE IN THE KIDNEY

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1 The effect of aspirin on the kidney has been investigated in mice and rabbits. [Acetyl-^{14}C]-aspirin was administered intraperitoneally in doses ranging from subtherapeutic to toxic. The degree of acetylation of protein was determined by the radioactivity remaining on protein precipitates of renal cortex and medulla after sequential washing designed to remove non-covalently bound material. Controls were established, by the use of [carboxyl-^{14}C]-aspirin.

2 The acetyl-^{14}C residue was bound to renal proteins in a linear manner in increasing amounts with increasing dosage up to 100 mg/kg. The [carboxyl-^{14}C]-aspirin was not bound and thus the salicylate portion of the molecule was not bound covalently to the renal protein. The time course of the acetylation was rapid, consistent with the rate of aspirin absorption. The disappearance of acetylated protein was slow, with a $T_{1/2}$ of 112.5 h in the renal cortex, and 129.5 h in the renal medulla.

3 Differential centrifugation, Sephadex chromatography and gel electrophoresis were carried out on tissue homogenates to determine the site of acetylation. The acetylation was greatest in the microsomal fraction, although all protein fractions showed some degree of acetylation.

4 The prostaglandin synthetase activity of a particulate preparation from rabbit kidney was determined by a spectrophotometric assay of malondialdehyde formation. Aspirin (10 mg/kg, i.v.) significantly inhibited prostaglandin synthetase in the renal cortex and medulla.

5 Aspirin and renal proteins undergo a transacetylation reaction resulting in stable acetylated protein, with acetylation being greatest in the microsomal fraction. Aspirin has been shown to inhibit prostaglandin synthetase and this could lead to functional impairment of the tissue.

Introduction

Aspirin has been implicated in the pathogenesis of analgesic nephropathy, acting synergistically with other drugs to induce renal damage (Duggin, 1977). We have investigated the effects of aspirin on the kidney to determine if kidney proteins are acetylated by aspirin and the possible functional significance of this acetylation.

Aspirin has been demonstrated to acetylate a variety of proteins including albumin (Hawkins, Pinchard, Crawford & Farr, 1969), haemoglobin (Shamsuddin, Mason, Ritchey, Honig & Klotz, 1974) and a particulate platelet protein (Roth & Majerus, 1975). In the case of albumin and haemoglobin, biological characteristics of the protein are not significantly altered, although physiochemical characteristics are. In the case of platelet particulate protein, Roth & Majerus (1975) have shown that platelet protein function is inhibited by aspirin by acetylation of an active site of an enzyme, resulting ultimately in failure of aggregation of platelets.

Methods

Protein acetylation

Male C57BL/6J mice were injected intraperitoneally with [acetyl-^{14}C]-aspirin (Radiochemical Centre, Amersham, sp. act. 24 mCi/mmol, 99% radiochemical purity) in a dose range from 0.0015 mmol/kg to 0.6 mmol/kg and were then killed 20 min after the injection. Another group of mice was injected with a dose of 0.003 mmol/kg and was killed from 5 min to 8 days following administration. The kidneys were removed, divided into cortex and medulla, homogenized in distilled water with the use of a Potter-Elvehjem

glass teflon homogenizer and the protein precipitated with trichloroacetic acid (TCA) solution to a final concentration of 4.5% TCA. Similar experiments were also done with the New Zealand white rabbit.

The precipitates were then washed twelve times, until all covalently bound aspirin was removed. These washes consisted of four with 4.5% TCA in water, three with acidified ether (1 M HCl) and five with absolute methanol. The supernatants from the last three washes showed no radioactivity above background. Further homogenates were subjected to prolonged equilibrium dialysis. They were dialysed in cellophane dialysis tubing against water for 48 h, phosphate buffer pH 4 for 48 h, and a saturated solution of aspirin for 72 h. The protein precipitate was then dissolved in an aqueous solution of 1 M NaOH, and disintegrations per mg of protein were determined. Counting was carried out in a Packard Tricarb (Model 3380) Liquid Scintillation Spectrometer and the scintillation solution was Insta-Gel (Packard).

All counts were corrected for quenching by automatic external standardization. Protein estimations were carried out by Lowry's method, with bovine serum albumin used as standard (Lowry, Rosebrough, Farr & Randall, 1951). Protein molecular weight was taken as 200,000. Control experiments identical to the above were performed, with [carboxyl-^{14}C]-aspirin (Radiochemical Centre, Amersham, specific activity 21 mCi/mmol, 99% radiochemical purity).

Site of acetylation

A further group of male C57BL/6J mice was injected with [acetyl-^{14}C]-aspirin 0.003 mmol/kg and killed after 20 min. The kidneys were removed, divided into cortex and medulla, homogenized and the homogenate subjected to differential centrifugation (Mahler & Cordes, 1968). The four fractions obtained from each homogenate were precipitated with a final solution of 4.5% TCA and were then washed as in previous experiments before determination of counts/mg of protein.

Another group of mice was injected with [acetyl-^{14}C]-aspirin 0.006 mmol/kg. The kidneys were removed and treated as above (experiment 1). These dissolved homogenates were then eluted from a Sephadex G200 superfine column (30 cm × 1 cm). The column had a void volume of 18 ml; 0.05 M Na_2HPO_4 adjusted to pH 7.4 with 0.05 M NaH_2PO_4 was used as eluant. Collection of samples (30 drops) was made in an LKB Bromma Fraction Collector. The radioactivity in each sample was counted and the protein concentration determined.

A sample of [acetyl-^{14}C]-aspirin was also run through the Sephadex column, samples were again collected, and counted.

Further mice were injected with [acetyl-^{14}C]-aspirin 0.006 mmol/kg and treated as in previous experiments. A small sample of homogenate was applied to 6% polyacrylamide gels and gel electrophoresis performed. The gels were made up and electrophoresis performed according to the method of Davis (1964). The gels were sliced into 0.5 cm pieces and the radioactivity in these pieces counted.

Prostaglandin synthetase inhibition

New Zealand white rabbits (3.0 to 3.5 kg) were injected with 0.056 mmol/kg (10 mg/kg) aspirin intravenously. Control rabbits were injected with an equal volume of 0.9% w/v NaCl solution (saline). Fifteen minutes after injection the rabbits were killed by a blow to the back of the neck and the kidneys were removed immediately and placed in ice cold 0.01 M Tris buffer, adjusted to pH 7.5 with 1 M HCl. The kidneys were divided into cortex and medulla, homogenized separately in 15 ml of buffer in a Polytron Kinematica at low speed. A preparation of lyophilized particulate prostaglandin synthetase from rabbit kidney cortex and medulla was prepared by the following method, which essentially follows that of Blackwell, Flower & Vane (1975). The homogenates were centrifuged at 12,000 *g* for 10 min (Sorvall RC2B) and the supernatant then centrifuged at 100,000 *g* for 1 h (Beckman L2, 42 rotor, 38,000 rev/min). The supernatant was decanted and the microsomal pellet was smeared around the sides of tubes, frozen in liquid N_2 and then lyophilized overnight. These lyophilized samples were then stored in a freezer at −20°C.

A measure of the activity of prostaglandin synthetase was then made by determining the formation of malondialdehyde (MDA) by the spectrophotometric assay of Flower, Cheung & Cushman (1973). The buffer used in these experiments was Tris (0.01 mol/l) adjusted to pH 7.5 with HCl (1 mol/l) and 10 mg of the lyophilized prostaglandin synthetase was used in each estimation. Estimations of prostaglandin synthetase were also carried out on rabbits killed 48 h after intravenous injection of 10 mg/kg of aspirin.

Statistical analysis was performed on a Compucorp 344. Linear regression analysis was carried out by the method of least squares; *t* value for paired and unpaired data was determined by Student's *t* test.

Results

The acetyl-^{14}C part of the molecule from the [acetyl-^{14}C]-aspirin was bound in increasing amounts in a linear manner with increasing dosage up to a dose of 0.01 mmol/kg in both the renal medulla and renal cortex (Figure 1). The [carboxyl-^{14}C]-aspirin was not bound, therefore the salicylate portion of the molecule does not bind covalently to the protein *in vivo*. When

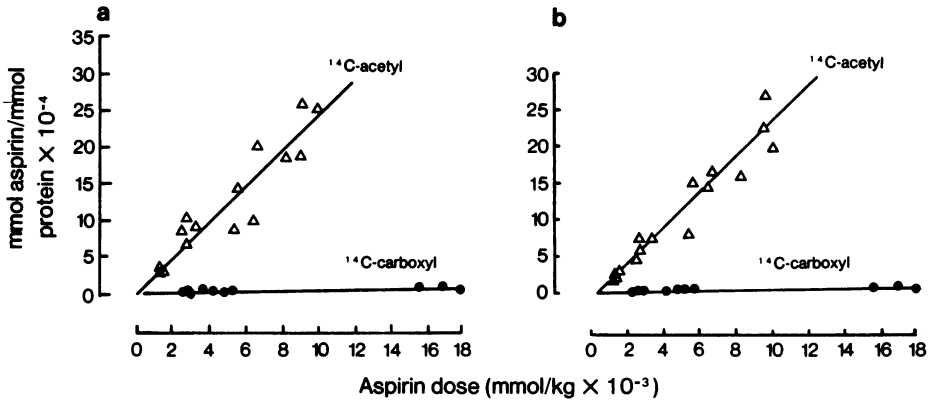

Figure 1 Acetyl-^{14}C residue of aspirin bound to mouse renal proteins as a function of low dose. (a) Cortex: $\bar{m}x = 4.8$, $\bar{m}y = 11.8$, $y = 2.4x + 0.3$, $r = 0.94$; (b) medulla: $\bar{m}x = 4.8$, $\bar{m}y = 10.8$, $y = 2.4x - 0.8$, $r = 0.95$.

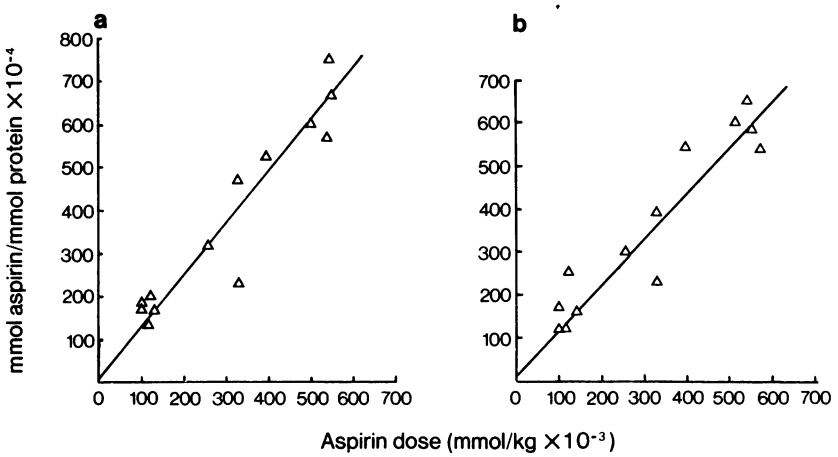

Figure 2 Binding of acetyl-^{14}C residues of aspirin to mouse renal proteins as a function high dose. (a) Cortex: $\bar{m}x = 132.3$, $\bar{m}y = 167.6$, $y = 1.2x + 9.2$, $r = 0.98$; (b) medulla: $\bar{m}x = 141.5$, $\bar{m}y = 163.7$ $y = 1.1x + 11.7$, $r = 0.98$.

the dosage of [acetyl-^{14}C]-aspirin was increased to 0.6 mmol/kg, this binding continued in a linear manner in both renal medulla and renal cortex (Figure 2). Similar results were obtained whether the homogenates were dialysed or washed.

There was an increase in the amount of acetyl-^{14}C residues bound with time after the dose was given up to 20 min. After this time, there was no significant difference in the amount bound up to 2 h. On a dose of 0.003 mmol/kg the maximum amount of acetyl-^{14}C residues bound at 20 min to the renal cortex was 2.46×10^{-3} mmol aspirin/mmol protein and to the renal medulla was 2.18×10^{-3} mmol aspirin/mmol protein (Figure 3). There was a gradual decrease in the amount of protein acetylated, until at 8 days there was 28% of the maximum bound in the medulla. The half life of the acetylated renal cortex was 122.5 h, and of the renal medulla was 129.5 h (Figure 4). Differential centrifugation experiments showed that acetyl-^{14}C residues were covalently bound to all of the four fractions obtained. There was, however, a significantly greater amount bound to the microsomal fractions (Table 1).

When both renal cortical and renal medullary protein acetylated by [acetyl-^{14}C]-aspirin was separated on a Sephadex G200 superfine column, two distinct

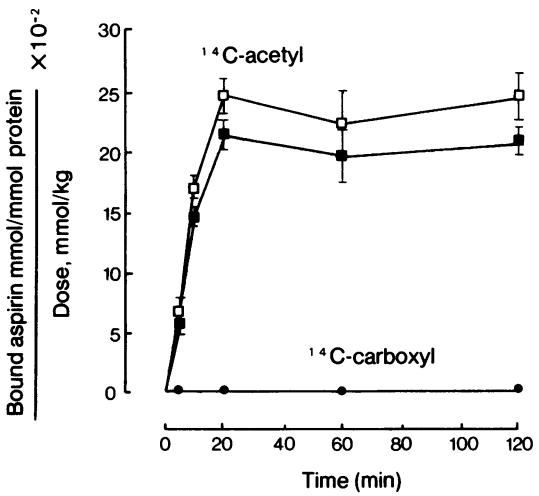

Figure 3 Acetyl-^{14}C residue of aspirin bound to mouse renal cortical and medullary proteins, normalised for dose and body weight, following a single dose as a function of time. (■) Medulla; (□) cortex. Acetylated protein expressed as mmol [acetyl-^{14}C]-aspirin bound per mmol protein/mmol per kg dose.

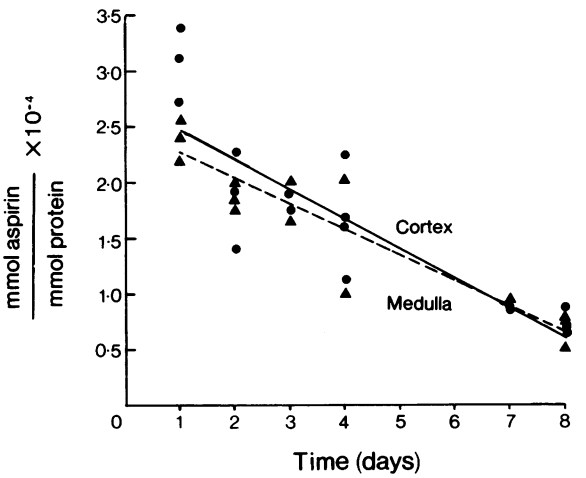

Figure 4 Persistence of binding of acetyl-^{14}C residue of aspirin to mouse renal proteins following a single dose with time. Unbroken line—cortex; broken line—medulla.

peaks of radioactivity were found. Corresponding peaks were found when the protein concentration of the samples was measured. When [acetyl-^{14}C]-aspirin alone was eluted, it gave a peak of activity in the same fractions as the second peak. It is unlikely however that the second peak obtained was due to free [acetyl-^{14}C]-aspirin as the radioactivity measured would be acetylated renal proteins and by the washing procedure no free aspirin would be in the solution applied to the column (Figure 5). The second peak contains small molecular weight proteins and peptides, some of which would be produced by the extraction procedure. Three distinct peaks of radioactivity were found when the polyacylamide gels were sliced and counted in both renal medulla and cortex. These peaks were also seen when washed homogenates were applied to the gels, although the counts were lower, consistent with the loss of protein in the washing procedure.

The prostaglandin synthetase activity as measured by inhibition of MDA formation in both renal medulla and cortex was significantly decreased after injection of aspirin in a dose of 0.056 mmol/kg when the rabbits were killed 15 min after injection. When the rabbits were killed 2 days after injection the activity of prostaglandin synthetase was still significantly decreased in the renal medulla, but although the activity was less in the renal cortex, the difference was not statistically significant (Table 2).

Discussion

Recent developments in studies of drug toxicity have led many investigators to examine the role of covalent binding of drug metabolites to tissue proteins and subsequent enzyme inactivation. It has been

Table 1 Differential centrifugation of mouse kidney homogenates demonstrating the binding of acetyl-^{14}C residues of aspirin to the different subcellular fractions.

Fraction	*Medulla*	*Cortex*
Microsomes	2.51 ± 0.49	2.87 ± 0.82
Mitochondria	1.42 ± 0.24	1.28 ± 0.24
Nuclei	1.77 ± 0.65	0.84 ± 0.22
Supernatant	1.05 ± 0.21	1.24 ± 0.17

Values ± s.e. of the mean are expressed as mmol aspirin/mol of protein.

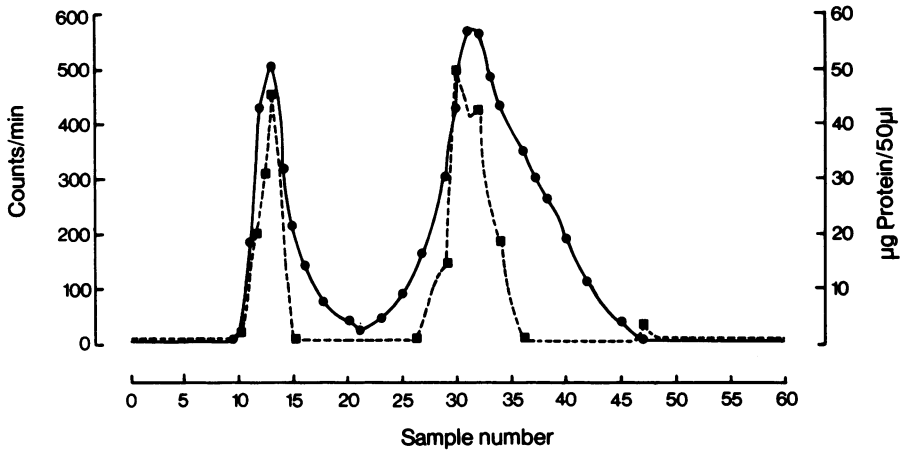

Figure 5 Sephadex G200 superfine column (30 cm × 1 cm) chromatography of acetylated renal medullary proteins from mouse kidney. (●) Disintegrations per min against time; (■) Protein concentration per sample in µg/50 µl.

demonstrated that the antipyretic analgesic, paracetamol, after metabolic 'activation' covalently combines with liver tissue proteins and this leads to liver necrosis (Mitchell & Jollow, 1975). The mechanism appears to be the binding of the drug metabolite to nucleophilic sites on enzymes, leading to their inactivation (Thorgersson, Sasame, Mitchell, Jollow & Potter, 1976). Other drug toxicity reactions have been examined and results indicate similar mechanisms.

The current experiments have demonstrated that renal cortical and medullary proteins are acetylated covalently by aspirin. Acetylation increased linearly with increasing dosage until animals exhibited signs of severe salicylism. The acetylation was rapid and the half life of the acetylated protein was long (130 h), suggesting that the protein remained acetylated until breakdown. This would result after continued high dosage in large accumulations of acetylated protein. Microsomal protein had the greatest concentration of acetylated protein, although all fractions contained some acetylated protein.

Prostaglandin biosynthesis involves the initial conversion of arachidonic acid to cyclic endoperoxide intermediates, designated prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) (Hamberg & Samuelsson, 1973; Hamberg, Svensson, Wakabayashi & Samuelsson, 1974). PGG_2 and PGH_2 are unstable and are metabolized enzymatically to form relatively stable prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Endoperoxide intermediates are broken down to form malondialdehyde (Hamberg & Samuelsson, 1973; Hamberg *et al.*, 1974). The microsomal enzyme that metabolizes arachidonic acid has been termed prostaglandin synthetase or cyclo-oxygenase.

It has been demonstrated previously that aspirin

Table 2 Formation of malondialdehyde (mu) from rabbit kidney

	Aspirin	*No aspirin*	*Time*	t
Cortex	0.06 ± 0.03	0.26 ± 0.06	15 min	4.55 $P < 0.01$
Medulla	0.13 ± 0.05	0.42 ± 0.04	15 min	3.63 $P < 0.05$
Cortex	0.37 ± 0.07	0.46 ± 0.13	2 days	1.53 NS
Medulla	0.21 ± 0.07	0.36 ± 0.05	2 days	4.13 $P < 0.01$

Values given are mean ± s.e. mean

is an irreversible inhibitor of prostaglandin synthetase, and that the inhibition is time- and concentration-dependent (Smith & Lands, 1971). Also arachidonic acid, the substrate for the reaction, will inhibit aspirin's effects at concentrations which correspond to the *Km* of the fatty acid for the enzyme (Rome & Lands 1975), and this suggests that aspirin acts by altering the enzyme's active site. These findings were confirmed in three tissues, sheep and bovine seminal vesicles and particulate platelet protein (Roth, Stanford & Majerus, 1975).

We have demonstrated that prostaglandin synthetase is inhibited in renal cortex and medulla after administration of aspirin intravenously in a dose of 10 mg/kg. This inhibition, in the case of the renal medulla, lasts at least 2 days after a single dose of aspirin. We have also demonstrated that aspirin is acetylated to renal proteins, particularly proteins in the microsomal fraction, the site of synthesis of prostaglandin in renal medullary cells. It is also likely that other enzymes are acetylated, and that aspirin could inhibit other essential enzymes or enzyme systems. Combination antipyretic analgesics induce the development of renal disease (Duggin, 1977) and this acetylation of proteins by aspirin may be important as a primary mechanism or more probably as an interaction with other drugs in the causation of this disease.

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