# 12-Hydroxyeicosatetraenoic acid upregulates P-selectin-induced tissue factor activity on monocytes

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**Abstract 12-Hydroxyeicosatetraenoic acid (12-HETE), a product of the platelet lipoxygenase pathway, amplifies tissue factor expression by P-selectin-stimulated monocytes in a time- and dose-dependent fashion. The same effect is observed when monocytes are incubated with Chinese hamster ovary cells transfected with the P-selectin cDNA. Both 5-HETE and leukotriene $C_4$ are inactive in this system. Furthermore, the effect is not dependent on non-specific monocyte adhesion, since monocytes incubated with CHO cells expressing E-selectin do not express tissue factor, either in the presence or in the absence of 12-HETE. These results show that 12-HETE is a cofactor for the expression of tissue factor by monocytes.**



*Key words:* Tissue factor; P-selectin; 12-Hydroxyeicosatetraenoic acid; Monocyte

## 1. Introduction

Tissue factor is constitutively expressed on non-vascular cells, and is therefore immediately available for binding to factor VIIa following vascular damage and activation of blood coagulation [1]. However, when appropriately stimulated, endothelial cells and monocytes can also synthesize tissue factor and express it on their surface. Numerous agonists have been shown to activate tissue factor synthesis by monocytes, including bacterial endotoxin [2,3], immune complexes [4], cytokines [5], malignant cells [6] and platelets [7].

The molecular basis for platelet-mediated stimulation of tissue factor by monocytes has been examined. P-selectin is a member of the selectin family of cell-cell adhesion molecules synthesized by platelets and endothelial cells. In resting cells, P-selectin is an integral component of the alpha-granule and the Weibel-Palade body membrane [8–10]. Upon cell stimulation and degranulation, P-selectin is translocated to the plasma membrane [8,11], where it functions as an adhesion receptor for neutrophils and monocytes [12,13] that constitutively express the mucin-like glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) [14]. More recently, P-selectin-mediated binding of Th2 lymphocytes to stimulated endothelial cells has also been reported [15]. We have demonstrated that in addition to mediating cell-cell adhesion, P-selectin mediates intracellular signaling events that lead to the synthesis of tissue factor by monocytes [16]. However, P-selectin is a relatively weak agonist when compared to intact platelets, suggesting that other platelet-derived molecules might be involved in monocyte stimulation.

12-Hydroxyeicosatetraenoic acid (12-HETE) is an arachidonic acid metabolite produced by platelets upon the action of the enzyme 12-lipoxygenase [17]. 12-HETE mediates numerous biologic functions, including enhancement of surface expression of integrins and of cell-cell adhesion, induction of endothelial cell retraction, and increase in metastases of tumor cells [18]. We have previously shown that 12-HETE also upregulates tissue factor synthesis by endotoxin-stimulated monocytes, while it has no effect in resting monocytes [19]. We therefore reasoned that 12-HETE could function as a costimulus in upregulating tissue factor by P-selectin-stimulated monocytes. In the present study, we demonstrate that 12-HETE enhances the expression of tissue factor on mononuclear cells exposed to P-selectin.

## 2. Materials and methods

### 2.1. Chemicals and cell lines

RPMI 1640 medium, Alpha medium, L-glutamine, penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Biochrom (Berlin, Germany). Dialyzed fetal bovine serum was from Gibco BRL (Grand Island, NY, USA). Sodium citrate solution 3.8%, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and sodium carbonate were obtained from Merck (Darmstadt, Germany). Adenosine, deoxyadenosine and thymidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methotrexate was obtained from Lederle Laboratories Division (Wayne, NJ, USA). Lymphoprep was from Nycomed Pharma AS (Oslo, Norway). Chromogenic *Limulus* Amebocyte assay was purchased from BioWhittaker Bioproducts (Walkersville, MD, USA). Sterile pyrogen-free microtubes were obtained from Sarstedt (Numbrecht, Germany). Tissue culture plates were obtained from Falcon Labware Division, Becton Dickinson Co. (Oxnard, CA, USA). P-selectin was purified from platelets as described [12]. 12-(*S*)-Hydroxyeicosatetraenoic acid (12-HETE), 5-(*S*)-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene $C_4$ ($LTC_4$), purchased from Biomol Research Labs (Philadelphia, PA, USA) were dissolved in alcohol, dried, then dissolved in 0.01 M $Na_2CO_3$ (10 μg/16.5 μl $Na_2CO_3$). Further dilutions were carried out in RPMI 1640 medium. All the steps were carried out in a nitrogen atmosphere in order to prevent oxidation. Chinese hamster ovary (CHO) cells expressing P-selectin (CHO:P-selectin) or E-selectin (CHO:E-selectin) adhere to leukocytes, in contrast to CHO cells [12].

### 2.2. Cell isolation and culture

Blood was obtained from healthy volunteers and anticoagulated with 0.1 vol of a 3.8% sodium citrate solution. Leukocytes were sedimented at 200×*g* for 15 min at 10°C. Platelet-rich plasma was discarded, and the sedimented cells were adjusted to the original volume with 0.38% sodium citrate solution/0.15 M NaCl. After centrifugation

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*Abbreviations:* 12-HETE, 12-hydroxyeicosatetraenoic acid; 5-HETE, 5-(*S*)-hydroxyeicosatetraenoic acid; $LTC_4$, leukotriene $C_4$


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and removal of the platelet-rich supernatant, the sedimented cells were collected and diluted to twice the original volume with citrate-saline, layered onto Lymphoprep and sedimented at 740×*g* for 20 min at 20°C. Mononuclear cells were collected, diluted in citrate-saline and sedimented at 680×*g* for 7 min at 8°C. Mononuclear cells were washed three times by resuspension in citrate-saline and sedimented at 250×*g* for 15 min at 8°C. Mononuclear cells were then resuspended in RPMI 1640 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at a cell concentration of $10^7$ cells/ml.

Cell viability exceeded 96% as measured by the trypan blue exclusion test. The monocytes were 25–30% in this cell population, as assessed by non-specific esterase staining. Mononuclear cells were incubated with purified P-selectin or CHO cells in sterile, pyrogen-free stoppered test tubes or in tissue culture plates at 37°C under 5% $CO_2$/95% air.

CHO, CHO:P-selectin, and CHO:E-selectin cells were cultured in 24 well tissue culture plate under 5% $CO_2$/95% air with Alpha medium supplemented with HEPES (10 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml). CHO cell culture medium required, in addition, heat-inactivated FBS (10%) and a mixture of adenosine, deoxyadenosine, and thymidine (0.01%). Dialyzed, heat-inactivated FBS (10%) and methotrexate (200 nM) were required for CHO:P-selectin and CHO:E-selectin growth. Confluent cells were washed three times with RPMI 1640 before mononuclear cell coincubation.

### 2.3. Control of endotoxin contamination

Sterile, pyrogen-free working conditions were observed in order to avoid any contamination by endotoxin. Solutions were prepared in glassware rendered pyrogen-free by heating at 180°C for 3 h. Reagents were dissolved in sterile pyrogen-free solvents, and, when tested for endotoxin contamination by the *Limulus* assay, were found negative at a sensitivity threshold of 0.1 endotoxin unit/ml, corresponding to 0.01 ng/ml.

### 2.4. Tissue factor assay

After incubation, cells were disrupted by three freeze-thaw cycles before testing for procoagulant activity by a one stage clotting time test as previously described [20]. Disrupted cells (100 μl) were mixed with 100 μl of normal human plasma at 37°C. After 30 s, 100 μl of 25 mM $CaCl_2$ at 37°C was added to the mixture and the time to clot formation was recorded. Results were expressed in arbitrary units (U) by comparison with a standard curve obtained using a human brain thromboplastin standard kindly donated by Dr. L. Poller, Manchester, UK. This preparation was assigned a value of 1000 U for a clotting time of 20 s. The standard curve was linear from 1000 to 0.01 U, corresponding to clotting times of 20 and 511 s, respectively. We have previously demonstrated that this coagulant activity represents the biological activity of tissue factor since the expression of coagulant activity on monocytes is blocked by antibodies to tissue factor, and correlates with the development of tissue factor mRNA and tissue factor antigen [16].

## 3. Results

Purified P-selectin stimulates procoagulant activity expression in mononuclear cells (Fig. 1) as previously shown [16]. The effect was inhibited by the monoclonal anti-P-selectin antibody, GA6. Heat denaturation of P-selectin abolished upregulation of the activity, indicating that endotoxin contamination was not responsible for tissue factor expression. Identity of the procoagulant activity elicited under these conditions with tissue factor was confirmed by inhibition with a specific monoclonal anti-tissue factor antibody [16].

When 12-HETE was added together with purified P-selectin, tissue factor expression was increased by 4–5-fold (Fig. 1). No effect could be observed when 12-HETE was incubated with mononuclear cells alone. The lack of effect of 12-HETE in the absence of other stimuli confirms previous results [19], and indicates that no endotoxin was contaminating the 12-HETE preparation since 12-HETE is capable of upregulating tissue factor synthesis in the presence of even small amounts of endotoxin [19].

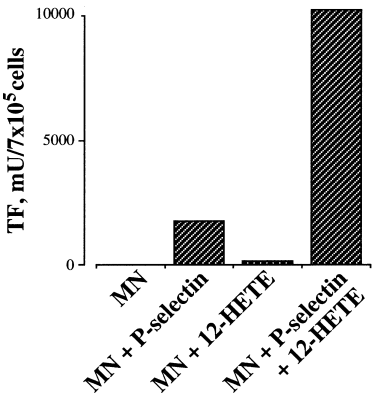


Fig. 1. Effect of P-selectin and 12-HETE on tissue factor expression in mononuclear cells. Tissue factor (TF) expression was measured in mononuclear cells (MN) that were untreated or exposed to P-selectin (250 ng/ml), 12-HETE (20 μM), or P-selectin and 12-HETE for 20 h at 37°C. At the end of incubation samples were frozen and thawed, and tissue factor activity was measured as described in Section 2.

In order to mimic physiologic conditions in which P-selectin is expressed within a cell membrane, as it is in activated platelets and endothelial cells, we used CHO:P-selectin cells. As shown in Fig. 2, in the absence of 12-HETE, CHO:P-selectin induced tissue factor activity in mononuclear cells, as previously shown [16]. 12-HETE, at concentrations up to 5 μM, potentiates the effect of P-selectin expressed by CHO:P-selectin cells. Higher concentrations of 12-HETE do not increase this effect. No increase in activity was observed when 12-HETE was added to mononuclear cells cultured in the presence of untransfected CHO. The viability of mononuclear cells incubated with these reagents, as assessed by trypan blue dye exclusion, was not affected (not shown).

The kinetics of 12-HETE enhancement of tissue factor expression by P-selectin-stimulated mononuclear cells is shown in Fig. 3. CHO:P-selectin-induced tissue factor expression peaked at 6 h. The time course of tissue factor expression resulting from addition of 12-HETE parallels that of CHO:P-selectin alone.

In addition to 12-HETE, we examined the effects on tissue factor expression of 5-lipoxygenase products that can be

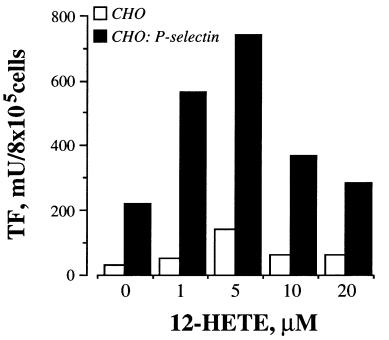


Fig. 2. 12-HETE enhances expression of tissue factor in mononuclear cells cultured with CHO:P-selectin cells. Mononuclear cells were added and incubated with confluent CHO and CHO:P-selectin for 6 h in the presence of different concentrations of 12-HETE. At the end of incubation, cells were treated as for Fig. 1.

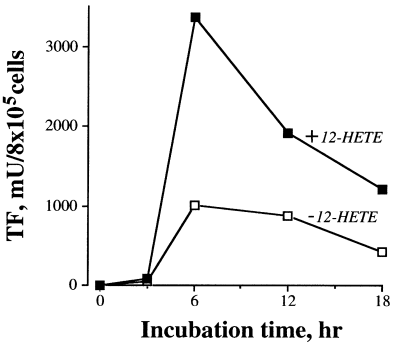


Fig. 3. Kinetics describing the development of tissue factor expression in mononuclear cells exposed to 12-HETE and CHO or CHO:P-selectin. Mononuclear cells were incubated with CHO or CHO:P-selectin in the presence of 12-HETE (5 μM). After the indicated times, the cells were assayed for tissue factor activity.

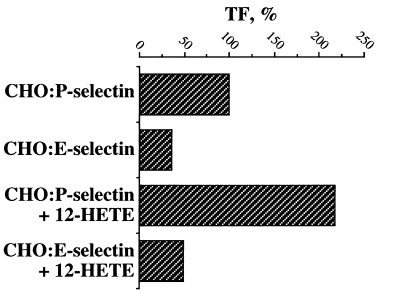


Fig. 5. Effect of 12-HETE on tissue factor activity of mononuclear cells incubated with CHO:P-selectin or CHO:E-selectin. Mononuclear cells were incubated with CHO:P-selectin or CHO:E-selectin with or without 12-HETE (5 μM) for 6 h, and then assayed for tissue factor activity. Results are expressed in percent of tissue factor activity. A 100% value was assigned to the activity expressed by mononuclear cells incubated with CHO:P-selectin.

formed by leukocytes, such as 5-HETE and $LTC_4$. When tested under identical conditions no effect on tissue factor activity could be observed (Fig. 4).

We have previously shown that monocyte stimulation induced by CHO:P-selectin was indeed due to P-selectin: E-selectin bearing CHO cells do not stimulate tissue factor expression in monocytes [16]. The data depicted in Fig. 5 confirm these results. Tissue factor expression was induced in mononuclear cells by CHO:P-selectin, and not by CHO:E-selectin cells. The presence of 12-HETE, responsible for an increase in tissue factor activity when incubated with CHO:P-selectin, had no effect on monocytes when added to CHO:E-selectin cells.

## 4. Discussion

Among the cells in contact with blood, monocytes and vascular endothelial cells, which do not constitutively express tissue factor activity under resting conditions, synthesize and express tissue factor on their membranes in response to activating agents [2–5]. In this way, activated monocytes and endothelial cells have the potential to promote the local generation of thrombin and the local deposition of fibrin. During activation, platelets translocate P-selectin from the alpha-granules to the external membrane [8,11]. P-selectin is an adhesion molecule which mediates binding of monocytes and neutrophils to activated platelets and endothelial cells [12,13]. It has been shown in an ex vivo model of thrombosis that P-selectin mediates leukocyte-platelet binding within thrombi, and that anti-P-selectin antibodies inhibit fibrin deposition after leukocyte accumulation on a vascular graft [21]. Moreover, we have shown that P-selectin upregulates tissue factor in monocytes [16]. This phenomenon is dependent upon binding of P-selectin to the ligand PSGL-1 since antibodies to PSGL-1 block the P-selectin effect [22]. Recently, monocyte tissue factor expression induced by platelet-monocyte binding was confirmed to be P-selectin dependent [23]. Since the stimulatory effect of P-selectin on monocyte tissue factor expression is relatively weak compared to that of platelets [16], it is possible that other molecules released at the sites of vascular injury might enhance the stimulation.

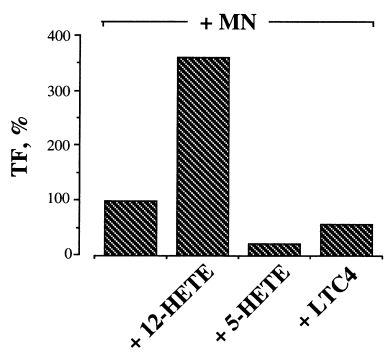


Fig. 4. Effect of 12-HETE, 5-HETE, and $LTC_4$ on tissue factor of P-selectin-stimulated mononuclear cells. Mononuclear cells (MN) were incubated with CHO:P-selectin in the presence of 12-HETE, 5-HETE, or $LTC_4$ (5 μM each) for 6 h. At the end of incubation, the cells were tested for tissue factor activity. Results are expressed in percent of tissue factor activity. A 100% value was assigned to the activity expressed by mononuclear cells incubated with CHO:P-selectin in the absence of the different agents.

In this report, we have demonstrated that 12-HETE amplifies tissue factor expression in P-selectin-stimulated mononuclear cells. The amplification occurs both with purified P-selectin and with CHO cells transfected with P-selectin. The effect of 12-HETE is specific, since 5-HETE and $LTC_4$ were inactive in our test system, in contrast to the chemotactic, chemokinetic and spasmogenic activities that leukotrienes exert [24].

12-HETE is the end product of the 12-lipoxygenase-mediated pathway of arachidonic acid metabolism [17]. In addition to its known chemotactic activities [25], 12-HETE has been shown to mediate several other biological functions. Evidence suggests that exogenous 12-HETE can increase surface expression of integrins, enhance cell-cell adhesion, induce endothelial cell retraction, and increase metastasis of tumor cells [26]. Moreover, we have shown that 12-HETE upregulates tissue factor synthesis by monocytes stimulated by endotoxin [19]. Although 12-HETE was originally reported to derive exclusively from platelets, recently the presence of a 12-lipoxygenase has also been reported in human aortic endothelial cells [27] and mononuclear cells [27,28].

The requirement for a second agonist for full expression of P-selectin-mediated signaling has been described in several recent studies which have focused on the role of selectins in monocyte activation and signal transduction. Binding of P-selectin regulates expression and secretion of the inflammatory cytokines monocyte chemotactic protein-1 (MCP-1) and tu-

mor necrosis factor-α by monocytes stimulated by platelet activating factor (PAF) [29]. In their experimental system, P-selectin alone was not sufficient to elicit these responses. P-selectin was able to cause a small but discernible translocation of NF-κB, a transcription factor involved in the activation of several immediate early genes. This phenomenon is greatly enhanced by PAF, which is required for full stimulatory function at the protein level [29]. Similarly, a priming effect of P-selectin was proposed for the synthesis of PAF by monocytes exposed to opsonized zymosan [30]. Finally, it has recently been reported that platelet-induced upregulation of synthesis and expression of MCP-1 and interleukin-1β by monocytes, although dependent upon P-selectin mediated cell-cell binding, requires the presence of the platelet chemokine RANTES [31]. These authors could not detect tissue factor activity when platelets were incubated with monocytes. This result is in disagreement with other reports [7,16,19,23,32], and may relate to their use of either thrombin-activated platelets or different experimental conditions.

In summary, activated platelets co-localize with monocytes at sites of vascular injury and inflammation. P-selectin on activated platelets and the damaged endothelial cells is most likely responsible for monocyte accumulation. 12-HETE, released from activated platelets, and possibly from endothelial cells or bound monocytes, enhances expression of tissue factor which is required for thrombogenesis and wound healing.

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