J. Pharm. Pharmacol. 1983, 35: 262–264
Received October 25, 1982

0022–3573/83/040262–03 $02.50/0

The influence of rhein on the biosynthesis of prostaglandin-like substances in-vitro

S. FRANCHI-MICHELI*, L. LAVACCHI, C. A. FRIEDMANN, L. ZILLETTI, *Istituto Interfacoltà di Farmacologia e Tossicologia dell'Università degli Studi di Firenze, Viale G. B. Morgagni 65, Florence, Italy*

It has recently been demonstrated that diacetylrhein (DAR; 1,8-diacetoxy-9,10-dioxo-dihydroanthracene-3-carboxylic acid) attenuates the symptoms of osteoarthritis in man (Kay et al 1980; Neuman 1980) and of various types of inflammatory arthritis (Neuman 1980).

Diacetylrhein is well absorbed from the gastrointestinal tract and is deacetylated to rhein, which is the principal urinary metabolite in animals and in man (Friedmann, personal communication). In addition to being an antipyretic and analgesic, DAR blocks the inflammation induced by carrageenan, beer yeast, Freund's adjuvant and cotton pellet granuloma. It also protects rabbit ear and knee cartilage from retinoic acid-induced degradation. It is suggested that protection from degradation of proteoglycans occurs as a result of inhibition of the liberation and/or of the activity of lysosomal enzymes. This view has also been suggested by the capacity of the molecule and of its deacetylated metabolite to inhibit certain proteinase enzymes in-vitro (trypsin, pepsin, carboxypeptidase A, elastase) (Raimondi et al 1982).

Other findings have indicated that DAR does not inhibit the synthesis of prostaglandins in-vivo. In fact it only possesses minimal effects in blocking the diarrhoea caused by castor oil using the methodology of Awouters et al (1978). In these conditions the anti-inflammatory drugs, which are active in inhibiting prostaglandin formation, also cause a delay in the production of diarrhoea.

On the contrary, when administered to rats, DAR and its metabolite, increase the prostaglandin content in the carrageenan-induced inflammatory exudate (Panarelli et al 1980). Bearing in mind that diacetylrhein is metabolized to rhein, which is a diphenolketone able to chelate divalent metallic ions and which possesses the biological property of specifically inhibiting NAD-dependent dehydrogenase (Kean 1968, 1970), we examined the possibility that rhein might also interfere with prostaglandin synthesis in-vitro. In this way we also hoped to avoid variations in the concentration of the deacetylated metabolite, which might have occurred when diacetylrhein itself was used.

Materials and methods

The supernatant layer of guinea-pig homogenated lung containing the microsomal fraction as enzyme source, was incubated with arachidonic acid according to Vane (1971) and subsequently according to Svensson et al (1975). Prostaglandin-like activity was determined as PGE_2 on strips of rat stomach fundus (Vane 1957) and as $PGF_{2\alpha}$ on rat colon (Regoli & Vane 1964) in the presence of mepyramine dimaleate, hyoscine hydrobromide, methysergide and indomethacin. Tests were carried out in duplicate and biological contents were determined at three dose levels.

The substances to be tested for their effects on prostaglandin synthesis (rhein, imidazole, indomethacin) were added in different concentrations to the incubation medium. In addition to being treated with the substances under investigation, parallel samples of supernatant from homogenized guinea-pig lung together with arachidonic acid were incubated to assess basal rates of biosynthesis. As rhein, at the concentration used, did not interfere with the amounts of prostaglandins found by biological assay, compared with the quantities found by thin layer chromatographic determination, its effects on prostaglandin formation were then directly determined on the medium after incubation.

In the experiments with imidazole, assays were carried out after chromatographic separation, because it had been observed in preliminary experiments, that this substance could cause variations in response of the tissue strips used for the biological assay.

The reaction between the enzyme suspension and arachidonic acid was effected by incubation as described above. After the required time (5 min) this was stopped by acidification of the mixture to pH 3 with 1 M HCl, and the prostaglandins so formed extracted by means of ethylacetate according to the method of Piper & Vane (1969). After agitation and separation by centrifuging, the organic phase was evaporated to dryness under reduced pressure. The residue was suspended in a small volume of ethanol (0·2 ml) which was then deposited on a silica gel plate.

Chromatographic separation was carried out by the method of Green & Samuelsson (1964) using system A II as running solvent. One part of the chromatogram was developed with the phosphomolybdate reagent, while from the non-developed part of the chromatogram, the fractions with R_F values corresponding to the spots obtained with PGE_2 and $PGF_{2\alpha}$ were collected, suspended in 2 ml of Krebs solution, centrifuged, and the amounts of prostaglandin-like substances in these determined biologically.

The results so obtained are reported as single values on as mean values of prostaglandins produced and expressed as ng ± s.e.m.; Student's *t*-test for paired results was calculated.

* Correspondence.

PGE_2 and $PGF_{2\alpha}$ was kindly supplied by Upjohn, rhein sodium by Proter (Milan), indomethacin (Sigma), imidazole (Merck, Darmstadt), hyoscine hydrobromide (BDH), mepyramine dimaleate (Gianni), methysergide (Sandoz).

Results

Prostaglandin synthesis in-vitro which is catalysed by prostaglandin synthetase present in guinea-pig lung microsomes, is influenced by rhein. Fig. 1 demonstrates that with our experimental conditions the phenomenon relates solely to PGF determined as $PGF_{2\alpha}$. The synthesis of this prostaglandin is greatly increased in the presence of rhein, $3{\cdot}2 \times 10^{-5}$ and $3{\cdot}2 \times 10^{-4}$ M, with a maximum increase of 133% compared with control, while the synthesis of PGE, determined as PGE_2, remains more or less unchanged. The action of rhein

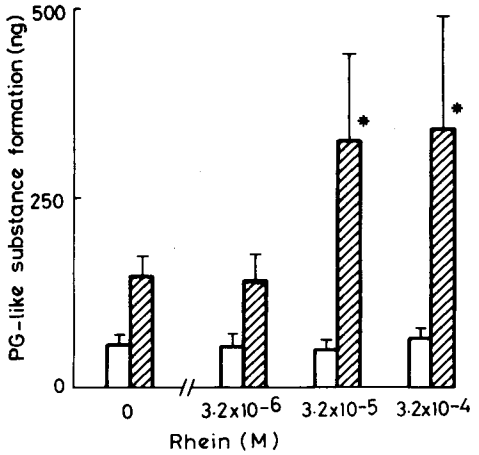

FIG. 1. The influence of rhein on the synthesis of prostaglandin-like substances by crude enzyme preparations of guinea-pig lung from exogenous arachidonic acid (20 μg ml^{-1}). Open columns PGL-substances bioassayed against PGE_2; hatched columns PGL-substances bioassayed against $PGF_{2\alpha}$. Mean values ± s.e.m. of duplicate samples; n = 4–8. $^*P < 0{\cdot}05$ for Student's *t*-test for paired data.

Table 1. Effect of imidazole and indomethacin upon production of prostaglandin-like substances by crude enzyme preparations of guinea-pig lung from exogenous arachidonic acid.

	PGE_2 ng	$PGF_{2\alpha}$ ng
Controls	176 ± 20	173 ± 15
Imidazole 10^{-3} M	332 ± 39†	322 ± 24**
Controls	93 ± 28	144 ± 16
Indomethacin 7×10^{-7} M	83 ± 28	115 ± 13*
Indomethacin $1{\cdot}4 \times 10^{-7}$ M	50 ± 18**	75 ± 10*
Indomethacin $2{\cdot}8 \times 10^{-7}$ M	14 ± 10*	30 ± 4‡

Mean ± s.e.m. (Imidazole n = 4; Indomethacin n 4–8) Student's *t*-test for paired data $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}02$; $\dagger P < 0{\cdot}01$; $\ddagger P < 0.001$.

Prostaglandin-substance production from arachidonic acid (20 μg) was standardized against PGE_2 and $PGF_{2\alpha}$.

appears to be different from that of imidazole. In Table 1 it can be seen that imidazole notably increases the synthesis of both PGE_2 and $PGF_{2\alpha}$ with increments of 118 and 127% respectively. The synthesis of both prostaglandins could be inhibited in a dose-dependent way by indomethacin (Vane 1971).

Discussion

Rhein accelerates the formation of PGF in-vitro. This increase does not appear to be caused by autoxidation of PGH_2 because such a mechanism would not have given rise to selectivity in the formation of PGF, but rather to a global increase of prostaglandins.

The possibility that anti-inflammatory molecules can facilitate prostaglandin synthesis has been known since 1977 when it was demonstrated that imidazole was able to inhibit selectively the synthesis of thromboxane, and thus, to direct the metabolism of arachidonic acid towards the synthesis of prostaglandins (Needleman et al 1977). Anti-inflammatory molecules (MK-427, phenol) which are able to block oxygen free radicals (superoxide and oxhydryl), increase prostaglandin synthesis by the prostaglandin synthetase derived from sheep seminal vesicles while they diminish the accumulation of PGH_2 endoperoxide (Kuehl et al 1977).

Recently it has been demonstrated that antioxidants such as propylgallate and ascorbic acid significantly increase the formation of 6-oxo-$PGF_{1\alpha}$ from arachidonic acid by means of the enzyme prostaglandin synthetase derived from sheep seminal vehicles (Beetens et al 1981).

The action of free radical scavengers or of antioxidants may be partly interpreted by their neutralization of the oxidizing species which are formed during peroxidation of arachidonic acid. Thus alteration of cyclo-oxygenase can be prevented and production of prostaglandins can pursue (Egan et al 1979).

The chemical structure of rhein suggests that it can act as a blocker of free radicals in a biological system and thus increase the synthesis of prostaglandins in the same way as the antioxidants.

However, the fact that it can inhibit NAD-dependent dehydrogenase suggests that the molecule could affect other enzymes involved in the metabolism of arachidonic acid.

The fact that rhein facilitates the formation in-vitro of prostaglandin F accords with the in-vivo effects of its diacetyl derivative, so that the hypothesis may therefore be advanced that DAR acts via its deacetylated metabolite, rhein.

The authors are grateful to Mrs Marigrazia D'Asta for her technical assistance.

REFERENCES

Awouters, F., Niemegeers, C. G. E., Leaerts, F. M., Janssen, P. A. J. (1978) J. Pharm. Pharmacol. 30: 41–45

Beetens, J. R., Clayes, M., Herman, A. G. (1981) Biochem. Pharmacol. 30: 2811–2815

Egan, R. W., Gale, P. R., Kuehl, F. A. (1979) J. Biol. Chem. 254: 3295–3302

Green, K., Samuelsson, B. (1964) J. Lipid Res. 5: 117–120

Kay, A. G. L., Griffiths, L. G., Volans, G. N., Grahame, R. (1980) Curr. Med. Res. Opin. 8: 548–551

Kean, F. A. (1968) Arch. Biochem. Biophys. 127: 528–533

Kean, F. A. (1970) Biochem. Pharmacol. 19: 2201–2210

Kuehl, F. A., Egan, R. W., Humes, J. L., Beveridge, G. C., Van Arman, G. C. (1977) Nature (London) 265: 170–173

Needleman, P., Raz, A., Ferrendelli, J. A., Minkes, M. (1977) Proc. Nat. Acad. Sci. U.S.A. 74: 1716–1720

Neuman, M. (1980) Drug Exp. Clin. Res. 6: 53–64

Panarelli, P., Berti, M., Gatti, M. T., Mosconi, P. (1980) Il Farmaco Ed. Sci. 35: 836–842

Piper, P. J., Vane, J. R. (1969) in: 'Prostaglandins, peptides and amines', Academic Press, London, 15–19

Raimondi, L. Banchelli, Soldaini, G., Buffoni, F., Ignest, G., Mussacesi, L., Amaducci, L., Friedmann, C. A. (1982) Pharmacol. Res. Commun. 14: 103–112

Regoli, D., Vane, J. R. (1964) Br. J. Pharmacol. 23: 351–359

Svensson, J., Hamberg, N., Samuelsson, B. (1975) Acta Physiol. Scand. 94: 222–228

Vane, J. R. (1957) Br. J. Pharmacol. 12: 344–349

Vane, J. R. (1971) Nature New Biol. 231: 232–235

J. Pharm. Pharmacol. 1983, 35: 264–265
Communicated September 27, 1982

0022–3572/83/040264–02 $02.50/0

In-vivo catecholamine formation from phenylephrine

JAN R. CROWLEY, CLYDE M. WILLIAMS*, MELVIN J. FREGLY, *Veterans Administration Hospital and Departments of Radiology and Physiology, University of Florida College of Medicine, Gainesville, Florida, 32610*

Exogenous phenylephrine (*m*-synephrine) is known to be metabolized by three major metabolic pathways: (1) conjugation as a sulphate (Bruce & Pitts 1968), (2) reduction to *m*-hydroxyphenylglycol (Rawlow et al 1980) and (3) oxidation to *m*-hydroxymandelic acid (Crowley et al 1981; Hengstmann & Goronzy 1982). Since phenylephrine can be converted to adrenaline by a non-specific hydroxylation enzyme system in liver (Axelrod 1963) we undertook to determine whether this alternate metabolic pathway occurred in-vivo and if so, to what extent.

Method

A 24 h control urine specimen was collected from 11 male Sprague-Dawley rats. Six of these were injected intraperitoneally with 300 μg of (±)-*N*-trideutero-methyl-*m*-synephrine HCl synthesized as described by Midgley et al (1980). The other 5 rats were injected with 0·9% NaCl (saline) solution. Twenty-four hour urine samples were converted to pH 1 with conc. HCl and heated at 95 °C for 1 h. After neutralization to pH 6 with 2 M NaOH, the mixture was passed through a strong cation exchange resin (AG 50W-X2, 100–120 mesh Bio-Rad). After washing the resin with water, the amines were eluted with 10 ml of 1 M NH_4OH in 65% ethanol. This eluate was then reduced to dryness by rotary evaporation and reconstituted in a small volume (1 ml) of 0·2 M ammonium acetate (pH 6) and placed on a weak cation exchange resin (Bio-Rex 70 200–400 mesh, 2 g). This column was washed with the ammonium acetate buffer and the 25–100 ml fraction containing phenylephrine and metanephrine was then passed through another column containing the cationic exchange resin (3 g) to remove the ammonium acetate buffer and the amines were eluted with 10 ml of 1 M NH_4OH in 65% ethanol. The amine fraction was then reduced to 100 μl by rotary evaporation, transferred to a 2 ml disposable vial and blown to dryness under N_2. The dried residue was treated with pentafluoropropionic acid anhydride (PFPA) for 15 min at 60 °C. The excess PFPA was evaporated under N_2 and the residue taken up in 25 μl of ethyl acetate. Of this, a 1 μl aliquot was injected into a g.c.-m.s. (Hewlett-Packard 5992A using a silanized glass column (1·8 m × 2 mm i.d.) packed with 5% OV-101 on Chromosorb-GHP 100/120 mesh (Supelco). The g.c. was operated isothermally at 220 °C using He as the carrier gas.

Identification of the PFP derivative of metanephrine was made by comparing the ratio of the base peak (*m/z* 190) to the molecular ion (*m/z* 635). The ratio of *m/z* 190/*m/z* 635 was 15 for authentic ($[^2H_0]$metanephrine standard (Regis Chemical Co.) and 15 for the biological unknown at the same retention time (4·4 min) in rat urine. After administration of $[^2H_3]$phenylephrine the same rat demonstrated peaks at *m/z* 193 and *m/z* 638 with the same ratio at the same retention time. The amount of $[^2H_3]$metanephrine is three times that of the endogenous $[^2H_0]$metanephrine found in the control urine specimens (Table 1). This provides unequivocal evidence for the presence in-vivo of the metabolic pathway phenylephrine → adrenaline → metanephrine after the intraperitoneal administration of phenylephrine.

Quantitative mass spectrometry may be carried out using deuterated analogues or chemical homologues. Since we could not use deuterated metanephrine as an internal standard, it was necessary to use a chemical homologue. For this purpose we chose $[^2H_0]$phenylephrine, which we had previously demonstrated does not occur naturally in rat urine in amounts > 1 ng mg^{-1} creatinine. The PFP derivative of phenylephrine has a retention time of 2·7 min and has a characteristic molecular ion *m/z* 605 and a base peak, *m/z* 190. After establishing that rats excreted no detectable phenyleph-

* Correspondence.