Differential metabolism of dihomo-γ-linolenic acid and arachidonic acid by cyclo-oxygenase-1 and cyclo-oxygenase-2: implications for cellular synthesis of prostaglandin E_1 and prostaglandin E_2

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Prostaglandin (PG) E_1 has been shown to possess anti-inflammatory properties and to modulate vascular reactivity. These activities are sometimes distinct from those of PGE_2, suggesting that endogenously produced PGE_1 may have some beneficial therapeutic effects compared with PGE_2. Increasing the endogenous formation of PGE_1 requires optimization of two separate processes, namely, enrichment of cellular lipids with dihomo-γ-linolenic acid (20:3 *n*–6; DGLA) and effective cyclo-oxygenase-dependent oxygenation of substrate DGLA relative to arachidonic acid (AA; 20:4 *n*–6). DGLA and AA had similar affinities (K_m values) and maximal reaction rates (V_{max}) for cyclo-oxygenase-2 (COX-2), whereas AA was metabolized preferentially by cyclo-oxygenase-1 (COX-1). To overcome the kinetic preference of COX-1 for AA, CP-24879, a mixed Δ^5/Δ^6 desaturase inhibitor, was used to enhance preferential accumulation of DGLA over AA in cells cultured in the presence of precursor γ-linolenic acid (18:3 *n*–6). This protocol was tested in two cell lines and both yielded a DGLA/AA ratio of approx. 2.8 in the total cellular lipids. From the enzyme kinetic data, it was calculated that this ratio should offset the preference of COX-1 for AA over DGLA. PGE_1 synthesis in the DGLA-enriched cells was increased concurrent with a decline in PGE_2 formation. Nevertheless, PGE_1 synthesis was still substantially lower than that of PGE_2. It appears that employing a dietary or a combined dietary/pharmacological paradigm to augment the cellular ratio of DGLA/AA is not an effective route to enhance endogenous synthesis of PGE_1 over PGE_2, at least in cells/tissues where COX-1 predominates over COX-2.

Key words: Δ^5 desaturase, Δ^6 desaturase, eicosanoids, fatty acid desaturase inhibitor.

INTRODUCTION

Alteration in the dietary content of fatty acids can lead to modulation of the structure/function of membrane-bound receptors, cell–cell interaction, enzyme activities, cellular signalling and eicosanoid production [1–4]. Eicosanoid synthesis is dependent on the size of the fatty acid precursor pool(s) [5–7] and on the availability of substrate fatty acids released from phospholipids [8]. Arachidonic acid (AA; 20:4 *n*–6) is a major long-chain polyunsaturated fatty acid in mammalian cell membrane phospholipids and a precursor for a plethora of oxygenated products, including the 2-series prostaglandins. Although the 2-series prostaglandins are generally thought of as being pro-inflammatory [9], anti-inflammatory properties have also been ascribed to them; prostaglandin (PG) E_2 may exert its anti-inflammatory effects by suppressing pro-inflammatory cytokine and lymphokine production by lymphocytes and Th1 cells respectively, and by promoting Th2 development [10–14]. The C_{20} polyunsaturated fatty acid, dihomo-γ-linolenic acid (DGLA; 20:3 *n*–6), is also a substrate for eicosanoid production and yields prostaglandins of the 1-series (e.g. prostaglandin E_1, PGE_1) [15], which are generally viewed as possessing anti-inflammatory properties. The question of whether PGE_1 and PGE_2 act on the same or different receptors is not yet resolved. Some reports provide data to indicate that PGE_1 binds to the PGE_2 receptors EP2 and EP4 [16], whereas data from earlier reports suggest the presence of separate PGE_1 and PGE_2 receptors [17] or even two distinct receptors for PGE_1 [18].

There is substantial evidence to suggest that overproduction of AA-derived eicosanoids, but not DGLA-derived eicosanoids, may play a detrimental role in atherothrombotic, inflammatory and autoimmune diseases. Unlike AA, which gives rise to thromboxane A_2, a potent platelet aggregator and vasoconstrictor, DGLA does not give rise to a thomboxane [15]. PGE_1 is distinct from PGE_2 in that it stimulates cAMP production more effectively than PGE_2 [10,19,20] and, thereby, could manifest enhanced cAMP-mediated downstream signalling. Additionally, DGLA-derived PGE_1 has been identified as possessing anti-inflammatory properties that differentiate it from AA-derived PGE_2. (1) PGE_1 was shown to be more powerful than PGE_2 in exerting anti-inflammatory effects in the rat adjuvant arthritis model [21,22] and in the mouse Lupus model [23]. (2) PGE_1 was found to be more potent than PGE_2 as a vasodilator [24] as well as a suppressor of synoviocyte proliferation [2,25]. (3) PGE_1, but not PGE_2 or $PGF_{2\alpha}$, was found to inhibit collagenase activity [19]. (4) In studies with human platelets, PGE_1 was shown to inhibit platelet aggregation *in vitro*, whereas PGE_2 at the same concentration either did not affect aggregation [26] or sometimes

Abbreviations used: AA, arachidonic acid; COX-1, cyclo-oxygenase-1; DGLA, dihomo-γ-linolenic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; LL, Lewis lung; LPS, lipopolysaccharide; PG, prostaglandin; NSAID, non-steroidal anti-inflammatory drug.

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enhanced it [27]. The anti-aggregatory effect of PGE_1 suggests that it could be a more potent anti-atherosclerotic agent compared with PGE_2. This suggestion was corroborated in studies in which PGE_1 was shown to inhibit vascular smooth-muscle-cell proliferation [28]. Furthermore, the major PGE_1 metabolite in blood, 13,14-dihydro-PGE_1, is relatively stable in blood and retains most of the anti-aggregatory activity [27], thus rendering PGE_1 as an overall longer acting anti-aggregatory agent [29].

Some of the biological effects of PGE_1 could be demonstrated *in vivo* by feeding the precursor dietary fatty acids, GLA (γ-linolenic acid) or DGLA. GLA or DGLA was shown to suppress inflammation and joint tissue injury in several animal models [23]. Furthermore, a GLA-enriched diet, but not a linoleic-enriched diet, was shown to cause subsequent inhibition of isolated vascular smooth-muscle-cell proliferation [4]. In humans, GLA supplementation produced a clear anti-inflammatory effect, as evidenced by decreased synthesis of LTB_4 in neutrophils [30]. However, the mechanism of action mediating these dietary GLA effects was not fully elucidated, because feeding of GLA or DGLA [as ethyl esters or triglycerides (triacylglycerols)] generally led to only a small increase in GLA or DGLA content in cell membrane lipids, often accompanied by a very significant increase in AA content. Furthermore, the ratio of PGE_1/PGE_2 produced in cells obtained from animals fed with GLA-containing oils was shown to be substantially lower than the cellular ratio of DGLA/AA [2]. Similarly, in studies with cultured mouse fibrosarcoma cells rendered deficient in essential fatty acids and then replenished with either DGLA or AA, the ratio of synthesized PGE_1/PGE_2 was considerably smaller than the cellular ratio of DGLA/AA [8]. It should, however, be pointed out that in both the *in vivo* dietary study [2] and the cell culture study [8], the cellular content of AA, even after significant enrichment with DGLA, was still 2.5–3-fold higher than DGLA, mainly due to effective desaturation of DGLA to AA.

The current study was conducted with two principal aims: first, to further elucidate the cellular determinants controlling the relative production of PGE_1 versus PGE_2 in cells and, secondly, to examine whether a dietary paradigm to increase PGE_1 synthesis at the expense of PGE_2 synthesis is viable. DGLA and AA were first compared as substrates for purified recombinant human cyclo-oxygenases-1 (COX-1) and -2 (COX-2). The mixed Δ^5/Δ^6 fatty-acid desaturase inhibitor CP-24879 was then utilized to assess whether substantial fatty-acid remodelling (i.e. DGLA/AA ratio > 2) would lead to preferential synthesis of PGE_1 over PGE_2.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline and RPMI-1640 culture media, lipopolysaccharide (LPS, type 055:B5), calcium ionophore A23187, anti-PGE_2 antisera, and Norit A-activated charcoal were purchased from Sigma. Foetal calf serum (FCS), non-essential amino acids, glutamine, trypsin/EDTA, antibiotic–antimycotic solution (penicillin, streptomycin and nystatin) were purchased from Biological Industries Co. (Kibbutz Beit Haemek, Israel). [^{3}H]PGE_2 (50 Ci/mmol) was purchased from Amersham International (Little Chalfont, Bucks., U.K.). Authentic fatty acids (AA, DGLA and GLA) were purchased from Nu-Chek Prep Inc. (Elysian, MN, U.S.A.). All solvents and reagents were of analytical grade. The mixed Δ^5/Δ^6 fatty-acid desaturase inhibitor CP-24879 was described previously [31]. This compound and SC-560 were gifts from Pharmacia (St Louis, MO, U.S.A.).

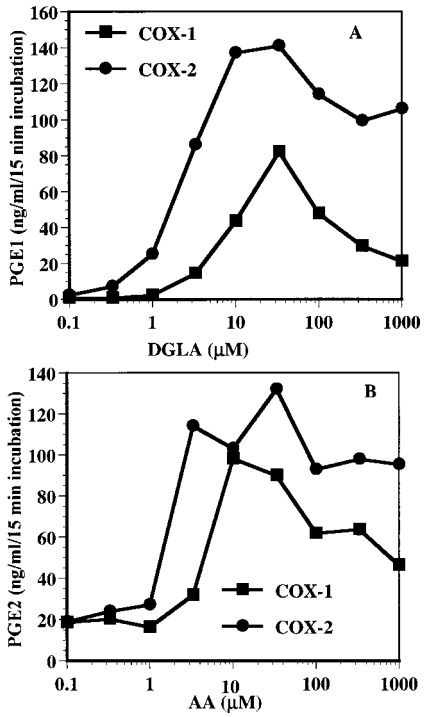

Figure 1 Concentration-dependent generation of PGE_1 (A) and PGE_2 (B), from DGLA and AA respectively, by recombinant hCOX-1 and hCOX-2

Recombinant hCOX-1 and hCOX-2 were incubated for 15 min at 37 °C with DGLA and AA at concentrations between 0.1 μM and 1 mM. The media were then extracted and PGE_1 and PGE_2 quantified by HPLC–mass spectrometry. Data are from a single experiment that was repeated twice with similar results.

Cell culture

Mouse Lewis lung (LL) carcinoma cells, variant 3LL-D122, were described previously [32]. The cells were kindly provided by Dr Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The cells were cultured in T-75 flasks in DMEM + 10 % FCS, 4 % 2 mM L-glutamine, 1 % penicillin–streptomycin solution and 2 % non-essential amino acids. HL-60 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were grown in RPMI-1640 + 10 % FCS, 1 % 2 mM L-glutamine and 1 % penicillin–streptomycin. All cells were maintained in a humidified 37 °C incubator with 5 % CO_2.

Fatty-acid incorporation into cells

Cells were seeded at 5×10^5 ml^{-1} in T-75 flasks and incubated for 72 h in the presence of 10 μM fatty acid $\pm$ 3.75 μM CP-24879. Stock solutions (10 mM) of GLA, DGLA or AA were prepared by evaporating a part of the fatty acid (ethanolic solution) to dryness with a stream of nitrogen and immediately dissolving it in 18.2 mM Na_2CO_3 (representing a 10-fold molar excess of Na^+ ions) to convert the fatty acids into their respective sodium salt. These fatty acid–sodium salt stock solutions were added to the cultured cells to yield a final concentration of 10 μM. CP-24879 was prepared in DMSO at a concentration of 3.75 mM and diluted 1000-fold in the culture medium. Control flasks received DMSO.

Assay of cyclo-oxygenase activity in cultured cells

Cells (5×10^5 cells/well) were seeded in 6-well plates and cultured for 48 h. The cells were then washed with PBS and incubated in

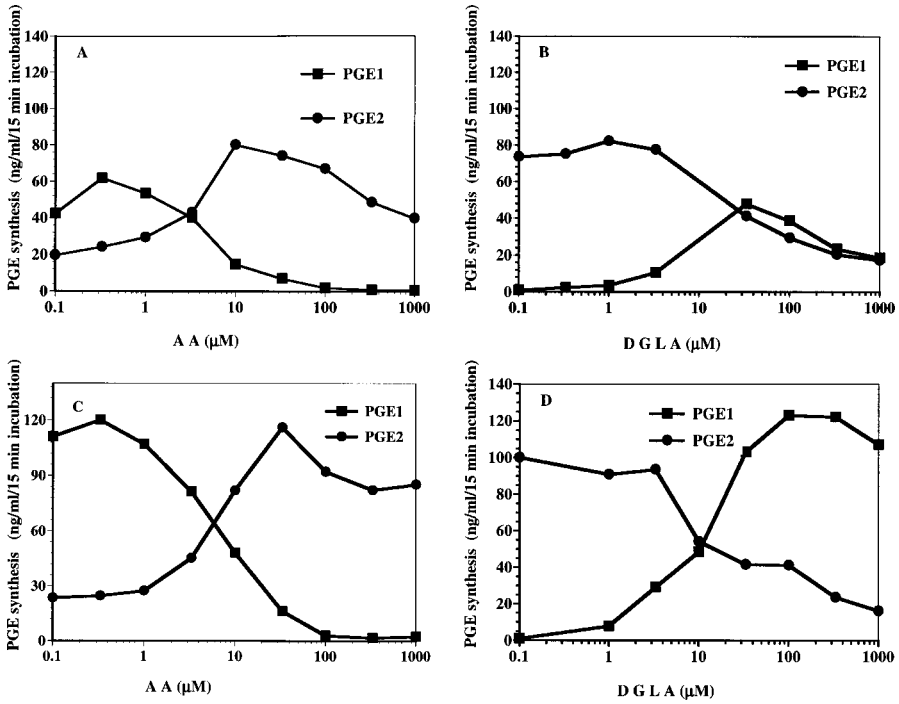

Figure 2 Generation of PGE_1 and PGE_2 by hCOX-1 and hCOX-2 in co-incubations with DGLA and AA

Recombinant hCOX-1 was incubated in the presence of either 10 μM DGLA and various (0–1 mM) concentrations of AA (**A**) or 10 μM AA and varied concentrations of DGLA (0–1 mM) (**B**). COX-2 was similarly incubated with 10 μM DGLA and various concentrations (0–1 mM) of AA (**C**) or 10 μM of AA and various concentrations (0–1 mM) of DGLA. (**D**) Following incubation, the amounts of PGE_1 and PGE_2 were quantified by extraction and HPLC–mass spectrometry. Data are from a single experiment that was repeated twice with similar results.

DMEM. LPS was added (final concentration, 1 μg/ml) for 16 h to stimulate the cells. Calcium ionophore A23187 was then added (final concentration, 0.5 μM) for 15 min, after which the medium was collected. PGE_1 and PGE_2 content in the media were quantified by HPLC–tandem mass spectrometry (MS/MS) (see below).

Assay of recombinant human COX-1 (hCOX-1) and hCOX-2 activity

Recombinant hCOX-1 and hCOX-2 were purified from insect cells [33]. The enzymes were preincubated for 10 min at room temperature in 100 mM Tris buffer (pH 8) containing 1 μM haem, 500 μM phenol and 300 μM adrenaline. The appropriate dilutions of stock solutions of DGLA and AA (sodium salts) were prepared in water and added to cofactor-activated COX-1 and COX-2 to start the reaction. After 10 min, the reaction was terminated by the addition of freshly prepared indomethacin (final concentration 25 μM). The amounts of PGE_1 and PGE_2, as determined by HPLC–MS/MS, were plotted as reaction velocity (V) versus substrate concentration (S) curves (Figures 1 and 2) or Lineweaver–Burk plots (results not shown) and the values of K_m and V_{max} were determined from the graphs.

Fatty-acid analysis

Cells were collected, washed three times with Hanks balanced salt solution, and then homogenized in 100 μl of PBS. The homogenate was processed by a single extraction–saponification–methylation step and the fatty-acid composition was determined as described previously [34]. Extraction of total cellular lipids was performed by the method of Duffin et al. [35]. The concentrated extract was subjected to TLC on silica gel plates developed in hexane/ether (70:30, v/v). The spots were scraped, extracted with chloroform/methanol (2:1, v/v) and subjected to fatty acid analysis.

Quantification of PGE_1 and PGE_2

Separation and quantification of PGE_1 and PGE_2 were achieved using HPLC–MS/MS. The culture medium was acidified to pH 3.5 with formic acid and then extracted twice with 3 vol. of ethyl acetate. The combined extract was dried over Na_2SO_4, evaporated to dryness and then dissolved in 30 μl of water/methanol/acetic acid (80:20:1, by vol.). The samples were applied to a 1 mm reverse-phase C-18 column (Keystone Scientific, Bellfonte, PA, U.S.A.). The column was eluted at 50 ml/min with a 50:50 ratio of water containing 10 mM ammonium acetate (solution A) and methanol containing 10 mM ammonium acetate (solution B). After 25 min the A/B ratio of the mixture was changed to 30:70 for 5 min, and then changed to methanol for 5 min. Eluted eicosanoids were injected into a PerkinElmer Sciex API III+triple-quadrupole electrospray mass spectrometer (Sciex Inc., Thornhill, Ont., Canada) operated in the negative-ion mode. Eicosanoids were detected and quantified by operating the mass spectrometer in the multiple-reaction monitoring mode as described previously [36].

RESULTS

Kinetic parameters for oxygenation of DGLA and AA by recombinant hCOX-1 and hCOX-2

Formation of PGE_1 and PGE_2 was used as a surrogate end point for comparing the rates of oxygenation of DGLA and AA respectively. K_m and V_{max} values were estimated from the V-

versus-*S* curves for the conversion of AA and DGLA to PGE_2 and PGE_1 respectively, using recombinant hCOX-1 and hCOX-2 (Figures 1 and 2, Table 1). The results showed a preferential catalysis (lower K_m) for oxygenation of AA over DGLA for COX-1, whereas COX-2 had approximately equal affinities for catalysis with AA and DGLA (Figure 1). For both DGLA and AA, hCOX-2 has a lower K_m value in comparison with hCOX-1 (Table 1). It should be noted that, due to the potential substrate inhibition of DGLA oxygenation by COX-1 (as evident at concentrations above 30 μM; Figure 1), the calculated V_{max} and K_m values represent the minimum apparent values.

Competition kinetics between DGLA and AA as co-substrates for hCOX-1 and hCOX-2

The synthesis of both PGE_1 and PGE_2 by hCOX-1 and hCOX-2 was determined in incubations where the two precursor fatty acids competed as substrates. Competition studies were carried out under conditions where one fatty acid was kept at a fixed concentration (10 μM) and the other was varied in concentration (0–1000 μM). Representative results are shown in Figure 2. The overall affinity ratio of AA/DGLA for each isoenzyme was calculated from the ratio of PGE_2/PGE_1 obtained in incubations with a 10 μM concentration of each fatty acid. Two main conclusions were drawn from these results. Firstly, COX-1 exhibited a preferential oxygenation of AA over DGLA when the two substrates were co-competing. This was observed in experiments in which equal concentrations (10 μM) of both fatty acids yielded a PGE_2/PGE_1 ratio of 2.3:1 (Figures 2A and 2B). Secondly, COX-2 had approximately equal rates for oxygenating AA and DGLA, as the synthesis of PGE_2 and PGE_1 was similar (Figures 2C and 2D). This conclusion is in accordance with the similar K_m values of hCOX-2 for AA and DGLA (Table 1).

Table 1 V_{max} and K_m values for DGLA and AA conversion into their corresponding PGEs by recombinant hCOX-1 and hCOX-2

The values of V_{max} and K_m were determined from the *V*-versus-*S* curves (Figure 1) or Lineweaver–Burk plots.

	DGLA		AA	
Isoenzyme	K_m (μM)	V_{max} (ng of $PGE_1 \cdot ml^{-1} \cdot 15\ min^{-1}$)	K_m (μM)	V_{max} (ng of $PGE_2 \cdot ml^{-1} \cdot 15\ min^{-1}$)
hCOX-1	9.1	84.5	4.2	98
hCOX-2	2.3	126	2.0	134

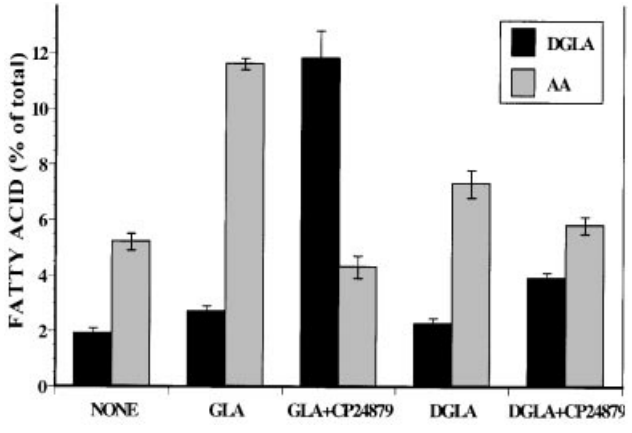

Figure 3 Effect of GLA or DGLA, added singly or together with the desaturase inhibitor CP-24879, on the content of DGLA and AA in cultured LL carcinoma cells

Cells were seeded at 5×10^5/ml in 6-well plates and cultured in DMEM with 10% FCS in the presence of 10 μM fatty acid $\pm$ 3.75 μM of the mixed Δ^5/Δ^6 desaturase inhibitor CP-24879 for 72 h. Values are means $\pm$ S.E.M. (5 wells) of the percentage of individual fatty acids in the total cellular lipids.

Effects of substrate concentration on V_{max} of hCOX-1 and hCOX-2

COX-2 activity is relatively insensitive to inhibition by high (1 mM) substrate concentrations (Figures 2C and 2D). In contrast, hCOX-1 activity is inhibited in a concentration-dependent manner by the two fatty acids, beginning at > 10 μM for AA (Figure 2A) and > 3 μM for DGLA (Figure 2B).

Increasing the cellular content of DGLA in LL carcinoma cells and HL-60 cells

Based on the relative affinities (compared as the reciprocal of the K_m values) of DGLA and AA for hCOX-1 and hCOX-2, we hypothesized that even in tissue or cells in which COX-1 predominates, greater synthesis of PGE_1 over PGE_2 might still occur if the DGLA/AA ratio was 1.5 or higher. This ratio was calculated from the data in Figure 2 that were obtained when equal concentrations of AA and DGLA were incubated with COX-1. An attempt was made to obtain a DGLA/AA ratio of 1.5 or higher by culturing cells with DGLA. Only limited incorporation of DGLA into cellular lipids was obtained; its relative percentage increased from 1.9% to only 2.3% (Figure 3). In LL carcinoma cells, the major consequence of DGLA addition was an increase in AA content from 5.2 to 7.5%, indicating significant Δ^5 desaturation of DGLA to AA. In an effort to block this conversion and thus further increase the cellular DGLA content, the cells were cultured with the mixed Δ^5/Δ^6 desaturase inhibitor CP-24879. This treatment inhibited most of the conversion of DGLA into AA, but the cellular DGLA content increased from 2.3% to only 4.0%, being still lower than that of AA (5.8%).

The small incorporation of DGLA into cellular lipids indicated that one or more steps in the acylation process (i.e. formation of DGLA-CoA and/or its utilization in subsequent acylation–transacylation reactions with the appropriate lysophospholipid) was considerably slower than the comparable reactions with AA. The DGLA precursor fatty acid, GLA, was therefore tested for cellular incorporation and subsequent elongation and Δ^5 desaturation to AA. The results showed that GLA was taken up very efficiently by the cells and metabolized to DGLA and further to AA (Figure 3) so that the total DGLA + AA content increased from 6 to 14%. When CP-24879 was combined with GLA, cells highly enriched with DGLA were obtained in which the DGLA content increased from 1.9 to 12% and the DGLA/AA ratio in cellular lipids increased from 0.4 to 2.8 (Figure 3).

To verify the general utility of this DGLA/AA remodelling protocol in human cells, we tested the same experimental paradigm in HL-60 cells. The relative incorporation of GLA versus DGLA and the desaturase-inhibiting effect of CP-24879 were similar to those seen in the LL carcinoma cells (Figure 4). The DGLA content was compared in cells cultured with 10% (normal) or 2% (low) FCS content to determine whether DGLA derived from added GLA would be more efficiently incorporated into cellular lipids under the low FCS condition. The results

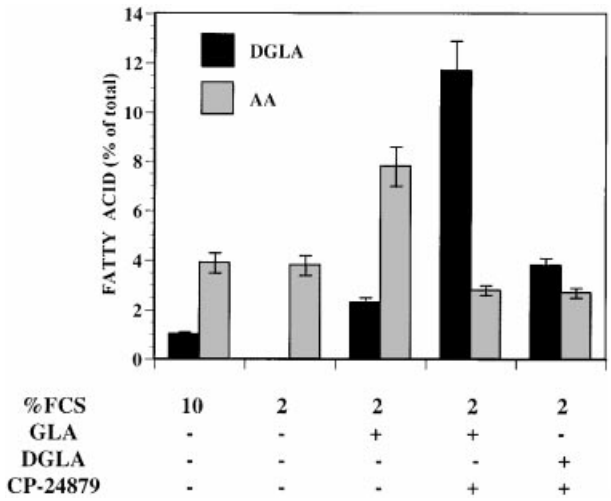

Figure 4 Content of DGLA and AA in HL-60 cells after addition of GLA ± CP-24879

HL-60 cells were seeded at 5×10^5 cells/ml in 6-well plates and grown in DMEM in the presence of 10 or 2% FCS with 10 μM GLA and 3.75 μM of the mixed Δ^5/Δ^6 desaturase inhibitor CP-24879 for 72 h. Values are means ± S.E.M. (5 wells) of the percentage fatty acids in the total cellular lipids.

Table 2 Effect of indomethacin (dual COX inhibitor) and SC-560 (selective COX-1 inhibitor) on COX activity in LL carcinoma cells

D122 cells were preincubated in the presence of indomethacin (10 μM) or SC-560 (0.1 μM, a concentration shown to inhibit only COX-1) for 30 min. AA (15 μM) was then added and the incubation continued for 15 min. Media PGE_2 and cell protein were determined as described in the Materials and methods section.

Inhibitor	PGE_2 synthesized (ng of PGE_2/mg of protein)	Inhibition of COX activity (%)
–	76.2 ± 3.5	–
Indomethacin	3.1 ± 0.4	96
SC-560	23.6 ± 1.9	69

showed that in comparison with 10% FCS, cells grown in 2% FCS contained no detectable DGLA in their lipids, but the AA content was unchanged (Figure 4).

COX-1 and COX-2 activities in cultured LL carcinoma cells

The capacity of LL carcinoma cells to produce PGE_2 versus PGE_1 when the cells were highly enriched with AA or DGLA was evaluated. Since the recombinant hCOX-1 enzyme appears to have some preference for AA over DGLA, the relative activities of COX-1 and COX-2 in intact LL carcinoma cells were first determined. For this purpose, the selective COX-1 inhibitor, SC-560 [37] as well as the combined COX-1/COX-2 inhibitor, indomethacin, were employed. Results showed that COX-1 accounted for approx. 70% of the total cellular COX activity in these cells (Table 2).

Distribution of AA and DGLA in cellular phospholipid and triacylglycerol fractions

The relative synthesis of PGE_1 versus PGE_2 is determined by the relative affinities of these substrate fatty acids for the individual COX enzymes as well as the relative content of the fatty acids in specific lipid pools from which the fatty acids are released by certain agonists. To evaluate the overall process, the relative content of DGLA and AA in the triacylglycerol and total phopholipid fractions from cells highly enriched with DGLA was determined (Table 3). Addition of GLA to cells yielded substantial conversion to DGLA that was incorporated into both triglyceride and phospholipid fractions. The resulting relative content of DGLA and AA in the two lipid fractions was, however, significantly different. In the triglyceride fraction, the relative content of DGLA and AA was approximately equal (DGLA/AA ratio 0.81), whereas in the phospholipid fraction AA was more enriched (DGLA/AA ratio 0.43). Unmetabolized GLA was detected only in the phospholipid fraction. Incubation of cells in the presence of the desaturase inhibitor CP-24879 produced a dramatic increase in the DGLA content of the phospholipid fraction, yielding a DGLA/AA ratio of almost 2. In contrast, the increase in DGLA in the triglyceride fraction was minimal (DGLA/AA ratio 1.08). Significantly, the GLA content in the phospholipid fraction was unchanged by CP-24879, whereas it increased in the triglyceride fraction.

Table 3 Changes in DGLA–AA composition in triglycerides and phospholipid fractions following treatment of LL carcinoma cells with CP-24879

Cells were grown in media containing 10 μM GLA for 3 days in the presence or absence of CP-24879. The cells were then harvested and their lipid extracts subjected to TLC to separate and isolate the triglycerides and phospholipid fractions. The two lipid fractions were then analysed for the fatty acid composition by GLC. Values are means ± S.E.M. of four replicate flasks of cells in each treatment.

		Fatty acid (% of total)			
CP 24789	Lipid	GLA	DGLA	AA	DGLA/AA ratio
–	Phospholipids	1.03 ± 0.14	3.52 ± 0.35	8.17 ± 0.40	0.43
–	Triglycerides	< 0.2	4.48 ± 0.22	5.55 ± 0.35	0.81
+	Phospholipids	1.01 ± 0.13	9.54 ± 0.71	4.95 ± 0.55	1.93
+	Triglycerides	1.72 ± 0.22	4.27 ± 0.44	3.95 ± 0.29	1.08

Synthesis of PGE_1/PGE_2 in cells highly enriched with DGLA

A potential benefit of a higher level of DGLA content in membrane phospholipids is the possibility for enhanced synthesis of PGE_1 by COX-1 and COX-2. To explore this possibility, the synthesis and release of PGE_1 and PGE_2 by DGLA-enriched cells (DGLA/AA ratio 2.8) were compared with non-enriched cells (DGLA/AA ratio 0.4). Cells enriched in DGLA released 0.36 ng of PGE_1/mg of protein and 0.88 ng of PGE_2/mg of protein compared with non-enriched cells that released 0.14 ng of PGE_1/mg of protein and 1.32 ng of PGE_2/mg of protein (Figure 5). Clearly, the large enrichment in DGLA did yield some increase in PGE_1 production together with a decrease in PGE_2 production. However, the PGE_1/PGE_2 ratio (0.41) in the DGLA-enriched cells was much lower than the DGLA/AA ratio (2.8) in these cells. Even allowing for the fact that most of the COX activity in these cells is COX-1 (Table 2) and that at equal concentrations of AA and DGLA the kinetic preference factor for PGE_2 over PGE_1 by COX-1 is 1.5 (Figure 1), the expected ratio of PGE_1/PGE_2 should have been approx. 1.9 (2.8/1.5), i.e. almost 2-fold more PGE_1 than PGE_2. The experimentally observed PGE_1/PGE_2 ratio of 0.41, almost 5-fold smaller, indicates that other factors contribute to the preferential formation of PGE_2.

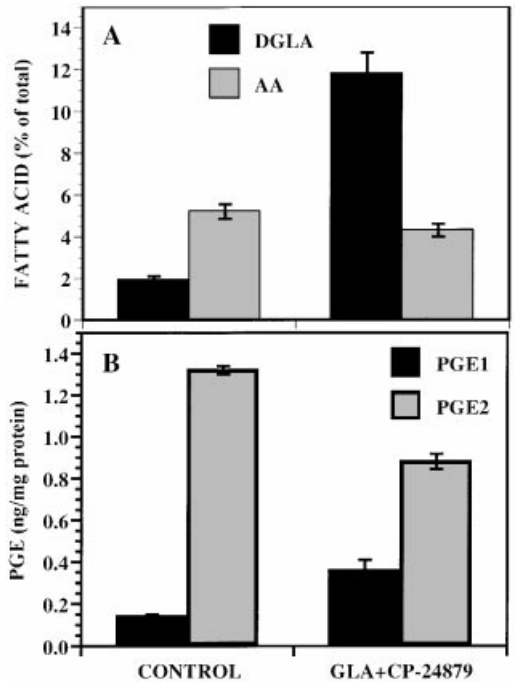

Figure 5 Synthesis of PGE_1 and PGE_2 in control and DGLA-enriched LL carcinoma cells

LL carcinoma cells were seeded at 5×10^5 cells/ml in 6-well plates in DMEM with 10% FCS $\pm$ 10 μM GLA and 3.75 μM CP-24879 for 48 h. The cells were then incubated with LPS (1 μg/ml) $\pm$ CP-24879 in a serum-free medium for 16 h. Ca ionophore A23187 (0.5 μM) was then added and the incubation was continued for 15 min. (**A**) Values are the means $\pm$ S.E.M. (5 wells) of the percentage of fatty acids in the cellular lipids. (**B**) The media were collected and total PGEs were quantified by RIA. Total prostaglandins were extracted from another aliquot of the media and PGE_1 and PGE_2 were separated and quantified by HPLC–MS/MS. Values are means $\pm$ S.E.M. (6 wells) of individual PGEs expressed as ng/mg of protein.

DISCUSSION

The present study demonstrated that in both mouse LL carcinoma and human monocytic HL-60 cells, incorporation of DGLA into cellular lipids was limited in comparison with the efficient incorporation and subsequent elongation and desaturation of GLA to yield AA (Figures 3 and 4). Addition of GLA together with a mixed Δ^5/Δ^6 fatty-acid desaturase inhibitor CP-24879 inhibited Δ^5 desaturase activity and the ensuing conversion of DGLA into AA. This led to a very substantial increase in the accumulation of DGLA from 2.3 % to almost 12 % of total fatty acids without a change in the level of AA (4–5 %, Figure 3). Significantly, the increased cellular DGLA level and the increased DGLA/AA ratio did not yield a proportional ratio of PGE_1/PGE_2. In order to explore the underlying reasons for this discrepancy, the kinetic parameters for metabolism of DGLA and AA were determined with *in vitro* assays using the individual recombinant hCOX-1 and hCOX-2 enzymes. The two fatty acids differed in their rates of oxygenation by the two COX isoenzymes. COX-2 had a higher affinity (lower K_m) than COX-1 for both DGLA and AA as substrates. Overall, COX-2 appeared to have approximately equal affinities for DGLA and AA, whereas AA was clearly the preferred substrate for COX-1. Consequently, when DGLA and AA were provided at equal (10 μM) concentrations, COX-1 produced approx. 1.5-fold more PGE_2 compared with PGE_1. Studies on the structures of COX-1 and COX-2 have yielded results which indicate that AA binding in the COX-1 active site is critically dependent on the precise interaction with Arg-120, the 'gatekeeper' residue of the active-site entrance, whereas for COX-2 the interaction of fatty acid substrates is dominated more by hydrophobic interactions with hydrophobic residues in the active site [38]. The presence of Δ^5 in AA, but not in DGLA, may impart an additional constraint on the steric movement of the carboxyl group, thereby helping to produce the correct conformation and distance for proper interaction of AA with Arg-120 and thus a more efficient catalysis of AA oxygenation by COX-1 [38,39].

In an effort to increase cellular PGE_1 synthesis, LL carcinoma cells were treated with CP-24879, which brought about a 7-fold increase in the DGLA/AA ratio to 2.8. This fatty acid remodelling led to a 2.5-fold increase in PGE_1 synthesis coupled with a 33 % decrease in PGE_2 production; the overall PGE_1/PGE_2 ratio was nevertheless only 0.41 (Figure 5), i.e. still favouring PGE_2 synthesis. One conclusion that can be drawn from these studies is that in addition to the differing kinetic parameters of the two COX isoenzymes for AA and DGLA, additional mechanisms operate which greatly favour the synthesis of PGE_2 over PGE_1. Such mechanisms could include preferential incorporation of AA into specific labile phospholipid pools, leading to sequential preferential hydrolysis of AA from such lipid pools by agonist-activated phospholipases. Previous studies with cells in culture [40–44] provide evidence for this possibility. Rosenthal and Candace Whitehurst [40] demonstrated that in cultured human skin fibroblasts DGLA was incorporated mostly into the triacylglycerol fraction, whereas AA was incorporated more selectively into the phospholipid fraction, particularly into phosphatidylserine and phosphatidylinositol. Similar results were obtained in human neutrophils incubated *in vitro* with GLA [45]. However, studies with humans given dietary GLA (supplied as borage oil) yielded different results in that DGLA was enriched in the phospholipid fraction [46]. The data presented here (Table 3) are similar, showing that DGLA was enriched more efficiently in the phospholipid fraction. DGLA either remained unmetabolized in cells treated with the desaturase inhibitor (DGLA/AA ratio almost 2) or was desaturated to AA in its absence (DGLA/AA ratio, 0.43) (Table 3). Hence, in LL carcinoma and HL-60 cells preferential distribution of AA over DGLA in the total phospholipid pool does not appear to exist and is therefore not a plausible explanation for the observed preferential formation of PGE_2. Two additional possible mechanisms may provide an explanation for the observed preferential PGE_2 synthesis in DGLA-enriched cells. (1) There is preferential hydrolysis of AA over DGLA from specific phospholipids. Evidence for this mechanism was obtained in studies using mouse fibrosarcoma cells that were rendered deficient in essential fatty acids and subsequently replenished with select individual fatty acids [8]. Results of these studies demonstrate a preferential release of AA over DGLA and an ensuing preferential PGE_2 synthesis. (2) There is preferential intracellular transport of AA from sites of lipid hydrolysis to sites where specific COX enzymes are located, namely in the ER lumen (predominantly COX-1) or the nuclear membrane (predominantly COX-2).

A significant difference was observed in the oxygenation rates of the two COX enzymes when DGLA and AA were provided at high concentrations (> 33 μM). Whereas COX-1 exhibited the frequently observed pattern of inhibition at high substrate concentrations, COX-2 was quite resistant to such inhibition, even at a substrate concentration of 1 mM, which is approx. 500-fold greater than the K_m values for DGLA and AA. This finding may have physiological relevance in connection with inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAIDS) at inflamed sites. Most of the NSAIDs are com-

petitive inhibitors of the COX enzymes and their inhibitory capacity is inversely correlated to the fatty acid substrate concentration. Resistance of COX-2 to inhibition by high fatty acid concentrations could also mean resistance to inhibition by NSAIDs when present along with high fatty acid substrate concentrations. A high concentration of AA at chronically inflamed sites (e.g. synovium of a rheumatoid arthritis patient) would compete with and reduce the interaction of NSAIDs with the COX-2 enzyme and therefore reduce their potency. Only very high doses of an NSAID that would be sufficient to compete with the high levels of available AA would be effective in arresting COX-2 activity. This would explain as to why under chronically severe rheumatoid arthritis conditions, high-dose salicylate is as effective as the same dose of aspirin for alleviating the COX-2-dependent inflammatory pain. Under such conditions, it would be the competition of the salicylate moiety and AA for binding to the enzyme and not the aspirin-dependent COX-2 acetylation that would effectively reduce the amount of AA binding at the COX-2 active site and thereby subsequent PGE_2 formation. Overall, our results suggest that dietary or combined dietary/pharmacological treatment aimed at augmenting the DGLA/AA ratio is not an effective means to increase substantially endogenous PGE_1 synthesis or decrease substantially PGE_2 synthesis in cells or tissues.

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