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Rapid Report

Endogenous hepoxilin A_3, produced under short duration of high shear-stress, inhibits thrombin-induced aggregation in human platelets

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Abstract

The effect of short duration of shear-stress (350 dyne/cm^2, 20 ms) on platelet-aggregation has been assessed. This treatment inhibits thrombin-induced but not ADP- or collagen-induced aggregation. The inhibitory effect is mediated by endogenous hepoxilin A_3. This conclusion is based on the following observations: (a) The shear-stress effect is abolished by lipoxygenase inhibitors. (b) Hepoxilin A_3 mimics the shear-stress effect.

Key words: Platelet; Aggregation; Hepoxilin A_3; Shear stress; (Human)

Although platelets 12-lipoxygenase was the first lipoxygenase to be discovered in animal tissue [1], its role in platelets behavior is currently under dispute. Some investigations do suggest a role for the 12-lipoxygenase metabolites 12-hydroperoxyeicosatetraenoic acid (12-HPETE) [2–4] and 12-hydroxyeicosatetraenoic acid (12-HETE) [5,6] in platelet responses, but since the effect of these metabolites is detected in the micromolar concentration range, it is not clear if they have any functional significance to platelets behavior under in-vivo conditions [7].

We reported recently that, in addition to 12-HPETE and 12-HETE, human platelets produce another 12-lipoxygenase product, hepoxilin A_3 [8]. This metabolite is the endogenous mediator opposing hypotonic swelling of human platelets and it affects platelet-regulatory volume decrease (RVD) at concentrations range of 50–200 nM [8]. This product is also produced by the platelets in response to other mechanical stresses such as centrifugation and short duration of flow [9]. Hepoxilin A_3 is formed by the platelets under shear-stress of 120–350 dyne/cm^2, for 5 and 20 ms, which are correlated to the shear-stresses at the site of sclerotic plaque in cardiovascular diseases [10]. In this communication we report that hepoxilin A_3 produced under these conditions inhibits thrombin-induced platelet aggregation.

In order to test the effect of short duration of shear-stress on platelets behavior, 500 μl of platelet-rich plasma (PRP) were exposed to shear-stress of 350 dyne/cm^2 for 20 ms utilizing the 'controlled-flow device', described previously [9]. Immediately thereafter, the platelets were tested for aggregation, in response to thrombin, 0.05 unit/ml (Fig. 1A), ADP, 2 μM (Fig. 1B) and collagen, 20 μg/ml (Fig. 1C). It is demonstrated that shear-stress inhibits platelets response to thrombin, but not to collagen or ADP. The shear-stress by itself had no effect on platelets aggregation (Fig. 1A). Pretreatment of the platelets with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) [11] abolishes this shear-stress associated effect (Fig. 1D). BWA4C, another inhibitor of the lipoxygenase pathway, produces a similar effect (data not shown). Addition of these inhibitors to the experimental system, did not, by itself, affect aggregation.

To verify that the inhibitory effect of short duration of shear-stress on thrombin is associated with hepoxilin A_3 function, platelets were treated with hypotonic-derived extract, which contains hepoxilin A_3 (Fig. 2A) or commercial hepoxilin A_3 (Fig. 2B). As shown in Fig. 2A, hypotonic derived hepoxilin A_3 inhibited thrombin-induced aggregation. When the hypotonic shock was applied to platelets in the presence of the lipoxyge-

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nase inhibitors NDGA or BWA4C, no inhibitory activity could be detected (data not shown). Commercial hepoxilin A_3 affected aggregation in a dose dependent manner (Fig. 2B). The threshold of its activity is about 50 nM. It is also shown that hepoxilin A_3 affects mainly the aggregation extant rather than its rate. Fig. 2C exhibits a plot of aggregation extent as a function of hepoxilin A_3 concentration. The calculated EC_{50} is 110 nM.

The main conclusion from our study is that hepoxilin A_3 which is produced by human platelets in response to short duration of shear-stress, inhibits thrombin induced aggregation. This conclusion is based on the following findings: (i) Shear stress, under condi-

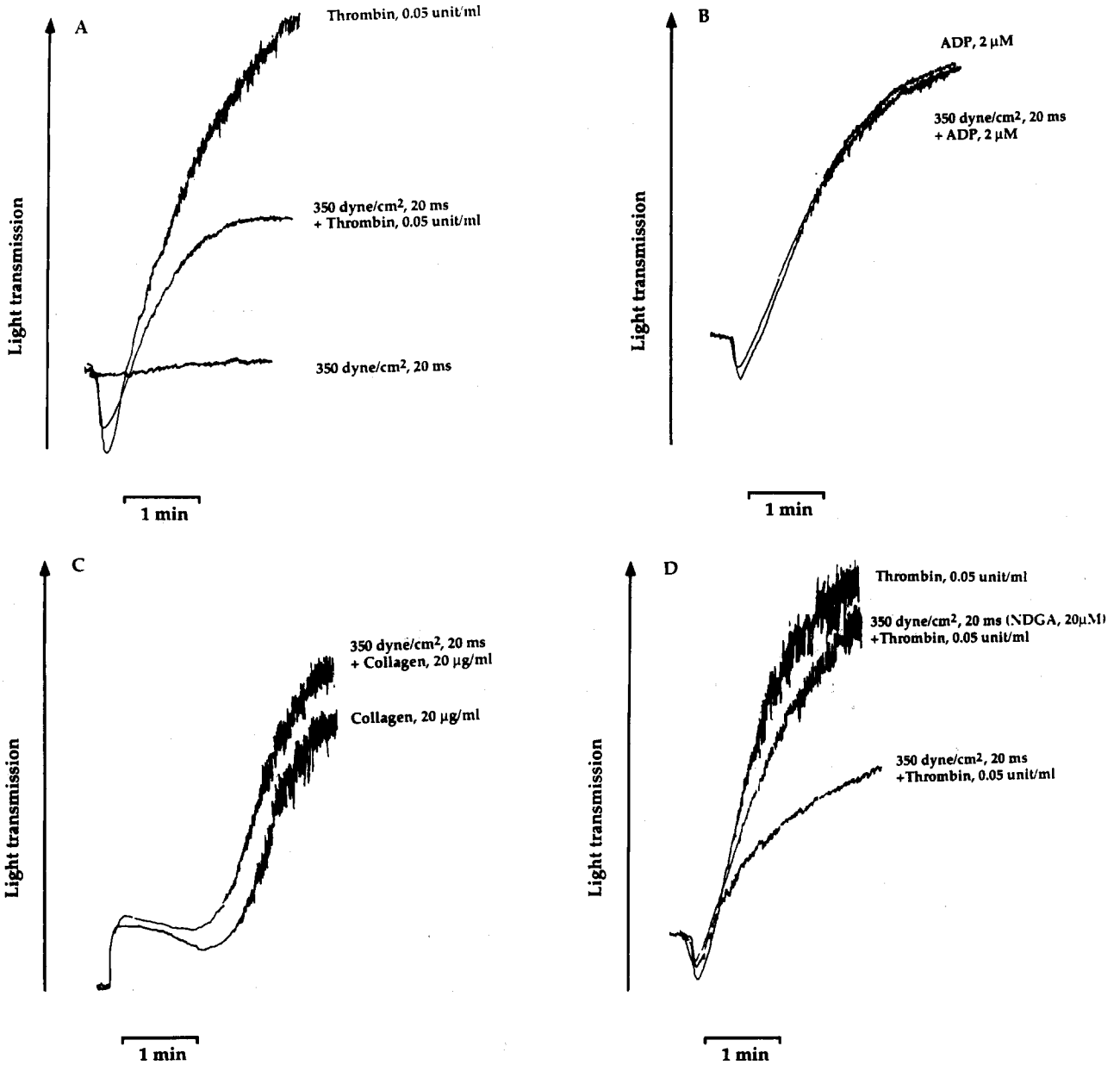

Fig. 1. Shear-stress effect on platelets aggregation in response to thrombin, 0.05 unit/ml (A); ADP, 2 μM (B); collagen, 20 μg/ml (C) and thrombin, 0.05 unit/ml on platelets pretreated with NDGA 20 μM (D). Platelet-rich plasma (PRP) was prepared as described [9], from healthy donors who had not taken any medication in the preceding ten days. For aggregation, 100 μl PRP were mixed with 400 μl standard medium containing: 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 290 mosM. Experiments were done by utilizing Chronolog S43D aggregometer (Havertown, PA). Shear-stress was applied by utilizing the 'controlled-flow device' as described previously [9]. For each experiment, 500 μl of PRP were exposed to 350 dyne/cm^2 for 20 ms and a sample of 100 μl was taken for aggregation. Diagnostic collagen was purchased from Stago (Asnieres-Sur-Siene, France). ADP, thrombin (human) and NDGA were obtained from Sigma (St, Lois, MO. USA). Traces are representative data. Each experiment was performed with three repetitions, from four different donors.

tions which promote hepoxilin A_3 production [9], inhibits thrombin induced aggregation. This inhibition is not detected when the platelets are pretreated with lipoxygenase inhibitors. (ii) Hypotonic derived hepoxilin A_3 mimics the shear-stress effect on thrombin-induced aggregation. (iii) Commercial hepoxilin A_3 inhibits thrombin-induced aggregation in a dose dependent manner, at concentrations range which are correlated to its effect on RVD reconstitution [8]. At concentrations of above 100 nM, hepoxilin A_3 affects both aggregation rate and extant and reverses aggregation. Hepoxilin A_3 effect at 100 nM concentration, can be related to the shear-stress effect on platelet-aggregation (compare Figs. 1A and 2B). Hepoxilin A_3 is susceptible to inactivation by epoxide hydrolases [12] and we have shown previously that its activity on RVD reconstitution is enhanced in about 5-fold in the presence of the epoxide hydrolase inhibitor TCPO [8]. It is possible, therefore, that less hepoxilin A_3 is actually produced by the platelets and since, it affects the platelets before its degradation, it yields similar effect as 100 nM of commercial hepoxilin.

The mechanism by which hepoxilin A_3 affects thrombin-induced aggregation is yet to be elucidated. However, it is probably not mediated by the PGI/PGE_1 receptor [13], since PGE_1 did not affect RVD [9]. It also seems that hepoxilin A_3 does not interfere with the binding of thrombin to its receptor since it does not affect the initial shape change detected by reduction of light transmission in the aggregometer [14] and Fig. 2B.

Rheological conditions may impact platelet behavior and therefore are suggested to play a major role in the events leading to thrombus formation in cardiovascular diseases [15]. Although it has been demonstrated that shear-stress activates the platelets and increases their sensitivity to aggregating agents, this phenomenon is not detected when platelets are exposed to shear-stresses for less then 10 s (for review, see Hellums et al. [16]). Our results indicate that endogenous hepoxilin A_3, which is formed in response to short duration

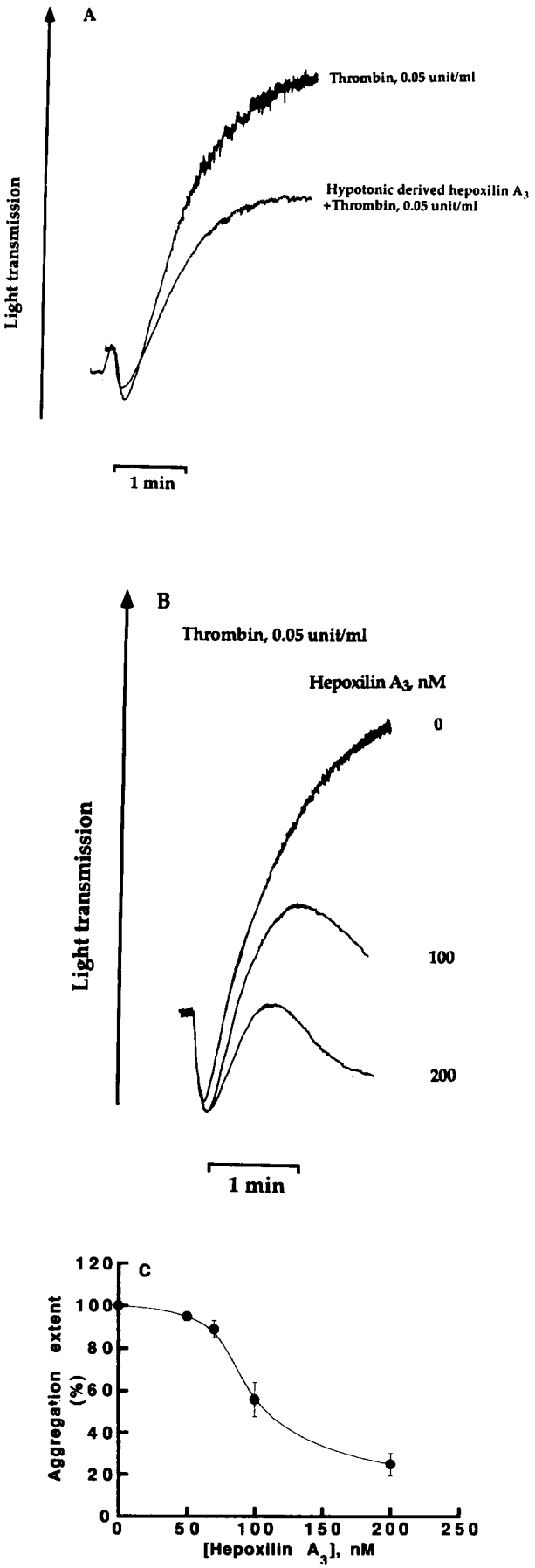

Fig. 2. Effect of hepoxilin A_3 on thrombin (0.05 unit/ml) induced aggregation of human platelets. Crude hepoxilin A_3 derived from platelets exposed to hypotonic treatment (A); commercial hepoxilin A_3 (B) and aggregation extent as a function of hepoxilin A_3 concentration, mean $\pm$SE, $n = 4$ (C). Crude hepoxilin A_3 was prepared by mixing 200 μl PRP with 200 μl of distilled water for hypotonic shock. After 15 s, NDGA was added and the cell-free eluates were prepared as described [9]. A sample of 50 μl was added to the aggregometer cuvette prior to thrombin. Commercial hepoxilin A_3 was purchased from Biomol (Plymouth Meeting, PA, USA). Stock solution of 100 μM was made in ethanol. Control samples were prepared with equal volume of ethanol. Total ethanol added did not exceed 0.1%. Commercial hepoxilin A_3 activity was assayed by the RVD reconstitution approach [8] prior to use. Traces are representative data. Each experiment was performed with three repetitions, from four different donors.

of high shear-stress, is a putative inhibitor of thrombin-induced aggregation. This type of shear-stresses is relevant to the conditions developed in stenosed arteries [17]. It has been recently reported that thrombin inhibition by hirudin analogue, reduces clot formation in canine model of myocardial ischemia [18]. On the basis of our findings, it is suggested that hepoxilin A_3 may be produced by platelets flowing through stenosed arteries and may have a relevance to the mechanisms which control clot formation and the development of myocardial ischemia.

This work is dedicated to the memory of Prof. A.A. Livne, it 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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