Differential metabolism of dihomo-ω-linolenic acid and arachidonic acid by cyclo-oxygenase-1 and cyclo-oxygenase-2: implications for cellular synthesis of prostaglandin E₁ and prostaglandin E₂

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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) E₂ 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 C₂₀ 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 E₁, PGE₁) [15], which are generally viewed as possessing anti-inflammatory properties. The question of whether PGE₁ and PGE₂ act on the same or different receptors is not yet resolved. Some reports provide data to indicate that PGE₁ binds to the PGE₂ receptors EP2 and EP4 [16], whereas data from earlier reports suggest the presence of separate PGE₁ and PGE₂ receptors [17] or even two distinct receptors for PGE₁ [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 A₂, a potent platelet aggregator and vasoconstrictor, DGLA does not give rise to a thromboxane [15]. PGE₁ is distinct from PGE₂ in that it stimulates cAMP production more effectively than PGE₂ [10,19,20] and, thereby, could manifest enhanced cAMP-mediated downstream signalling. Additionally, DGLA-derived PGE₁ has been identified as possessing anti-inflammatory properties that differentiate it from AA-derived PGE₂ (1) PGE₁ was shown to be more powerful than PGE₂ in exerting anti-inflammatory effects in the rat adjuvant arthritis model [21,22] and in the mouse Lupus model [23]. (2) PGE₁ was found to be more potent than PGE₂ as a vasodilator [24] as well as a suppressor of synovocyte proliferation [2,25]. (3) PGE₁, but not PGE₂ or PGE₂, was found to inhibit collagenase activity [19]. (4) In studies with human platelets, PGE₁ was shown to inhibit platelet aggregation in vitro, whereas PGE₂ 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 PGE₂ suggests that it could be a more potent anti-atherosclerotic agent compared with PGE₁. This suggestion was corroborated in studies in which PGE₁ was shown to inhibit vascular smooth-muscle-cell proliferation [28]. Furthermore, the major PGE₁ metabolite in blood, 13,14-dihydro-PGE₁, is relatively stable in blood and retains most of the anti-aggregatory activity [27], thus rendering PGE₁ as an overall longer acting anti-aggregatory agent [29].

Some of the biological effects of PGE₁ 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 LTB₅ 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 PGE₁/PGE₂ 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 PGE₁/PGE₂ 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 PGE₁ versus PGE₂ in cells and, secondly, to examine whether a dietary paradigm to increase PGE₁ synthesis at the expense of PGE₂ 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 Δ⁹/Δ⁷ 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 PGE₁ over PGE₂.

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-PGE₂ antisera, and Norit A-activated charcoal were purchased from Sigma. Foetal calf serum (FCS), non-essential amino acids, glutamine, trypsin, antibiotics–antimycotic (penicillin, streptomycin and nystatin) were purchased from Biological Industries Co. (Kibbutz Beit Haemek, Israel). [¹⁴C]PGE₂ (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 Δ⁹/Δ⁷ 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.).

Fatty-acid incorporation into cells

Cells were seeded at 5 × 10⁶ ml⁻¹ in T-75 flasks and incubated for 72 h in the presence of 10 μM fatty acid ± 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 Na₂CO₃ (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 × 10⁶ cells/well) were seeded in 6-well plates and cultured for 48 h. The cells were then washed with PBS and incubated in...
Synthesis of prostaglandin E₁/prostaglandin E₂ by cyclo-oxygenases-1 and -2

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. PGE₁ and PGE₂ 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 PGE₁ and PGE₂, 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 PGE₁ and PGE₂

Separation and quantification of PGE₁ and PGE₂ 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 Na₂SO₄, 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 PGE₁ and PGE₂ 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\(_3\) 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 \(\mu\)M; Figure 1), the calculated \(V_{\text{max}}\) 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 \(\mu\)M) and the other was varied in concentration (0–1000 \(\mu\)M). 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 \(\mu\)M 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 \(\mu\)M) 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\(_1\) and PGE\(_2\) 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).

**Effects of substrate concentration on \(V_{\text{max}}\) 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 \(\mu\)M for AA (Figure 2A) and > 3 \(\mu\)M 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 \(\Delta^\beta\)/\(\Delta^\gamma\) desaturation of DGLA to AA. In an effort to block this conversion and further increase the cellular DGLA content, the cells were cultured with the mixed \(\Delta^\beta\)/\(\Delta^\gamma\) 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 \(\Delta^\alpha\) 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

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

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>(V_{\text{max}}) (ng of PGE (_2)/ml(^{-1})·15 min(^{-1}))</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{\text{max}}) (ng of PGE (_2)/ml(^{-1})·15 min(^{-1}))</th>
<th>(K_m) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCOX-1</td>
<td>91.1</td>
<td>9.1</td>
<td>84.5</td>
<td>4.2</td>
</tr>
<tr>
<td>hCOX-2</td>
<td>2.3</td>
<td>2.3</td>
<td>126</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**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 \(\mu\)M fatty acid ± 3.75 \(\mu\)M of the mixed \(\Delta^\beta\)/\(\Delta^\gamma\) desaturase inhibitor CP-24879 for 72 h. Values are means±S.E.M. (5 wells) of the percentage of individual fatty acids in the total cellular lipids.

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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 phospholipid 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.

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\(_1\) over PGE\(_2\) 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\(_2\).

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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PGE(_2) synthesized (ng of PGE(_2)/mg of protein)</th>
<th>Inhibition of COX activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>76.2 ± 3.5</td>
<td>–</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3.1 ± 0.4</td>
<td>96</td>
</tr>
<tr>
<td>SC-560</td>
<td>23.6 ± 1.9</td>
<td>69</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Lipid</th>
<th>DGLA/AA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids</td>
<td>0.43</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.81</td>
</tr>
<tr>
<td>+ Phospholipids</td>
<td>0.43</td>
</tr>
<tr>
<td>+ Triglycerides</td>
<td>1.08</td>
</tr>
</tbody>
</table>

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

HL-60 cells were seeded at 5 × 10^6 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\(^1\)/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.

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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 Δ² 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 PGE₁ 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 remodeling led to a 2.5-fold increase in PGE₁ synthesis coupled with a 33% decrease in PGE₂ production; the overall PGE₁/PGE₂ ratio was nevertheless only 0.41 (Figure 5), i.e. still favouring PGE₁ 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 PGE₁ over PGE₂. 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 PGE₁. Two additional possible mechanisms may provide an explanation for the observed preferential PGE₁ 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 from specific phospholipids. (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 Kₘ 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-

Figure 5  Synthesis of PGE₁ and PGE₂ in control and DGLA-enriched LL carcinoma cells

LL carcinoma cells were seeded at 5 × 10⁵ cells/ml in 6-well plates in DMEM with 10% FCS ± 10 μM GLA and 3.75 μM CP-24879 for 48 h. The cells were then incubated with LPS (1 μg/ml) ± CP-24879 in a serum-free medium for 16 h. An isoprophor A23187 (0.5 μM) was then added and the incubation was continued for 15 min. (A) Values are the means ± 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 PGE₁ and PGE₂ were separated and quantified by HPLC–MS/MS. Values are means ± 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 Δ⁵/Δ⁶ fatty-acid desaturase inhibitor CP-24879 inhibited Δ² 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 PGE₁/PGE₂. 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 Kₘ) 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 PGE₁ compared with PGE₂. Studies on the structures of COX-1 and COX-2 have yielded results which indicate that AA binding in the COX-1

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itive 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 to 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\(_2\) synthesis or decrease substantially PGE\(_2\) synthesis in cells or tissues.

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