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RAPID REDUCTION OF PROSTAGLANDIN 15-HYDROXY DEHYDROGENASE ACTIVITY IN RAT TISSUES AFTER TREATMENT WITH PROTEIN SYNTHESIS INHIBITORS

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1 The capacity of rat kidneys to metabolize prostaglandins is rapidly lost when *de novo* protein synthesis is reduced by cycloheximide and puromycin. Enzyme activity declines to about half control values 45-75 min after a single dose of cycloheximide. Prostaglandin metabolism by the lung is also diminished.

2 These inhibitors apparently act by preventing the synthesis of new prostaglandin 15-hydroxy dehydrogenase enzyme.

3 The RNA synthesis inhibitor actinomycin D has no effect on metabolism.

4 It is concluded that prostaglandin dehydrogenase is a short-lived enzyme in the cell whose replacement depends upon *de novo* protein (but not RNA) synthesis. The implications of this finding are discussed.

Introduction

The initial step in the metabolic degradation of prostaglandins is oxidation of the C-15 alcohol (Änggård & Samuelsson, 1964, 1966; Hamberg & Samuelsson, 1971a). This reaction is catalyzed by prostaglandin 15-hydroxy-dehydrogenase (PGDH), an enzyme found in high concentrations in the lung and kidney (Änggård, Larsson & Samuelsson, 1971). This oxidation reaction can result in a substantial loss of biological activity (Änggård, 1966) and plays an important part in the inactivation of prostaglandins in man and animals (Green & Samuelsson, 1971; Hamberg & Samuelsson, 1971b, 1972).

Proteins are synthesized by all viable cells, and a state of dynamic equilibrium exists such that the continual degradation which occurs is balanced by *de novo* synthesis. The 'lifespan' of an enzyme, as determined by the rapidity of its synthesis and destruction, may be an important regulatory mechanism. One way of estimating this lifespan is to inhibit *de novo* protein or RNA synthesis using specific blocking agents, such as cycloheximide, puromycin or actinomycin D (see Reich, 1963; Vazquez, 1974).

Whilst investigating the effect of protein synthesis inhibitors on the ability of rat kidney homogenates to synthesize prostaglandins, we observed that the rate of prostaglandin metabolism apparently fell. We now present evidence that PGDH is a short-lived enzyme and that its continued activity depends on continual protein, but not RNA synthesis within the cell.

A preliminary account of this work has been presented to the Society (Blackwell, Flower, Parsons & Vane, 1975).

Methods

Measurement of prostaglandin metabolism

Rats (male, Wistar strain, weighing between 150-250 g) were killed by a blow on the head. The kidneys (or in one experiment, the lungs) were removed, immediately placed on ice and homogenized within 5 min in 4 vol of ice-cold 100 mM pH 7.5 Tris buffer. The resultant suspensions were then cetrifuged at 1,000 g_{av} to remove coarse cellular debris and the supernatant re-centrifuged for 1 h at 100,000 g_{av} in a Beckman Spinco ultra-centrifuge. The resultant pellet was discarded, and the supernatant, which contained PGDH, was used for assays of prostaglandin metabolism. The supernatant was stored on ice until required for use since enzyme activity was very labile; when kept at room temperature, about 50% activity was lost within 30 min of centrifugation. Reaction sets for the radioisotope

assay comprised the following components: supernatant, 1 ml; NAD^+, 280 pmol; [^{3}H]-prostaglandin E_2, 0.1 μCi; unlabelled prostaglandin E_2, 28 pmol. After the contents were thoroughly mixed the tubes containing the reaction mixture were incubated in a shaking waterbath at 37° C for varying time periods. The reaction was terminated by acidifying the mixture to pH 3 with 1 N HCl and the unreacted prostaglandin E_2 and its metabolites were extracted by mixing for 30 s with 1.5 ml of ethyl acetate. This procedure reproducibly extracts more than 95% of labelled products. After mixing, the phases were separated by centrifugation, 1 ml of the organic layer was removed and mixed with 5 μg each of unlabelled prostaglandin E_2 and 15-keto prostaglandin E_2. The organic solvents were removed in a vacuum dessicator and the dried residue redissolved in 50 μl of ethanol and spotted quantitatively onto a plastic-backed silica gel thin layer chromatography (t.l.c.) plate. After development in iso-octane, ethyl acetate, acetic acid, and water (5:11:2:10), the plates were dried and zones corresponding to authentic prostaglandin markers were visualized by brief exposure to iodine vapour. Those zones corresponding to prostaglandin E_2 and its 15-keto metabolite were cut out with scissors and after spontaneous evaporation of the iodine, the radioactivity in each zone was estimated by liquid scintillation counting using Beckman cocktail 'D' and a Beckman LS-150 scintillation counter; ct/min were converted to d/min using a quench correction curve and external standards.

In one experiment prostaglandin metabolism was quantitated biologically. Reaction sets in this instance comprised 2.8 nmol prostaglandin E_2 and 28 nmol NAD^+. After solvent extraction the prostaglandins were bioassayed on the rat fundic strip, treated with a mixture of antagonists to make the assay more specific (see Gilmore, Vane & Wyllie, 1966).

Estimation of protein synthesis

Rat kidneys were finely chopped with a scalpel blade and incubated in test tubes containing 1 ml of 100 mM pH 7.5 Tris buffer and 0.1 μCi of [^{14}C]-lysine. After 1 h, the slices were homogenized in the buffer using a glass homogenizer, and the proteins precipitated with 5 volumes of ice-cold 1 M perchloric acid. The precipitate was sedimented in a bench centrifuge, and the supernatant discarded. The precipitate was washed twice with 5 volumes of 0.5 M perchloric acid and 5 volumes ethanol/ether (1/1), and the final pellet solubilized with 'NCS' or concentrated sodium hydroxide. The radioactivity in aliquots of the digest was estimated by liquid scintillation counting after neutralization with acetic acid, and the protein content was estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

Estimation of RNA synthesis

The procedure was identical to that outlined in the previous section except that the incorporation of 0.1 μCi of [^{3}H]-uridine was measured. Radioactivity in the acid insoluble material was again estimated by liquid scintillation counting techniques.

Drugs

The following compounds were used: cycloheximide, puromycin dihydrochloride, actinomycin D and β-NAD^+, (Sigma); [5,6,8,11,12,14,15-^{3}H]-prostaglandin E_2 (batch 4, 160 Ci/mmol); [5-^{3}H]-uridine (batch 93, 29 Ci/mmol); [U-^{14}C]-(L)-lysine (batch 52, 348 mCi/mmol), (Radiochemical Centre, Amersham); plastic-backed silica gel (type 6060) t.l.c. plates (Eastman Kodak); unlabelled prostaglandin E_2 and $F_{2\alpha}$ (Cambrian Chemicals); and NCS (Amersham-Searle). The 15-keto derivative of prostaglandin E_2 was prepared by oxidation of the parent compound in chloroform by manganese dioxide. All other reagents were of 'Analar' grade, or the highest purity obtainable.

Results

Time course for inhibition of protein and RNA synthesis

In an initial series of experiments the time course for inhibition of protein (see Figure 1) and RNA synthesis was established. A single dose of cycloheximide (20 mg/kg i.p.) was sufficient to produce a progressive fall in protein synthesis which reached about 50% of control values 1-2 h after injection and fell to about 20% after 4 hours. Most rats were dead within 10 h and even at 2-4 h exhibited lassitude and diarrhoea. Puromycin was a much less effective inhibitor but hourly doses of 50 mg/kg (i.p.) produced a similar effect to that of cycloheximide, and the incidence of observable side-effects was reduced. The inhibition of protein synthesis by both drugs using the dose schedule above was quite reproducible but was checked for each experiment (see Tables1-3). PGDH activity was usually measured 2 h after treatment (i.e. when protein synthesis was less than 50% of control values).

The inhibitory effect of actinomycin D on RNA synthesis had a much longer latency. It was

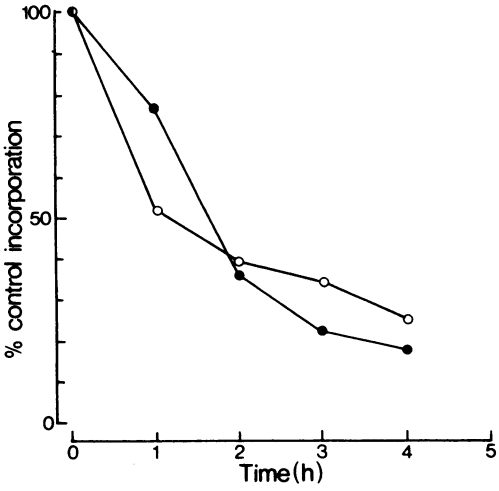

Figure 1 Time course of the effect of protein synthesis inhibitors puromycin (○) and cycloheximide (●) on *de novo* protein synthesis by rat kidney. For dosage see results section. The results are expressed as % control incorporation of [^{14}C]-lysine/mg protein. Each point is the mean of 2-4 determinations.

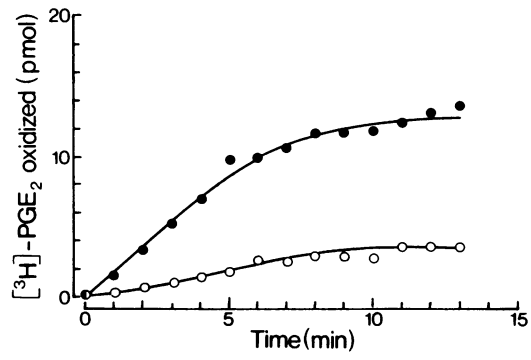

Figure 2 Oxidation of prostaglandin E_2 by high-speed supernatant of rat kidney homogenates of control rats (●) and of rats treated with cycloheximide 2 h before the experiment (○). Incubation conditions are described in the methods section. Each point is the mean of duplicate determinations.

necessary to dose rats (2.5 mg/kg i.p.) 12 h prior to the experiment in order to achieve a greater than 50% reduction of [^{3}H]-uridine incorporation. The effect of actinomycin D on protein synthesis at this time was very small (less than 5%) or undetectable. A high mortality rate was observed in rats treated for longer than 12 h with actinomycin D.

In these initial experiments the kidney protein content of control rats was compared with those receiving protein or RNA synthesis inhibitors but no differences were detected.

Kinetics of [^{3}H]-prostaglandin E_2 oxidation

Under the reaction conditions described in the methods section the oxidation of [^{3}H]-prostaglandin E_2 was linear for 4 to 5 min during which one third to one half of the labelled substrate was consumed; thereafter the rate of oxidation declined (Figure 2). Reaction mixtures were, therefore, routinely incubated for 4 minutes. At this time the labelled substrate was transformed into a single less polar product (as judged by t.l.c.) which had an R_F value identical to that of authentic 15-keto prostaglandin E_2. The formation of this product was NAD$^+$-dependent. The reduction products obtained after treatment of the isolated metabolite with sodium borohydride (principally $F_{2\alpha}\beta$) had chromatographic mobilities identical to those products obtained after reduction of the authentic compound. It is virtually certain, therefore, that this product was the 15-keto metabolite of prostaglandin E_2.

Effect of cycloheximide on [^{3}H]-prostaglandin E_2 oxidation

Figure 2 shows the rates of [^{3}H]-prostaglandin E_2 oxidation in the high-speed supernatant of kidneys from control rats, and kidneys from rats pretreated with cycloheximide. There was a dramatic reduction in oxidation rate from 1.75 pmol [^{3}H]-prostaglandin E_2/min for control rats, to 0.31 pmol [^{3}H]-prostaglandin E_2/min for rats treated with cycloheximide.

In two sets of experiments the loss of oxidizing activity with time was calculated. Seven rats were injected with cycloheximide and killed at 20 min intervals. The kidneys were removed, processed and the enzyme activity measured. The ability to oxidize [^{3}H]-prostaglandin E_2 was reduced to half of the control levels 45-75 min after a single dose of the drug.

Direct action of cycloheximide on PGDH

The reduction in PGDH activity by cycloheximide could have been due to a direct antagonism of the drug or a metabolite on the enzyme rather than an effect on protein synthesis. To exclude the former possibility, control experiments were performed with high-speed supernatants of kidney homogenates from untreated rats with the addition of cycloheximide directly to the reaction

mixture. In concentrations as high as 2.5 mg/ml cycloheximide caused less than 5% inhibition of PGDH activity. Thus, the effects of cycloheximide *in vivo* (at a maximum dose of 20 mg/kg) were not due to a direct inhibition of the enzyme.

Effect of puromycin on PGDH activity

To exclude the possibility that the effect of cycloheximide was due to formation of a metabolite, puromycin, a protein synthesis inhibitor of a different chemical structure and mode of action (Vazquez, 1974) was tested. Table 1 shows that this drug had qualitatively the same effect as cycloheximide; there was a reduction in protein synthesis (69% inhibition) as well as in oxidizing capacity (56% inhibition).

Effect of cycloheximide on PGDH in the lung

To discover whether the action of cycloheximide was restricted to the kidney we performed one experiment in which the PGDH activity in rat lung was measured after cycloheximide. Table 2 shows that when lung protein synthesis was inhibited by 64%, PGDH activity was reduced by 67%.

Effect of actinomycin D on kidney PGDH activity

The effect of actinomycin D, an inhibitor of RNA (but not protein) synthesis, is shown in Table 3. Using the dose schedule described above, RNA synthesis (as quantitated by [^{3}H]-uridine incorporation) was inhibited by 53%. Protein synthesis, however, fell by only 4% and there was no reduction in PGDH activity.

Controls for isotope dilution artifacts

An inherent flaw in all isotope assays using crude enzyme preparations is that the extent to which the isotope is metabolized may be influenced by the pre-existing pool size of unlabelled material in the enzyme preparation. In the present experiments for example, the amount of [^{3}H]-prostaglandin E_2 metabolism could be influenced by the amount of unlabelled prostaglandin E_2 (or other prostaglandins) present

Table 1. Effect of puromycin on rat kidney protein synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE_2 oxidized (pmol) (mean ± s.e. mean)	[^{14}C]-lysine incorporated (pmol)
Control	14.08 (± 0.12) (n = 4)	33.71 (n = 2)
Plus puromycin	6.18 (± 0.14) (n = 5)	10.23 (n = 2)

Table 2. Effect of cycloheximide on rat lung protein synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE_2 oxidized (pmol)	[^{14}C]-lysine incorporated (pmol)
Control	8.79 (n = 2)	42.40 (n = 2)
Plus cycloheximide	3.26 (n = 2)	15.39 (n = 2)

Table 3. Effect of actinomycin D on rat kidney protein and RNA synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE_2 oxidized (pmol) (mean ± s.e. mean)	[^{3}H]-uridine incorporated (fmol)	[^{14}C]-lysine incorporated (pmol)
Control	15.96 (± 0.19) (n = 5)	224.29 (n = 2)	30.44 (n = 2)
Plus actinomycin	16.12 (± 0.18) (n = 5)	104.73 (n = 2)	28.79 (n = 2)

in the high-speed kidney supernatant. Rat kidneys are rich in prostaglandin synthetase and it could be argued that if cycloheximide were stimulating renal prostaglandin synthesis the amount of [^{3}H]-prostaglandin E_2 metabolized might be reduced thus giving apparently similar results to the findings reported here.

We have therefore checked the results of the cycloheximide experiments using bioassay techniques to measure the total prostaglandin content of the reaction mixture. Similar results were obtained to those obtained by the isotope method; the metabolism in the control samples was 457.1 pmol ($n = 3$) and in samples from cycloheximide-treated rats it was 174.4 pmol ($n = 4$).

Discussion

The addition of [^{3}H]-prostaglandin E_2 to high-speed supernatants of rat kidney resulted in rapid metabolism to the 15-keto derivative. This metabolism was greatly depressed when rats were pretreated with the protein synthesis inhibitor, cycloheximide. This effect was not due to an isotope dilution artifact nor to a direct inhibition of the enzyme by cycloheximide. The fact that puromycin was also effective despite its different chemical structure and mode of action (Vazquez, 1974) makes it unlikely that the effect was due to formation of an inhibitory metabolite. The fact that we have obtained the same effect on kidney slices *in vitro* (Blackwell *et al.*, 1975), makes it even more unlikely that cycloheximide is metabolized to an active inhibitor of PGDH. When measured under constant conditions a reaction rate is proportional to the amount of enzyme present, and we suggest, therefore, that the results are due to a decreased synthesis of new PGDH enzyme. This effect is probably a general one, for lung as well as kidney PGDH activity was depressed by pretreatment with cycloheximide. The inability of actinomycin D to affect PGDH activity (despite its powerful inhibitory action on RNA synthesis) suggests that the RNA template for PGDH synthesis is relatively stable.

The time course studies indicate that PGDH is a very short-lived enzyme with a half-life of approximately 1 h after cycloheximide injection. The life of proteins within cells may vary from less than 1 h to 20 days or more (Schapira, Kruh, Dreyfus & Schapira, 1960; Kenney, 1962; Nicolette & Mueller, 1966; Gorski & Morgan, 1967). The short life span of PGDH may be due to an inherent instability of the protein molecule. Certainly, the enzyme deteriorates rapidly after purification and even preparations stored at 4°C have a life of only 60 to 80 days (Marazzi & Andersen, 1974). Our own observations support the concept that PGDH is unstable, for when the crude enzyme was kept at room temperature (about 21°C) it rapidly lost its capacity to oxidize [^{3}H]-prostaglandin E_2. Metabolism fell to about half the control values in 30 min and was virtually absent after 1 hour. The enzyme is thought to contain sulphydryl groups (Marazzi & Andersen, 1974) and there is a possibility, therefore, that the loss of activity occurs as a result of oxidative damage. Recently, Parkes & Eling (1975) have shown that exposure of guinea-pigs to 100% oxygen for 48 h results in a considerable loss of lung PGDH activity. Another possibility is that PGDH is degraded by cytoplasmic proteolytic enzymes for Katunuma, Kito & Kominami (1971) have described a proteolytic enzyme which specifically inactivates NAD^+-dependent dehydrogenases.

What are the physiological implications of this work? PDGH belongs to that group of enzymes (which includes phosphodiesterase, acetylcholinesterase, monoamine-oxidase and kininase) responsible for the inactivation of endogenous substances of high biological activity (Marazzi & Andersen, 1974). Thus the mechanism by which its catalytic activity is controlled is clearly of interest. One possibility is that the synthesis of PGDH is controlled by blood concentrations of steroid or other hormones (Bedwani & Marley, 1975).

Another implication is that any agent which decreases cellular protein synthesis would decrease PGDH activity and thus result in elevated prostaglandin levels. Many antibiotics as well as some other compounds such as diphtheria toxin block protein synthesis (Vazquez, 1974). In this connection it is interesting to note that Nakano & Prancan (1973) have demonstrated that PGDH activity in the lung and kidney was impaired in rats after endotoxin administration.

A further implication of the work described here is that irreversible inhibitors of PGDH are likely to have short duration of action.

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