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Detection of Antiinflammatory Agents from Natural Products as Inhibitors of Cyclooxygenase I and II

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Abstract – Constitutive cyclooxygenase (COX-I) is present in cells under physiological conditions, whereas inducible cyclooxygenase (COX-II) is induced by some cytokines, mitogens, and endotoxin presumably in pathological conditions such as inflammation. We have evaluated the inhibitory effects of solvent fractionated extracts of natural products on the activities of COX-I and COX-II. Oxygen uptake COX assay was performed, as a primary screening from the tissue extracts of bovine seminal vesicles (BSV), by monitoring the initial rate of oxygen uptake using an oxygen electrode. Additionally, we evaluated plant extracts for the inhibitory effects of COX-I (in HEL cells) and COX-II (in lipopolysaccharide activated J774A.1 macrophages) using thin layer chromatography of prostanoids produced from ^{14}C-labelled arachidonic acid (AA). The use of such models of COX-I and COX-II assay will lead to the identification of specific inhibitors of cyclooxygenases with presumably less side effects than present therapies. Inhibitory effects of 50 kinds of plant extracts on the COX-I and COX-II activities were determined and the active fractions were found in the ethyl acetate fractions of *Dryopteris crassirhizoma* (roots), *Amomum cardamomum* (roots), *Triticum aestivum* (seeds), *Perilla sikokiana* (leaves), *Anemarrhena asphodeloides* (roots). Especially, the ethyl acetate fraction of *Dryopteris crassirhizoma* (roots), which exhibited the strong inhibition against BSV COX (IC_{50}, 65.4 μg/ml), COX-I (IC_{50}, 8.5 μg/ml), and COX-II (IC_{50}, 17.2 μg/ml), is under investigation to isolate active principles using activity-guided fractionation method.

Keywords – Cyclooxygenase I, Cyclooxygenase II, Inflammation, Antiinflammation, HEL cells, J774A.1 cells, Arachidonic acid, Lipopolysaccharide.

Introduction

Biosynthesis of the prostanoids such as prostaglandins, thromboxane and prostacyclin is enhanced by a widely divergent physical, chemical, and hormonal stimuli, and these prostanoids contribute importantly to the genesis of the signs and symptoms of inflammation (Moncada *et al.*, 1979). Upon membrane perturbation or cellular activation induced by trauma or biochemical stimulation, arachidonic acid (AA) is liberated from the phospholipid stores within the plasma membrane by the action of phospholipase A_2. Once liberated, AA readily metabolized by several enzymatic pathways into numerous biologically active mediators such as prostanoids. The first step in the biosynthesis of prostanoids is the oxygenation of AA to the hydroperoxy endoperoxide prostaglandin G_2. This reaction is a rate-limiting step and catalyzed by a heme containing prostaglandin endoperoxide synthase/cyclooxygenase (EC 1.14.99.1). Prostanoids, products

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of cyclooxygenase pathway of AA metabolism, possess potent vasoactive properties which account for some of the vascular leakage and tissue edema associated with inflammation. Prostanoids also play a significant role in the hyperalgesia which accompanies inflammation and can enhence bone resorption (Davies *et al.*, 1984, Dietrich *et al.*, 1975). Due to this central function in the AA cascade, COX is of physiological and pharmacological impor- tance in the regulation of prostanoids synthesis. COX exists in at least two isoforms with similar molecular weights. The isoforms of COX-I and COX-II are encoded by seperate genes and differ in their regulation and tissue distribution (Hla *et al.*, 1992). COX-I is expressed constitutively and was first characterized, purified and cloned from sheep vesicular glands (Van Der Ouderaa *et al.*, 1977). Activation of COX-I leads, for instance, to the production of prostacyclin, which is considered to be the target of antithrombogenesis of endothelium (Smith *et al.*, 1994) and it also plays a role in gastric cytoprotection (Whittle *et al.*, 1980). COX -II is induced in cells exposed to proinflammatory agents such as cytokines (Arias *et al.*, 1995), mitogens (Herschman *et al.*, 1993) and endotoxin (O' Sullivan *et al.*, 1992). Nonsteroidal antiinflammatory drugs (NSAIDs) inhibit the activity of COX, a property that accounts for their shared therapeutic and side effects (Vane, 1971). Thus, the ability of NSAIDs to inhibit COX-II may well explain their therapeutic utility as antiinflammatory drugs, whereas inhibition of COX-I by NS AIDs may explain their unwanted side effects, such as gastric and renal damage (Schlondorff, 1993). Therefore, we have evaluated the solvent fractionated extracts of natural products on the inhibitory effects against COX-I (in HEL cells) and COX-II (in LPS activated J774A.1 macrophages) in intact cell systems and in tissue extract system (COX-I in BSV). The use of this model systems for COX-I and COX-II will lead to the identification of selective inhibitors of cyclooxygenases with presumably less side effects than present therapies.

Experimental

Instrumentations and reagents – COX activity of BSV was measured by YSI model 5300 biological oxygen monitor equipped with a Clarke-type electrode (Yellow Springs Instrument Co., Inc., USA). BSV was homogenized with a electric blender (Biospec Products Inc., Switzerland). The radioactivities of prostanoid bands were scanned by BAS-1500 bio-imaging analyzer (Fuji Photo Film Co., Ltd., Japan). Autoradiography was performed by placing the TLC plate in contact with Fuji BAS-IIIs imaging plate (Fuji Photo Film Co., Ltd., Japan). Radiolabelled prostanoids were applied to TLC aluminium sheets silica gel 60W (E. Merck, Germany). [1-^{14}C]arachidonic acid (56 mCi/mmol), 5-S-[5,6,8,9,11,12,14,15-^{3}H(N)]hydroxy-6,8,11, 14-eicosatetraenoic acid (182 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H(N)]thromboxane B_2 (152 Ci/mmol), [5,6,8,9,12,14,15-^{3}H(N)]prostaglandin D_2 (115 Ci/mmol), [5,6,8,11,12,14,15-^{3}H(N)]prostaglandin E_2 (171 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H(N)]prostaglandin $F_{2\alpha}$ (170 Ci/mmol) were purchased from DuPont Company, USA. Arachidonic acid, indomethacin, aspirin, hematin, lipopolysaccharide and calcium ionophore A 23187 were purchased from Sigma Chem. Co., USA.

Plant materials – Plant materials were purchased from a herb market in Seoul, and voucher specimens have been deposited at Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Each of dried plant parts were sliced, and then extracted 3 times with methanol at room temperature. The methanol extracts were concentrated under reduced pressure below 40℃, and then the concentrated methanol extracts were partitioned into n-hexane, ethyl acetate, and water layers.

Cells and cell culture – The human erythroleukemia cell line (HEL) and the mouse macrophage cell line (J774A.1) were purchased from the American Type Culture Collection. Cells were grown at 37℃ in a humidified atmosphere (5% CO_2) in RPMI 1640 medium containing 5% fetal bovine serum.

Preparation of COX from BSV – All procedures were carried out between 0 and 4℃. Deep-frozen BSVs (-70℃) were homogenized for 30 sec with a electric blender in a homogenizing buffer containing 0.05 M Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM diethyldithiocarbamate (DDC). The homogenate was centrifuged for 30 min at 13,000 g, and then the supernatant was centrifuged for 60 min at 130,000 g. The pellets were re-homogenized, using a electric blender, in homogenizing buffer containing 0.05 M Tris-HCl (pH 8.0), 0.1 M sodium perchlorate, 1 mM EDTA and 0.1 mM DDC, and centrifuged again for 60 min at 130,000 g. The perchlorate-washed microsomes were then homogenized in a buffer of 0.05 M Tris-HCl (pH 8.0), 0.1 mM EDTA and 1% Tween-20. The Tween-20 homogenate was centrifuged for 90 min at 130,000 g. The clear supernatant was used as the enzyme source of COX (Van Der Ouderaa *et al.*, 1977). Protein contents of the enzyme preparations were determined by Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as a standard. Specific activity of the resulting enzyme preparation was about 500 units/mg, where one unit of COX activity is defined as that amount of enzyme which will catalyze the uptake of 1 nmol of oxygen/min/ml of reaction mixture at 37℃.

Oxygen uptake cox assay – The cox activity of prostaglandin endoperoxide synthase was estimated by measuring the maximal rate of oxygen uptake in the presence of enzyme and AA by using YSI model 5300 biological oxygen monitor equipped with a Clarke-type electrode (DeWitt *et al.*, 1990). Enzyme reaction was initiated by adding an aliquot of the enzyme solution (ca. 50 μg protein) to a reaction mixture of 1 ml of 0.2 M sodium phosphate buffer (pH 7.4), 3 μM hematin, 0.4 mM hydroquinone, 0.3 mM AA and a designated concentration of test samples. The velocity was determined from a tangent value to the linear portion of the oxygen uptake curve. The inhibitory effects of test samples on BSV COX-I were indicated by the initial rate of oxygen uptake compared with that of DMSO control (% of control).

Radiometric COX-I assay – Cultured HEL cells were plated at 2×10^6 cells per well in a 24-well microtiter plate and incubated for 24 hr at 37℃ in a humidified atmosphere (5% CO_2) with the test samples or vehicle solvent (final 0.5% DMSO). Cultured 24-well microtiter plate was centrifuged for 10 min at 3,000 g. After the supernatant was poured out, the pellet was mixed with phosphate-buffered saline (PBS, pH 7.4). After a further centrifugation, the supernatant was poured out and resuspended in 200 μl PBS. The resuspended cells were incubated for 10 min at 37℃ in the presence of 1 μM calcium ionophore A23187 and [1-^{14}C]AA solution (0.05 μCi) and acidified with 10 μl of 1 M citric acid to pH 3.5. After shaking the 24-well plate, 200 μl of reaction mixture was transferred to microcentrifuge tube and extracted twice with 500 μl of ethyl acetate to isolate the metabolic products of cyclooxygenase. Ethyl acetate layers were applied to aluminium foil-backed silica gel TLC sheets, and then chromatographed for 90 min at room temperature in a solvent containing chloroform-methanol-acetic acid-water (v/v, 90:10:1:1). Authentic prostanoid standards were co-developed in the same condition. For detection of ^{14}C-labelled prostanoids, autoradiography was performed by placing the TLC plate in contact with Fuji BAS-IIIs imaging plate for 24 hr (Najid *et al.*, 1992). The imaging plate was scanned by BAS-1500 bio-imaging analyzer and then radioactivities of prostanoid bands were quantified. The inhi-

bitory effects of test samples on COX-I activity were indicated by the amount of ^{14}C-labelled prostanoids produced compared with that of DMSO control (% of control).

Radiometric COX-II assay – J774A.1 macrophages were cultured in 150 cm^2 tissue culture flask until confluent. Cultured J 774A.1 macrophages were plated at 2×10^5 cells per well in a 24-well microtiter plate and preincubated for 24 hr at 37℃ in a humidified atmosphere (5% CO_2). The basal release of prostanoids from untreated J774A. 1 macrophages was blocked by pretreatment of aspirin solution (final concentration, 0.4 μg/ml) so as to exclude basal COX-I effect. Aspirin pretreated cells were added with test samples (final 0.5% DMSO) and treated with LPS (2.5 μg/ml) for 18 hr to induce COX-II (O'Sullivan *et al.*, 1992). The inhibitory effects of test samples against COX-II were determined by radiometric COX-I assay method as described above.

Results

In order to investigate the antiinflammatory effects of some natural products, we evaluated these samples using different assay systems for COX-I and COX-II. As shown in Fig. 3 (Lane D), HEL cells, known from various studies to host only COX-I (Funk *et al.*, 1991; Tanabe *et al.*, 1993), express COX-I activity and COX-II activity was not induced by LPS (data not shown) in HEL cells. In case of J774A.1 macrophages, COX-II activity was greatly induced by LPS (2.5 μg/ml) as shown Fig. 4A (Lane 3). LPS-untreated J 774A.1 macrophages contained no COX-II activity and low levels of COX-I activity (Fig. 4A, Lane 1). The basal release of prostanoids from untreated J774A.1 macrophages was blocked by pretreatment of aspirin solution (final concentration, 0.4 μg/ml) so as to exclude basal COX-I effect (Fig. 4A, Lane 2). With the use of these cells as models for COX-I and COX-II assay systems and semi-

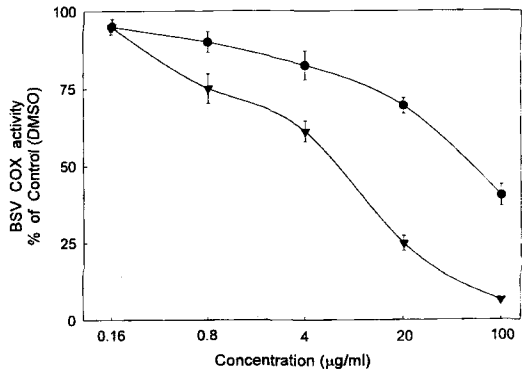

Fig. 1. Inhibitory effects of indomethacin (▼) and the ethyl acetate fraction of the root of *Dryopteris crassirhizoma* (●) against BSV COX-I. Data are expressed as mean±S.D. from triplicate determinations at each concentration.

purified enzymes from BSV for COX-I assay system, we evaluated the plant extracts on the inhibitory effects aganist COX-I and COX-II. Table 1 and Fig. 1 show data for the dose-dependent inhibition of COX-I in BSV by ethyl acetate fractions of *Dryopteris crassirhizoma* (roots; IC_{50}, 65.4 μg/ml) and *Triticum aestivum* (seeds; IC_{50}, 79.4 μg/ml). Indomethacin, a positive control, shows the dose-dependent inhibition (IC_{50}, 6.2 μg/ml) of BSV COX-I (Fig. 1, Table 2). Ethyl acetate fractions of *Dryopteris crassirhizoma* (roots; IC_{50}, 8.5 μg/ml), *Amomum cardamomum* (roots; IC_{50}, 16.5 μg/ml), *Anemarrhena asphodeloides* (fruits; IC_{50}, 12.1 μg/ml) and *Perilla ikokiana* (leaves; IC_{50}, 20.0 μg/ml) exhibited dose dependent inhibition of COX-I activity in HEL cells (Table 1, Fig. 2, Fig. 3). In this radiometric COX-I assay, the enzyme activity was measured by the formation of thromboxane B_2 after incubation with exogenous [1-^{14}C]AA for 10 min and the inhibitory effects of aspirin and indomethacin were determined (Table 2). With this assay system, indomethacin (IC_{50}, 5.0 ng/ml) was 30 times more potent than aspirin (IC_{50}, 0.16 μg/ml) against COX-I activity in HEL cells. In order to investigate the inhibitory effects of test samples against COX-II, the formation of

Table 1. Inhibitory effects of plant extracts on the activities of COX-I (in HEL cells) and COX-II (in LPS activated J774A.1 macrophages) in intact cells and semi-purified enzyme preparations (COX in bovine seminal vesicles).

Scientific name / Family	Plant parts	Inhibition of COX activity (IC_{50}, μg/ml)								
		BSV COX			HEL COX-I			J774A.1 COX-II		
		Hexane	EtOAc	Water	Hexane	EtOAc	Water	Hexane	EtOAc	Water
Acanthopanax senticosus/Araliaceae	Rb	>100	>100	>100	>20	>20	>20	>20	>20	>20
Achyranthes bidentata/Amaranthaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Aconitum kusnezoffii/Ranunculaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Albizzia julibrissin/Leguminosae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Alisma orientale/Alismataceae	Bk	>100	>100	>100	>20	>20	>20	>20	>20	>20
Amomum cardamomum/Zingiberaceae	Rt	>100	>100	>100	>20	16.5	>20	>20	15.7	>20
Anemarrhena asphodeloides/Liliaceae	Fr	>100	>100	>100	>20	12.1	>20	>20	20.0	>20
Angelica gigas/Umbelliferae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Artemisia argyri/Compositae	Lf	>100	>100	>100	>20	>20	>20	>20	>20	>20
Atractylodes japonica/Compositae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Chrysanthemum sinese/Compositae	Fl	>100	>100	>100	>20	>20	>20	>20	>20	>20
Cnidium officinale/Umbelliferae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Cucumis melo var. markuwa/Cucurbitaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Dictammus dasycarpus/Rutaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Dryopteris crassirhizoma/Polypodiaceae	Rt	>100	65.4	>100	>20	8.5	>20	>20	17.2	>20
Epimedium koreanum/Berberidaceae	Lf, St	>100	>100	>100	>20	>20	>20	>20	>20	>20
Evodia officinalis/Rutaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Gardenia jasminoides/Rubiaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Gastrodia elata/Orchidaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Gentiana scabra var. buergeri/Oganiaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Gleditschia japonica/Leguminosae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Kalopanax pictum/Araliaceae	Bk	>100	>100	>100	>20	>20	>20	>20	>20	>20
Kochia scoparia/Chenopodiaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Ligusticum delavayi/Umbelliferae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Ligustrum japonicum/Oleaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Liriope platyphylla/Liliaceae	Tu	>100	>100	>100	>20	>20	>20	>20	>20	>20
Lonicera japonica/Caprifoliaceae	Fl	>100	>100	>100	>20	>20	>20	>20	>20	>20
Lycium chinense/Solanaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Lysimachia foenum graecum/Primulaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Nelumbo nucifera/Nymphaeaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Orostachys japonicus/Orobanchaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20

Table 1. Continued.

Scientific name / Family	Plant parts	Inhibition of COX activity (IC_{50}, μg/ml)								
		BSV COX			HEL COX-I			J774A.1 COX-II		
		Hexane	EtOAc	Water	Hexane	EtOAc	Water	Hexane	EtOAc	Water
Pachyma hoelen/Polyporaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Perilla sikokiana/Labiatae	Lf	>100	>100	>100	>20	20.0	>20	>20	>20	>20
Phlomis umbrosa/Labiatae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Plantago asiatica/Plantaginaceae	Sd	>100	>100	>100	>20	>20	>20	>20	>20	>20
Platycodon grandiflorum/Campanulaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Poncirus trifoliata/Rutaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Prunella vulgaris var Liachina/Labiatae	Fl	>100	>100	>100	>20	>20	>20	>20	>20	>20
Prunus armeniaca/Hamamelidaceal	Sd	>100	>100	>100	>20	>20	>20	>20	>20	>20
Pueraria thunbergiana/Leguminosae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Sanguisorba officinalis/Rosaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Saussurea lappa/Compositae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Seseli mairei/Umbelliferae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Scrophularia ningpoensis/Scrophulariaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Taraxacum platycarpum/Compositae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Trichosanthes kirilowii/Cucurbitaceae	Rt	>100	>100	>100	>20	>20	>20	>20	>20	>20
Triticum aestivum/Gramineae	Sd	>100	79.4	>100	>20	>20	>20	>20	>20	>20
Typha orientalis/Typhaceae	Fl	>100	>100	>100	>20	>20	>20	>20	>20	>20
Xanthium strumarium/Compositae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20
Zanthoxylum bungeanum/Rutaceae	Fr	>100	>100	>100	>20	>20	>20	>20	>20	>20

Abbreviations are bark (Bk), flower (Fl), fruits (Fr), leaves (Lf), root bark (Rb), roots (Rt), seeds (Sd), stem (St) and tuber (Tu). IC_{50} values based on tests at 5 different concentrations; each in triplicate. Inhibition of COX-I and COX-II were determined in terms of the production of thromboxane B_2 and prostaglandin D_2, respectively.

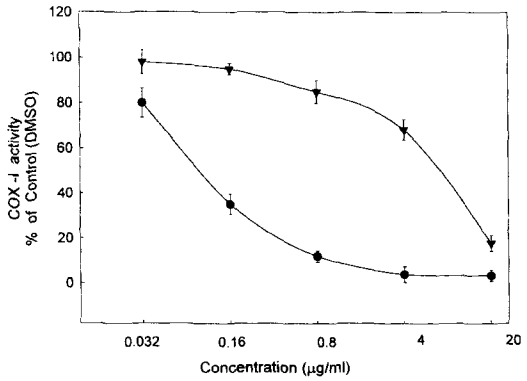

Fig. 2. Inhibitory effects of aspirin (●) and the ethyl acetate fraction of the root of *Dryopteris crassirhizoma* (▼) against COX-I in HEL cells. Data are expressed as mean ± S.D. from triplicate determinations at each concentration. Inhibition of COX-I was determined in terms of the production of thromboxane B_2.

Table 2. Effects of aspirin, indomethacin, dexamethasone and *Dryopteris crassirhizoma* (roots) on cyclooxygenases activities

	Inhibition of COX activity (IC_{50}, μg/ml)			Relative selectivity value
	BSV COX	HEL COX-I	J774A.1 COX-II	(IC_{50}, COX-I/ IC_{50}, COX-II)
Aspirin	>100	0.16	12.0	0.001
Indomethacin	6.2	0.05	0.1	0.05
Dexamethasone	>100	>20	25.0	>0.8
Dryopteris crassirhizoma	65.4	8.5	17.2	0.49

IC_{50} values were determined using 5 different concentrations in triplicate. Inhibition of COX-I and COX-II were determined in terms of the production of thromboxane B_2 and prostaglandin D_2, respectively.

prostaglandin D_2 was used as an indicator of COX-II activity in LPS-activated J774A.1 macrophages. As a result of the radiometric COX-II assay, ethyl acetate fractions of *Dryopteris crassirhizoma* (roots; IC_{50}, 17.2 μg/ml), *Amomum cardamomum* (roots; IC_{50}, 15.7 μg/ml) and *Anemarrhena asphodeloides* (fruits; IC_{50}, 20.0 μg/ml) exhibited dose dependent inhibition on COX-II activity in LPS-activated J774A.1 macrophages (Table 1, Fig. 4). Inhibitory effects of dexamethasone, aspirin and indomethacin against COX-II were also determined (Table 2) and indomethacin (IC_{50}, 0.1 μg/ml) was the most potent inhibitor of COX-II and was about 120 times more potent than aspirin (IC_{50}, 12.0 μg/ml).

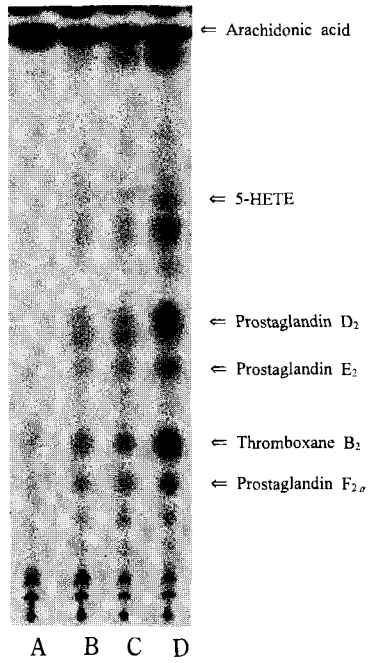

Fig. 3. Autoradiographed TLC-chromatogram of inhibitory effects against COX-I by the ethyl acetate fractions of *Dryopteris crassirhizoma* (roots) and *Anemarrhena asphodeloides* (fruits) in HEL cells. For detection of ^{14}C-labelled prostanoids, autoradiography was performed by placing the TLC plate in contact with Fuji BAS-IIIs imaging plate for 24h and the imaging plate was scanned by BAS-1500 bio-imaging analyzer. Lane A, aspirin (2.0 μg/ml); Lane B: ethyl acetate fraction of *Dryopteris crassirhizoma* (roots, 20.0 μg/ml); Lane C, ethyl acetate fraction of *Anemarrhena asphodeloides* (fruits, 20.0 μg/ml); Lane D, DMSO (control)

Discussion

The discovery of an inducible isoform of prstaglandin H synthase (COX-II) raised the possibility that the constitutive enzyme (COX-I) was responsible for many of the protective features of prostanoids, while prostanoids generated from the induced enzyme (COX-II) largely participate in pathological, inflammatory conditions (Griswold *et al.*, 1996).

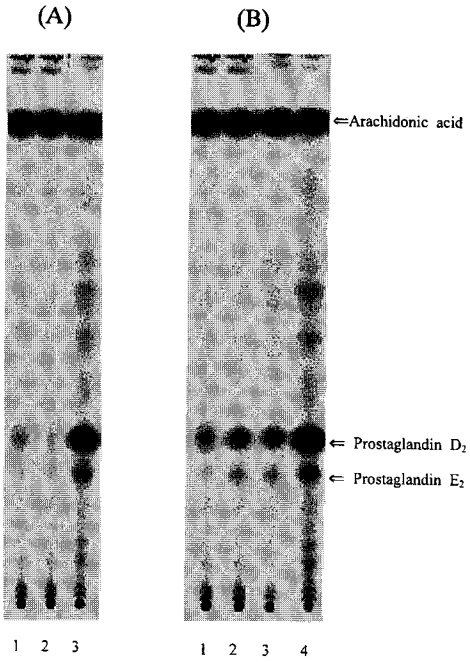

Fig. 4. Autoradiographed TLC-chromatogram of COX-II induction (A) and inhibitory effects against COX-II by the ethyl acetate fractions of *Dryopteris crassirhizoma* (roots) and *Amomum cardamomum* (roots) in J774A.1 macrophages (B). For detection of ^{14}C-labelled prostanoids, autoradiography was performed by placing the TLC plate in contact with Fuji BAS-IIIs imaging plate for 24h and the imaging plate was scanned by BAS-1500 bio-imaging analyzer. Induction of COX-II was achieved by the stimulation of J 774A.1 cells with LPS (2.5 μg/ml) for 18 hr. The basal release of prostanoids from untreated J774A.1 macrophages was blocked by pretreatment of aspirin (0.4 μg/ml) so as to exclude basal COX-I effect. (A) Lane 1, untreated cells (COX-I activity); Lane 2, cells were preincubated with aspirin (0.4 μg/ml); Lane 3, cells were preincubated with both aspirin (0.4 μg/ml) and LPS (2.5 μg/ml). (B) Lane 1, indomethacin (0.5 μg/ml); Lane 2, ethyl acetate fraction of *Dryopteris crassirhizoma* (roots, 20.0 μg/ml); Lane 3, ethyl acetate fraction of *Amomum cardamomum* (roots, 20.0 μg/ml); Lane 4, DMSO (control).

Practically, COX-II is induced in migr- atory and other cells by inflammatory stimuli presumably through cytokine production. The facts that both of these two types of enzymes are inhibited by NSAIDs attractively suggested that the antiinflammatory actions of the NSAIDs are due to inhibition of COX-II, whereas the unwanted side effects such as gastric toxicity and nephrotoxicity are due to inhibition of the COX-I (Griswold *et al.*, 1996).

Aspirin, indomethacin and dexamethasone, which were used as standard compounds, were evaluated with these systems and they were much less active against COX-II than against COX-I (Table 2). Indomethacin was about 1,200 times more active as an inhibitor of COX-I in intact cells (HEL cells) than as an inhibitor of purified BSV COX-I. It is interesting that the inhibitory effect of dexamethasone against COX-II (25.0 μg/ml) is much lower than that of aspirin and indomethacine (12.0 and 0.1 μg/ml, respectively) with this system. It was reported that dexamethasone affects the prostanoids synthesis potently by the inhibiton of COX-II expression without affecting COX-I synthesis or activity (Masferrer *et al.*, 1994). Although the cellular mechanism involved in this glucocorticoid effect is unclear, another report showed that dexamethasone did not inhibit the transformation of exogeneously supplied AA into prostanoids, either by the untreated or mitogen-induced cell line (Kujubu *et al.*, 1992). In consistent with the above report, our results showed that COX-II activity in exogeneously supplied [1-^{14}C]AA source was weakly inhibited ($\approx$35%) by the high concentration of dexamethasone used (20 μg/ml). However, COX-I activity was not inhibited at 20 μg/ml concentration of dexamethasone in HEL cells (Table 2).

In summary, we observed that two NS AIDs, aspirin (relative selectivity value: IC_{50}, COX-I/IC_{50}, COX-II, 0.013) and indomethacin (relative selectivity value, 0.05) represented poor selectivity for COX-II but the ethyl acetate fraction of *Dryopteris crassirhizoma* showed high selectivity for COX-II (relative selectivity value, 0.49) as compared to those NSAIDs (Table 2). Therefore, the ethyl acetate fraction of *Dryopteris crassirhizoma*

(roots), which exhibited the strong inhibition against BSV COX, COX-I (HEL cells) and COX-II (J774A.1 macrophages), is under investigation to isolate active principles using activity-guided fractionation method. On the basis of these results, the use of COX-I and COX-II assay systems will lead to the identification of selective inhibitors of cyclooxygenases and these active principles can to be used as effective antiinflammatory agents devoid of the side effects associated with the inhibition of COX-I and thus can provide a significant improvement to current therapies.

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References

Arias, N. S., Keller, K. and Chadee, K., Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. *Biochem. Biophys. Res. Commun.*, **17**, 582-589 (1995).

Davies, P., Bailey, P. J., Gordenberg, M. M. and Ford-Hutchinson, A. W., The role of arachidonic acid oxygenation products in pain and inflammation. *Annu. Rev. Immunol.*, **2**, 335-357 (1984).

DeWitt, D. L., El-Harith, E. A. Kraemer, S. A. and Andrews, M. J. The aspirin and heme-binding sites of bovine and murine prostaglandin endoperoxide synthase. *J. Biol. Chem.*, **265**, 5192-5198 (1990).

Dietrich, J. W., Goodson, J. M. and Raisz, L. G., Stimulation of bone resorption by various prostaglandins in organ culture. *Prostaglandins*, **10**, 231-238 (1975).

Funk, C. D., Funk, L. B., Matthew, E. K., Amy, S. P. and Garret, A. F., Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.*, **5**, 2304-2312 (1991).

Griswold, D. E. and Adams, J. L., Constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2): Rational for selective inhibition and progress to date. *Med. Res. Rev.*, **16**, 181-206 (1996).

Herschman, H., Fletcher, B. and Kujubu, D., TIS10, A mitogen-inducible glucocorticoid-inhibited gene that encodes a second prostaglandin synthase/cyclooxygenase enzyme. *J. Lipid Med.*, **6**, 89-99 (1993).

Hla, T. and Neilson, K., Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA*, **89**, 7384-7388 (1992).

Kujubu, D. A. and Herschman, H. R., Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene. *J. Biol. Chem.*, **267**, 7991-7994 (1992).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).

Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leathy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K., Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. USA*, **91**, 3228-3232 (1994).

Moncada, S. and Vane, J. R., Pharmacology and endogeneous roles of prostaglandin endoperoxides, thromboxane A2 and prostacyclin. *Pharmacol. Rev.*, **30**, 293-331 (1979).

Najid, A., Simon, A., Cook, J., Chable-Rabinovitch, H., Delage, C., Chulia, A. J. and Rigaud, M., Characterization of ursolic acid as a lipoxygenase and cyclooxygenase inhibitor using machrophages, platelets and differentiated HL60 leukemic cells. *FEBS*, **3**, 213-217 (1992).

O'Sullivan, M. G., Huggins, Jr., E. M., Meade, E. A., DeWitt, D. L. and McCall, C. E., Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem. Biophys. Res. Commun.*, **187**, 1123-1127 (1992).

Schlondorff, D., Renal complications of nonsteroidal anti-inflammatory drugs. *Kidney Int.*, **44**, 643-653 (1993).

Smith, W. L. and DeWitt, D. L. Differential interactions of prostaglandin endoperoxide synthase with nonsteroidal anti-inflammatory drugs. *Curr. Opin. Invest. Drugs*, **3**, 1-11 (1994).

Tanabe, T., Yokoyama, C., Miyata, A., Ihara, H., Ko-

saka, T., Suzuki, K., Nishikawa, Y., Yoshimoto, T., Yamamoto, S., Nusing, R. and Ullrich V., Molecular cloning and expression of human thromboxane synthase. *J. Lipid Med.*, **6**, 139-144 (1993).

Van Der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H. and Van Dorp, D. A., Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim. Biophys. Acta,* **487**, 315-331 (1977).

Vane, J. R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, *Nature,* **231**, 232-235 (1971).

Whittle, B. J. R., Higgs, G. A., Eakins, K. E., Moncada, S. and Vane, J. R., Selective inhibition of prostaglandin production in inflammatory exudates and gastric mucosa. *Nature,* **284**, 271-273 (1980).

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