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# THE RELEASE OF PROSTAGLANDIN $E_2$ FROM THE SKIN OF THE PLAICE, *Pleuronectes platessa L.*

A.A. ANDERSON, THELMA C. FLETCHER* & G.M. SMITH

School of Pharmacy, Robert Gordon's Institute of Technology, Aberdeen,
N.E.R.C. Institute of Marine Biochemistry*, Aberdeen

**1** A fungal extract which produces a cutaneous hypersensitivity reaction in the plaice, *Pleuronectes platessa L.*, was incubated *in vitro* with the skin of this teleost fish. Samples of incubation media were assayed for smooth muscle stimulating activity.

**2** Prostaglandin $E_2$ was identified by bioassay, thin-layer chromatography, ultraviolet absorption spectroscopy and gas chromatography–mass spectrometry. Release from challenged skin was maximum after 60 min incubation.

**3** Analysis of the fatty acid composition of plaice skin showed that although arachidonic acid was present (3% of total fatty acids), the precursor of prostaglandin $E_3$, eicosapentaenoic acid contributed 9% of total.

**4** Indomethacin (50 mg/kg i.p.) did not inhibit the erythema induced by the fungal extract, whilst a dose of 1 mg/kg maximally inhibited prostaglandin release from skin on incubation *in vitro*.

**5** It is concluded that prostaglandins do not have an exclusive role in the mediation of the hypersensitivity reaction.

## Introduction

An immediate erythematous response has been observed in the skin of the plaice (*Pleuronectes platessa*), a marine teleost fish, following the intradermal injection of certain fungal extracts (Fletcher & Baldo, 1974). A similar response was not observed in the closely related flounder (*Platichthys flesus*) unless it received an intravenous injection of plaice serum, at least 24 h before fungal challenge. The evidence for systemic anaphylaxis in fish is controversial (Dreyer & King, 1948; Clem & Leslie, 1969) but the skin reaction was considered to be a hypersensitivity response, since it was transferrable to the flounder with plaice serum containing precipitins to the fungal extracts. The flounder normally contained no detectable precipitins and the common factor in the fungal extracts eliciting the response was C-substance (Baldo, Fletcher & Pepys, 1977).

The mediators implicated in hypersensitivity responses in mammals have been extensively studied but little is known of such mediators in poikilotherms. The skin reaction in the plaice can be inhibited *in vivo* by disodium cromoglycate (Baldo & Fletcher, 1975) and preliminary work using an *in vitro* system has identified prostaglandin-like substances released from challenged plaice skin (Anderson, Fletcher & Smith, 1977). The present work extends the findings on the *in vitro* release of mediators and the characterization of the prostaglandin involved.

## Methods

### *Preparation of skin*

Skin from freshly-killed normal plaice was chopped, washed and incubated at 18°C with 2 volumes (1 g/2 ml) of a specific plaice ringer (Cobb, Fox & Santer, 1973) in the presence and absence of the fungal extract, *Epidermophyton floccosum* (1 mg/ml) prepared as described by Baldo *et al.* (1977), hereafter referred to as challenged and non-challenged skin respectively. Samples of the incubation mixtures were withdrawn at timed intervals for biological assay.

For the identification of active material, large-scale incubations were performed and a standard extract prepared.

### *Extraction and characterization of prostaglandin-like material*

Incubation was terminated by the addition of 2 volumes of ethanol. Petroleum ether was added and

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the mixture inverted. After removal of the petroleum ether, each sample was acidified to pH 3 to 3.5 with formic acid and extracted three times with 4 volumes of chloroform. The pooled chloroform layers were dried under a stream of nitrogen at 30°C. Preparative thin-layer chromatography (t.l.c.) was carried out on silica gel G (0.25 μm) coated glass plates using 4 solvent systems (Table 1). Methanolic solutions of prostaglandin standards and skin extracts were applied to the plates and developed in solvent mixture. Following visualization of standards, zones (1 cm) of silica gel were scraped off the plate and eluted with a mixture of chloroform/methanol (1:1). Eluates were dried under nitrogen, the residue resuspended in Krebs solution and tested for contractile activity on the rat fundus strip.

The use of 2 further solvent systems (Table 1) with silver nitrate impregnated plates enabled separation of the individual members of the E series prostaglandins. As above, zones of silica were extracted and the eluates tested for contractile activity. Amounts of prostaglandin-like material were expressed as prostaglandin $E_2$ equivalents.

*Bioassay*

Assay of smooth muscle stimulating activity was performed on the following isolated preparations: rat stomach strip, chick rectum, gerbil colon, rat colon and guinea-pig ileum. Preparations were mounted in organ baths in Krebs solution (composition (mM): NaCl 118.2, KCl 4.75, $CaCl_2$ 2.54, $KH_2PO_4$ 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.2) at 37°C ± 0.5°C gassed with a mixture of 95% $O_2$ and 5% $CO_2$. Isometric contractions were detected by strain gauge transducers and displayed on a Devices Recorder.

For the identification of the active material, 3 + 3 quantitative assays were performed in parallel using a randomized block design on 3 different smooth muscle preparations which vary in their relative sensitivity to authentic prostaglandin standards. An analysis of variance was performed on the results of the parallel assays to obtain potency ratios and fiducial limits.

In subsequent experiments, active material was assayed by the bracketing technique against prostaglandin $E_2$ on rat fundus strips (Vane, 1957) in Krebs solution containing a drug combination which increases the sensitivity and selectivity of the assay (Gilmore, Vane & Wyllie, 1968, as modified by Bennett, Stamford & Unger, 1973). Differences in prostaglandin levels were analysed by paired *t* test.

The following drugs were used: bradykinin triacetate (Sigma), α-chymotrypsin (Worthington Biochemical Corporation); FPL 55712, 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid sodium salt (Fisons); (−)-hyoscine hydrobromide (BDH); indomethacin (Merck, Sharpe & Dohme), methysergide bimaleate (Sandoz), mepyramine maleate (May & Baker), phenoxybenzamine hydrochloride (Smith Kline and French); propranolol hydrochloride (ICI); prostaglandins $E_1$, $E_2$, $E_3$, $F_{1\alpha}$ and $F_{2\alpha}$ (Upjohn).

*Spectrometry*

Ultraviolet spectra of partially purified skin extract prepared as above were recorded on a Unicam SP 1800 by the method of Yoshimoto, Ito & Tomita (1970). For mass spectrometric analysis, samples of incubation media from challenged and non-challenged skin were acidified to pH 3 and extracted twice with equal volumes of ethyl acetate. Following evaporation of the combined ethyl acetate extracts, the residue was subjected to preparative t.l.c. using the solvent system, ethyl acetate–acetone–acetic acid (90:10:1) (Andersen, 1969). The zones of silica gel corresponding to authentic $E_2$ were eluted with methanol. The eluates were evaporated under vacuum and the residue derivatized for analysis using a Finnigan 3200 gas chromatograph–mass spectrometer. The samples were run as the methyl ester, methyloxime,

**Table 1** Solvent systems used in preparative thin-layer chromatography of prostaglandin-like material from plaice skin

| *Composition* | *Absorbant* |
|---|---|
| Chloroform–methanol–acetic acid–water (90:10:1:0.75) | Silica Gel G |
| Ethyl acetate–formic acid (400:5) | Silica Gel G |
| Chloroform–ethyl acetate–ethanol–acetic acid (20:20:4:1) | Silica Gel G |
| Benzene–chloroform–butanol–ethanol (4:10:5:1) | Silica Gel G |
| Chloroform–methanol–acetic acid (18:3:1) | Silica Gel G–$AgNO_3$ (10:1) |
| Ethyl acetate–ethanol–acetic acid (100:1:1) | Silica Gel G–$AgNO_3$ (10:1) |

trimethylsilyl ether and compared with the corresponding authentic $E_2$ derivatives.

*Fatty acid composition*

Finely-chopped skin (10 g) was transferred to a mortar containing liquid nitrogen and ground finely with a pestle. Lipids were extracted by the method of Bligh & Dyer (1959) as modified by Allen, Good, Davis, Chisum & Fowler (1966). Samples of lipid extract were transesterified with methanolic hydrochloric acid for gas chromatographic analysis (g.l.c.) of methyl esters (Farquhar, 1962). A Pye 104 gas chromatograph equipped with a flame ionization detector was used for g.l.c. Two different glass columns (1.5 m × 4 mm) were used to separate the fatty acids: EGSS-X on gas chrom. Q and 10% EGSS-Y on DMCS-treated P (Field Instrument Company Ltd.). Both columns were operated at 200°C. The relative proportion of each fatty acid was found by calculating the product of peak height and the retention time at half-peak width and expressed as a percentage of the total.

## Results

Incubation media from challenged and non-challenged skin contracted all the tissues studied. The fungal extract of *E. floccosum*, incubated with Ringer alone, did not change the smooth muscle tone. The rat stomach strip gave the most reproducible and dose-dependent responses and therefore was used to assay the active principle from skin in subsequent experiments. The contractile response to skin extract persisted in the presence of atropine, mepyramine, methysergide and FPL 55712 which antagonize the effects of acetylcholine, histamine, 5-hydroxytryptamine and slow-reacting substance of anaphylaxis (Augstein, Farmer, Lee, Sheard & Tattersall, 1973) respectively. In addition, incubation with chymotrypsin, which destroyed activity due to bradykinin, did not reduce the response to the skin extract.

**Table 2** 3 + 3 parallel assays using prostaglandin standards $E_1$, $E_2$, $E_3$, $F_{1\alpha}$ and $F_{2\alpha}$.

| | *Concentration of unknown in terms of standard (ng/ml)* | | | | |
|---|---|---|---|---|---|
| *Tissue** | $E_1$ | $E_2$ | $E_3$ | $F_{1\alpha}$ | $F_{2\alpha}$ |
| RSS | 446 | 208 | 782 | 1648 | 882 |
| CR | 269 | 197 | 1065 | 6425 | 1355 |
| RC | 343 | 185 | 3220 | 913 | 815 |

* RSS: rat stomach strip, CR: chick rectum, RC: rat colon.

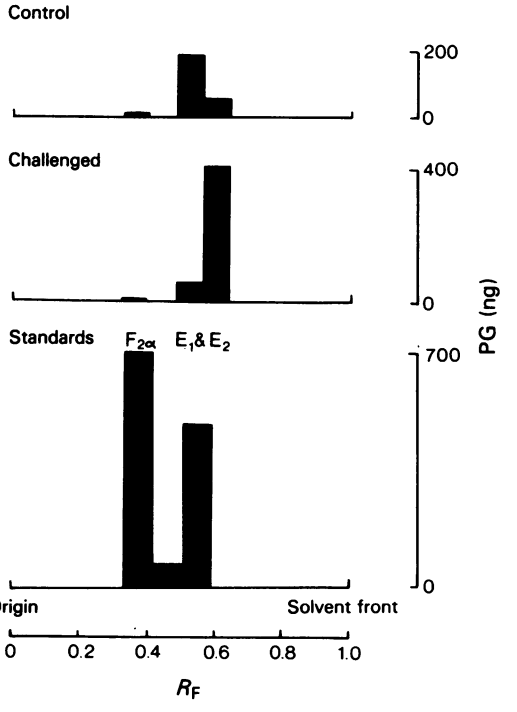


**Figure 1** Thin-layer chromatography of prostaglandin-like material from plaice skin using the solvent system ethyl acetate–formic acid (400:5). The 3 histograms represent migration of smooth muscle contracting material released from challenged skin, from non-challenged skin and from a mixture of standard prostaglandins $F_{2\alpha}$ (1.5 μg), $E_1$ and $E_2$ (0.5 μg each).

*Identification of prostaglandin $E_2$*

The activity could be partitioned from an acid aqueous phase into chloroform, from there into Krebs solution (pH 7) and finally back into chloroform at pH 3. This behaviour is consistent with the properties of a prostaglandin.

Preparative t.l.c. using 4 different solvent systems indicated that the active material co-chromatographed almost exclusively with the E-series prostaglandins (Figure 1). An aliquot of the skin extract, treated with alkali showed an absorption maximum of 278 nm in methanol. Rechromatography using 2 further solvent systems on thin-layer plates impregnated with silver nitrate showed that the prostaglandin-like material migrated in the $R_F$ range corresponding closely with the distance travelled by standard $E_2$ but not with $E_1$ or $E_3$ (Figure 2).

Table 2 shows the results of quantitative assays of the prostaglandin-like material performed in parallel. When the unknown was assayed in terms of standard

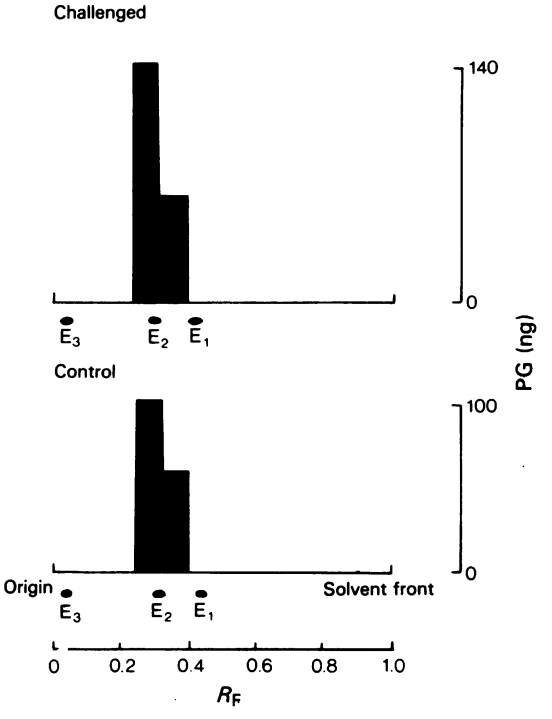


**Figure 2** Argentation thin-layer chromatography of prostaglandin-like material from plaice skin using the solvent system ethyl acetate–ethanol–acetic acid (100:1:1). The 2 histograms represent migration of smooth muscle contracting material released from challenged and from non-challenged skin. The distance travelled by standard $E_1$, $E_2$ and $E_3$ is indicated under each chromatogram.

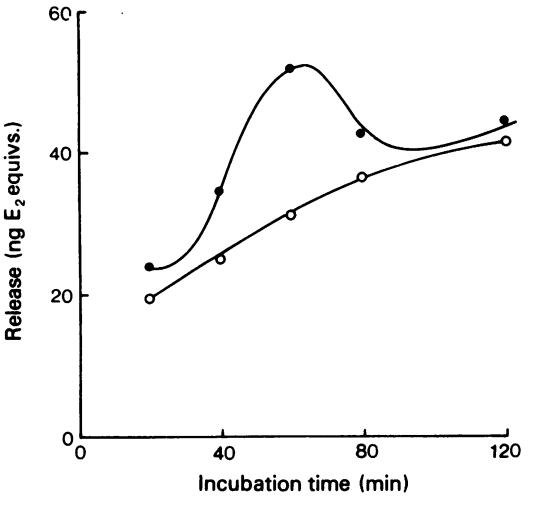


**Figure 3** Time course of release of prostaglandin-like material from challenged (●) and from non-challenged plaice skin (○). Each point represents the mean of 6 observations of the amount of prostaglandin-like material released from plaice skin (1 g) expressed as ng $PGE_2$ equivalents.

$E_2$, consistent potencies were obtained on the three tissues. In contrast, the potency varied considerably when assayed as $E_1$, $E_3$, $F_{1\alpha}$ or $F_{2\alpha}$. The presence of $E_2$ in the challenged skin extract was confirmed by comparison with the mass spectra of a standard preparation of $E_2$. There was insufficient material present in the non-challenged skin extract to obtain a satisfactory spectrum.

*Time course of release from place skin*

Release of prostaglandin-like material from challenged and non-challenged skin was studied over a period of 120 min (Figure 3). Release of active material from challenged skin was significantly ($P < 0.05$) greater than that from non-challenged skin from 20 to 80 min incubation and was maximum (50 ng/g) after about 60 min.

*Effect of indomethacin on prostaglandin release*

The effect of indomethacin was studied on *in vitro*

**Table 3** Fatty acid composition of plaice skin

| *Fatty acid* | *% of total fatty acids* | |
|---|---|---|
| | *Column 1 (EGSS-Y)* | *Column 2 (EGSS-X)* |
| $E_1$ Precursor (20:3 ω6) | Trace | 0.2 |
| $E_2$ Precursor (20:4 ω6) | 2.9 | 2.8 |
| $E_3$ Precursor (20:5 ω3) | 9.0 | 8.7 |
| Others | 88.1 | 88.3 |

The methyl esters were analysed by gas chromatography on two phases, 10% EGSS-Y and 10% EGSS-X (methods: fatty acid composition).

release of prostaglandin-like material from skin after 60 min incubation. Indomethacin (1 to 1000 μg/kg), given to plaice by intraperitoneal injection 2 h before killing, produced a 10 to 90% inhibition of release of active material from both challenged and non-challenged skin. While indomethacin maximally inhibited *in vitro* release of prostaglandin-like material in a dose of 1 mg/kg, even 50 mg/kg did not reduce the erythema induced by *E. floccosum in vivo*.

*Fatty acid composition*

The fatty acid composition of the skin of the plaice is summarized in Table 3. There was good agreement between the results of the two columns. The fatty acids which were major components were C16:0, C18:1 and C22:6, contributing about 60% of total. The precursor of prostaglandin $E_2$, namely C20:4 $\omega$6 fatty acid was present in a concentration of 2.8%. The level of the C20:3 $\omega$6 fatty acid was very low but the precursor of prostaglandin $E_3$, C20:5 $\omega$3 contributed about 9% of total.

## Discussion

This investigation has provided evidence that one active substance is released from plaice skin following challenge *in vitro* with extracts of fungus which will produce an immediate erythema in the skin of teleost fish (Fletcher & Baldo, 1974). Incubation media from challenged and from non-challenged skin were shown to contain prostaglandin $E_2$, identified by bioassay, thin-layer chromatography and ultraviolet absorption spectrometry. Unequivocal evidence for the release of prostaglandin $E_2$ from challenged skin was obtained by mass-spectrometric analysis.

Although prostaglandin $E_2$ was released from skin incubated with Ringer solution alone, the linear pattern of release suggests a simple leakage of prostaglandin out of skin as a result of a sudden synthesis stimulated by the removal and chopping process. The increased prostaglandin output from skin incubated in the presence of *E. floccosum* indicates a further synthesis of prostaglandin on challenge which, when superimposed on the simple basal release, leads to the observed more complex pattern of release. The fall in prostaglandin release after 60-min incubation is probably due to diminished synthesis, possibly accompanied by enzymatic degradation of released prostaglandin.

Work in progress using a skin perfusion technique similar to that employed by Greaves & Søndergaard (1970) indicates that acidic lipid activity is present in perfusates of plaice skin challenged intradermally with *E. floccosum*, whereas no activity has been observed in perfusates of non-challenged skin. *E. floccosum* therefore appears to stimulate prostaglandin synthesis.

Prostaglandins of the 1, 2 or 3 series are synthesized from the $C_{20}$ unsaturated fatty acids, eicosatrienoic acid (20: 3 $\omega$6), eicosatetraenoic acid (20:4 $\omega$6) and eicosapentaenoic acid (20:5 $\omega$3) respectively. In these experiments we were unable to detect prostaglandin $E_1$ or $E_3$. The absence of $E_1$ is probably due to the fact that there is a low concentration of 20:3 $\omega$6 in plaice skin. It is interesting, however, that 20:5 $\omega$3 is abundant in the skin. Using a particulate enzyme fraction from sheep vesicular glands, Struijk, Beerthuis & Van Dorp (1967) showed that whereas $E_1$ and $E_2$ are converted at a high rate from their respective precursor fatty acids, $E_3$ is formed at a much lower rate from 20:5 $\omega$3. It would be worthwhile, therefore, to study the comparative aspects of prostaglandin biosynthesis in more detail, preferably using an enzyme fraction of plaice skin.

Prostaglandins are synthesized or released by almost all mammalian tissues studied. In lower animals, Christ & Van Dorp (1972) demonstrated prostaglandin biosynthesis from tissue homogenates of a number of fish and Ogata & Nomura (1975) identified prostaglandin $E_2$ from the gastrointestinal tract of the shark. Hall, O'Regan & Quigley (1977) have indicated that prostaglandins could have an important role in regulating osmolal changes in frog skin; indeed their ubiquitous occurrence has led to the suggestion that prostaglandins may be involved in the regulation of fundamental biological processes (Christ & Van Dorp, 1972). In humans, the intradermal injection of prostaglandin $E_1$ causes increased vascular permeability and erythema (Søndergaard & Greaves, 1971; Crunkhorn & Willis, 1971a, b) and increased concentrations of prostaglandins have been found in skin inflamed as a result of various injuries (Anggard, Arthurson & Jonsson, 1970; Greaves, Søndergaard & McDonald-Gibson, 1971; Søndergaard, Greaves & Jorgenson, 1974). However, since prostaglandins seem to be synthesized whenever cell membranes are distorted (Gilmore, Vane & Wyllie, 1969), it is difficult to ascertain whether they are formed primarily to mediate the inflammation or as a result of tissue injury caused by other mediators.

Challenge of plaice skin *in vitro* with fungal extracts caused an increased release of prostaglandin $E_2$ in the incubation mixtures, whereas in preliminary experiments a similar challenge of flounder skin did not lead to an increased release. This correlates well with the observation that intradermal injection of fungal extracts caused an immediate erythema in the skin of plaice but not of flounders and suggests that prostaglandins may therefore have some role in the mediation of the skin reaction. However, the inability of indomethacin, which inhibited prostaglandin synthesis in plaice skin *in vitro*, to suppress the erythema

induced by fungal extracts *in vivo* suggests that prostaglandins may not be the only mediators of the reaction.

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