Barettin: A marine natural compound with potent anticoagulant and anti-inflammatory properties

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Summary

Background: Vascular inflammation is the prominent feature of atherosclerotic lesion formation. In search of bioactive products from the sea, we found barettin, isolated from the marine sponge *Geodia barretti*, to possess potent bioactive immunomodulatory effects. *Objectives:* The aim of this study was to examine the biological functions of barettin using an ex-vivo whole blood model stimulated with LPS. *Methods:* Blood was collected in Fragmin (10 U/ml). Aliquots were immediately stimulated with LPS (5 ng/ml) for 2 hours at $37^{\circ}C$ in a rotary incubator (190 rpm) in the presence and absence of barettin. TF activity was measured in isolated, frozen and thawed mononuclear cells, and plasma samples were assessed for TNFα, MCP-1, IL-10 and high mobility group box protein 1 (HMGB1) by ELISA . *Results:* Barettin was recently found by our group to selectively inhibit protein kinases, in particular receptor-interacting serine-threonine kinase 2 (RIPK2) and calcium/calmodulin-dependent protein kinase 1α (CAMK1α). Barettin attenuated LPS-induced TF activity and TNFα in a dose-dependent manner. At 5, 10 and 20 µg/ml, TF activity was reduced 34.8, 36.6, and 60.7 % and TNFα 35.1, 63.0 and 89.2%, respectively. Similarly, LPS-induced MCP-1 (CCL2) was inhibited 46.6, 55.5 and 46.1 % by barettin at 2.5, 5.0 and 10.0 µg/ml. The production of the anti-inflammatory IL-10, which preferably should be high, was also reduced by barettin, (45.6, 54.5 and 34.6 % at 2.5, 5.0 and 10 µg/ml, respectively), probably through the inhibition of CAMK1α. Activated monocytes/macrophages secrete HMGB1 as a cytokine mediator of inflammation. There was a non-significant reduction of HMGB1 by barettin in LPS-stimulated blood, but much less pronounced (21.3 % at 10 µg/ml) compared to the other test products. *Conclusions:* Barettin has a very potent anticoagulant and anti-inflammatory effect with the potential to prevent inflammatory based diseases including atherosclerosis.

Introduction

Atherosclerosis is a pro-inflammatory disease, and it is well known that the pro-inflammatory function of circulating monocytes is associated with enhanced risk of coronary heart disease (CHD). For many years, we have observed that the reactivity of monocytes, as judged by production of tissue factor in lipopolysaccharide (LPS) stimulated blood, is very different between individuals, ranging from low activity to very high activity (high responders) (1). This property of the monocytes appears to be inherited, and hyperactive peripheral blood monocytes were suggested to be associated with a significant risk factor in developing coronary heart disease (CHD) (2). Support for an important role of monocytes in atherosclerosis was reported several years ago as monocyte count was found to be a predictor for novel plaque formation (3, 4).

The emerging notion that chronic infections may unleash atherogenic trigger mechanisms is suggestive of a very important role of monocytes in lesion formation by the way of their proficiency in generating pro-inflammatory products. This warrants focus on the cellular signal transduction network of monocyte activation and potential products capable of suppressing the expression of pro-inflammatory products in blood cells, particularly monocytes. Low-grade inflammation can become detrimental if it persists for a prolonged period of time (chronic inflammation), and this occurs not only in atherosclerosis, but in a variety of chronic illnesses, including diabetes, cancer, obesity, asthma, Alzheimer's disease, Parkinson's disease and autoimmune disorders (5).

Monocytes are the major source of cytokines in blood, but also the only cells in the circulation synthesizing tissue factor (TF) (5). TF is a major initiator of blood coagulation. Being a transmembrane glycoprotein, TF binds to Factor VII (FVII)/FVIIa. The complex between TF and FVIIa activates FIX and FX leading to generation of thrombin, and subsequent activation of platelet and fibrin deposition (6). In addition to its role in coagulation, TF–FVIIa-dependent signaling pathways contribute to a variety of pathologic processes, including inflammation, atherosclerosis, angiogenesis and tumor metastasis (7, 8).

Obviously, products possessing anti-inflammatory properties may have the potential to prevent diseases associated with aging and other diseases connected to lifestyle and diet. In a search for such compounds, barettin isolated from the marine sponge *Geodia barretti*, was found to have

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strong antioxidant activities in biochemical assays (9). Since we also have seen that barettin reduced LPS-induced IL-1β in THP-1 cells and inhibited particularly three kinases, RIPK2, CAMK1α and SIK2 (10), it was of great interest to examine its effect in a more physiological test-system. From earlier experience, we have observed that isolated monocytes in cell cultures or monocyte cell line cells behave differentially from monocytes in whole blood. Thus, PMA, TNFα, IL-6 and several other cellular activation products are inducing tissue factor (TF) and cytokine expression in isolated monocytes in cell cultures (11), whereas monocytes of heparinized (hirudinized) whole blood do not express these cell activation products after being exposed to the agonists (14). Further discrepancies between these cell systems have been observed as several hormones/drugs had the opposite effects in whole blood compared to cell cultures (11). This study was therefore undertaken to assess the effect of a barettin product on the production of TF and cytokines, relevant to the development of atherosclerosis, in LPS-stimulated whole blood. Since activated monocytes and macrophages secrete HMGB1 as a cytokine mediator of inflammation (12), it was also of interest to examine the effect of barettin on the production of HMGB1 in LPS-stimulated blood.

Materials and methods

Escherichia coli serotype 026-B6 LPS was obtained from Difco (Detroit, Mi, USA). Lymphoprep was purchased from Amersham (Oslo, Norway). The chromogenic substrate-peptide Pefachrome TH (Pefa-5114) for the determination of thrombin in the TF assay was obtained from Pentapharm (Basel, Switzerland). PMA was from Sigma Chemical Company (St Louis, MO, USA).

Barettin

Two forms of barettin (*Z*-barettin and *E*-barettin) were isolated from the sponge *G. barretti* as described in a previous article (9, 13). In the present study, we have examined the effect of *E*-barettin.

Blood samples

Venous blood was withdrawn from healthy volunteers using a plastic syringe with a 19 G needle. The blood samples were immediately dispensed into 5 ml polycarbonate tubes containing Fragmin at a final concentration of 10 U/ml blood. The study was approved by the Regional Committee for Medical Health Research Ethics in Norway.

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Ex vivo stimulation of whole blood.

Aliquots of Fragmin anticoagulated blood (1 ml) were immediately after collection dispersed into polycarbonate tubes and added the various reagents.

Ex vivo stimulation of whole blood with lipopolysaccharide (LPS)

Aliquots of Fragmin anticoagulated blood (1 ml) were placed in polycarbonate tubes and stimulated with LPS (5 ng/ml) in the presence and absence of barettin at various concentrations for 2 hours incubation at 37°C in a rotary incubator (190 rpm). Thereafter, the LPS-stimulation was stopped by adding 100 µl 0.005 M EDTA. Parts of the blood aliquots were centrifuged at 1200 g for 10 min, and the plasma obtained was stored frozen at -70°C until ELISA testing/cytokine testing. The remaining aliquots were diluted 1:1 with saline, and mononuclear cells were then isolated as described earlier (14). The mononuclear cell samples were frozen at -20°C until being thawed for testing the TF activity. The highest concentration of barettin used (20 µg/ml) had no adverse effect on the viability of the monocytes.

Measurement of TF activity

TF was quantified in frozen and thawed mononuclear cells in a two-stage amidolytic assay based on the ability of TF to accelerate the activation of FX by FVIIa, followed by the FXa conversion of prothrombin to thrombin in the presence of activated FV (15). Thrombin was quantified using the Th-1 substrate, and the amount of color generated was determined spectrophotometrically at 405 nm using a microplate reader. Crude rabbit brain extract was used as a standard for TF activity, with an undiluted activity assigned at 1 U/ml. Details of the assay have previously been described (15)

TNFα, MCP-1, IL-10, HMGB1, TxB$_2$ and LTB$_4$ measurements in plasma samples

TNFα was measured by using an ELISA system with anti-human TNFα and anti-human TNFα Biotin from eBioscience, whereas MCP-1 was tested in an ELISA kit named Human CCL2 (MCP-1) ELISA Ready-Set-Go, also from eBioscience. TxB$_2$, the stable product of TxA$_2$, and LTB$_4$ were determined by means of enzyme immunoassays (Amersham Pharmacia Biotech, Buckinghamshire, England). The detection limits of the assays for the quantification of TNFα, IL-6, TF antigen, LTB$_4$ and TxB$_2$ were 1.0 pg/ml, 0.2 pg/ml, 10.0 pg/ml, 6 pg/ml, and 3.4 pg/ml, respectively.

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Cell count

Cell counts were performed in a Sysmex K 1000 (TOA Medical Electronics Co. Ltd, Kobe, Japan).

Statistical analysis

All results are reported as means ± SEM. The statistical analysis was performed by Student's t-test. Results were considered statistically different in the case of $P<0.05$.

Results

The inhibitory effect of barettin on LPS-induced TF activity and TNFα in monocytes from whole blood.

To investigate the effect of barettin on LPS-induced TF and TNFα in monocytes, whole blood anticoagulated with Fragmin was incubated with various concentrations of barettin as indicated and then stimulated with 5 ng LPS/ml for 2 hours at 37°C in a rotary shaker. After stopping the incubation with EDTA, mononuclear cells were isolated, frozen and thawed before measuring TF activity. Separately incubated test samples were subjected to centrifugation, and the collected plasma was used for measurement of LPS-induced TNFα and IL-10. Fig 1 shows that barettin dose dependent reduced LPS-induced TF activity in monocytes of two different persons tested repeatedly. Whereas the average reduction of LPS-induced TF activity at 5 μg/ml barettin was 34.8 %, it was reduced by 60.7 % at 20 μg/ml ($p<0.01$). Similarly, TNFα was reduced by 35.1 % when blood was challenged with LPS in the presence of 5 μg /ml barettin and by 89.2 % at 20 μg/ml ($p<0.001$) (Fig. 2).

Reduction of LPS-induced TxB_2 and LTB_4 by barettin in whole blood

TxB_2, the stable product of TxA_2, is mainly produced by platelets in LPS-stimulated blood, whereas LTB_4 is produced both in monocytes and neutrophils. Barettin reduced the production of TxB_2 by 32.8 % at 5ug/ml and by 50.6 % at 10 ug/ml barettin ($p<0.05$) (Fig. 3), whereas the production of LTB_4 was reduced respectively 24.5 % and 26.7 % ($p<0.01$) (Fig.4).

*Inhibition of IL-10 synthesis by barettin*IL-10 is a cytokine that has anti-inflammatory effect by its inhibitory effect on LPS-activation of monocytes. Thus, a reduction in IL-10 does outweigh some of the beneficial anti-inflammatory effect of barettin. As shown in Fig. 5, barettin reduced LPS-induced IL-10 in the whole blood regime by 45.6 %, 54.5 % and 34.6 % at 2.5, 5.9 and 10.0 μg/ml, respectively ($p<0.05$).

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The effect of barettin on MCP-1 production in monocytes

Since the production of MCP-1 is slower in LPS-induced monocytes of whole blood than for the products tested above, blood was stimulated with LPS ±barettin for 4 hours before plasma was prepared. As observed with the other activation products from monocytes, LPS-induced MCP-1 was reduced by 47.6 % at 2.5 µg/ml barettin and by 55.4 % at 5µg/ml ($p < 0.06$) (Fig.6).

The effect of barettin on LPS-induced HMGB1

HMGB1, which is a mediator of inflammation and recently shown to be implicated in the development of atherosclerosis (12), is known to be mainly produced in monocytes/macrophages. The observation that Barettin inhibited CAMK1α, a kinase that has been shown to regulate IL-10 as well as HMGB1 (16), it was of interest to see whether barettin had any effect on this product. There was some reduction, but the effect on the concentration of HMBG1 in plasma of LPS-stimulated blood was less pronounced compared to the other test parameters above as the reduction was 21.3 % at 10 µg/ml barettin ($p < 0.05$) (Fig. 7).

Discussion

In an ex vivo whole blood model we show that barettin has a remarkable anti-inflammatory effect by inhibiting LPS-stimulation of monocytes and thereby attenuate TNFα, MCP-1, TxB_2 and LTB_4 as well as TF activity in LPS-stimulated blood at a level which we never have seen before. These are all cellular activation products from monocytes known to play a central role in several inflammatory-induced diseases, including the development of atherosclerosis where MCP-1, TNFα and TF are important parts of the reactions leading to lesion formation and CHD (16). Although IL-10, an anti-inflammatory cytokine, is also significantly reduced by barettin, the overall reduction in several of the pro-inflammatory products may be more significant for the suppression of inflammation-mediated diseases (17). Z-barettin was also tested, but in contrast to results from studies on THP-1 cells (9), no effect or only a trend of enhanced LPS-induced monocyte activation was observed in the whole blood system by this barettin isomer.

TF, which is the physiological trigger of blood coagulation, is expressed by activated monocytes in whole blood through an interaction between monocytes, neutrophils and activated platelets in a P-selectin-PSGL-1-dependent reaction (14). In addition, we have previously shown that the TF activity expression of LPS-stimulated monocytes is mediated through signaling by thromboxane receptor, PAF-receptor and a protease (18), whereas at the gene level TF synthesis is regulated by NF-κB and AP-1 (19). TF, in addition to being mandatory for

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initiating activation of the coagulation cascade, has important signaling effects on inflammatory reactions. Thus a potent inhibitor of the expression of TF may be of great interest not only for suppressing expression of TF under pathophysiological conditions, e.g. diseases associated with monocyte activation, but also TF-mediated pro-inflammatory reactions as seen in diabetes and obesity (20). The relatively weaker effect observed of barettin on LPS-induced TxB_2 and LTB_4 might stem from the long incubation of 2 hours, as both of them are synthesized quite rapidly and probably bind to the blood cells.

Compared to other anti-inflammatory products, barettin appears to be a very potent drug and more efficient than any other product we have tested previously in the whole blood model, e.g. prednisone, estrogen, melatonin, histamine and aspirin, where the latter product in whole blood upregulated TF activity in LPS-stimulated monocytes in contrast to its inhibitory effect in monocyte cell cultures.

As referred to above, protease(s) appeared to be involved in the expression of TF in monocytes of blood stimulated with LPS. It was therefore not surprising, as reported previously, that when screening barettin for inhibition of a large number of kinases, it showed a significant inhibition of particularly three kinases, RIPK2, CAMK1 and SIK2. Both CAMK1α and RIPK2 have regulatory effects on the innate immune system (16, 21, 22) and may therefore play an important role in the inhibitory effect of barettin of the production of inflammatory products in LPS-stimulated monocytes. Earlier studies showed that RIPK2 deficient mice has impaired activation of the transcription factor NF-κB in response to TLR signaling and are more resistant to LPS-induced lethal sepsis (23, 24). Thus, a study documented that RIPK2 interacts with the kinase TAK1, which leads to activation of NF-κB and mitogen-activated protein kinases (MAPKs) and induction of the expression of pro-inflammatory cytokines. Interestingly, RIPK2 has also been demonstrated to contribute to the induction of pro-inflammatory responses and susceptibility to gram-negative bacteria after exposure to LPS, a condition that was suggested to be associated with reduced Toll-like receptor signaling (25). Apparently, the RIPK2 pathway has likely key roles in the increased lethality and morbidity that clinically is observed in secondary bacterial infections.

Although inhibition of CAMK1α did not alter LPS-induced TNFα release in murine macrophages, it was shown that in a CLP model of sepsis, NT[RNAi] mice displayed significant elevation of IL-10, IL-6, TNFα and HMGB1 concentrations, which were attenuated in

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CaMK1α^{RNAi} mice (17). It was concluded that in a poly-microbial sepsis model, inhibition of CaMK1α had a broader effect with reductions in cytokines, including IL-10. The discrepancy of *in vitro* and *in vivo* was suggested to be attributed to the additional microorganisms, processes, cells and organs involved in the *in vivo* response to poly-microbial infection, in contrast to the LPS alone. However, it might also be argued that in contrast to the cell culture system, *in vivo* environment also includes all blood cells and thereby possible regulatory effects through interactions between the blood cells.

In the present study, we have seen a striking inhibitory effect of *E*-barettin, whereas it had no or only weak effect on LPS-induced TNFα in THP-1 cells (data not reported). In contrast, *Z*-barettin was found to have weak pro-inflammatory effect in the whole blood system (data not shown), but exerted significant inhibition of TNFα, IL-1β, MCP-1 and IL-10 in LPS-stimulated THP-1 cells subjected to adherence by incubation with PMA prior to LPS stimulation (10). This confirms our earlier observations of differential behavior of pro- and anti-inflammatory products tested in the whole blood model (11, 26, 27). Furthermore, in whole blood stimulated with the combination of LPS and PMA, the *E*-barettin tested in this study enhanced the production of monocyte activation products in a similar way as we have observed for melatonin (25) and histamine (26). This phenomenon has previously been demonstrated in studies where rabbit alveolar macrophages were stimulated with LPS. Whereas adherent cells treated with LPS (10 ng/ml) elicited a 26-fold increase in TNFα production, non-adherent cells did not elicit significant TNFα in response to LPS (28). It was concluded that adherence primes monocytes/macrophages for activation and regulation of MAPK signal transduction pathways. This could at least partially account for our observations of the barettin behavior.

In conclusion, this study demonstrates that barettin has a very potent anticoagulant and anti-inflammatory effect in an ex vivo whole blood model with probably great potential in prevention of e.g. atherosclerosis. This study needs to be followed up by experiments exploring the biochemical and cellular mechanisms involved, including studies on lesion formation in transgenic mice.

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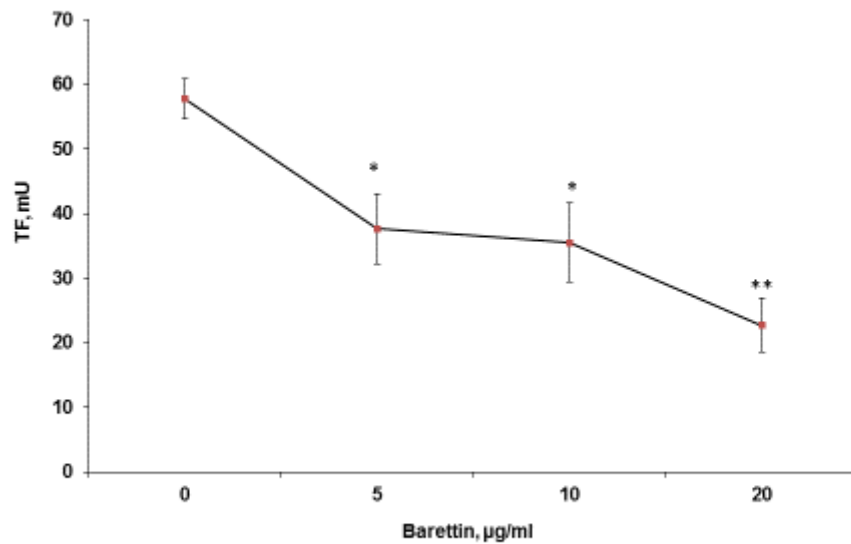
Figure 1

Dose-dependent effect of barettin on LPS-induced TF activity in monocytes of whole blood. Heparinized plasma was incubated with increasing concentrations of barettin, LPS (5 ng/ml) for 2 hours at 37 °C. The isolated mononuclear cells were frozen and thawed before testing for TF activity. Results from two individuals tested separately on four occasions are presented as mean ± SEM, *p < 0.05 and **p < 0.01. The values are respectively compared to the TF activity obtained without any barettin added.
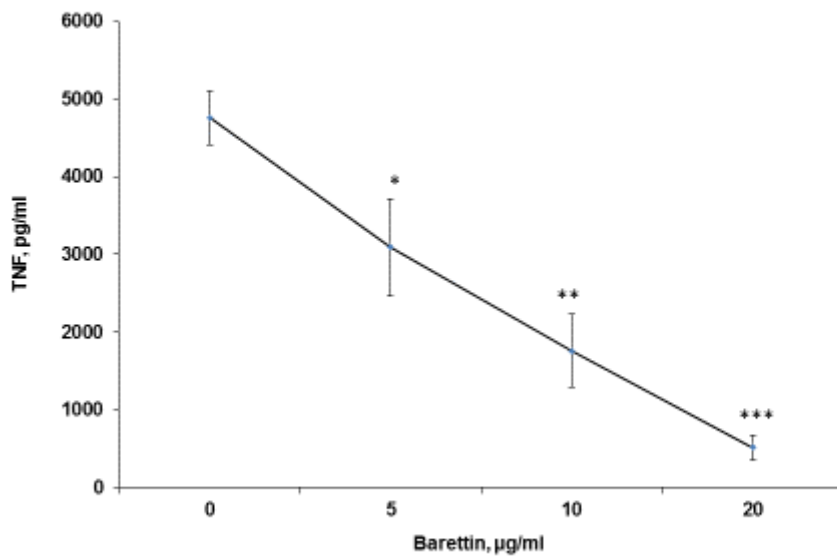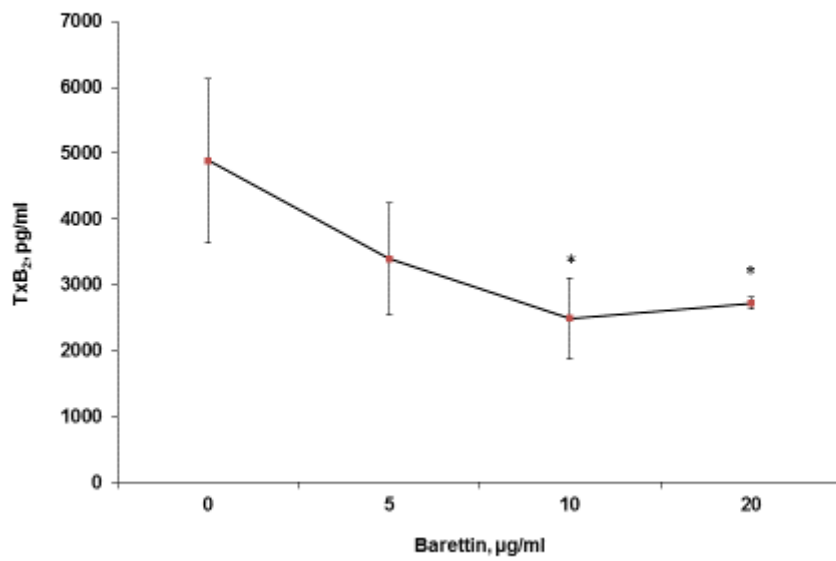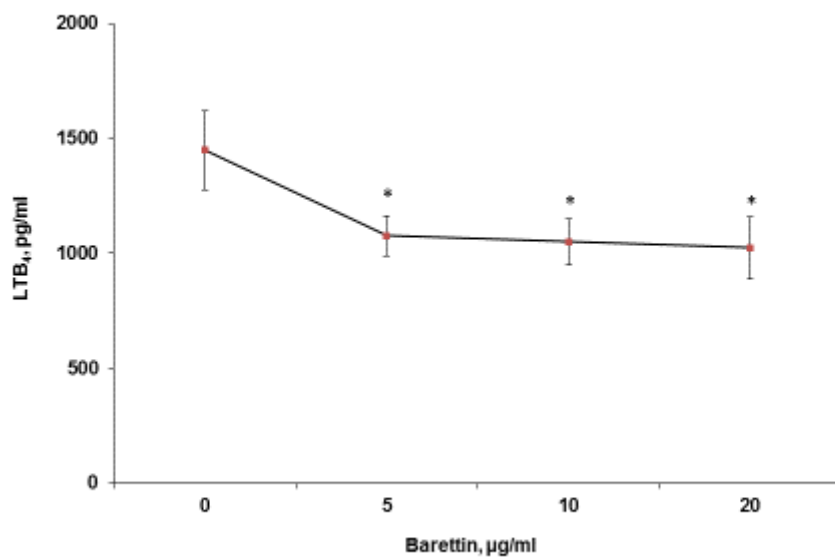
Figure 2

Dose-dependent effect of barettin on LPS-induced TNFα in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from two individuals tested on four different occasions, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. The values are respectively compared to the TNFα obtained without any barettin added.

Figure 3

Dose-dependent effect of barettin on LPS-induced TxB_2 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from two different individuals tested twice, $*p < 0.05$. The values are respectively compared to the TxB_2 obtained without any barettin added.
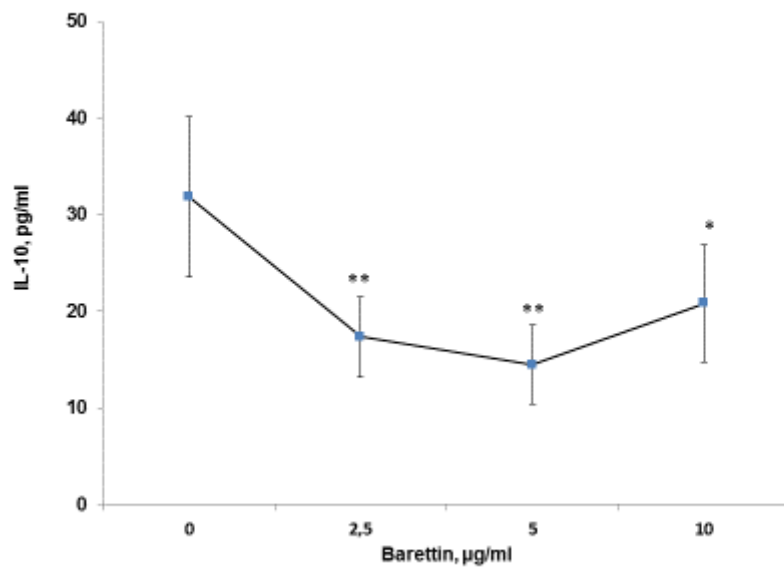
Figure 4

Dose-dependent effect of barettin on LPS-induced LTB4 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean $\pm$ SEM from 2 different individuals tested twice, $*p < 0.05$. The values are respectively compared to the LTB_4 obtained without any barettin added.

Figure 5

Dose-dependent effect of barettin on LPS-induced IL-10 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from 2 different individuals tested twice in duplicates, $*p < 0.05$, $**p < 0.01$. The values are respectively compared to the IL-10 obtained without any barettin added.
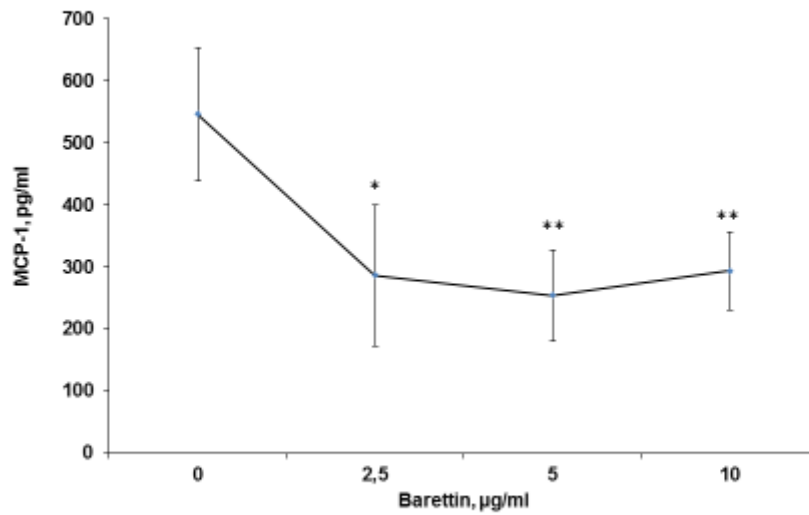
Figure 6

Dose-dependent effect of barettin on LPS-induced MCP-1 in monocytes of whole blood. The incubation was as described in Figure 1 except that the incubation time was 4 hours. The results are presented as mean ± SEM from 2 different individuals tested twice in duplicates, *p < 0.05, **p < 0.01. The values are respectively compared to the MCP-1 obtained without any barettin added.
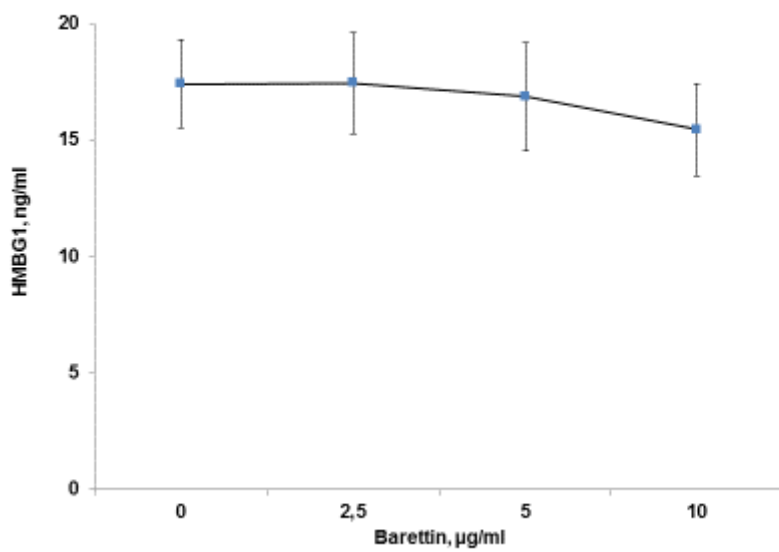
Figure 7

Dose-dependent effect of barettin on LPS-induced HMGB1 in monocytes of whole blood. The incubation was as described in Figure 1 except the incubation time was 4 hours. The results are presented as mean ± SEM from 2 different individuals tested on three different occasions (non significant). The values are respectively compared to the HMGB1 obtained without any barettin added.