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Effects of apocynin, a drug isolated from the roots of *Picrorhiza kurroa*, on arachidonic acid metabolism

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Apocynin is a constituent of root extracts of the medicinal herb *Picrorhiza kurroa* and has been shown to possess anti-inflammatory properties. We investigated the effects of apocynin on the production of arachidonic acid-derived inflammatory mediators by guinea pig pulmonary macrophages. Apocynin concentration-dependently inhibited the formation of thromboxane A_2, whereas the release of prostaglandins E_2 and $F_{2\alpha}$ was stimulated. Apocynin potently inhibited arachidonic acid-induced aggregation of bovine platelets, possibly through inhibition of thromboxane formation. The present results suggest that apocynin might, beside its therapeutic effects in inflammatory conditions when given in a root extract of *P. kurroa*, also be a valuable tool in the development of new anti-inflammatory or anti-thrombic drugs.

Apocynin; Arachidonic acid metabolism; Prostaglandin; Thromboxane; Platelet aggregation

1. INTRODUCTION

Picrorhiza kurroa is an Himalayan herb that has been extensively used in Oriental medicine for a variety of conditions, including liver and lung diseases, fever, skin lesions, worm infections, rheumatic diseases, urinary disorders, heart failure and snake and scorpion bites. One of the active principles of the root extract of *P. kurroa* is apocynin (4-hydroxy-3-methoxyacetophenone) [1] or its glycoside derivative, androsin [2]. Apocynin was proven to be effective in vivo. Orally administered apocynin protected WAG/Rij rats to autoimmune-arthritis [3] and to ulcerative skin lesions induced with tubercle bacteria (submitted for publication). In vitro experiments have shown that apocynin inhibits the generation of reactive oxygen species from human neutrophils [4], which might be responsible for the beneficial effects of *P. kurroa* in inflammatory conditions. In order to further explore the basis of the anti-inflammatory properties of apocynin, we have studied the effects of this drug on the formation of arachidonic acid-derived mediators, which are also of importance in the inflammatory process [5,6]. Our data indicate that apocynin inhibits the formation of thromboxanes (Tx), but stimulates the generation of prostaglandins (PG) E_2 and $F_{2\alpha}$. The results also indicate that apocynin potently inhibits arachidonic acid-induced platelet aggregation, possibly through an inhibitory effect on thromboxane formation.

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2. MATERIALS AND METHODS

2.1. *Isolation of guinea pig pulmonary macrophages*

Pulmonary macrophages (PM) were obtained from male, specified pathogen free guinea pigs (Dunkin Hartley strain, 400–600 g, Harlan Olac Ltd., Bicester, UK) through lung lavage in situ, as described previously [7]. Combined lavage fluids were centrifuged at 400×g for 10 min at 4°C. The cells were subsequently washed twice and finally resuspended in Krebs–bicarbonate solution of the following composition: 118.1 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4.7H_2O$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 8.3 mM glucose (pH 7.4). The cell preparations thus obtained contained ≤2% neutrophils and 10–20% lymphocytes. Viability was ≥95% as judged by Trypan blue dye exclusion. PM were identified by morphology and diffuse staining for nonspecific esterase.

2.2. *Effect of apocynin on the generation of arachidonic acid metabolites by pulmonary macrophages*

PM (5×10^6 cells/ml) were incubated for 15 min at 37°C in a shaking water bath with different concentrations of apocynin. The cells were subsequently stimulated by addition of exogenous arachidonic acid (30 μM) and phorbol myristate acetate (PMA, 10 ng/ml). The incubations were continued for another 15 min, after which the cells were sedimented by centrifugation at 4°C for 10 min at 1225×g. Part of the supernatants was stored at −20°C for the analysis of TxB_2 by specific radioimmunoassay, which was carried out according to the manufacturer's instructions. The remaining part of the supernatants was subjected to an initial extraction using disposable reverse phase C_{18} extraction columns (J.T. Baker, Deventer, The Netherlands). Prior to extraction, the internal standards PGE_1 and PGB_2 were added to correct for extraction losses. The C_{18} extracts were fractionated on Silica Gel 60 columns according to the method of Claeys et al. [8], yielding fractions containing apolar products (A), monohydroxy acids (B), dihydroxy acids (C), and prostaglandins (D), respectively. Fraction B, containing 12-hydroxy-heptadecatrienoic acid (HHT), and fraction D, containing PGE_2 and $PGF_{2\alpha}$, were finally analyzed by reverse phase HPLC as described previously [9,10].

2.3. *Isolation of bovine platelets*

Citrated bovine blood was centrifuged at 240×g for 20 min at room

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temperature. The upper phase was withdrawn and used as platelet rich plasma (PRP), whereas the remaining fluid was further centrifuged at 1760×g for 10 min at room temperature. The upper phase was again collected and represented platelet poor plasma (PPP). All experimental procedures were carried out using plastics to prevent spontaneous platelet aggregation.

2.4. *Effect of apocynin on platelet aggregation*

Platelet aggregation was measured in a Payton dual channel aggregometer. PRP and PPP were used to adjust 0% and 100% readings of the apparatus, respectively. PRP was incubated for 10 min with different concentrations of apocynin, after which aggregation was induced by addition of 1.5 mM arachidonic acid. The aggregatory responses were recorded on a dual channel pen recorder (BBC Goerz Servogor 220).

2.5. *Materials*

Apocynin was obtained from Carl Roth GmbH, Karlsruhe, Germany. Apocynin solution was prepared freshly every day by dissolving it in a minimal volume of ethanol, and subsequently diluting it in Krebs–bicarbonate solution. The final ethanol concentration did not exceed 0.1%. A specific radioimmunoassay for TxB_2 was obtained from NEN du Pont de Nemours, Den Bosch, The Netherlands (NEK-007). Arachidonic acid was purchased from Sigma Chemical Co., St. Louis, MO, as a free acid and was stored at −20°C in hexane. Prior to each experiment, the arachidonate salt was prepared by evaporation of hexane with nitrogen and addition of an equal volume of 0.5 M KOH in ethanol. After evaporation of the ethanol with nitrogen, the salt was dissolved in Krebs–bicarbonate solution. PMA was also obtained from Sigma.

2.6. *Statistics*

The results were statistically evaluated using Student's *t*-test. Differences were considered significant at $P<0.05$.

3. RESULTS

Incubation of guinea pig PM with increasing concentrations of apocynin revealed that the production of HHT was dose-dependently inhibited (Fig. 1). At 3125 μg/ml (≈20 mM) almost total inhibition of HHT production was achieved. A similar pattern was observed when the formation of TxB_2 was measured (see Fig. 1). At the highest concentration used, apocynin significantly inhibited TxB_2 formation, whereas it was not inhibitory at the lower concentrations. At 25 μg/ml it even stimulated TxB_2 formation slightly. The formation of TxB_2 directly reflects TxA_2 generation, since TxB_2 is the biologically inactive breakdown product of TxA_2, a substance which induces e.g. aggregation of platelets [11–13]. HHT formation is also considered to closely parallel TxA_2 production [14,15].

In contrast to the inhibitory effect towards HHT and TxB_2, apocynin stimulated the formation of PGE_2 and $PGF_{2\alpha}$ (Fig. 2). In the absence of apocynin, about 300 ng $PGE_2/5\times10^6$ cells were formed, whereas $PGF_{2\alpha}$ formation was 590 ng/5×10^6 cells. Increasing concentrations of apocynin remarkably increased the generation

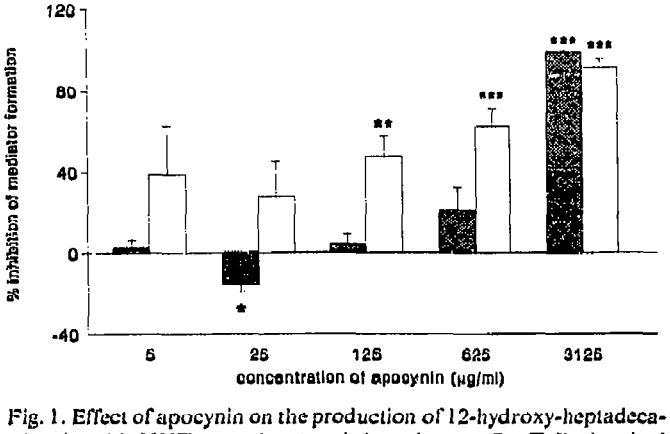

Fig. 1. Effect of apocynin on the production of 12-hydroxy-heptadecatrienoic acid (HHT, open bars) and thromboxane B_2 (TxB_2, hatched bars) by guinea pig pulmonary macrophages. HHT was measured by reverse phase HPLC and TxB_2 was measured by specific radioimmunoassay, as described in Materials and Methods. The data represent the mean ± SEM of 7 and 4 separate experiments, respectively. Basal HHT production was 1.59±0.51 μg/5×10^6 cells, and basal TxB_2 production amounted to 0.78±0.04 μg/5×10^6 cells. $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$.

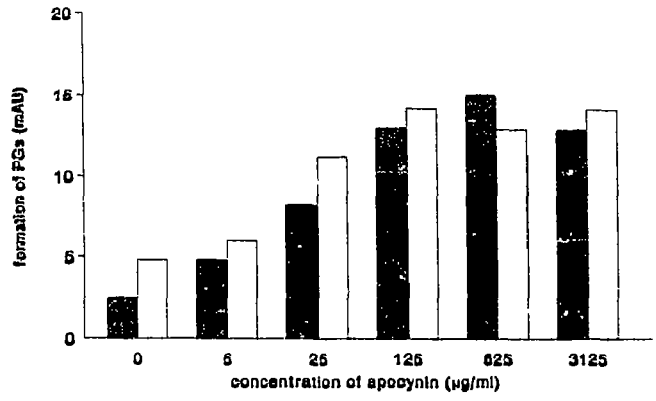

Fig. 2. Effect of apocynin on the production of prostaglandin E_2 (PGE_2, hatched bars) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, open bars) by guinea pig pulmonary macrophages. PGE_2 and $PGF_{2\alpha}$ were measured by reverse phase HPLC, as described in Materials and Methods. The data are an example from one experiment, and are expressed as milli-absorbance units (mAU). One mAU corresponds to approximately 120 ng of the respective prostaglandins.

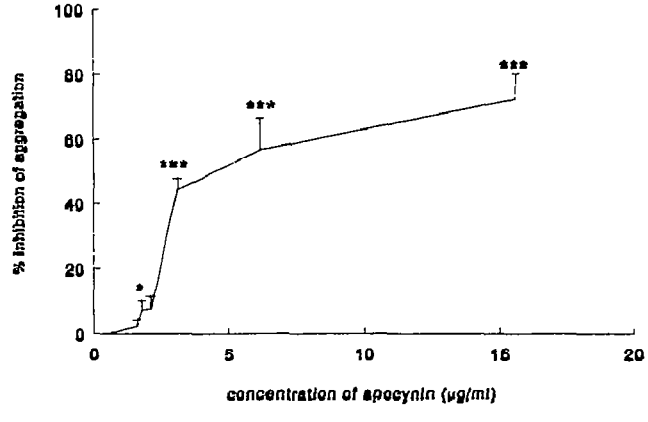

Fig. 3. Effect of apocynin on arachidonic acid-induced aggregation of bovine platelets. The data are expressed as the mean±SEM of 5–9 separate experiments. $^*P<0.05$, $^{***}P<0.0005$.

of PGE_2 and $PGF_{2\alpha}$. The formation of other prostaglandins was not detectable.

In order to investigate whether the inhibitory activity of apocynin towards HHT and TxB_2 is also reflected in thromboxane-dependent cellular responses, we determined the effects of apocynin on arachidonic acid-induced platelet aggregation. Platelet aggregation induced by arachidonic acid is largely dependent on the conversion to TxA_2 [16]. The concentration range of apocynin used in this series of experiments was between 0.5 and 15 μg/ml (≈4–100 μM), much lower than in the metabolic studies. It can be seen from Fig. 3 that apocynin inhibited arachidonic acid-induced aggregation of bovine platelets in a concentration-dependent fashion. The estimated IC_{50} was about 15 μM. As a control, the cyclooxygenase inhibitor indomethacin (1 μM) totally inhibited platelet aggregation (results not shown).

4. DISCUSSION

Our present results indicate that apocynin may be involved in the anti-inflammatory properties of root extracts of *P. kurroa* not only via an effect on oxygen radical production by inflammatory cells [4], but also via an effect on the generation of arachidonic acid-derived inflammatory mediators. Thus, apocynin inhibited the formation of the pro-inflammatory compound TxA_2, whilst it enhanced the formation of the anti-inflammatory PGE_2. This opposite effect on TxA_2 and PGE_2 suggests that apocynin does not affect the cyclooxygenase enzyme, but may rather inhibit thromboxane synthetase. When TxA_2 synthesis would be inhibited by apocynin, one would expect accumulation of the PGH_2 intermediate to result in increased formation of other prostaglandins, including PGE_2 and $PGF_{2\alpha}$ as observed in our experiments. The inhibitory activity of apocynin towards TxA_2 formation in PM was not particularly potent, considering that total inhibition was only achieved at 3125 μg/ml (≈20 mM). Many other drugs have been shown to inhibit thromboxane synthetase with IC_{50} values of 10^{-6} M to 10^{-10} M [16]. However, our functional studies with bovine platelets demonstrate that apocynin may actually be effective at micromolar concentrations when looking at platelet aggregatory reponses. The difference in potency between inhibition of HHT/TxB_2 formation and inhibition of platelet aggregation can be explained in several ways. It is conceivable that the kinetics of apocynin action towards guinea pig PM are different from those of the drug towards bovine platelets, e.g. the uptake of the drug may be different in the two cell types. Also, it is possible that apocynin is degraded by reactive oxygen species produced by the stimulated PM. This is supported by the observation that apocynin is a scavenger of H_2O_2 (unpublished observation).

The effect of apocynin on the release of reactive oxygen species by neutrophils has been reported to be dependent on metabolic activation of the drug by reactive oxygen species formed through myeloperoxidase activity [4]. Since PM do not contain myeloperoxidase, we can conclude that apocynin either (i) directly affects the generation of arachidonic acid-derived mediators in PM, or (ii) is metabolically activated by reactive oxygen species which are not formed through myeloperoxidase activity. Future research will be needed to clarify this point.

The present results with apocynin suggest that, beside its therapeutic effects in inflammatory conditions [3] and possibly in asthmatic diseases [2], the drug may also be a valuable tool in the development of new anti-inflammatory or anti-thrombic drugs.

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