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THE EFFECT OF SOME FLAVONE DRUGS ON THE CONVERSION OF PROSTACYCLIN TO 6-OXOPROSTAGLANDIN E_1

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Abstract—Flavonoid drugs (rutin, naringenin and quercetin) were compared with indomethacin and sulphasalazine as inhibitors of rat and rabbit renal 9-hydroxyprostaglandin dehydrogenase, prostaglandin synthesis (bovine seminal vesicle microsomes) and inactivation (rabbit colon 100,000 *g* supernatant), vascular PGI_2 formation (rat aortic rings) and for effects on platelet aggregation and on the isolated rat stomach strip. Rutin and naringenin potently inhibited rabbit renal conversion of PGI_2 and $PGF_{2\alpha}$ to 6-oxoPGE_1 and PGE_2, respectively, but had no effect on rat renal 9-hydroxyprostaglandin dehydrogenase activity, prostaglandin breakdown, vascular PGI_2 synthesis, platelet aggregation or the anti-aggregatory effect of PGI_2 and 6-oxoPGE_1. High concentrations (100 μM) of both drugs inhibited the spasmogenic effect of PGI_2 on the rat stomach strip. Naringenin and quercetin (1 mM) inhibited whilst rutin (1 mM) stimulated microsomal prostaglandin synthesis. These results suggest that rutin and naringenin may be useful experimental tools to study the biological roles of 6-oxoPGE_1.

Prostacyclin (PGI_2) is enzymatically converted to 6-oxoprostaglandin E_1(6-oxoPGE_1) by the enzyme 9-hydroxyprostaglandin dehydrogenase (9-PGDH) in human platelets [1, 2], rabbit [3], rat[4], pig [5] and human [6] kidney and in the perfused rabbit liver[7]. 6-oxoPGE_1 has also been demonstrated in plasma following i.v. infusion of prostacyclin in dogs [8] and has been detected by gas chromatography–mass spectrometry in blood obtained by venepuncture of healthy human volunteers [9]. Unlike other metabolites of prostacyclin, 6-oxoPGE_1 has considerable biological activity as an inhibitor of platelet aggregation [10], vasodilator [11], bronchodilator [12] and spasmogen on gastrointestinal smooth muscle [13]. It has been suggested that conversion to chemically stable 6-oxoPGE_1, which is resistant to inactivation in the pulmonary circulation [14], may explain the prolonged inhibition of platelet aggregation following i.v. infusion of PGI_2 in man [15]. However, Jackson et al. [9] have failed to demonstrate an increase in plasma 6-oxoPGE_1 concentration in human volunteers during or after i.v. injection of prostacyclin suggesting that any synthesis of 6-oxoPGE_1 which may occur is unlikely to contribute to the biological activity of exogenous PGI_2. This does not rule out the possibility that endogenous PGI_2 may be enzymatically converted to 6-oxoPGE_1. Indeed, recent experiments in this laboratory have shown spontaneous release of a substance with pharmacological activity and chromatographic mobility identical to that of authentic 6-oxoPGE_1 from non-aggregating human platelets [16].

One approach to the elucidation of the biological roles of 6-oxoPGE_1 in the body is the development of potent and selective inhibitors of 9-PGDH activity. Chang and Tai [5] have shown inhibition of pig kidney 6-oxoPGE_1, formation from precursor 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo$PGF_{1\alpha}$) by the flavone drug rutin. We have now studied the effect of rutin and two other flavone derivatives, quercetin and naringenin on 9-PGDH and other enzymes of prostaglandin synthesis and breakdown as well as their effect on the action of PGI_2 and 6-oxoPGE_1 on platelets and gastrointestinal smooth muscle. For comparison, we have also studied the effect of indomethacin (an inhibitor of arachidonic acid cyclooxygenase) and sulphasalazine (an inhibitor of 15-hydroxyprostaglandin dehydrogenase, 15-PGDH). Some of these results have been published in preliminary form [17].

MATERIALS AND METHODS

Drugs

Rutin, naringenin, quercetin, indomethacin, arachidonic acid, ADP, NAD^+, NADH, PGE_2, $PGF_{2\alpha}$ and 13,14-dihydro-15-oxo$PGF_{2\alpha}$ were purchased from Sigma (London) Ltd., Poole, U.K. Bovine seminal vesicle microsomes were obtained from Miles Research Laboratories, Stoke Poges, U.K. Sulphasalazine was a gift from Pharmacia Ltd., Uppsala, Sweden. PGI_2 and 6-oxoPGE_1 were gifts from Dr. J. O'Grady, Wellcome Ltd., Beckenham, U.K. and Dr. J. Pike, Upjohn Co., Kalamazoo, U.S.A., respectively. All drugs were dissolved in 0.5% (w/v) Na_2CO_3. PGI_2 was stored in 0.1 M NaOH (pH 12) at −20°C and diluted in Tris–HCl buffer (50 mM, pH 8.5) and kept on ice during the experiment. All other prostaglandins were stored at −20°C in ethanol.

Effect of drugs on rabbit renal 9-PGDH

Kidney cortex from male, New Zealand White rabbits (1.5–3.5 kg) was separated from medulla and homogenised in 4 vol phosphate buffer (pH 7.4, com-

position : K_2HPO_4, 40 mM; KH_2PO_4, 10 mM) using 3–4 strokes of an Ultra–Turrax homogeniser (type 18/2N). The homogenate was centrifuged twice at 4°C (first at 3000 *g* for 10 min then at 100,000 *g* for 45 min) to prepare cytosolic, high speed supernatant (HSSN). Aliquots (0.2 ml) were incubated at 37°C with 1 μg/ml prostacyclin containing 0.05 μCi 9β-[^{3}H]- prostacyclin tetramethylammonium (New England Nuclear, specific activity 12 Ci/mmole) or 10 μg/ml $PGF_{2\alpha}$ containing 0.05 μCi 9β-[^{3}H]$PGF_{2\alpha}$ (Radiochemical Centre, Amersham, specific activity 20 Ci/mmole) with NAD^+ (5 mM) as cofactor. After 60 min ($PGF_{2\alpha}$) or 150 min (PGI_2), incubates were acidified with 1 M formic acid, 1 vol. of ethanol added and extracted twice into 2 vol of ethyl acetate. The combined organic phase was evaporated to dryness under a stream of air at 30°C and the dried residue resuspended in 200 μl methanol, 20 μl of which was transferred to scintillation vials and counted in a Beckmann LS230 liquid scintillation counter. Extracted radioactivity was corrected for background and quenching as appropriate and compared with counts obtained from aliquots extracted on ice at zero time. The difference in radioactivity represents loss of the [^{3}H-9β] label following oxidation of PGI_2 (or $PGF_{2\alpha}$) to 6-oxoPGE_1 (or PGE_2). This loss of radioactivity method for assaying 9-PGDH in tissue homogenates has been described in detail previously [18].

Effect of drugs on rat kidney 9-PGDH

High speed supernatants prepared from rat kidney were incubated (37°C, 60 min) with 10 μg/ml 13, 14-dihydro-15-oxo$PGF_{2\alpha}$ containing 0.07 μCi [5, 6, 8, 11, 14-*n*-^{3}H]13,14-dihydro-15-oxo$PGF_{2\alpha}$ (Radiochemical Centre, Amersham, specific activity 75 Ci/mmole) and NAD^+ (5 mM) as cofactor. After acidification and extraction as described above enzymatic conversion to 13, 14-dihydro-15-oxoPGE_2 was determined by radio thin-layer chromatography. The position of unmetabolised prostaglandin was determined by reference to authentic 13, 14-dihydro-15-oxo$PGF_{2\alpha}$ which has an R_f value in the solvent system used (ethyl acetate : acetone : acetic acid, 90/10/1 v/v) of 0.39 ± 0.06 (n = 10).

Effect of drugs on rabbit colon 15-PGDH

Rabbit colon HSSN was incubated with 10 μg/ml $PGF_{2\alpha}$ containing 0.05 μCi radiolabel and 5 mM NAD^+. After 20 min incubation at 37°C aliquots were extracted and conversion to 15-oxo$PGF_{2\alpha}$ determined by radio thin-layer chromatography in the above solvent. The R_f values of authentic $PGF_{2\alpha}$ and 15-oxo$PGF_{2\alpha}$ were 0.19 ± 0.03 (n = 6) and 0.33 ± 0.04 (n = 9), respectively.

Effect of drugs on prostaglandin synthesis

Bovine seminal vesicle microsomes (BSVM) were resuspended in Tris–HCl buffer (50 mM, pH 7.4, 5 mg/ml) and incubated with 10 μg/ml arachidonic acid and 3 mM reduced glutathione (GSH). Aliquots were extracted on ice and after 60 min incubation at 37°C. Dried residues were resuspended in 400 μl Krebs' solution and 2 vol (usually 50 and 200 μl) bioassayed against authentic PGE_2 on the rat stomach strip preparation as described previously [18].

Action of drugs on vascular PGI_2 formation

In these experiments PGI_2 was assayed by its ability to inhibit human platelet aggregation. Blood (20 ml) was obtained by venepuncture from male, drug-free volunteers and anticoagulated (1 : 9 v/v) with 3.8% (w/v) trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifuging anticoagulated blood (200 *g*, 20 min) at room temperature. Platelet-poor plasma (PPP)was prepared by centrifugation of PRP (1000 *g*, 10 min). Rat aortic rings (5–15 mg) were incubated at room temperature in 0.3 ml Tris–HCl buffer with stirring for 5 min in the presence or absence of drug. Five to ten μl aliquots were transferred to cuvettes containing warmed (37°C), stirred (1100 rev/min) human PRP (0.1 ml) in a Payton dual channel aggregometer (model 300BD). After 1 min pre-incubation aggregation was induced by addition of ADP (2–10 μM) and calculated as the increase in light transmittance after 3 min. Anti-aggregatory activity was assayed in terms of authentic PGI_2.

Effect of drugs on human platelet aggregation and on the anti-aggregatory and spasmogenic effects of PGI_2 and 6-oxoPGE_1

Platelet aggregation was carried out as above. To test their effect on platelet aggregation each drug (100–500 μM) was pre-incubated with PRP for 1 min at 37°C. The effect of drugs on the anti-aggregatory action of PGI_2 and 6-oxoPGE_1 was also determined. In these experiments drugs were added to stirred PRP followed 1 min later by PGI_2 (0.01–0.1 ng) or 6-oxoPGE_1 (0.1–2.0 ng) and 1 min thereafter by ADP. All drugs were tested at concentrations of 100–500 μM.

The effect of PGI_2 and 6-oxoPGE_1 on the isolated rat stomach strip was measured in the presence and absence of each of the 5 drugs tested. These experiments were performed in the absence of antagonists to other spasmogens which were normally incorporated into the Krebs' solution to improve the selectivity of the tissue for prostaglandins. Results are expressed as the percentage maximum tension developed over the 1 min contact period and repredent experiments on at least 4 stomach strips.

Results show mean ± S.E. with the number of observations in parentheses. The concentration of drugs required for 50% inhibition of enzyme activity (IC_{50}) were obtained from log concentration–inhibition curves in which each drug was tested at 5 concentrations.

RESULTS

The flavone derivatives rutin, naringenin and quercetin inhibited rabbit kidney 9-PGDH in a dose dependent manner. The concentrations of each drug required for 50% inhibition of the conversion of PGI_2 to 6-oxoPGE_1 were 82.7 ± 2.5 μM (n = 8), 45.4 ± 1.4 μM (n = 8) and 751.9 ± 25.0 μM (n = 8), respectively. All three drugs were more potent when $PGF_{2\alpha}$ was used as substrate. In this case IC_{50} values were 2.57 ± 0.11 μM (n = 16) for rutin, 0.56 ± 0.02 μM (n = 8) for naringenin and 104.5 ± 6.3 μM (n =8) for quercetin. Dose response curves for the

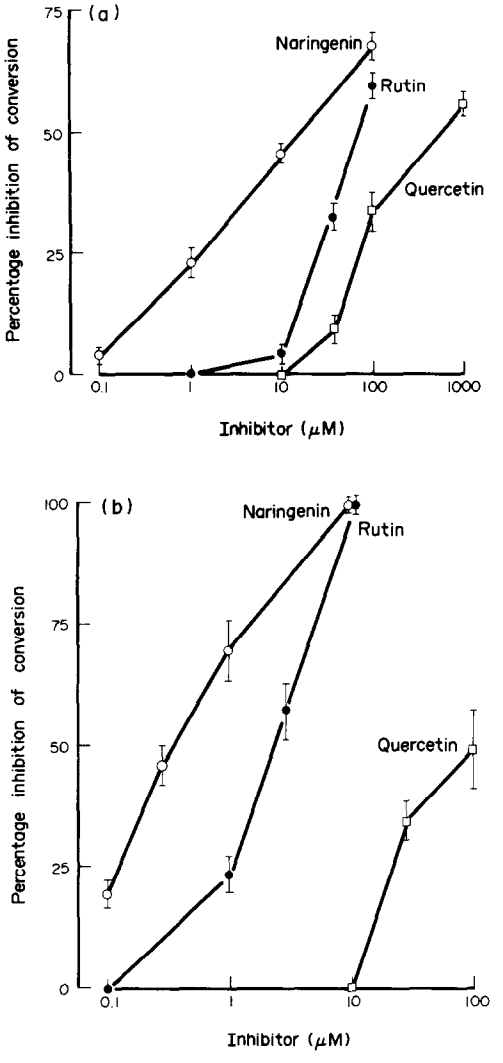

Fig. 1. Percentage inhibition of rabbit renal PG-9HDH by flavonoid drugs. (a) Conversion of PGI_2 to 6-ketoPGE$_1$, (b) conversion of $PGF_{2\alpha}$ to PGE_2. Results show mean ± S.E., $n = 8$.

three flavones on rabbit renal 9-PGDH are shown in Fig. 1. Incubation of 9β-[^{3}H]PGI_2 or 9β-[^{3}H]$PGF_{2\alpha}$ in Tris–HCl buffer did not result in a significant reduction in extracted radioactivity. After 150 min (PGI_2) or 60 min ($PGF_{2\alpha}$) incubation at 37°C extracted radioactivity was 98.2 ± 1.6 and 96.2 ± 3.8% (both $n = 8$) of radioactivity detected in zero time samples. Similarly, no loss of extracted radioactivity was detected in boiled rabbit kidney supernatant (100°C, 5 min) incubated with PGI_2 (97.2 ± 3.9%, $n = 8$) or $PGF_{2\alpha}$ (98.1 ± 2.2%, $n = 8$). These figures represent the percentage of the radioactivity in samples extracted on ice at zero time. Rat kidney 9-PGDH efficiently converted 13, 14-dihydro-15-oxo$PGF_{2\alpha}$ to the corresponding PGE_2 metabolite. After 60 min incubation at 37°C conversion was 74.6 ± 6.7%, $n = 18$. No catabolism of 13, 14-dihydro-15-oxo$PGF_{2\alpha}$ was observed following incubation in Tris–HCl buffer (37°, 60 min). None of the drugs tested inhibited rat renal 9-PGDH at concentrations up to 300 μM (flavones) or 1 mM (indomethacin and sulphasalazine). Both indomethacin (50 μM) and sulphasalazine (50 μM) potentiated the conversion of PGI_2 to 6-oxoPGE$_1$by rabbit kidney 9-PGDH by 51.6 ± 2.9% ($n = 8$) and 51.6 ± 6.2% ($n = 8$), respectively. This action of both drugs is probably secondary to inhibition of 15-PGDH and preservation of PGI_2 for conversion to 6-oxoPGE$_1$ by 9-PGDH.

In control experiments conversion of $PGF_{2\alpha}$ to 15-oxo$PGF_{2\alpha}$ by rabbit colon 15-PGDH was 71.2 ± 6.1%, $n = 8$. As expected, both sulphasalazine and indomethacin inhibited rabbit colon 15-PGDH with IC_{50} values of 49.0 ± 6.1 μM, ($n = 8$) and 241.0 ± 36.4 μM, ($n = 8$) respectively. Log dose inhibition curves from which these IC_{50} values were obtained are not shown. In contrast, rutin, naringenin and quercetin were without effect even at high concentrations (up to 1 mM).

Bovine seminal vesicle microsomes resuspended in buffer and incubated with arachidonic acid synthesised 310 ± 21 ng/ml PGE_2 equivalents, $n = 8$ assayed on the rat stomach strip. The effect of flavone derivatives on bovine seminal vesicle microsomal prostaglandin synthesis were complex. Low concentrations of these drugs were without effect. At high concentrations (1 mM) rutin produced a small potentiation of prostaglandin synthesis (by 35.1 ± 10.1%, $n = 6$), whilst naringenin at the same concentration produced an almost equal reduction of prostaglandin formation (33.1 ± 10.1%, $n = 6$). Quercetin (1 mM) also inhibited BSVM prostaglandin synthesis (71.0 ± 6.8%, $n = 8$). As expected indomethacin potently inhibited the conversion of arachidonic acid to prostaglandins (IC_{50} = 0.21 ± 0.09 μM, $n = 6$). Sulphasalazine was a weak cyclooxygenase inhibitor (IC_{50} = 1402 ± 86 μM, n =9).

Rat aortic rings incubated in Tris–HCl buffer generated 0.50 ± 0.07 ng PGI_2 equivalents/mg/5 min, $n = 16$. All antiaggregatory activity was lost on boiling the incubate for 1 min or after warming to 60°C for 10 min, suggesting that biological activity was due to PGI_2. Indomethacin (IC_{50} = 2.8 ± 0.12 μM, n =16) inhibited vascular PGI_2 synthesis. Rutin, naringenin and quercetin (up to 300 μM) and sulphasalazine (up to 1 mM) were without effect.

Platelet aggregation to ADP was unaffected by rutin, naringenin and quercetin at concentrations up to 500 μM or sulphasalazine up to 1 mM. Indomethacin (50 μM) prevented ADP induced platelet aggregation. Inhibition of ADP induced platelet aggregation by PGI_2 (IC_{50} = 0.49 ± 0.06 ng/ml, $n = 8$) was unaffected by pre-incubation with 500 μM rutin (IC_{50} = 0.50 ± 0.05ng/ml, $n = 6$), naringenin (IC_{50} = 0.47 ± 0.06 ng/ml, $n = 6$), quercetin (IC_{50} = 0.42 ± 0.09 ng/ml, $n = 6$) or sulphasalazine (IC_{50} = 0.42 ± 0.09 ng/ml, $n = 6$). Lower concentrations (10–1000 μM) were also ineffective. Additionally, these drugs did not affect the anti-aggregatory effect of 6-oxoPGE$_1$ (data not shown).

Both PGI_2 and 6-oxoPGE$_1$ contracted the isolated rat stomach strip in a dose dependent manner. The concentration of each prostaglandin required for half maximal spasmogenic effect on this preparation were 45.1 ± 5.6 ng/ml, $n = 11$ and 14.5 ± 2.3 ng/ml, n = 8, respectively. Pre-incubation of rat stomach strips

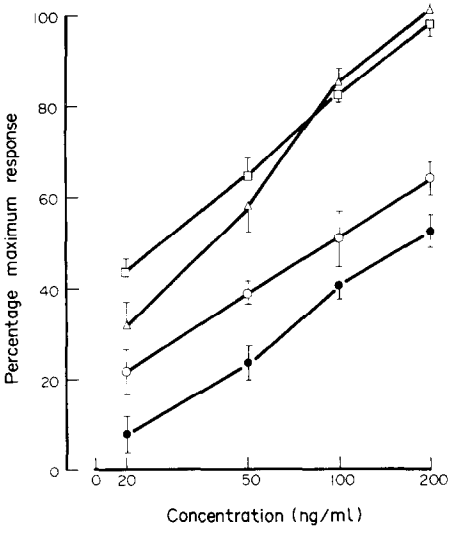

Fig. 2. Effect of pre-incubation with flavonoid drugs (100 μM) on the spasmogenic action of PGI_2 on the rat stomach strip preparation. Control (△), quercetin (□), rutin (○) and naringenin (●). Results show mean ± S.E., $n = 8$–11.

for 15 min in Krebs' solution containing rutin or naringenin, but not quercetin, (all 100 μM) inhibited the responses to PGI_2 (Fig. 2) and 6-oxoPGE_1 (data not shown). Lower concentrations of rutin and naringenin (10 μM) did not affect the contractile response of either prostaglandin.

DISCUSSION

In this paper we have assessed the suitability of three flavone derivatives as experimental tools to study the contribution of 6-oxoPGE_1 to the biological actions of endogenous PGI_2. Oxidation of PGI_2 and its stable hydrolysis product 6-oxo$PGF_{1\alpha}$ to biologically active 6-oxoPGE_1 is brought about by the enzyme 9-PGDH which is present in large amounts in cell-free homogenates of rabbit kidney. In addition to PGI_2, rabbit kidney 9-PGDH also converts $PGF_{2\alpha}$ to PGE_2 while rat kidney enzyme converts 13, 14-hydro-15-oxo$PGF_{2\alpha}$, but not $PGF_{2\alpha}$ or PGI_2, to the corresponding PGE_2 derivative. The different substrate specificity of renal 9-PGDH from the two species may reflect the presence of at least two forms of isoenzyme which in turn may explain why the rabbit (but not rat) 9-PGDH enzyme is potently inhibited by rutin and naringenin.

Both indomethacin and frusemide [19] have been reported to inhibit 9-PGDH activity. However, neither of these drugs has the necessary potency or selectivity of action to be of use for the study of 6-oxoPGE_1 synthesis. From our results it is clear that naringenin is the most potent inhibitor of rabbit renal 9-PGDH discovered to date. Furthermore the selectivity of this drug is indicated by its inability to influence metabolism of $PGF_{2\alpha}$ by rabbit colon 15-PGDH or the synthesis of PGI_2 by rat aorta. Naringenin also had no action on microsomal prostaglandin formation at concentrations which maximally inhibited rabbit renal 9-PGDH. In contrast, high concentrations of rutin stimulated BSVM microsomal prostaglandin production from arachidonic acid. Flavonoid drugs have previously been shown to affect prostaglandin biosynthesis. Rutin derivatives either increased or decreased microsomal human skin prostaglandin formation [20], whilst Baumann *et al.* [21] studied a large number of flavonoid drugs and observed either potent inhibition or no effect on rabbit kidney cyclooxygenase activity. It has been suggested that some flavonoid drugs increase prostaglandin synthesis by acting as a cofactor for the cyclooxygenase enzyme [26].

Even at high concentrations none of the flavonoid drugs tested affected platelet aggregation or the antiaggregatory effect of either PGI_2 or 6-oxoPGE_1. This is in contrast to a recent report in which quercetin inhibited ADP induced human platelet aggregation and potentiated the anti-aggregatory and increase in platelet cAMP concentration produced by prostacyclin [22]. The discrepancy between this study and the present results is difficult to explain, although in our experiments aggregation was determined in platelets suspended in plasma whilst the previous authors had prepared washed platelets.

Flavonoid drugs are known to influence other enzymes associated with arachidonic acid metabolism. For example, quercetin decreased zymosan stimulated release of tritiated arachidonic acid from pre-labelled human polymorphonuclear leukocytes by inhibiting membrane phospholipase A_2 activity [23]. In addition rutin and quercetin [24, 25] inhibit arachidonic acid lipoxygenase. However, these effects occur at concentrations greater than those required to inhibit 9-PGDH.

The conversion of PGI_2 to 6-oxoPGE_1 may be physiologically important for the regulation of platelet aggregability [16] and for the control of renal function since 6-oxoPGE_1 is a potent renal vasodilator [26] and renin secretogogue [27]. The results obtained in this study suggest that naringenin, and to a lesser extent rutin may have the necessary potency and selectivity for inhibition of 9-PGDH to be useful experimental tools to study the role of this prostaglandin in platelet and renal function *in vivo*.

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