Novel prostaglandin dehydrogenase in rat skin

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Present evidence suggests that skin is an important organ of prostaglandin metabolism. To clarify its role, the basic kinetics of 15-hydroxyprostaglandin dehydrogenase (PGDH) from rat skin were investigated with either NAD^+ or NADP^+ as co-substrate. Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) and prostaglandin E_2 (PGE_2) were used as substrates and preliminary studies were made of the inhibitory effects of the reduced co-substrates NADH and NADPH. A radiochemical assay was used in which $[^3\text{H}]\text{PGF}_{2\alpha}$ or $[^{14}\text{C}]\text{PGE}_2$ were incubated with high-speed supernatant of rat skin homogenates. The substrate and products were then extracted by solvent partition, separated by t.l.c. and quantified by liquid-scintillation counting. At linear reaction rates and at an NAD^+ concentration of 10 mM the mean apparent K_m for $\text{PGF}_{2\alpha}$ was $24\text{ }\mu\text{M}$ with a mean apparent V_{max} of 9.8 nmol/s per litre of reaction mixture. For PGE_2 the mean apparent K_m was $8\text{ }\mu\text{M}$, with a mean apparent V_{max} of 2.7 nmol/s per litre of reaction mixture. With NADP^+ as a co-substrate at a concentration of 5 mM a mean apparent K_m of $23\text{ }\mu\text{M}$ was obtained for $\text{PGF}_{2\alpha}$ with a mean apparent V_{max} of 5.2 nmol/s per litre. For PGE_2 values of $7.5\text{ }\mu\text{M}$ and 3.0 nmol/s per litre were obtained respectively. These results show that skin contains NAD^+ - and NADP^+ -dependent PGDH. An important finding was that the NADP^+ -linked enzyme gave K_m values for PGE_2 that were considerably lower than those reported for NADP^+ -linked PGDH from other tissues. Furthermore, preliminary inhibition studies with the NAD^+ -linked PGDH system indicate that this enzyme is not only inhibited by NADH, but also by NADPH, a property not previously reported for NAD^+ -linked PGDH derived from other tissues.

The first step in the enzymic degradation of $\text{PGF}_{2\alpha}$ and PGE_2 is catalysed by PGDH. The mode of action is by oxidation of the 15(*S*)-hydroxy group, converting the substrate prostaglandin into its biologically less active 15-oxo-metabolite (Anggard & Larsson, 1971; Anggard *et al.*, 1971; Hamberg & Samuelsson, 1971). PGDH has been isolated from several tissues (Hansen, 1976) and various 'types' have been identified, each type possessing differing specificities for the nicotinamide nucleotide co-substrates NAD^+ and NADP^+ . Type-I PGDH uses NAD^+ but not NADP^+ as a co-substrate (Anggard & Samuelsson, 1966), whereas the type-II enzyme uses NADP^+ as a co-substrate (Kaplan *et al.*, 1975; Lee & Levine, 1975). Both these types are reported to be inhibited by their respective reduced co-substrates (Lee & Levine, 1975; Ruckrich *et al.*,

1975). These enzymes also show differing specificities for their prostaglandin substrates. A major difference between the two enzymes is that PGE_2 has been found to be a poor substrate for the type-II enzymes that have been investigated, as reflected by the high K_m values reported (Lee & Levine, 1975; Westbrook *et al.*, 1977; Jarabak & Fried, 1979). Previous studies of NAD^+ -linked PGDH in rat skin, with $\text{PGF}_{2\alpha}$ as substrate, suggested that skin may be an important organ of prostaglandin catabolism (Camp & Greaves, 1980). These studies have now been extended by determining the basic kinetics of PGDH in rat skin, with both $\text{PGF}_{2\alpha}$ and PGE_2 as substrates, and NAD^+ and NADP^+ as co-substrates.

Experimental

Materials

The materials used have been described previously (Camp & Greaves, 1980) with the exception of the following. $[1\text{-}^{14}\text{C}]\text{PGE}_2$, with a specific

Abbreviations used: PGDH, 15-hydroxyprostaglandin dehydrogenase (EC 1.1.1.141), $\text{PGF}_{2\alpha}$, prostaglandin $\text{F}_{2\alpha}$ (9 α ,11 α ,15 α -trihydroxyprosta-5-*cis*,13-*trans*-dienoic acid); PGE_2 , prostaglandin E_2 (11 α ,15 α -dihydroxy-9-oxoprosta-5-*cis*,13-*trans*-dienoic acid).

radioactivity of 14.06 Ci/mol, was from Amersham International, Amersham, Bucks., U.K. All prostaglandins were stored in methanol at -20°C . NADP⁺ (disodium salt, 98%) and NADH (disodium salt, grade 1) were from Boehringer, Lewes, East Sussex, U.K. NADPH (tetrasodium salt) was from Sigma, Poole, Dorset, U.K. Silica gel G t.l.c. plates were supplied by Anachem, Luton, Beds., U.K., and were of 0.25 mm thickness. 2,5-Diphenyl-oxazole was from Koch-Light, Colnbrook, Bucks., U.K., and was of Puriss quality.

Methods

Preparation of subcellular fractions. The 120000 g supernatant of rat skin homogenate was freshly prepared as described previously (Camp & Greaves, 1980) and was used immediately.

Reaction conditions. Exact quantities of either [9β - ^3H]PGF_{2 α} or [1 - ^{14}C]PGE₂ in methanol were added to test tubes and the methanol was evaporated in a vacuum desiccator. To each tube was added saturating concentrations of NAD⁺ (10 mM) or NADP⁺ (5 mM) dissolved in 60 μl of 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.06% (v/v) 2-mercaptoethanol. The reaction was initiated by the addition of 120 μl of crude enzyme extract (120000 g supernatant) and the tubes were incubated at 37°C in a shaking water bath. Incubations were continued for 15 min at 37°C , the reaction rates being linear for this time under the conditions used. The only exception to this was when PGF_{2 α} and NADP⁺ were used as substrate and co-substrate respectively, when the reaction rates were linear for 6 min only. In these experiments an incubation time of 6 min was thus used. The reaction was then terminated by the addition of 0.5 M-HCl to a final pH of approx. 3.5. For the determination of blank rates, acidification was carried out before the addition of 120000 g supernatant. In some experiments [11β - ^3H]PGF_{2 α} with a specific radioactivity of 4.27 Ci/mol was used as described by Camp & Greaves (1980). In the product inhibition experiments, which were necessarily performed under subsaturating conditions with resultant low reaction rates, the incubation volume was doubled and the concentrations of all reagents were adjusted accordingly. By increasing the amount of radioactivity, greater accuracy of scintillation counting was achieved.

Because the report of Wright & Corder (1979) suggested that 2-mercaptoethanol inhibited PGDH activity, the effects of this compound in 6 min incubations containing both low (2.45 μM) and high (78.4 μM) concentrations of PGF_{2 α} and 5 mM-NADP⁺ were determined. Incubations were carried out with 120000 g supernatant prepared with or without the addition of 0.06% (v/v) 2-mercaptoethanol to the homogenization buffer.

During experiments to investigate summation of NAD⁺- and NADP⁺-linked activity, 120000 g supernatant was incubated for 6 min with 100 μM -PGF_{2 α} and with 10 mM-NAD⁺ or 5 mM-NADP⁺ separately, and with both 10 mM-NAD⁺ and 5 mM-NADP⁺ combined. Initial inhibition studies were performed by using high concentrations of PGF_{2 α} (117 μM) as substrate and subsaturating concentrations of co-substrates (0.5 mM-NAD⁺ or 1.0 mM-NADP⁺). NADH and NADPH were used as product inhibitors at the concentrations indicated in Fig. 4. An incubation time of 6 min gave linear reaction rates. In subsequent experiments to determine the type of inhibition a range of concentrations of NAD⁺ (0.25–1.0 mM) and of both NADH (0.25 mM and 0.5 mM) and NADPH (0.2 mM and 5.0 mM) were used. Substrate concentration (PGF_{2 α}) was 50 μM .

Extraction of substrates and products. Extraction was always performed immediately the reaction had been stopped. Saturating amounts of NaCl were added to the acidified samples to lessen protein emulsification and simplify extraction. Ethyl acetate (0.8 ml) was added to each sample, which was then vortex-mixed for 10 s and centrifuged at full speed for 2 min in a bench centrifuge. The ethyl acetate layer was then removed and the process repeated with a further 0.8 ml of ethyl acetate. The two organic phases were pooled and taken to dryness under a stream of N₂ at 40°C . The dried extracts were vacuum-desiccated for 15–20 min, after which they were immediately subjected to t.l.c.

T.l.c. Samples were dissolved in 40 μl of methanol and were applied to t.l.c. plates. Two t.l.c. systems to separate substrate and products were used in the initial experiments involving PGF_{2 α} tritiated either in the 9β - or 11β -positions. These experiments were carried out to ensure that the radiolabel would not be lost and that no interfering products were being formed by the oxidation of 9α - or 11α -hydroxy groups of the substrates or by reduction of 15-oxo groups of the products. The first system consists of a double development to 15 cm in ethyl acetate/acetone/acetic acid (90:10:1, by vol.) (Anderson, 1969). The second system consisted of a double development to 15 cm in chloroform/methanol/acetic acid (18:3:1, by vol.) on t.l.c. plates impregnated with 3% silver nitrate in 95% methanol. Reference standards were applied alongside the samples and were ultimately visually detected by I₂ vapour or by spraying with 5–10% molybdophosphoric acid in ethanol and heating at 100–120 $^{\circ}\text{C}$. The section of each plate containing the radioactive samples was scraped into scintillation vials as thirty 0.5 cm bands.

In subsequent experiments in which reaction rates were determined the prostaglandin reference standards and their 15-oxo and 15-oxo-13,14-dihydro

metabolites were spotted on top of the applied samples. The plates were developed twice to a distance of 15 cm with the first solvent system described above (Anderson, 1969) and the positions of the reference standards were detected by using I_2 vapour and marked. The I_2 staining was allowed to fade and substrate and product areas were scraped into scintillation vials and 10 ml of scintillant was added. The product area was scraped as a single band extending from 1 cm before and after the reference marks of the 15-oxo and 15-oxo-13,14-dihydro metabolites respectively. The substrate was removed as a 2 cm band.

Liquid-scintillation counting. This was carried out as described by Camp & Greaves (1980).

Determination of enzyme activity. Activity of PGDH was determined by the percentage conversion of prostaglandin into total products, and velocities were expressed as nmol of prostaglandin converted into 15-oxo and 15-oxo-13,14-dihydro metabolites/s per litre of reaction mixture. Blank rates were subtracted.

Determination of protein. The concentration of protein in 120 000 g supernatants was estimated by the method of Lowry *et al.* (1951) with human plasma albumin as standard.

Results and discussion

Concurrent oxidation of either the 9 α - or the 11 α -hydroxy groups of the prostaglandin substrate or reduction of the 15-oxo group of the prostaglandin metabolites in reactions containing $NADP^+$ were excluded as detailed in the Experimental section. It was shown that the only two prostaglandin products were the 15-oxo and 15-oxo-13,14-dihydro metabolites. Similar findings for reactions containing NAD^+ were reported by Camp & Greaves (1980). It was found that, in common with other tissues (Hansen, 1976), PGDH in skin is associated with a Δ^{13} -reductase. It has been shown that oxidation of the 15-hydroxy group precedes saturation of the 13,14-double bond (Anggard & Larsson, 1971; Anggard *et al.*, 1971; Hamberg & Samuelsson, 1971). This sequence has been confirmed in skin (Camp & Greaves, 1980), and thus the method of determination of enzyme activity used here is justified. To allow comparison with previous work in skin (Camp & Greaves, 1980), reaction rates are expressed as nmol/s per litre of reaction mixture. However, to determine consistency of enzyme preparations, total protein concentrations in samples of 120 000 g supernatant were found to be 10.2 ± 0.9 mg per ml (mean $\pm$ s.d., $n = 15$).

In contrast with the report of Wright & Corder (1979) that 2-mercaptoethanol inhibited PGDH from rat kidney, it was shown that this compound did not inhibit skin PGDH activity under the conditions described in the Experimental section,

and that enzyme activity was slightly increased by the addition of 2-mercaptoethanol.

Preliminary experiments indicated that with either prostaglandin substrate, saturating co-substrate concentrations were obtained with 10 mM- NAD^+ and 5 mM- $NADP^+$. In all experiments an incubation time of 15 min provided linear reaction rates, with the exception of incubations with $PGF_{2\alpha}$ and $NADP^+$, when reactions were linear for 6 min only.

At linear reaction rates and saturating concentrations of the appropriate co-substrate, apparent K_m and V_{max} values for type-I (NAD^+ -linked) and type-II ($NADP^+$ -linked) PGDH were determined with $PGF_{2\alpha}$ and PGE_2 as substrates. Figs. 1 and 2 show the effects on reaction rate of prosta-

Fig. 1. (a) Dependence of NAD^+ -linked PGDH activity on $PGF_{2\alpha}$ (●) and PGE_2 (○) concentration and (b) linear plot of the effect of substrate concentration on NAD^+ -linked PGDH activity

(a) Different concentrations of substrate were incubated at 37°C with 120 000 g supernatant of rat skin and 10 mM- NAD^+ for 15 min. Experimental details are given in the text. (b) Experimental details were as described for (a). Plots of $[PGF_{2\alpha}]/v$ versus $[PGF_{2\alpha}]$ (●) and $[PGE_2]/v$ versus $[PGE_2]$ (○) are shown. The mean kinetic constants of several such experiments are given in the Results and discussion section. Straight lines were constructed by simple linear regression.

Fig. 2. (a) Dependence of NADP⁺-linked PGDH activity on PGF_{2 α} (●) and PGE₂ (○) concentration and (b) linear plot of the effect of substrate concentration on NADP⁺-linked PGDH activity

(a) Different concentrations of substrate were incubated at 37°C with 120000 g supernatant of rat skin homogenate and 5 mM-NADP⁺ for 6 min with PGF_{2 α} as substrate and 15 min with PGE₂ as substrate. Experimental details are given in the text. (b) Experimental details were as described for (a). Plots of $[\text{PGF}_{2\alpha}]/v$ versus $[\text{PGF}_{2\alpha}]$ (●) and $[\text{PGE}_2]/v$ versus $[\text{PGE}_2]$ (○) are shown for rat skin. The mean kinetic constants for several such experiments are given in the Results and discussion section. Straight lines were constructed by simple linear regression.

glandin substrate concentration in the presence of 10 mM-NAD⁺ and 5 mM-NADP⁺. Figs. 1(a) and 2(a) show the corresponding Hanes plots (Hanes, 1932), and demonstrate Michaelis–Menten kinetics. Straight lines were constructed by simple linear regression. With NAD⁺ the apparent K_m for PGF_{2 α} was $24.0 \pm 6.0 \mu\text{M}$ (mean $\pm$ s.d., $n = 6$) and the apparent V_{max} was 9.8 ± 2.0 nmol/s per litre (mean $\pm$ s.d., $n = 6$). With PGE₂ as substrate the apparent K_m was $8.0 \pm 3.3 \mu\text{M}$ (mean $\pm$ s.d., $n = 8$) with an apparent V_{max} of 2.7 ± 1.6 nmol/s per litre (mean $\pm$ s.d., $n = 8$). With NADP⁺ as a co-sub-

strate, an apparent K_m of $23 \pm 5.0 \mu\text{M}$ (mean $\pm$ s.d., $n = 10$) was obtained for PGF_{2 α} with an apparent V_{max} of 5.2 ± 3.0 nmol/s per litre (mean $\pm$ s.d., $n = 10$). With PGE₂ as a substrate, values of $7.5 \pm 6.0 \mu\text{M}$ (mean $\pm$ s.d., $n = 7$) and 3.0 ± 2.6 nmol/s per litre (mean $\pm$ s.d., $n = 7$) were obtained respectively. K_m values obtained for the NAD⁺-linked system agree with those reported for the NAD⁺-linked enzymes obtained from other tissues (Hansen, 1976). The low mean apparent K_m obtained for PGE₂ with NADP⁺ as co-substrate ($7.5 \mu\text{M}$) is noteworthy, since all other reports on type-II PGDH prepared from other tissues indicate that PGE₂ is a poor substrate for this enzyme, K_m values of 80–200 μM being reported (Lee & Levine, 1975; Hansen, 1976; Jarabak & Fried, 1979).

In the summation experiments, where saturating concentrations of both NAD⁺ and NADP⁺ were included in the same incubations, reaction rates were no greater than in incubations containing either co-substrate alone (Fig. 3). A preliminary series of product inhibition experiments were then performed to elucidate this apparent anomaly. It is known that type-I PGDH is competitively inhibited by NADH and that the type-II enzyme is competitively inhibited by NADPH (Kaplan *et al.*, 1975; Lee & Levine, 1975; Hansen, 1976). Thus initial inhibition experiments using the conditions described in the Experimental section were carried out. These showed that, apart from the expected product inhibition, NADPH also inhibited the NAD⁺-linked PGDH and NADH inhibited the NADP⁺-linked activity (Fig. 4). Subsequent studies were performed using PGF_{2 α} as a substrate and a range of

Fig. 3. Evidence for a lack of summation of enzyme activity

Experimental details are given in the Experimental section. The columns show the mean velocities ($\pm$ s.d., $n = 3$) obtained with the co-substrates indicated. No increase in activity was seen in incubations containing both co-substrates.

subsaturating concentrations of NAD^+ , with NADH and NADPH as product inhibitors as described in the Experimental section. The results, when transferred on to a double-reciprocal plot (Lineweaver & Burk, 1934), indicated that, as expected, NADH was acting as a competitive inhibitor (Fig. 5). However, it was found that NADPH, which should not be an inhibitor (Kaplan *et al.*, 1975; Lee & Levine, 1975) was acting as an uncompetitive inhibitor (Fig. 6). Attempts were made to carry out similar inhibition experiments with the NADP^+ -linked enzyme. However, at the subsaturating concentrations of NADP^+ used, the rate of reaction was too slow to be measured accurately by the method described.

The evidence from this study, including the summation and inhibition experiments, raises the possibility that rat skin contains a single PGDH enzyme capable of utilizing either NAD^+ or NADP^+ as co-substrate. The finding that NADP^+ -linked PGDH activity in rat skin gives a K_m for PGE_2 which is much lower than that reported for type-II dehydrogenase in other tissues is of interest. PGE_2 has potent biological effects in skin, particularly with regard to inflammation (Camp, 1982). The ability of skin PGDH to catabolise this biologically active compound efficiently adds to past evidence (Camp & Greaves, 1980) of the importance of skin in prostaglandin catabolism.

Fig. 4. Inhibition of both NAD^+ -linked and NADP^+ -linked PGDH by reduced nicotinamide nucleotides. 120000g supernatant of rat skin homogenate and $117\mu\text{M-PGF}_{2\alpha}$ were incubated for 6 min with both NAD^+ or NADP^+ and the reduced co-substrates as indicated. Inhibition by the reduced co-substrates is represented as percentage loss of enzyme activity.

Fig. 5. Double-reciprocal plot indicating competitive inhibition of NAD^+ -linked PGDH by NADH.

120000g supernatant was incubated with $50\mu\text{M-PGF}_{2\alpha}$ and a range of subsaturating concentrations of NAD^+ . NADH was used as a product inhibitor. Further experimental details are given in the Experimental section. Straight lines were constructed using weighted linear regression. ●, No NADH; ■, 0.25 mM-NADH; ▲, 0.5 mM-NADH.

Fig. 6. Double-reciprocal plot indicating uncompetitive inhibition of NAD^+ -linked PGDH by NADPH.

120000g supernatant was incubated with $50\mu\text{M-PGF}_{2\alpha}$ and a range of subsaturating concentrations of NAD^+ . NADPH was used as an inhibitor. Experimental details are given in the Experimental section. Straight lines were constructed using weighted linear regression. ●, No NADPH; ■, 0.2 mM-NADPH; ▲, 5.0 mM-NADPH.

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References

- Anderson, N. H. (1969) *J. Lipid Res.* **10**, 316–319
- Anggard, E. & Larsson, C. (1971) *Eur. J. Pharmacol.* **14**, 66–70
- Anggard, E. & Samuelsson, B. (1966) *Ark. Kemi* **25**, 293–300
- Anggard, E., Larsson, C. & Samuelsson, B. (1971) *Acta Physiol. Scand.* **81**, 396–404
- Camp, R. D. R. (1982) *Clin. Exp. Dermatol.* **7**, 435–444
- Camp, R. D. R. & Greaves, M. W. (1980) *Biochem. J.* **186**, 153–160
- Hamberg, M. & Samuelsson, B. (1971) *J. Biol. Chem.* **246**, 1073–1077
- Hanes, C. S. (1932) *Biochem. J.* **26**, 1406–1421
- Hansen, H. S. (1976) *Prostaglandins* **12**, 647–679
- Jarabak, J. & Fried, J. (1979) *Prostaglandins* **18**, 241–246
- Kaplan, L., Lee, S.-C. & Levine, L. (1975) *Arch. Biochem. Biophys.* **167**, 287–293
- Lee, S.-C. & Levine, L. (1975) *J. Biol. Chem.* **250**, 548–552
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ruckrich, M. F., Wendel, A., Schegel, W., Jackisch, R. & Jung, A. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 799–809
- Westbrook, C., Lin, Y.-M. & Jarabak, J. (1977) *Biochem. Biophys. Res. Commun.* **76**, 943–949
- Wright, J. T. & Corder, C. N. (1979) *Prostaglandins* **17**, 431–440