Characterization of leukotriene A₄ synthase from murine mast cells: Evidence for its identity to arachidonate 5-lipoxygenase

TAKAO SHIMIZU*, TAKASHI IZUMI‡, YOUSUKE SEYAMA*, KENJI TADOKORO‡, OLOF RÅDMARK§, AND BENGT SAMUELSSON§

*Department of Physiological Chemistry and Nutrition and ‡Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; §Department of Physiological Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden; and †Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Contributed by Bengt Samuelsson, February 13, 1986

ABSTRACT  Leukotriene A₄ synthase was purified from the cytosolic fraction of murine mast cells. The enzyme converted 5-hydroperoxy-6-trans-8,11,14-cis-icosatetraenoic acid (5-HPETE) to leukotriene A₄. This unstable product was identified by demonstration of two epimers of 6-trans-leukotriene B₄, methanol trapping, as well as further transformation to leukotriene B₄ by leukotriene A₄ hydrolyase. Leukotriene A₄ synthase stereospecifically eliminated the δ-hydrogen at C-10 (pro-R) in the synthesis of leukotriene A₄ when incubated with [10n-H;3,14C]-HPETE. The purified enzyme also exhibited 5-lipoxygenase activity toward arachidonic acid and 8-lipoxygenase activity towards 8,11,14-cis-icosatrienoic acid. All of these activities required Ca²⁺ and ATP for their maximal velocities. The effects of heat treatment and of several lipoxigenase inhibitors on these enzyme activities as well as coelution in various chromatographic systems strongly suggest that lipoxigenase and leukotriene A₄ synthase activities reside in the same enzyme molecule.

The leukotrienes (LTs) (1) constitute a family of arachidonic acid metabolites involved not only in inflammatory or pathologic processes but also in neuroendocrine functions. Formation of LTs is initiated by the 5-lipoxygenation of arachidonic acid to yield (5S)-5-hydroperoxy-6-trans-8,11,14-cis-icosatetraenoic acid (5-HPETE). Lipoxigenase catalysis involves stereospecific abstraction of hydrogen, and in this case the δ-hydrogen at C-7 of arachidonic acid is preferentially removed (2). In the following conversion [5-HPETE to (5S)-5-trans-5,6-oxido-7,9-trans-11,14-cis-icosatetraenoic acid (LT₄)] the δ-hydrogen at C-10 is also removed (3, 4). Thus, there are mechanistic similarities between these two reactions, and recently we demonstrated that a single enzyme (from potato tubers) catalyzed both reactions by virtue of its dual lipoxigenase activities (5-HPETE and 8-lipoxygenase) (5). In this paper we present data showing that also a single enzyme from a mastocytoma cell line converts arachidonic acid to 5-HPETE and sequentially to LT₄.

EXPERIMENTAL PROCEDURES

Materials. [1-14C]Arachidonic acid (56 mCi/mmol; 1 Ci = 37 GBq) was purchased from Amersham. [10n-H;3,14C]Arachidonic acid was kindly gifted from A. Panossian at the Karolinska Institutet (4). Arachidonic acid and 8,11,14-cis-icosatrienoic acid (C20:3) were from Nu Chek Prep; linoleic acid was from Sigma. 5-HPETE, [1-14C]5-HPETE, and [10n-H;3,14C]-5-HPETE were prepared by using potato lipoxigenase (5). 13-Hydroxylinoleic acid was synthesized by using soybean lipoxigenase (Sigma). AA-861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) (6) was kindly provided by Takeda Pharmaceutical (Osaka, Japan); 5,6-methano-LTA₄ (7), (5S,12R)-5,12-dihydroxy-6,14-cis-8,10-trans-icosatetraenoic acid (LTB₄), and prostaglandin B₂ (PGB₂) were from Ono Pharmaceutical (Osaka, Japan). The FPLC system equipped with columns Mono Q (HR 5/5) and Superose TM 12 (HR 10/30) was a product of Pharmacia. A hydroxyapatite column (Bio-Gel HPH T 0.78 × 10 cm, Bio-Rad) was used in connection with the FPLC system.

Assay of LT₄ Synthase. HPLC method. The standard assay mixture contained 0.1 M Tris-HCl buffer (pH 7.5), 2 mM CaCl₂, 2 mM ATP, the microsomal protein from PB-3c cells (1 mg/ml), and enzyme in a total volume of 0.5 ml. The microsomal fraction, an activator of 5-lipoxygenase (8), also stimulated the LT₄ synthase reaction by 10–30%. After preincubation (30°C, 2 min) the incubation (5 min) was initiated by addition of 5-HPETE (50 μM, 25 nmol in 3 μl of ethanol). The reaction was terminated by addition of 1 ml of methanol (−20°C) with 800 pmol of PGB₂ as internal standard. Following centrifugation, the supernatant was evaporated to dryness and reconstituted in 0.1 ml of solvent A (acetonitrile/methanol/H₂O/acetatic acid, 3:1:3:0.006 vol/vol), containing 0.05% EDTA. HPLC analysis was performed with a TSK gel ODS column 80-TM (4.6 × 150 mm, Toyo Soda) eluted with solvent A (1 ml/min), and the absorbance at 270 nm was monitored. Enzyme activity was estimated from the ratio of peak areas (LTA₄-derivatized products to PGB₂). When C20:3 was included in the reaction mixture as an inhibitor, [1-14C]-5-HPETE (25 nmol, 86,000 cpm) was used as a substrate, and the radioactivity corresponding to hydrolysis products of LTA₄ was measured. The recoveries of LTs and PGB₂ were constant (95–97%).

Spectrophotometric assay. The incubation mixture was described above except that ATP was omitted. After addition of 5-HPETE, the increase in the absorbance at 275 nm was followed. This method was less appropriate, since the omission of ATP diminished the activity by 30–50%.

Assay of Lipoxygenases. The standard assay mixture (0.2 ml; composition, see above) was preincubated (2 min, 30°C) and arachidonic acid (80 μM, 16 nmol in 2 μl of ethanol) was added. 15-Hydroperoxyicosatetraenoic acid (5 μM) was added to the assay mixture together with the arachidonic acid. The incubation (5 min) was terminated with 0.5 ml of methanol (−20°C) containing 3 nmol of 13-hydroxylinoleic acid (internal standard). Following centrifugation, the supernatant was evaporated to dryness and reconstituted in 0.2 ml of methanol. For HPLC analysis, the ODS column was eluted with methanol–water 80:20.

Abbreviations: LT, leukotriene; 5-HPETE, (5S)-5-hydroperoxy-6-trans-8,11,14-cis-icosatetraenoic acid; LTA₄, (5S)-5-trans-5,6-oxido-7,9-trans-11,14-cis-icosatetraenoic acid; LTB₄, (5S,12R)-5,12-dihydroxy-6,14-cis-8,10-trans-icosatetraenoic acid; 5,12-DHETE, (5S,12R)-5,12-dihydroxy-6,10-trans-8,14-cis-icosatetraenoic acid; PG, prostaglandin; C20:3, 8,11,14-cis-icosatrienoic acid.
Table 1. Subcellular distribution of LTA\(_4\) synthase in PB-3c cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein, mg</th>
<th>Total activity, units</th>
<th>Specific activity, units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>120</td>
<td>336</td>
<td>2.8</td>
</tr>
<tr>
<td>800 (\times) g</td>
<td>85</td>
<td>382</td>
<td>4.5</td>
</tr>
<tr>
<td>10,000 (\times) g</td>
<td>78</td>
<td>250</td>
<td>3.2</td>
</tr>
<tr>
<td>105,000 (\times) g</td>
<td>12</td>
<td>—</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Homogenates of PB-3c cells (5 \(\times\) 10\(^6\) cells in buffer A; 10 ml) were subjected to centrifugation as indicated. The pellet was resuspended in 1 ml of buffer A.

Clinical Immunology, University of Bern, Switzerland. The mouse bone marrow–derived mast cell line (PB-3c) (10) was generously provided by J.-F. Conscience of Friedrich Miescher Institute, Basel, Switzerland, and was further subcloned for more satisfactory IL-3-dependent proliferation. WEHI-3b-conditioned medium was prepared as described (9). The subclone of PB-3c was maintained by regular passages in 50 vol of fresh RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 10% WEHI-3b-conditioned medium at intervals of 3–4 days. Cells at confluence were harvested and used as the enzyme source. After washing, cells (2.5 \(\times\) 10\(^6\)) were suspended in 50 ml of 20 mM potassium phosphate buffer, pH 7.4/0.1 M NaCl/2 mM EDTA/0.5 mM dithiothreitol (buffer A) saturated with N\(_2\) gas.

**Purification of LTA\(_4\) Synthase from PB-3c Cells.** All procedures were carried out at 0–4°C, except for FPLC chromatographies (room temperature). Buffers were saturated with N\(_2\) gas and degassed thoroughly before use. Protein concentrations were determined according to Bradford (11) with bovine serum albumin as standard. The cell suspension was homogenized by sonication (Branson B-12, output 4, 5 \(\times\) 10 sec). After centrifugation (10,000 \(\times\) g, 15 min) the supernatant (referred to as crude extracts) was subjected to ammonium sulfate fractionation. The precipitate between 35% and 60% was reconstituted in 7.5 ml of 20 mM Tris-HCl, pH 8/1 mM EDTA/0.5 mM dithiothreitol/20% glycerol (buffer B) and applied to three PD-10 columns (Pharmacia) equilibrated with the same buffer. The pooled macromolecule fractions (10.5 ml) were concentrated to 3 ml (ultrafiltration, PM 10, Amicon). After filtration (Millipore, 0.22 \(\mu\)m) the sample (3 \(\times\) 1 ml) was applied to the Mono Q column equilibrated with buffer B. The enzyme was eluted by increasing the ionic strength as shown in Fig. 1A. Active fractions were combined and concentrated, and the buffer was changed (PD-10) to 5 mM potassium phosphate buffer, pH 7.6/1 mM EDTA/1 mM dithiothreitol/20% glycerol (buffer C).

This sample was applied to the hydroxyapatite column equilibrated with buffer C. The enzyme was eluted by increasing the concentration of potassium phosphate (up to 0.3 M) at a flow rate of 0.4 ml/min. The pool of active fractions was concentrated to 0.4 ml by using a Centricon 10 (Amicon).

**Fig. 1.** (A) Anion-exchange chromatography (Mono Q) of ammonium sulfate fraction (35–60%) from PB-3c cells. The solid line indicates the absorbance at 280 nm; the dashed line denotes the concentration of buffer (B) (100% = 1 M KCl). Active fractions between arrows were combined for further purification. (B) Gel filtration on Superose TM 12. Vertical arrows indicate the positions of markers [blue dextran (M, 2,000,000), bovine serum albumin (M, 68,000), ovalbumin (M, 45,000), soybean trypsin inhibitor (M, 22,000), and cytochrome c (M, 13,000); from left to right]. The active fractions between horizontal arrows were combined and are referred to as the purified enzyme.

with methanol/H\(_2\)O/acetic acid, 75:25:0.01 (vol/vol/1 ml)/min, and the absorbance at 234 nm was monitored. Lipoxygenase activity was determined from the ratio of peak areas [5S]-5-hydroxy-6-trans-8,11,14-cis-icosatetraenoic acid (+ 5-HPETE) to the internal standard; recoveries >88%. C20:3 8-lipoxygenase activity was measured essentially as described above, except that arachidonic acid was replaced by C20:3. In all assays, 1 unit of activity was defined as that producing 1 nmol of product per 5 min at 30°C.

**Cells.** The interleukin-3 (IL-3)-producing murine WEHI-3b cell line (9) was donated by B. M. Stadler of the Institute of

**Fig. 2.** HPLC analyses of products formed in incubations with 80 \(\mu\)M 5-HPETE. Reactions were carried out under standard assay conditions. (A) Ten thousand \(\times\) g supernatant fraction of PB-3c cell homogenates (0.8 mg of protein). (B) As in A except that Ca\(^{2+}\) and ATP were omitted. (C) The purified enzyme (4.3 \(\mu\)g). Peak 1, PGB; peak 2, 5-trans-LTB\(_4\); peak 3, 12-epi-5-trans-LTB\(_4\); peak 4, LTB\(_4\); peak 5, 5,12-DiHETE.
Table 2. Purification of LTA₄ synthase from PB-3c cells

<table>
<thead>
<tr>
<th>Purification</th>
<th>Protein, mg</th>
<th>Specific activity, units/mg</th>
<th>5-Lipoxygenase</th>
<th>Ratio*</th>
<th>Yield, † %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extracts</td>
<td>889</td>
<td>LTA₄ synthase 3.1</td>
<td>29</td>
<td>0.11</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>255</td>
<td></td>
<td>8.5</td>
<td>0.16</td>
<td>79</td>
</tr>
<tr>
<td>Mono Q</td>
<td>10.1</td>
<td></td>
<td>59</td>
<td>0.09</td>
<td>22</td>
</tr>
<tr>
<td>HPHT (Bio-Rad)</td>
<td>1.0</td>
<td></td>
<td>179</td>
<td>0.10</td>
<td>6.5</td>
</tr>
<tr>
<td>Superoxide TM 12</td>
<td>0.05</td>
<td></td>
<td>310</td>
<td>0.08</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The ratio was expressed as the LTA₄ synthase activity/5-lipoxygenase activity determined under standard assay conditions.
† Yield was calculated on the basis of the LTA₄ synthase activity.

The final purification step was gel filtration on Superose TM 12 eluted with 20 mM potassium phosphate buffer, pH 7.4/0.2 M NaCl/1 mM EDTA/0.5 mM diethiothreitol/20% glycerol (buffer D, 0.3 ml/min). Active fractions as indicated by the arrows were combined and are referred to as the purified enzyme (Fig. 1B).

RESULTS

Enzymatic Synthesis of LTA₄. When 5-HPETE was incubated with crude extracts of cell homogenates, as shown in Fig. 2A, 6-trans-LTB₄, 12-epi-6-trans-LTB₄, LTB₄, and (5S,12S)-5,12-dihydroxy-6,10-trans-8,14-cis-icosatetraenoic acid (5,12-DiHETE) were formed. Ca²⁺ and ATP were required for the formation of the former three compounds (peaks 2-4), whereas the formation of 5,12-DiHETE was only slightly affected (Fig. 2B). When the purified enzyme was used instead of crude extracts, only the isomers of 6-trans-LTB₄ were observed. These results indicate that the cell homogenates contained LTA₄ synthase, LTA₄ hydrolase, and 12-lipoxygenase; the latter two enzymes were removed during purification. The existence of an epoxide intermediate (LTA₄) was demonstrated as follows. First, 1-min incubations of 5-HPETE (25 nmol) with enzyme (0.1 mg of protein, 180 units/mg) were terminated by addition of 10 vol of acidic methanol (12). The products were extracted with diethyl ether and subjected to reverse-phase HPLC in solvent A. A pair of peaks (18.2 and 19.8 min) showed UV spectra with maxima at 258, 269, and 280 nm. The compounds were converted to methyl ester trimethylsilyl ether derivatives and analyzed by GC/MS. Both compounds appeared with a C value of 24.2 (2% OV-1), showing ions at m/z 421 (M-15), 404, 389, 293, 234, 203, 171, 159 (base peak), and 129, in fair agreement with reported data for the epimers at C-12 of (5S)-5-hydroxy-12-O-methyl-6-trans-LTB₄ (12). Second, the addition of partially purified LTA₄ hydrolase (human leukocytes) to the standard reaction mixture produced LTB₄. These results indicate the presence of LTA₄ in the synthesis of 6-trans-LTB₄ and its 12-epimer.

Copurification of LTA₄ Synthase and Arachidonate 5-Lipoxygenase: Some Properties of the Enzyme. Upon subcellular fractionation, LTA₄ synthase was recovered in the cytosol fraction (Table 1). The purification procedure gave a protein of about 85% purity, as judged by NaDodSO₄/PAGE, and the yield was 0.6%. LTA₄ synthase and 5-lipoxygenase copurified, and the ratio of the two activities was 0.08–0.1 after Mono Q column chromatography (Table 2). The diverging ratio in the crude preparations may reflect nonenzymatic formation of LTA₄ (13). A major band (M, 75,000) always appeared on the NaDodSO₄ gel in parallel with the enzyme activity, whereas the Mₓ was estimated to be 50,000–55,000 by gel filtration (Superose TM 12). The enzyme was unstable especially after Mono Q column chromatography, even in the presence of glycerol, dithiothreitol, and EDTA, and under anaerobic conditions, which all partially stabilized the enzyme. Addition of microsomal protein from the PB-3c cells back to the purified enzyme (or to the 105,000 × g supernatant of the homogenate, Table 1) stimulated enzyme activities, as shown for 5-lipoxygenase from human leukocytes. In contrast, there was no similar stimulatory effect of the protein nonadsorbing to the Mono Q column (8, 14).

Synthesis of LTA₄ proceeded almost linearly for the first 2–3 min (Fig. 3A). It was dependent of the amount of enzyme (up to 10 μg), and was diminished by boiling for 3 min (Fig. 3B). The enzyme required Ca²⁺ for full activity; the half-effective dose was ≈ 0.1 mM (Fig. 3C). Omission of ATP from the reaction mixture decreased the activity by almost 50%. When cells were treated with 2 μM Ca ionophore (A23187, Calbiochem) for 5 min in Tyrode solution and washed with buffer A prior to homogenization, the enzyme activity of the

Fig. 3. (A) Time course of LTA₄ synthase determined by HPLC assay (○) and spectrophotometric assay. Approximately 8 μg of enzyme (180 units/mg, after HPHT column) was incubated with 5-HPETE under standard assay conditions. The solid line indicates the increase in the absorbance at 275 nm with the same amount of enzyme. (B) Effect of enzyme amount (0–15 μg) either in the presence (●) or absence (○) of 2 mM Ca²⁺ with 2 mM ATP. LTA₄ was not formed with an enzyme boiled for 3 min (×). (C) Requirement of Ca²⁺ for LTA₄ synthase either in the presence (○) or absence (●) of ATP.
10,000 × g supernatant was lowered by 25–30% compared to the control. The inclusion of EDTA during all steps of the enzyme purification was in fact required for obtaining reproducible enzyme activities.

When the purified enzyme (2.4 μg) was incubated with 80 μM arachidonic acid (with Ca²⁺ and ATP), about 7.4 nmol of 5-HPETE and 2.1 nmol of LTA₄ were produced. The enzyme produced about 2.5 times more LTA₄ when incubated with arachidonic acid than with 5-HPETE (50 μM), although less 5-HPETE was formed in the arachidonate incubation.

Also under standard assay conditions, the purified enzyme (2.4 μg) acted on C20:3 to give 0.8 nmol of 8-hydroperoxy-9,11,14-icosatrienoic acid (identified by HPLC, UV spectroscopy, and GC/MS; data not shown).

Heat Inactivation and Inhibitors. When the enzyme was treated for 3 min at various temperatures, 5-lipoxygenase and LTA₄ synthase activities decreased concomitantly (Fig. 4).

In the crude extracts, however, about 25% of the LTA₄ synthase activity was heat-resistant (13). The lipoxygenase inhibitors AA-861 (Fig. 4) and 5,6-methano-LTA₄ inhibited both enzyme activities. IC₅₀ values of the latter compound for LTA₄ synthase and 5-lipoxygenase activities were 25 and 18 μM, respectively. Finally, C20:3, another substrate of the enzyme, competitively inhibited (Kᵢ = 0.15 mM) the arachidonate 5-lipoxygenase and inhibited the formation of LTA₄ from 5-HPETE in a dose-dependent manner (data not shown).

**Incubation of the Purified Enzyme with [10n-³H;³¹C]5-HPETE.** The purified enzyme as well as crude extracts of cell homogenates were incubated with 10 μM [10n-³H;³¹C]5-HPETE (20,000 cpm for ³H and 51,000 cpm for ³¹C). Metabolites were isolated by HPLC and the radioactivity ratio was determined for each compound. As shown in Table 3, LTA₄ (6-trans-LTB₄, 12-epi-6-trans-LTB₄) synthesized with the purified enzyme, and LTB₄ (formed only in crude extracts) lost >92% of the d (pro-R)-hydrogen when compared to the ³H/³¹C ratio of the precursor 5-HPETE. On the other hand, 5,12-DIHETE retained the d-hydrogen at C-10. The apparent partial retention of 10n (pro-R) in the formation of LTA₄ by crude homogenates is explained by hemoproteins catalyzing synthesis of LTA₄ and isomeric compounds by a different mechanism (13). Interestingly, however, the ³H/³¹C ratio of LTB₄ formed in the crude extracts was significantly lower, probably reflecting the preference of LTA₄ hydrolysis for enzymatically formed LTA₄, with the correct stereochemistry.

![Graph](image)

**Table 3.** ³H/³¹C ratios of products from incubations with [10n-³H;³¹C]5-HPETE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purified enzyme</th>
<th>Crude extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-trans-LTB₄</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>12-epi-6-trans-LTB₄</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>LTB₄</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>5,12-DIHETE</td>
<td>—</td>
<td>98</td>
</tr>
</tbody>
</table>

Experiments in duplicate were carried out with crude extracts (3.1 units/mg) and the purified enzyme (310 units/mg) from PB-3c cells. The ³H/³¹C ratio of the precursor 5-HPETE (0.392) was defined as 100%.

**DISCUSSION**

Biologically active LTs are synthesized by way of the unstable intermediate LTA₄. Several studies have shown the enzymatic nature of LTA₄ formation from 5-HPETE (3, 4, 12) and indicated that the enzyme(s) involved were cytosolic (15). Here, we describe a cytosolic, Ca²⁺-requiring LTA₄ synthase from murine mast cells. The PB-3c cell line was chosen because of the prominent conversion of arachidonic acid by the 5-lipoxygenase pathway. In our hands, the specific activity of LTA₄ synthase in these cells was about 6 times higher than in RBL cells and 2.5 times higher than in human leukocytes (data to be published elsewhere).

The LTA₄ synthase from PB-3c cells was purified to about 85% homogeneity. Many properties were similar to those of mammalian arachidonate 5-lipoxygenases (8, 14–19): the molecular weight (75,000), the acidic isoelectric point, and the requirements for Ca²⁺ and ATP. In addition, there are mechanistic similarities between the reactions catalyzed by 5-lipoxygenase and LTA₄ synthase (5). Thus, several criteria suggested the identity of LTA₄ synthase with arachidonate 5-lipoxygenase. This concept was further supported by the following findings: (i) both enzyme activities copurified (Table 2) and coeluted in three principally different FPLC chromatographies; (ii) both enzyme activities responded practically identically to heat treatment and to inhibitory agents (Fig. 4); (iii) C20:3 was a substrate for the enzyme (oxygenated at C-8), which inhibited LTA₄ synthase and 5-lipoxygenase activities; (iv) when the purified enzyme was incubated with [10n-³H;³¹C]5-HPETE, a selective elimination of the 10n-hydrogen was observed, in agreement with data on the mechanism of LTA₄ synthase in intact cells (3, 4).

The data presented here indicate that in murine mast cells, as was demonstrated recently for LTA₄ formation in human leukocytes (20), a single enzyme catalyzes 5-lipoxygenation and further conversion to LTA₄. These findings are of considerable interest in connection with physiologic and therapeutic control of LT formation.

We are grateful to Dr. O. Ohara and Dr. T. Miyamoto for helpful discussions. This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and by grants from The Japanese Foundation on Metabolism and Diseases, from the Research Foundation for Cancer and Cardiovascular Diseases, from the Advance Co. Ltd. (Tokyo), and from the Swedish Medical Research Council (03X-217 and 03X-07467).

Biochemistry: Shimizu et al.