Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 2589–2592, April 1993
Biochemistry

Hepoxilin A_3 is the endogenous lipid mediator opposing hypotonic swelling of intact human platelets

(retraction/lipoxygenase)

ALON MARGALIT*, YOSSI SOFER†, SHLOMO GROSSMAN†, DENIS REYNAUD‡, CECIL R. PACE-ASCIAK‡§¶, AND AVINOAM A. LIVNE‖

*Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel; †Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel; ‡Research Institute, Hospital for Sick Children, Toronto, Canada; and §Department of Pharmacology, Faculty of Medicine, Toronto, Canada

Communicated by Josef Fried, December 21, 1992

ABSTRACT **When human blood platelets are exposed to hypotonic medium they swell first but, shortly thereafter, revert toward their original volume in a process termed regulatory volume decrease (RVD). RVD is the result of an enhanced efflux of K^+ and Cl^- ions and associated water. Platelet RVD is controlled by a short-lived lipoxygenase-derived product (LP). By using a combination of high-performance liquid chromatography, gas chromatography–mass spectrometry, and RVD reconstitution bioassay, we show that LP is identical with hepoxilin A_3. In addition we demonstrate that authentic hepoxilin A_3 possesses the same biological properties on RVD reconstitution as LP and that the activity of both compounds is amplified through epoxide hydrolase inhibition with 3,3,3-trichloropropene-1,2-oxide. Therefore, we report here that volume expansion causes the formation and release of hepoxilin A_3 from intact human platelets and that this hepoxilin plays a major role in volume regulation.**

Regulation of cell volume is an important physiological process expressed by many cell types (for review, see ref. 1). Platelets respond to hypotonic-induced swelling by an increase in K^+ conductance which occurs simultaneously with an increase in an independent conductive Cl^- transport. The outward movement of KCl, driven by the K^+ gradient, results in an osmotically obliged water efflux causing a volume loss designated as regulatory volume decrease (RVD) (2). Margalit and Livne (3) have shown that volume retraction of human platelets after hypotonic swelling is controlled by an unidentified lipoxygenase-derived product (LP). LP is released into the medium in response to hypotonic shock, and when added to volume expanded cells whose retraction has been inhibited by the presence of a lipoxygenase inhibitor, it initiates volume retraction by promoting exclusively K^+ permeability. The identity of this metabolite with hepoxilin A_3 [8-hydroxy-11,12-epoxyeicosa-5(*Z*),9(*E*),14(*Z*)-trienoic acid], a metabolite of arachidonic acid formed via the 12-lipoxygenase pathway, is the subject of this communication.

MATERIALS AND METHODS

Materials. The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) was obtained from Sigma. 3,3,3-Trichloropropene-1,2-oxide (TCPO) was kindly provided by A. Ford-Hutchinson and R. Zamboni (Merck Frosst, Pointe Claire, PQ, Canada). Methyl 2-[(3,4-dihydro-3,4-dioxo-1-naphthalenyl)amino]benzoate (CGS 8515) was a gift of A. Lovell (CIBA–Geigy), and *N*-(3-phenoxycinnamyl)acetohydroxamic acid (BW A4C) was provided by L. G. Garland (Wellcome). Hepoxilin A_3 was kindly provided by E. J. Corey (Harvard University, Cambridge, MA). [1-^{14}C]Hepoxilin A_3 was prepared in our laboratories as previously described (4).

Solutions. Acid citrate/dextrose solution was composed of 65 mM citric acid, 11 mM glucose, and 85 mM trisodium citrate. The standard isotonic medium contained 137 mM NaCl, 1 mM KCl, 0.42 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4, adjusted to 285 mOsm. Hypotonic solutions were prepared by 1:1 dilution of the standard isotonic medium with distilled water. Media were filtered through a 1.2-μm membrane filter (Schleicher & Schuell, AE 9S) to remove particles that would interfere with the cell sizing measurement. Stock solution of NDGA (20 mM) was made in ethanol, BW A4C (20 mM) was made in dimethyl sulfoxide, and CGS 8515 was made in dimethylformamide. Fresh stock solutions (0.5 and 0.1 mM) of hepoxilins were made daily in dimethyl sulfoxide.

Platelet Preparation. Venous blood was obtained from healthy volunteers, 20–40 years of age, who had not taken any medication in the preceding 14 days. Blood was collected in plastic tubes and anticoagulated with acid citrate/dextrose solution at a blood/anticoagulant volume ratio of 6:1. Platelet-rich plasma was obtained by centrifugation at 120 × *g* for 10 min and had a pH of 6.5 ± 0.1. Platelets were used within 4 hr of collection.

Volume Measurements. Cell volume distribution curves were obtained with Coulter Counter (model ZM) with Coulter Channelizer 256 (orifice diameter, 70 μm). The mean cell volume was calculated as the median of the cell volume distribution curves. The relative volume relates to the cell volume in isotonic medium.

RVD Reconstitution Assay. The sample to be assayed for the presence of LP was mixed rapidly with 10 ml of hypotonic solution containing 20 μM NDGA, and 10 μl of platelet-rich plasma was added immediately. Volume measurements commenced within 20 sec after addition of platelets and lasted 4 min. The presence of NDGA in the assay medium abolishes platelet RVD, so that only the addition of substances which supplement the missing LP reconstitutes RVD (3). All experiments presented were conducted at 22–24°C and were performed at least three times. Unless stated otherwise, results of a representative experiment are shown.

Solid-Phase Extraction of LP. Solid-phase extraction employed C_{18} Sep-Pak cartridges (Waters) essentially as described by Powell (5). Briefly, 10 ml of platelet-rich plasma (≈3 × 10^9 cells) was mixed with the same volume of medium.

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Abbreviations: RVD, regulatory volume decrease; LP, lipoxygenase-derived product; 12-HPETE, 12(*S*)-hydroperoxyeicosa-5(*Z*),8(*Z*),10(*E*),14(*Z*)-tetraenoic acid; TCPO, 3,3,3-trichloropropene-1,2-oxide; NDGA, nordihydroguaiaretic acid.

¶To whom reprint requests should be addressed at: Division of Neurosciences, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.
‖Deceased April 23, 1992.

After 15 sec of incubation, the cell suspension was percolated through the Sep-Pak cartridge and the cartridge was washed further with 4 ml of isotonic solution followed by 4 ml of petroleum ether. The fraction containing LP was eluted with 4 ml of absolute ethanol. The latter fraction was evaporated to near dryness with a stream of nitrogen gas, and the residue was dissolved in 1 ml ethanol. A 10-μl aliquot of this extract was assayed for RVD reconstitution activity.

HPLC Purification. The ethanol eluate from Sep-Pak was purified by straight-phase HPLC on a Waters μBondapak column with hexane/2-propanol/acetic acid, 98:2:0.1 (vol/vol), as solvent at a flow rate of 1.5 ml/min. Fractions of 2 min were collected and the presence of LP was assessed through the RVD reconstitution assay. Authentic [1-^{14}C]hepoxilin A_3 was used to evaluate the retention time for this compound. Radioactivity was monitored with an on-line radioactivity monitor (Berthold, Wildbad, Germany) after mixing (2:1) with PCS scintillation mixture, and fractions were quantified through conventional scintillation counting.

Gas Chromatography–Mass Spectrometry (GC–MS). The fraction from HPLC with RVD reconstitution activity was acidified with 1 M HCl and extracted with diethyl ether. After centrifugation, the diethyl ether phase was washed to neutrality with water, and the ether phase was then taken to complete dryness. Authentic hepoxilin A_3 was treated in a similar fashion. The residue was converted to the pentafluorobenzyl ester, trimethylsilyl ether as previously described (6). The derivatized samples were analyzed by GC–MS (Hewlett–Packard model 5890) in the negative-ion chemical ionization mode. Samples were injected on a 60-m DB-1 fused silica column (J & W Scientific, Rancho Cordova, CA) with hydrogen as carrier gas and methane as reactant gas. Column temperature at time of injection was 80°C, followed by a rapid temperature program at 20°C/min to a final temperature of 300°C. Injector and transfer lines were maintained at 275°C.

RESULTS

Purification and Characterization of the Platelet-Derived LP as Hepoxilin A_3. Since the RVD reconstituting LP was found to be very unstable ($t_{1/2}$ of 8 and 120 sec in the presence and absence of cells, respectively) (3), our initial effort was aimed toward its stabilization. LP stabilization was attained through use of solid-phase extraction on C_{18} Sep-Pak. Ten milliliters of platelet-rich plasma was mixed with an equal volume of isotonic solution (isotonic experiment) or distilled water (hypotonic experiment). After 15 sec of isotonic or hypotonic exposure, the mixture was percolated through a Sep-Pak cartridge and the ethanol eluate was tested for the presence of LP with the RVD reconstitution assay. As seen in Fig. 1, the ethanol fraction eluted from the Sep-Pak from the hypotonic experiment displayed RVD reconstitution activity. In contrast, the corresponding fraction derived from the isotonic experiment was devoid of activity. When a lipoxygenase inhibitor (NDGA, CGS 8515, or BW A4C) was present during the hypotonic exposure of the platelets, no RVD reconstitution activity was detected (data not shown). This activity was relatively stable and could be preserved for 2 weeks at −20°C. The active factor was further characterized by subjecting it to HPLC purification.

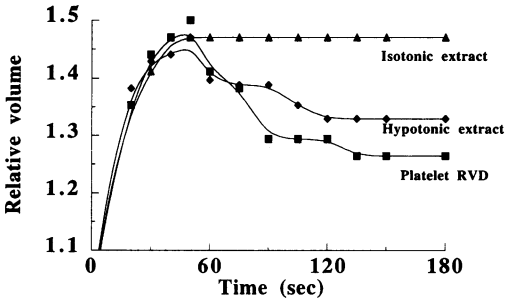

FIG. 1. RVD reconstitution activity of C_{18} Sep-Pak ethanol extracts derived from platelets exposed to isotonic or hypotonic solution. The normal pattern of RVD (hypotonic medium without NDGA) is shown for comparison.

Fractions of 2 min were collected and analyzed for the presence of LP by the RVD reconstitution assay. As shown in Fig. 2*A*, the main biological activity was associated with the fraction at 16–18 min (*Bottom*), which corresponded with authentic [^{14}C]hepoxilin A_3 (*Top*). The absence of correlation of the biological activity with UV absorption (Fig. 2*A Middle*) is consistent with the absence of any conjugated double bonds in hepoxilins (6). Further characterization of the material in the fraction at 16–18 min was made with GC–MS after acidifying this fraction. For comparison, similar treatment of authentic hepoxilin A_3 was carried out (acidification converts hepoxilin A_3 into its stable trihydroxy metabolite). As shown in Fig. 2*B*, the single-ion chromatogram (m/z 569) resulting from the purified HPLC fraction 16–18 is virtually identical to the chromatogram derived from authentic hepoxilin A_3, both in the multiplicity of peaks and in their retention times.

Authentic Hepoxilins Reverse Swelling of Platelets. A separate approach was taken to further confirm that hepoxilin A_3 possesses the same RVD-reconstituting biological activity as the platelet-derived LP. As seen in Fig. 3*A*, a dose-dependent reduction of hypotonic-induced cell volume expansion was observed with chemically derived hepoxilin A_3, with an apparent threshold of 50 nM. Thus, authentic hepoxilin A_3 mimicked the platelet-derived extract (LP) in opposing the volume expansion of human platelets induced by hypotonic stress in the presence of the lipoxygenase inhibitor NDGA.

Hepoxilins possess an epoxide moiety which is subject to hydrolysis by epoxide hydrolases in platelets (7). TCPO inhibits hepoxilin epoxide hydrolase (8). When hepoxilin A_3 was tested in the presence of 1 mM TCPO, its activity was found to be 4- to 5-fold higher (Fig. 3*B*) than in the absence of TCPO (Fig. 3*A*). To test the effect of TCPO on the platelet-derived LP, the RVD reconstitution activity of the Sep-Pak ethanol extract was tested in the presence and absence of TCPO. As shown in Fig. 4*A*, addition of TCPO enhanced the activity of the extract. To verify whether the TCPO effect is correlated with LP stabilization, blood platelets were exposed for different time intervals to hypotonic medium in the presence and absence of TCPO. At the end of the incubation, NDGA was added, the suspension was centrifuged for 30 sec to sediment the cells, and the cell-free medium was tested for RVD reconstitution activity. As shown in Fig. 4*B*, TCPO enhanced and stabilized the RVD reconstitution activity, confirming structural identity of LP and hepoxilin. Using radiolabeled hepoxilin A_3 methyl ester incubated with intact platelets in the presence of TCPO to block hydrolysis of the epoxide group, we determined through thin-layer chromatography that 47 ± 7% (n = 2) of the compound was saponified to the free acid during 5 min at 23°C, indicating that platelet esterases are capable of converting the methyl ester of hepoxilin A_3 to the free acid (data not shown). This experiment also indicates that the biological activity shown for the synthetic compound is underestimated by a factor of 2.

Hepoxilins (A_3 and B_3) are formed by intramolecular rearrangement of 12(S)-hydroperoxyeicosa-5(Z),8(Z),10(E),14(Z)-tetraenoic acid (12-HPETE) (6). Previously, it was demonstrated that 100 nM 12-HPETE restores RVD of human platelets in the presence of NDGA (3). We therefore tested hepoxilin B_3 [10-hydroxy-11,12-epoxyeicosa-5(Z),8(Z),14(Z)-trienoic acid] as well, to determine whether both hepoxilins A_3 and B_3 share in RVD reconstitution activity.

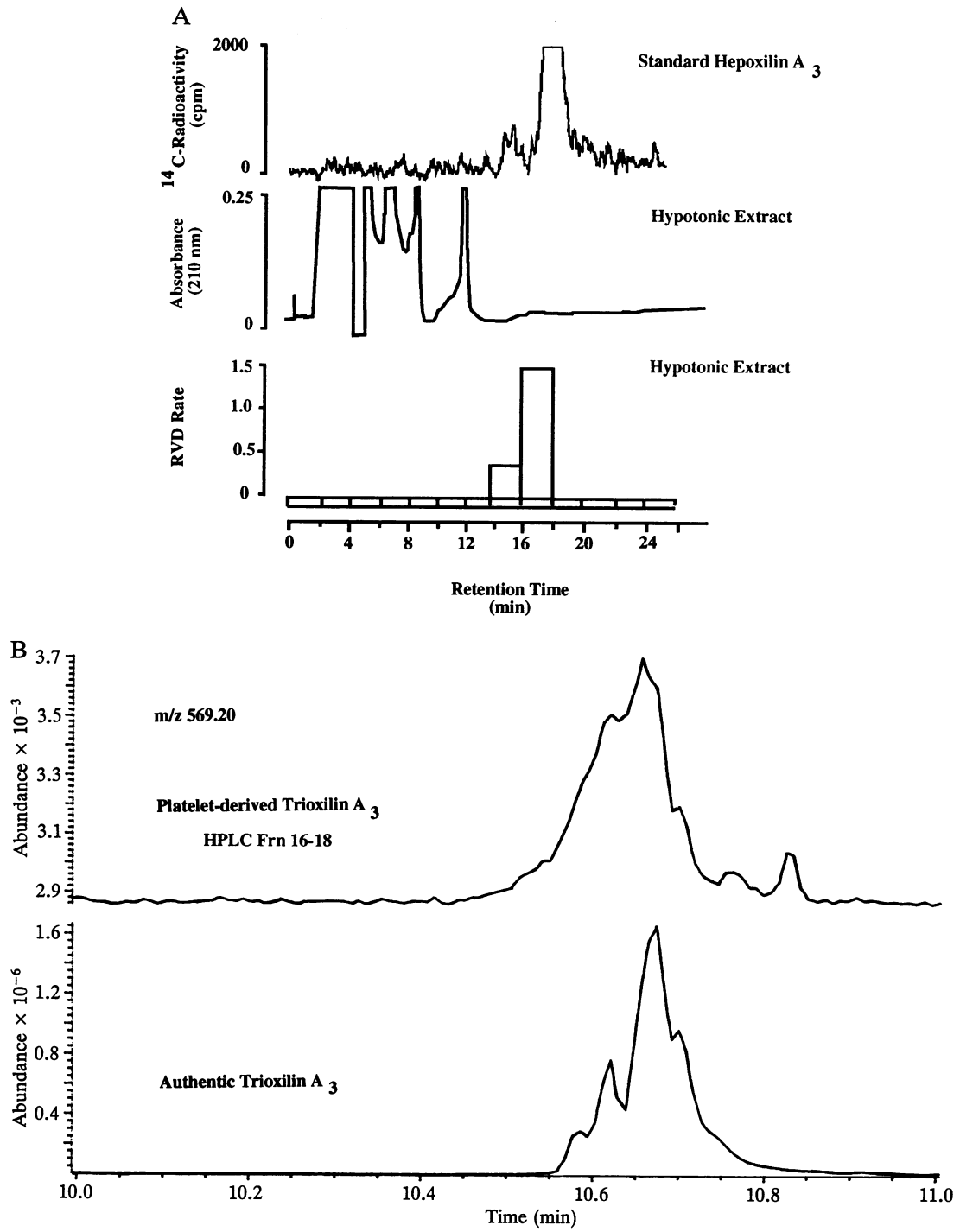

FIG. 2. Correspondence with hepoxilin A_3 of RVD biological activity present in platelet-derived hypotonic extract. (*A*) HPLC data showing the retention time of standard [^{14}C]hepoxilin A_3 (*Top*), the UV absorption of the hypotonic extract from the C_{18} Sep-Pak cartridge (*Middle*), and the biological activity of the collected fractions as tested by the RVD reconstitution assay (*Bottom*). RVD rate was determined from the cell retraction rate as the log(relative volume/sec) × 10^3. (*B*) GC–MS single-ion chromatogram (*m/z* 569) of derivatized acidified HPLC fraction 16–18, with negative-ion chemical ionization detection.

We found that hepoxilin B_3 was without effect at concentrations below 100 nM (data not shown).

DISCUSSION

This study demonstrates that intact human platelets synthesize and excrete hepoxilin A_3 from endogenous precursors in response to hypotonic shock and that this compound in turn controls platelet RVD. This conclusion is based on the following findings: (*i*) the RVD reconstitution activity detected after HPLC purification of the Sep-Pak ethanol extract derived from platelets exposed to hypotonic medium is coeluted with hepoxilin A_3, (*ii*) the active HPLC fraction possesses a gas chromatographic pattern and mass spectral characteristics identical to those of authentic hepoxilin A_3, (*iii*) authentic hepoxilin A_3 restores RVD of human platelets in the presence of the lipoxygenase inhibitor NDGA, and (*iv*) the RVD reconstitution activities of both hepoxilin A_3 and the platelet-derived LP are enhanced in the presence of the epoxide hydrolase inhibitor TCPO at 1 mM.

12-Lipoxygenase metabolites of arachidonic acid have been found to play a role in neuronal signaling by activation

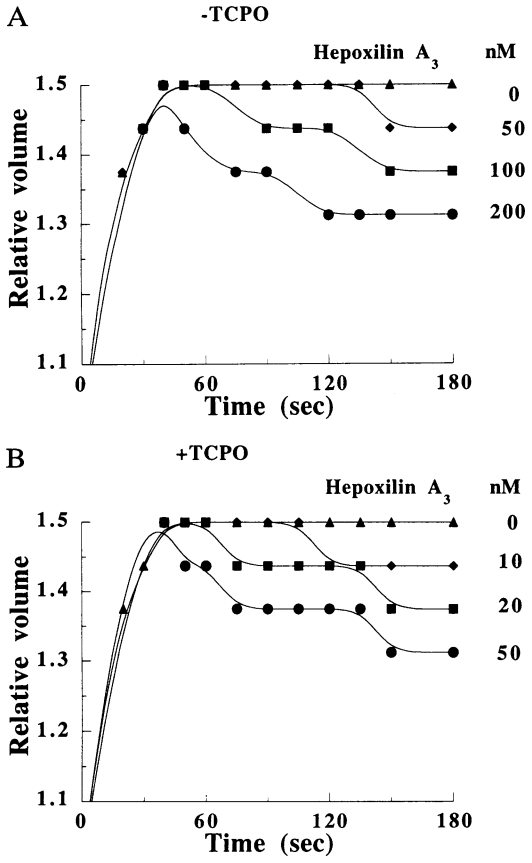

FIG. 3. RVD reconstitution activity of chemically derived hepoxilin A_3 in the absence (*A*) or presence (*B*) of 1 mM TCPO.

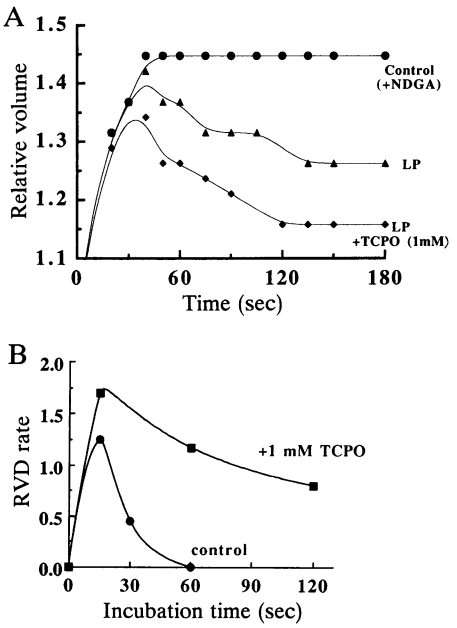

FIG. 4. RVD reconstitution activity of platelet-derived LP in the presence of 1 mM TCPO. (*A*) Sep-Pak hypotonic ethanol extract (10 μl). (*B*) Hypotonic eluate. The hypotonic eluate was prepared as follows: 200 μl of platelet-rich plasma containing 3–4.5 × 10^7 platelets was mixed with 200 μl of distilled water and 100 μl of hypotonic solution. At the indicated times, NDGA was added to terminate further formation of LP and the suspension was centrifuged at 15,000 × *g* for 30 sec. The cell-free supernatant was collected and a sample of 200 μl was assayed for RVD reconstitution. RVD rate was determined from the cell retraction rate as log[relative volume/sec] × 10^3.

of distinct potassium channels (for review see ref. 9). In the marine mollusc *Aplysia californica*, neuronal potassium channels have been found to be affected by hepoxilins (10). Our data correlate well with these studies and show that, like neurons, platelets apparently possess potassium channels that are activated by hepoxilins. Hepoxilins have been shown to exert a number of biological actions—e.g., release of insulin (11), potentiation of norepinephrine-induced aortic vasoconstriction (12), potentiation of vascular permeability in the skin (13), activation of neutrophils (14), and modulation of neurotransmission in the mammalian central nervous system (8, 15) and the *Aplysia* brain (16). Platelets are considered to be the main source of 12-lipoxygenase metabolites in the bloodstream, but their role is currently under dispute since no activity has been demonstrated for its main metabolites, 12(*S*)-hydroxyeicosa-5(*Z*),8(*Z*),10(*E*),14(*Z*)-tetraenoic acid and 12-HPETE, at submicromolar concentrations (17–19). The present experiments indicate that platelets form and are activated by hepoxilins, metabolites derived from the 12-lipoxygenase pathway. A previous study showed that platelets excrete LP (herein identified as hepoxilin A_3) in response to mechanical stimuli, such as shear stress, which appear under extreme conditions in the bloodstream (20). It is possible therefore that platelet-derived hepoxilins produced under *in vivo* conditions play a major role in hemostasis.

This work is dedicated to the memory of Professor A. A. Livne, who died after this research was completed. This study was supported in part by funds from the Medical Research Council of Canada (MT#4181 to C.R.P.-A.) and by a special grant from Dr. Aser Rothstein (A.M.). Part of this work was carried out at the Amelia (Mimi) Rose Laboratory for Cellular Signal Transduction at the Department of Life Sciences, Ben-Gurion University of the Negev.

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