Evidence for Coordinate, Selective Regulation of Eicosanoid Synthesis in Platelet-derived Growth Factor-stimulated 3T3 Fibroblasts and in HL-60 Cells Induced to Differentiate into Macrophages or Neutrophils*

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We used Swiss 3T3 fibroblasts stimulated with platelet-derived growth factor and HL-60 cells induced to differentiate into macrophages or neutrophils to study the regulation of prosstaglandin and leukotriene synthesis. Addition of platelet-derived growth factor to quiescent 3T3 fibroblasts led within 4 h to a dramatic and preferential increase in prostacyclin synthesis from endoperoxide prostaglandin H₂, and microsomal assays showed a strong platelet-derived growth factor-dependent increase in the maximal velocities (Vₘₐₓ) of both prostaglandin H synthase and prostacyclin synthase. In contrast, addition of phorbol ester to HL-60 cells to induce differentiation into macrophages led within 4 h to a strong and preferential increase in thromboxane synthesis from prostaglandin H₂, and microsomal assays disclosed a major rise in Vₘₐₓ for both prostaglandin H synthase and thromboxane synthase. No comparable changes occurred in HL-60 cells that were differentiating into neutrophils, though upregulation of 5-lipoxygenase pathway enzymes occurred in both differentiation systems. Actinomycin D and cycloheximide prevented the appearance of all of these enzymes of eicosanoid synthesis in all three model systems. Thus, the distinctive patterns of eicosanoid synthesis that are seen in replicating fibroblasts and in differentiating macrophages and neutrophils appear to depend on a coordinate, selective upregulation of several enzymes of eicosanoid biosynthesis that is specific for each cell system.

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¶¶The abbreviations used are: PDGF, platelet-derived growth factor; Me₂SO, dimethyl sulfoxide; LT, leukotriene; PBS, Dulbecco's phosphate-buffered saline; PDS, platelet-derived serum; PC, prostaglandin; RIA, radioimmunoassay; RP, reversed phase; HPLC, high pressure liquid chromatography; TPA, 12-O-tetradecanoylphorbol-13-acetate; TX, thromboxane; EGTA, [(ethylenebis(oxyethylenenitrilo)] tetracetic acid.

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and the 5-lipoxygenase pathway. In contrast, the addition of MeSO to HL-60 cells mainly causes an increase in the activity of the 5-lipoxygenase pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were obtained from Gibco; standard eicosanoids from Seragen, Boston, MA; [3H]TXB2 (specific activity, 130 Ci/mmol), [3H]PGE2 (specific activity, 120 Ci/mmol), [3H]6-keto-PGF1α (specific activity, 120 Ci/mmol), [3H]LTB4 (specific activity, 32.0 Ci/mmol), [3H]LT-C4 (specific activity, 40.0 Ci/mmol) from Du Pont-New England Nuclear; 12-O-tetradecanoylphorbol-13-acetate (TPA) and arachidonic acid from Pharmacia, Federal Republic of Germany; antibodies against TXB2, PGE2, and 6-keto-PGF1α from the Institute Pasteur, Paris, France; antibodies against LT from Amersham Buchler, Braunschweig, Federal Republic of Germany; antibodies against LTC4 from Calbiochem (Frankfurt, Federal Republic of Germany); dimethyl sulfoxide, actinomycin D, cycloheximide, and all other reagents from Sigma Chemie, Munich, Federal Republic of Germany. Stock solutions of TPA at 10 mM in acetone were stored under argon at -80 °C.

**Cell Culture**—Diploid human fibroblasts and Swiss 3T3 cells were cultured as described (23). The human promyelocytic leukemia cell line, HL-60 was obtained from American Type Culture Collection (Rockville, Md.) and cultured as described (23). The human promyelocytic leukemia cell line, HL-60, was obtained from American Type Culture Collection and maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 units/liter penicillin, 100 μg/ml streptomycin, and maintained as described previously (17). Human platelets were obtained from healthy volunteers and prepared as described (11). Cells were incubated for 15 min at 37 °C in the presence of ethanol as control. Then L-cysteine which had been dissolved in 10 μl of ethanol at 5 mg/ml was added to a final concentration of 5 μM. Interactions between 8-15 pg/ml. Intraassay variation was 11.8-13.5% for LTB4 and 10-14% for LTC4. Interassay variation was 14-18% for LTB4 and 11-20% for LTC4. Total recoveries for LTC4 ranged between 38-54% and for LTC4 between 35-56%.

**Assays of Microsomal PGH Synthase, TX Synthase, and Prostacyclin Synthase Activities**—PGH synthase activity was determined as described before (17). TX synthase and prostacyclin synthase activities were determined according to Sheng et al. (37). Briefly, microsomes of cells were prepared as described (17). 20 μg of microsomal protein, suspended in 20 μl of homogenization buffer (50 mM Tris, 2 mM EGTA, 0.3 mM MeSO4, 25% sucrose, 1% fatty acid-free albumin, pH 7.5), were added to 477 μl of assay buffer (50 mM Tris, pH 7.4) that had been prewarmed to 37 °C in a water bath and incubated at 37 °C or to 22 °C for prostacyclin synthase activity. The respective reactions were then started by addition of buffer containing the microsomes to siliconized glass tubes containing PGG2 dissolved in 3 μl of MeSO4 (the solution of PGG2 had been kept under argon until the start of the experiment). The reaction was stopped after 30 s for TX synthase and after 45 s for prostacyclin synthase by addition of 2.5 ml of ice-cold PBS that had been acidified to pH 3.4. and eicosanoids were immediately extracted using octadecyl silica gel columns. At concentrations of PGG2 between 4-128 μM, the formation of TXB2 was linear with respect to microsomal protein (determined between 5-50 μg) for 60 s. Control microsomes that had either been boiled or treated with the competitive inhibitor of TX synthase, dazoxiben, at 300 μM for 10 min did not contain measurable TX synthase activity. At concentrations of PGG2 between 4-128 μM the formation of 6-keto-PGF1α was linear with respect to microsomal protein (determined between 5-50 μg) for 80 s at 22 °C as described by Salmon et al. (38). Protein measurements were performed according to Lowry et al. (39).

**RESULTS**

**Selective Induction of PGH Synthase and Prostacyclin Synthase in 3T3 Fibroblasts and HL-60 Cells**—In previous studies of 3T3 fibroblasts (11) and in unpublished studies of diploid human fibroblasts we showed that PGH synthase is upregulated in human skin fibroblasts when we showed the possibility that PDGFB might be upregulating a specific pattern of eicosanoid synthesis by selectively activating subsequent enzymes in the PGH synthase pathway as well. In the present study we used several different approaches to examine the latter possibility. First, we determined the synthesis rates of prostacyclin and TX from exogenously added endoperoxide PGH2 in quiescent and PDGFB-stimulated 3T3 fibroblasts. We found that quiescent cells synthesized low amounts of prostacyclin (≤20 ng/106 cells/30 min) even when incubated with concentrations of PGH2 as high as 32 μM (Fig. 1, Table I). Likewise, quiescent cells formed low or undetectable concentrations of TX from PGH2 (≤5 ng of TXB2/106 cells/30 min). PDGFB-stimulated cells that were exposed to the same high concentration of PGH2...
trations of PGH₂ also formed low amounts of TXB₂ (≤18 ng of TXB₂/10⁶ cells/30 min), but PDGF-stimulated cells did synthesize 380–1100 ng of prostacyclin/10⁶ cells/30 min in four independent experiments (Table I, Figs. 1 and 2). Because no additional stimulation of the formation of prostacyclin occurred at concentrations of PGH₂ that exceeded 10 μM, we used this concentration in all further experiments.

The cell cycle kinetics of prostacyclin synthesis from PGH₂ is shown in Fig. 2. PDGF stimulated prostacyclin synthesis within 2–4 h, and plateau levels were reached at 4 h. This time course closely resembled that of the PDGF-dependent induction of PGH synthase activity observed earlier in the same cell system (11). It is noteworthy in this connection that PDGF-stimulated cells that had been pretreated with 100 μM aspirin and were unable to produce PGE₂ or prostacyclin from exogenous arachidonic acid showed rates of prostacyclin synthesis from exogenous PGH₂ that were as high as those shown in Figs. 1 and 2 (data not shown). This clearly indicates that the latter rates directly reflect prostacyclin synthase activity.

A comparison of the kinetic parameters of PGH synthase, prostacyclin synthase, and TX synthase in microsomes prepared from PDGF-stimulated 3T3 fibroblasts provided evidence regarding the mechanism of upregulation of prostacyclin synthesis. PDGF caused a marked increase in the maximal velocity (Vₘₐₓ) of PGH synthase without altering the apparent affinity of the enzyme for its substrate (Kₘ) (Fig. 3). Moreover, PDGF induced a striking increase in the Vₘₐₓ values of TX synthase.
of prostacyclin synthase (Fig. 4A). In contrast, the growth factor did not induce a comparable change in the kinetic parameters of TX synthase (Fig. 4B and see below).

A study of the effects of actinomycin D and cycloheximide on the ability of PDGF-stimulated cells to incorporate exogenous arachidonic acid or PGH₂ into eicosanoids showed that these agents completely prevented the PDGF-dependent up-regulation of the activities of PGH synthase and prostacyclin synthase (Table I). Thus, transcriptional and translational mechanisms affecting the activities of both enzymes appear to be required for the PDGF-dependent up-regulation of prostacyclin synthase in 3T3 fibroblasts.

Inactivation of TX Synthase, Prostacyclin Synthase, and 5-Lipoxygenase in HL-60 Cells Induced to Differentiate into Macrophages—In a previous study of undifferentiated HL-60 cells (17), we found that these cells were unable to convert exogenous arachidonic acid into eicosanoids. However, when we added TPA to the cells to stimulate them to differentiate into macrophages, they rapidly developed a marked ability to convert exogenous arachidonic acid into TXB₂ in association with an increased mass and activity of PGH synthase. In the present study we sought to determine whether this pattern of eicosanoid synthesis also was associated with an induced up-regulation of TX synthase and other enzymes of eicosanoid synthesis. We first incubated control HL-60 cells or HL-60 cells that were differentiating into macrophages in the presence of increasing concentrations of PGH₂ and measured the cells’ ability to convert the PGH₂ into TXB₂ and prostacyclin. We found that undifferentiated HL-60 cells formed low amounts of either eicosanoid (<1.5 ng of TXB₂/10⁶ cells/30 min and <1.1 ng of 6-keto-PGF₁α/10⁶ cells/30 min), but that TPA-stimulated HL-60 cells that had differentiated for 12 h along the macrophage differentiation pathway formed 50–70 ng of TXB₂/10⁶ cells/30 min and 5–7 ng of prostacyclin/10⁶ cells/30 min (Fig. 5).

The time course of induction of TX and prostacyclin synthase from exogenous PGH₂ is shown in Fig. 6. Significant synthesis of TX occurred within 1 h after the addition of TPA, and the rate of TX synthesis reached a plateau level after 4 h (Fig. 6). This time course of induction resembled that observed for prostacyclin synthase in PDGF-stimulated 3T3 fibroblasts as well as that found for PGH synthase in our previous study of HL-60 cells that were differentiating into macrophages (17).

We next determined the kinetic parameters of TX synthase and prostacyclin synthase in microsomes prepared 12 h after the cells had been stimulated to differentiate into macrophages. We found that the Vₘₐₓ values of TX synthase increased by a factor of 9–13 in six independent experiments (Fig. 7A). The Vₘₐₓ values of prostacyclin synthase also increased significantly, although by a factor of only 1.6–3.2 in four independent experiments (Fig. 7B). A comparison of the kinetic parameters of PGH synthase, TX synthase, and prostacyclin synthase in PDGF-stimulated Swiss 3T3 fibroblasts and TPA-stimulated HL-60 cells illustrates the striking differences in Vₘₐₓ values that are seen in these two culture systems (Table III). However, experiments with actinomycin D and cycloheximide yielded analogous results in the two systems. As mentioned earlier, each inhibitor was able to prevent the PDGF-induced conversion of exogenous arachi-

![Fig. 4. Kinetic parameters of microsomal prostacyclin and TX synthase in quiescent or PDGF-stimulated 3T3 fibroblasts. Cells were cultured and partially purified PDGF at 2 µg/ml was added as described in Fig. 1. 16 h later, microsomes from quiescent or PDGF-stimulated cells were prepared, and prostacyclin and TX synthase activities were determined as described under "Experimental Procedures." The results were subjected to linear regression analyses. The data points represent means of duplicate determinations of two experiments for prostacyclin synthase and of one experiment for TX synthase.](image-url)
The data points represent means of duplicate dishes ± S.D.

donic acid or PGH₂ into prostacyclin in 3T3 fibroblasts (Table 1), and the same was true for the TPA-induced conversion of these precursors into TX and prostacyclin in HL-60 cells (Table II). Note that the results obtained for TX generally resemble the results of Honda and co-workers (28), who found that homogenates prepared from HL-60 cells, 3 days after the cells had been treated with 1α, 25 (OH)₂ vitamin D₃ showed an increase in the conversion of radiolabeled PGH₂ into TXB₂ that was sensitive to cycloheximide (28).

To obtain further evidence regarding the upregulation of enzymes of eicosanoid synthesis during the differentiation of HL-60 cells into macrophages we used the assay method described by Borgeat and Samuelsson (32; see also "Experimental Procedures") to examine the activity of the 5-lipoxygenase pathway in intact cells. We first validated the assay for use in HL-60 cells by incubating the cells with Ca²⁺ ionophore and arachidonic acid for different periods of time and measuring the amounts of LTB₄ and LTC₄ that were formed (Fig. 8). We found that undifferentiated control HL-60 cells were unable to produce significant amounts of LTs even when incubated with high concentrations of Ca²⁺ ionophore and arachidonic acid. In contrast, HL-60 cells that were differentiating into macrophages formed significant amounts of LTs. LT synthesis during the assay peaked at 10 min, then declined in the case of LTB₄ (Fig. 8), or remained constant for up to 20 min in the case of LTC₄ (Fig. 8). Concentrations of 20-50 μM arachidonic acid and 10-20 μM ionophore gave maximal synthesis rates of LTs, and the effects of arachidonic acid and ionophore were synergistic (data not shown). A similar synergism was demonstrated previously in polymorphonuclear leukocytes by Borgeat and Samuelsson (32) and in macrophages by Tripp and co-workers (40). It is noteworthy that the chemotactic peptide, formyl-methionyl-leucyl-phenyl-alanine or opsonized zymosan could effectively sub-

stitute for the ionophore, but only in differentiating cells (data not shown).

Having validated the assay, we measured the time course of upregulation of the 5-lipoxygenase pathway in HL-60 cells that were differentiating into macrophages (Fig. 9). We found that it closely resembled the time courses of induction of TX synthase and prostacyclin synthase (Fig. 6) as well as that of PGH synthase (17) in the same cell system. In all four cases, enzyme activity rose sharply to reach a plateau within 4 h after the cells had begun to differentiate along the macrophage differentiation pathway.

Fig. 6. Kinetics of induction of TX and prostacyclin synthases in HL-60 cells that were differentiating into macrophages. Cells were cultured and TPA was added as described in Fig. 5. 30 min before the time points indicated in the figure, 10 μM PGH₂ was added to the cultures. Concentrations of TXB₂ and 6-keto-PGF₁α were determined as described under "Experimental Procedures." The data points represent means of duplicate dishes ± S.D.

Fig. 7. Kinetic parameters of microsomal TX and prostacyclin synthase in control or TPA-treated HL-60 cells. Cells were cultured and TPA was added as described in Fig. 5. 12 h later, microsomes from control or TPA-treated cells were prepared and microsomal TXB₂ (A) and 6-keto-PGF₁α (B) synthesis was determined as described under "Experimental Procedures." The results were subjected to linear regression analyses. The data points represent means of duplicate determinations in each of six independent experiments for TX synthase and in each of four independent experiments for prostacyclin synthase.

Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>PGH synthase</th>
<th>TX synthase</th>
<th>Prostacyclin synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/10⁶ cells</td>
<td>μg/min/mg</td>
<td>ng/10⁶ cells</td>
</tr>
<tr>
<td>Control</td>
<td>0.06 ± 0.04</td>
<td>0.41 ± 0.06</td>
<td>0.04 ± 0.02</td>
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<tr>
<td>TPA</td>
<td>2.29 ± 0.17</td>
<td>68.42 ± 5.87</td>
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<td>TPA + act.</td>
<td>0.04 ± 0.60</td>
<td>0.57 ± 0.29</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>TPA + cyc.</td>
<td>0.11 ± 0.45</td>
<td>0.16 ± 0.85</td>
<td>0.75 ± 0.40</td>
</tr>
</tbody>
</table>
**DISCUSSION**

The results of this investigation provide new information about the regulation of eicosanoid synthesis in replicating fibroblasts and differentiating HL-60 cells (Fig. 10).

They show for the former that up-regulation of prostacyclin synthesis during the cell cycle is associated with an increase in the activities of both PGH synthase and prostacyclin synthase. They demonstrate also that the activities of the two enzymes increase in parallel, early in the G1 phase of the cell cycle, and that an increase in $V_{max}$ is involved in each case. Furthermore, they indicate that the changes in the two enzymes are selective because a third PGH synthase pathway enzyme, TX synthase is not significantly affected. This demonstration of a cell cycle-dependent up-regulation of prostacyclin synthase activity provides the first direct evidence for specific regulation of this enzyme in any cell culture system.

The results of the present investigation show that HL-60 cells that are differentiating into macrophages up-regulate three different enzymes of the PGH synthase pathway, including not only PGH synthase itself (17, 28) and TX synthase, but also prostacyclin synthase. They demonstrate that the up-regulation of TX synthase and prostacyclin synthase, like that of PGH synthase is associated in each case with an increase in $V_{max}$, and that it is sensitive to actinomycin D and cycloheximide. Furthermore, they establish that the activities of all three enzymes as well as the activity of the 5-lipoxygenase pathway are up-regulated concomitantly within 4 h after the cells have been stimulated to enter the macrophage differentiation pathway.

The results demonstrate for HL-60 cells that are differentiating into neutrophils, that up-regulation of the 5-lipoxygenase pathway occurs with a time course that is initially quite similar to that observed for differentiating macrophages. Finally, they show that the up-regulation of 5-lipoxygenase activity is sensitive to actinomycin D and cycloheximide.

When taken together, these results support three major possibilities. First, the ability of fibroblasts, macrophages, and neutrophils to form specific patterns of eicosanoids depends at least in part on the selective induction of enzymes that control specific branchpoints in the pathways of eicosanoid synthesis.
TABLE III
Kinetic parameters of TX and prostacyclin synthase of quiescent or PDGF-stimulated fibroblasts and undifferentiated HL-60 cells or HL-60 cells induced to differentiate into macrophages or neutrophils

Cells were cultured, and PDGF, TPA, and Me2SO was added as described in Figs. 1, 5, and 10. The results were subjected to linear regression analysis. Data represent means of duplicate determinations in n experiments ± S.D.

<table>
<thead>
<tr>
<th>Cells</th>
<th>PGH synthase</th>
<th>TX synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{max} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Control (n = 1)</td>
<td>155 ± 65</td>
</tr>
<tr>
<td></td>
<td>PDGF (n = 1)</td>
<td>1791 ± 286</td>
</tr>
<tr>
<td>HL-60 cells</td>
<td>Control (n = 4)</td>
<td>79 ± 13</td>
</tr>
<tr>
<td></td>
<td>TPA (n = 4)</td>
<td>894 ± 162</td>
</tr>
<tr>
<td></td>
<td>Me2SO (n = 2)</td>
<td>244 ± 61</td>
</tr>
<tr>
<td></td>
<td>PDGF (n = 1)</td>
<td>41 ± 5</td>
</tr>
<tr>
<td></td>
<td>Control (n = 6)</td>
<td>70 ± 29</td>
</tr>
<tr>
<td></td>
<td>TPA (n = 6)</td>
<td>894 ± 162</td>
</tr>
<tr>
<td></td>
<td>Me2SO (n = 2)</td>
<td>244 ± 61</td>
</tr>
</tbody>
</table>

TABLE IV
Actinomycin D and cycloheximide prevent up-regulation of the 5-lipoxygenase pathway in HL-60 cells induced to differentiate into macrophages or neutrophils

HL-60 cells were cultured and TPA or Me2SO was added as described in Figs. 5 and 8. The time of TPA or Me2SO addition, cultures were incubated with 2 \( \mu g/ml \) actinomycin D (act.) or 5 \( \mu g/ml \) cycloheximide (cyc.). 6 h later cells were incubated under "incubation conditions." Data represent means of duplicate dishes ± S.D.

<table>
<thead>
<tr>
<th>Additions</th>
<th>( LT_B )</th>
<th>( LT_C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>TPA</td>
<td>1.15 ± 0.08</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>TPA + act.</td>
<td>0.02 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>TPA + cyc.</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Me2SO</td>
<td>2.15 ± 0.10</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>Me2SO + act.</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Me2SO + cyc.</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.00</td>
</tr>
</tbody>
</table>

synthesis (Fig. 10). Second, the enzymes that contribute to any one pattern of eicosanoid synthesis are up-regulated concomitantly. Third, the up-regulation occurs at an early time point, well before DNA synthesis occurs in replicating fibroblasts or conventional differentiation markers appear in differentiating macrophages or neutrophils.

One caveat should be kept in mind when considering these possibilities, the results were all obtained in experiments with model cell culture systems. This is a potential problem particularly in relation to the HL-60 cell experiments. In HL-60 cells we used an unphysiological stimulus, TPA to trigger the macrophage differentiation pathway, and TPA may have effects of its own that are independent of this pathway. Thus, it will be important to use other differentiation systems to confirm the results that we have reported here. We have recently taken a step in this direction by showing that an early up-regulation of the 5-lipoxygenase pathway occurs in a chicken macrophage differentiation system that does not require activation by TPA.

A question for future research will be to identify the molecular mechanisms that underlie the differential enzyme responses that we have observed. These mechanisms are likely to be rather complicated. On the one hand, a common mechanism presumably regulates the timing of enzyme responses in a given cell system because enzyme activities seem to be up-regulated in parallel. On the other hand, separate mechanisms appear to regulate different enzymes or groups of enzymes because the pattern of enzyme upregulation in replicating fibroblasts differs from that which occurs in differentiating macrophages or differentiating neutrophils. At least some of these mechanisms appear to operate at the level of gene activation and translation because the enzyme up-regulation is sensitive to actinomycin D and cycloheximide. Studies of the molecular biology of eicosanoid synthesizing enzymes have recently been initiated (31, 41-45). Dixon et al. (31) have shown that HL-60 cells treated with Me2SO for 5 days contain significant amounts of 5-lipoxygenase mRNA whereas control cells did not. It will therefore be possible to apply the techniques and approaches of molecular biology to the model systems of cell replication and differentiation that we have used here. A strategy of this type might well be fruitful because the present study has already identified enzymes that are differentially up-regulated in the three systems, and defined a window in time, very early in the pathways.

of replication and differentiation, during which critical regulatory events are likely to occur.

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6-keto-PGF1,
 and TXB,
 by mass spectrometry. We acknowledge the technical assistance of I. Drozdel, E. Specht, and I. Tomić.

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