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Upregulation of the 5-lipoxygenase pathway in human monocytes by growth and differentiation factors

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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5-Lipoxygenase (5-LO) is the key enzyme in leukotriene biosynthesis. Leukotrienes are key mediators of inflammation, allergic and innate immune reactions. 5-LO expression is mainly restricted to a variety of immune competent cells including B-lymphocytes, granulocytes and monocytes/macrophages. Here, we studied the effects of the growth or differentiation factors TGFβ, $1,25(OH)_2D_3$, GM-CSF and TNFα on 5-LO mRNA and protein expression and on 5-LO activity in human monocytes. We found that cultivation of monocytes under standard culture conditions downregulates 5-LO mRNA expression which could be prevented by addition of the four factors. Monocyte 5-LO activity was serum-dependent and cultivation of the cells in serum-free medium strongly downregulated cellular 5-LO activity which could be prevented by TGFβ, $1,25(OH)_2D_3$, GM-CSF and TNFα to different extents. The protein kinase A activator dibutyryl-cAMP blocked the effects of the four factors. The data suggest that 5-LO expression and activity in monocytes is strongly regulated by pro- and anti-inflammatory growth or differentiation factors.

1. Introduction

The enzyme 5-lipoxygenase (arachidonate:oxygen 5-oxidoreductase EC 1.13.11.34) catalyzes the conversion of arachidonic acid to (5S)-hydroperoxy-6-trans-6,11,14-cis-eicosatetraenoic acid (5-HPETE) and further to leukotriene A_4 ((5S) - 6-oxido-7,9,11,trans-14-cis-eicosatetraenoic acid) (Ford-Hutchinson et al. 1994; Samuelsson et al. 1987). 5-Lipoxygenase (5-LO) expression is mainly restricted to a variety of immune competent cells including B-lymphocytes, granulocytes, monocytes/macrophages, mast cells and dendritic cells (for review, see Steinhilber 1999) whereas LTA_4 hydrolase which converts 5-LO-derived LTA_4 into LTB_4 is almost ubiquitously expressed (Haeggström et al. 2011). Leukotrienes are involved in host defence reactions against microorganisms and have been associated with several disorders such as asthma, arthritis, atherosclerosis and cancerogenesis (Rådmark et al. 2007; Steinhilber et al. 2010).

Thus, it is not surprising that both, 5-LO expression and cellular LT biosynthesis are tightly regulated (Steinhilber 1994). Cellular formation of these mediators is controlled by the liberation of arachidonic acid by cytosolic phospholipase ($cPLA_2$), nuclear translocation as well as the allosteric regulation of 5-LO activity (Peters-Golden et al. 1993; Rådmark et al. 2007). It has been shown that cellular 5-LO activity is strongly stimulated by activation of the p38 MAPK-regulated MK-2 and by ERK1/2 which were identified as 5-LO kinases that phosphorylate 5-LO *in vitro* at Ser271 and Ser663, respectively (Werz et al. 2002, 2000). ERKs and p38 MAPKs are activated by a number of pro-inflammatory cytokines, chemotactic factors, phorbol esters and Ca^{2+} mobilizing agents, but also by cell stress, such as osmotic shock, genotoxic stress (sodium arsenite (SA)), UV light and heat shock as well as TGFβ (Blanchette et al. 2001). Both phosphorylation reactions seem to induce nuclear translocation of 5-LO (Boden et al. 2000; Flamand et al. 2009; Luo et al. 2003) whereas 5-LO phosphorylation at Ser523 by protein kinase A inhibits 5-LO activity and prevents nuclear translocation (Luo et al. 2005, 2004).

Depending on the cell type, several cytokines have been shown to be inducers of 5-LO protein expression. In granulocytes, 5-LO expression is stimulated by GM-CSF (Stankova et al. 1995) whereas IL-3 regulates the development of the 5-LO pathway in mouse mast cells (Murakami et al. 1995). In the human myeloid leukemic cell lines HL-60 and Mono Mac 6, cell differentiation by calcitriol ($1,25(OH)_2D_3$) and transforming growth factor-beta (TGFβ) leads to a strong induction of the 5-LO pathway (Brungs et al. 1994, 1995). In addition, differentiation of HL-60 cells into granulocytes by DMSO or retinoic acid also induces 5-LO mRNA and protein expression as well as cellular 5-LO activity (Anthes et al. 1986; Brungs et al. 1994). Also for mature blood leukocytes, upregulation of 5-LO has been reported. An increased 5-LO expression under pro-inflammatory conditions

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CM, compound mix of TGFβ; $1,25(OH)_2D_3$, GM-CSF and TNFα; DMSO, dimethyl sulfoxideFCSfetal calf serum; FLAP, 5-lipoxygenase-activating protein; GM-CSF, granulocyte macrophage colony-stimulating factor; 5-HPETE, (5S)-hydroperoxy-6-trans-6,11,14-cis-eicosatetraenoic acid; IL-3, interleukin 3; LT, leukotriene; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; 5-LO, 5-lipoxygenase; TGFβ, transforming growth factor-beta; TNFα, tumor necrosis factor-alpha.

was indicated by the increase in 5-LO mRNA in peripheral blood leukocytes from asthmatics (Koshino et al. 1998). During another differentiation process, when blood monocytes enter tissues and differentiate into macrophages, 5-LO expression increases. This was described for lung macrophages (Bigby et al. 1987; Pueringer et al. 1992), with the highest amount of protein found in alveolar macrophages (Covin et al. 1998). Interestingly, human monocytes lose 5-LO and FLAP, when kept in cell culture for seven days (Ring et al. 1996). Thus, it appears that additional (growth) factors not present in a typical cell culture are required to maintain and increase 5-LO expression.

There is only a limited correlation between 5-LO protein expression and cellular 5-LO activity in B-cells and myeloid cells. B cells express 5-LO protein but do not produce significant amounts of 5-LO products when stimulated by arachidonic acid and Ca^{2+}-ionophore (Jakobsson et al. 1992). Reactive oxygen species or stimulation of p38 MAPK were shown to trigger 5-LO activity in B-lymphocytes (Werz et al. 2001, 2000). Similar discrepancies were observed in myeloid cells where low 5-LO activity was observed in immature myeloid cells (Werz et al. 1996). Cellular 5-LO activity in monocytes is regulated by the cellular redox status and diacylglycerols (Pergola et al. 2011), and glutathione peroxidase-1 has been identified as endogenous 5-LO inhibitor (Straif et al. 2000). Although monocytes and the 5-LO pathway play an important role in the development of atherosclerosis (Spanbroek et al. 2003), little is known about the regulation of 5-LO expression in these cells. Therefore, we studied the effects of proinflammatory cytokines and of inducers of myeloid cell maturation on 5-LO mRNA and protein expression as well as on 5-LO activity of human monocytes.

2. Investigations and results

2.1. Cultured monocytes downregulate 5-LO activity

In order to study whether 5-LO activity in monocytes depends on extracellular factors, 5-LO activity in intact cells and cell homogenates was determined directly after cell isolation from buffy coats as well as after cultivation of the isolated monocytes for 40 h in serum-free medium. As shown in Fig. 1, cultivation of the cells in serum-free medium leads to a significant reduction of 5-LO activity in intact cells and cell homogenates. The data suggest, that additional factors are required for maintenance of 5-LO activity.

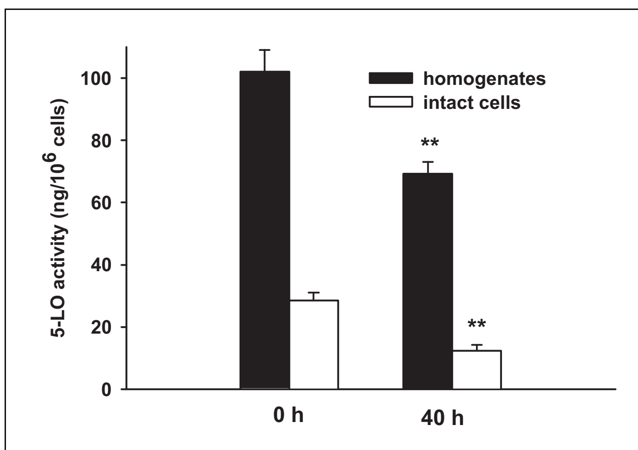

Fig. 1: Effect of cultivation of monocytes on 5-LO activity in intact cells and cell homogenates. Monocytes were isolated from buffy coats and either first cultured for 40 h a density of 0.3×10^6 cells/ml in serum free RPMI medium or directly used for further analysis of 5-LO activity in intact cells and in cell homogenates. Values are given as mean ± S.E. of three independent experiments.

2.2. Effects of serum and a mix of growth and differentiation factors on expression and activity of 5-LO

Since our data showed that 5-LO activity decreases after cultivation of monocytes in serum-free medium, the effects of serum and a compound mix (CM) of growth and differentiation factors consisting of TGFβ (3 ng/ml), $1{,}25(OH)_2D_3$ (50 nM), GM-CSF (5 ng/ml) and TNFα (1 ng/ml) on 5-LO activity in intact cells, cell homogenates and 5-LO protein expression were investigated. TGFβ and $1{,}25(OH)_2D_3$ were previously identified as agents that strongly induce the 5-LO pathway in the monocytic cell line Mono Mac 6 (Brungs et al. 1995). Furthermore GM-CSF and TNFα have been shown to increase the TGFβ /$1{,}25(OH)_2D_3$-dependent induction of cellular 5-LO activity and protein expression in the human myeloid cell line HL-60 (Steinhilber et al. 1993). In PMNL, GM-CSF has been shown to increase 5-LO gene transcription and protein expression (Stankova et al. 1995).

When monocytes were cultured in serum-containing medium for 40 h, no significant effects of the CM were observed on 5-LO activity in cell homogenates, whereas cellular 5-LO activity was stimulated by about 3-fold (Fig. 2A). Regarding 5-LO expression, no significant changes were observed after cell stimulation with CM as can be seen from the Western blot data (Fig. 2A) which correlates with the enzyme activity in the cell

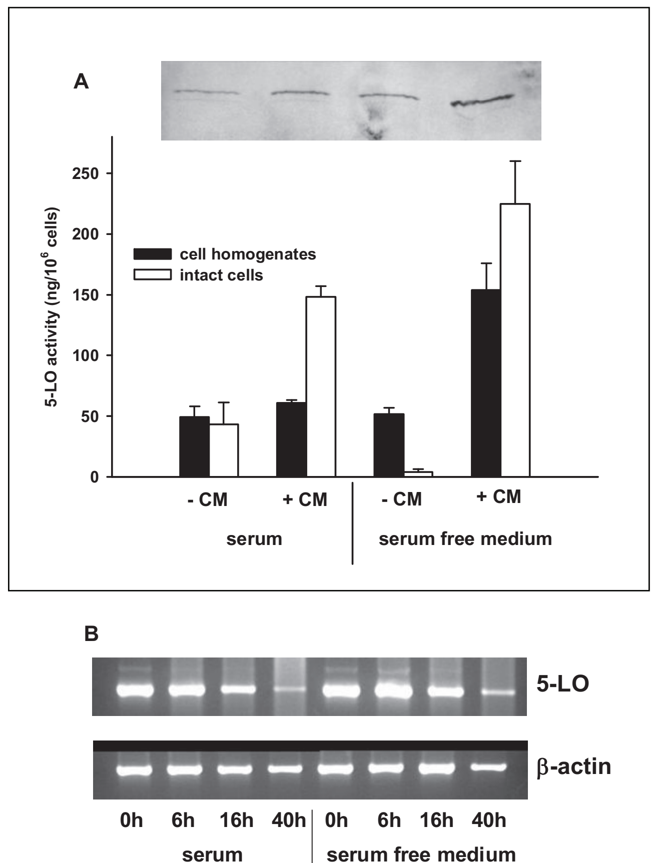

Fig. 2: Effect of serum and a compound mix (CM) of TGFβ (3 ng/ml), $1{,}25(OH)_2D_3$ (50 nM), GM-CSF (5 ng/ml) and TNFα (1 ng/ml) on 5-LO activity, protein and mRNA expression in monocytes. A) Human monocytes were cultured for 40 h in RPMI supplemented with 10% FCS or in serum-free medium in the absence or presence of CM. Then, cells were harvested and 5-LO activity of intact cells and cell homogenates was determined. Values are given as mean ± S.E. of three independent experiments. 5-LO protein expression was analyzed by Western blot. Aliquots of cellular protein extracts corresponding to 10^6 cells were applied. B) Determination of 5-LO mRNA expression by RT-PCR. After harvest of the monocytes, total RNA was isolated, reverse transcribed into cDNA. 30 PCR cycles were applied for analysis of 5-LO whereas 24 cycles were used for β-actin which served as reference.

homogenates. Therefore, the CM may induce cellular 5-LO-stimulating factors that lead to the increased 5-LO activity in the intact cells without elevating the amount of 5-LO protein. Interestingly, in the absence of serum, cellular 5-LO activity was strongly affected by the CM. Thus, in the absence of CM, 5-LO activity of the intact cells was very low and addition of the CM led to a 55-fold increase in cellular enzyme activity. No such concomitant effects were observed when 5-LO activity was analyzed in the corresponding cell homogenates. In this case, the enzyme activity was hardly reduced in serum-free *versus* serum containing medium and the CM led to an about 3-fold induction of 5-LO activity. A similar induction was observed on 5-LO protein levels as can be judged from the corresponding Western blot analysis (Fig. 2A).

In parallel, the effect of various cell culture conditions on cellular 5-LO mRNA expression in monocytes was analyzed by RT-PCR. As can be seen from Fig. 2B, cultivation of monocytes for 40 h in serum or under serum free conditions leads to a strong decrease in 5-LO mRNA expression. Interestingly, this loss of 5-LO mRNA expression can be counteracted by addition of CM. In cells, grown for 40 h in the serum-free medium with and without CM, addition of CM leads to a 7 ± 2 fold ($n = 7$) induction of 5-LO mRNA expression (data not shown). Taken together, the CM very efficiently prevents downregulation of the 5-LO pathway in cultured monocytes.

2.3. *Effects of components of the compound mix (CM) on 5-LO activity*

Since the CM (TGFβ, $1,25(OH)_2D_3$, GM-CSF and TNFα) strongly induces cellular 5-LO activity in monocytes and prevents loss of the leukotriene producing capacity during cell culture, the contribution of each component of the CM on induction of 5-LO activity in intact cells and cell homogenates was investigated. Monocytes were cultured for 40 h in serum-free medium in the presence of the indicated components of the CM (Fig. 3). Then, the cells were harvested and 5-LO activity was determined. The most prominent induction of 5-LO activity was observed when all four compounds (TGFβ, $1,25(OH)_2D_3$, GM-CSF and TNFα) were combined. As found previously for the human monocytic cell line Mono Mac 6 (Brungs et al. 1995), the combination of TGFβ and $1,25(OH)_2D_3$ upregulated 5-LO activity in intact cells and in cell homogenates as compared to the control (without CM). The combination of GM-CSF and TNFα was not as effective as TGFβ and $1,25(OH)_2D_3$. Interestingly, in combination with TGFβ and $1,25(OH)_2D_3$, GM-CSF was more potent than TNFα to upregulate cellular 5-LO activity, whereas similar effects were observed when 5-LO activity of the corresponding cell homogenates were analyzed.

Taken together, combination of all four components of the CM was most effective in the upregulation of 5-LO activity. The most prominent differences of the various combinations were observed when 5-LO activity of intact cells was analysed.

2.4. *Dibutyryl-cAMP blocks the upregulation of 5-LO activity by the compound mix*

Previous data suggest that in myeloid cells, cellular 5-LO is regulated by 5-LO kinases such as ERK and the p38 MAPK downstream kinase MK2 which leads to 5-LO activation. On the other hand, activation of protein kinase A strongly inhibits cellular 5-LO activity and counteracts the MK2 and ERK effects. Since GM-CSF and TNFα are prominent inducers of ERK and MK2 activation, it was of interest to check whether the protein kinase A activator db-cAMP can inhibit CM-induced upregulation of cellular 5-LO activity in monocytes. Monocytes were cultured for 40 h in the absence or presence of the CM and db-cAMP. As shown in Fig. 4, db-cAMP efficiently blocks the CM-induced upregulation of 5-LO activity in human monocytes.

3. Discussion

The regulation of cellular 5-LO activity has been studied in human neutrophils and in myeloid cell lines such as Mono Mac 6 or HL-60 cells after differentiation to granulocytic or monocytic cells (Rådmark et al. 2007). Differentiation of these myeloid cells by TGFβ and $1,25(OH)_2D_3$ leads to a prominent induction of 5-LO mRNA and protein expression and to the upregulation of cellular 5-LO activity. Several studies on the regulation of cellular 5-LO activity have revealed that stimuli that activate the p38 MAPK and the ERK pathways upregulate cellular enzyme activity and it was found that ERKs and the p38 MAPK downstream kinase MK2 can phosphorylate the 5-LO protein

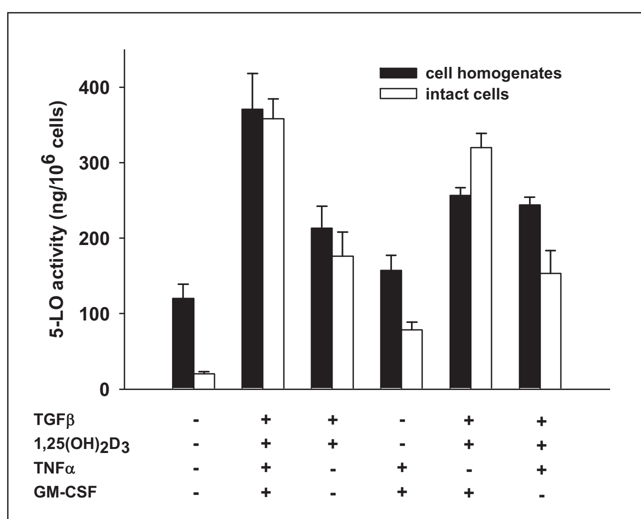

Fig. 3: Effects of components of growth and differentiation factors on 5-LO activity in intact monocytes and cell homogenates. Monocytes were cultured for 40 h in serum-free medium in the presence of the indicated agents. The applied concentrations were TGFβ = 3 ng/ml, $1,25(OH)_2D_3 = 50$ nM, GM-CSF = 5 ng/ml and TNFα = 1 ng/ml. Then, the cells were harvested and 5-LO activity of intact cells and cell homogenates was determined. Values are given as mean ± S.E of three independent experiments.

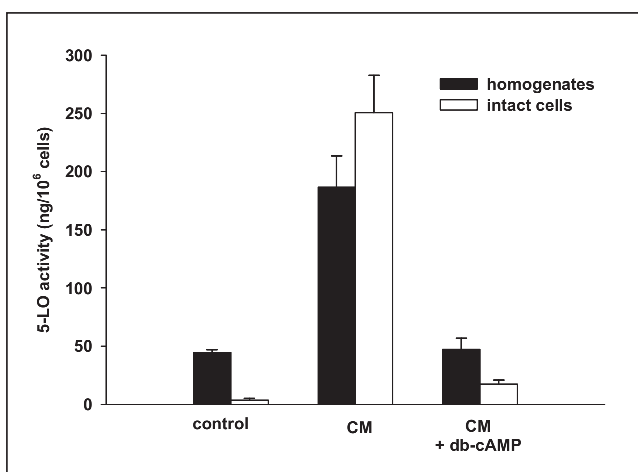

Fig. 4: Dibutyryl cAMP (db-cAMP) blocks upregulation of 5-LO activity by the growth and differentiation factors. Monocytes were cultured for 40 h in serum-free medium in the presence of growth and differentiation factors (see legend to Fig. 3) and with db-cAMP (1 mM) as indicated. Then, the cells were harvested and 5-LO activity of intact cells and cell homogenates was determined. Values are given as mean ± S.E of three independent experiments.

(Werz et al. 2002, 2000). Conversely, protein kinase A also phosphorylates 5-LO at another site which leads to an inhibition of its activity. Indeed, several cellular stimuli such as histamine and adenosine that activate Gs-coupled receptors and thus stimulate protein kinase A have been shown to inhibit cellular 5-LO activity (Flamand et al. 2004, 2002; Luo et al. 2004). Thus, activation of pro-inflammatory and/or mitotic signalling pathways such as p38 MAPK or ERK leads to enhanced leukotriene formation whereas anti-inflammatory signalling pathways involving protein kinase A seem to inhibit 5-LO activity. This is in line with the general perception that leukotrienes are mediators of inflammation and allergic responses and that they are involved in host defence reactions (Peters-Golden et al. 2007). In this context, it is of interest that the 5-LO pathway has been linked to cardiovascular disease. It has been shown that there is increased 5-LO expression in atherosclerotic lesions which correlates with the disease progression (Spanbroek et al. 2003) and that 5-LO knockout mice are partially protected against the development of atherosclerosis (Mehrabian et al. 2002).

Since monocyte/macrophages are a key players in atherosclerosis development and have been identified as one of the main 5-LO sources, it was of interest to study the regulation of the 5-LO pathway in human monocytes. Whereas the 5-LO pathway was extensively studied in neutrophils, our knowledge about the regulation of 5-LO in monocytes is limited. It is known that 5-LO expression is increased when monocytes differentiate into macrophages and that GM-CSF stimulates monocytic 5-LO protein expression (Brock et al. 1996; Coffey et al. 1993; Ring et al. 1996). In contrast to neutrophils, it was observed by us that cellular 5-LO activity in monocytes is highly variable. This is due to an endogenous inhibitor of cellular 5-LO activity in monocytes (Coffey et al. 1998) which could be identified as glutathione peroxidase-1 (Straif et al. 2000). The inhibitory effects of glutathione peroxidase-1 on cellular 5-LO activity in monocytes can be antagonized by an increase in intracellular Ca^{2+} concentration (Bürkert et al. 2002), diacylglycerols (Hörnig et al. 2005) or by priming agents such as GM-CSF.

In order to study the regulation of the 5-LO pathway in human monocytes, we determined 5-LO protein and mRNA expression, and we analyzed 5-LO activity of intact cells and cell homogenates. When the cells were transferred into the culture medium and cultured for 40 h, there was a strong time-dependent decrease in 5-LO mRNA expression which was independent from the presence of serum. Interestingly, 5-LO activity in the cell homogenates was much less affected by the cell culture and by addition of the mix of growth and differentiation inducers (TGFβ, $1,25(OH)_2D_3$, GM-CSF and TNFα) and the maximal changes in enzyme activity were around 3-fold. 5-LO activity in cell homogenates correlated with the 5-LO protein expression as determined by Western blot which is in line with previous observations (Steinhilber et al. 1993). The discrepancy between the decline in 5-LO mRNA and protein expression could be explained by the fact that the 5-LO protein has a half life of about 24 h (Reid et al. 1990) whereas the half life of the 5-LO mRNA is about 7 h (Härle et al. 1998). Thus, the weak reduction in 5-LO protein expression could simply be due to the fact that there is a slow turnover of the 5-LO protein in monocytes leading to persisting protein expression although the mRNA is degraded. The most striking effects during cell culture of monocytes were observed when 5-LO activity of intact cells was determined. Cultivation of the cells in serum-free medium led to a strong downregulation of cellular activity which was partially prevented by addition of serum. Similar effects were observed in HL-60 cells where we found that serum strongly induces cellular activity without concomitant changes in 5-LO protein expression or enzyme activity in cell homogenates (Steinhilber et al. 1993). In subsequent studies the active serum components were identified as TGFβ and $1,25(OH)_2D_3$ (Brungs et al. 1995). Indeed, addition of TGFβ and $1,25(OH)_2D_3$ to the monocytes grown under serum-free conditions strongly increased cellular 5-LO activity (Fig. 3). This induction could be further elevated when both agents where combined with GM-CSF and TNFα. Although we did not perform mechanistic studies of this effect, it is reasonable to speculate that the upregulation of cellular activity by these four factors could involve ERK and p38 MAPK pathways. Addition of the protein kinase A activator db-cAMP strongly suppressed the induction of cellular 5-LO activity by the CM. This is of particular interest since intracellular cAMP is a general suppressor of innate immune functions and inflammatory mediator generation (Serezani et al. 2008).

Taken together, our investigation revealed that cellular 5-LO activity in monocytes is strongly affected by signals controlling inflammatory and immune reactions. Pro-inflammatory mediators upregulate cellular 5-LO activity and maintain 5-LO enzyme expression whereas the anti-inflammatory mediator cAMP suppresses cellular 5-LO activity. The data suggest that 5-LO expression and activity in monocytes is strongly regulated by pro- and anti-inflammatory mediators and that (pharmacological) modulation of the 5-LO pathway might significantly affect monocyte-mediated inflammatory and immune reactions.

4. Experimental

4.1. Materials

Molecular biology reagents were from MBI Fermentas, Sigma, GIBCO, Promega or other sources indicated in the text. Insulin was a gift from Hoechst-Marion-Roussel. Human TGFβ1 was purified from outdated platelets according to (Werz et al. 1996). HPLC solvents were from Merck (Darmstadt, Germany). 5-LO antiserum (AK7, 1551) was obtained from Olof Rådmark (Karolinska Institute, Stockholm).

4.2. Isolation of human monocytes

Human leukocytes were freshly isolated from leukocyte concentrates obtained from St. Markus-Hospital in Frankfurt. 8-10 leukocyte concentrates (obtained from 500 ml peripheral blood, each) were collected and diluted 1:2 with Dulbecco's phosphate-buffered saline pH 7.4 (PBS). Dextran sedimentation was carried out by the addition of 10 ml 5% dextran solution to 40 ml of the diluted leukocyte concentrates. After 30–40 min of sedimentation, the supernatants were collected and subjected to density gradient centrifugation. 40 ml of the cell suspension were layered on top of 10 ml of Nycoprep 1.077 solution and centrifuged at 800 × g for 10 min. The mononuclear cells were collected and diluted with PBS with Ca^{2+}, pH 5.9, centrifuged (300 × g, 10 min) and washed three times with PBS with Ca^{2+}, pH 5.9. The cell pellet was finally taken up in PBS with Ca^{2+}, pH 5.9 and layered on top of 20 ml Opti-Prep® solution. After centrifugation at 350 × g for 15 min, the cell pellets were taken up in serum-free RPMI 1640 medium. After addition of human plasma (20% final concentration) the cells were grown for 1 h under standard culture conditions. Then, the supernatant was removed and the adherent monocytes were washed three times with PBS without Ca^{2+} and were finally taken up in culture medium as indicated.

4.3. Cell culture

Monocytes (0.3×10^6 cells/ml) were grown in RPMI 1640 medium supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml), 10 % fetal calf serum, 1 × nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM) and insulin (10 μg/ml) at 37 °C in a humified atmosphere with 5% CO_2. For serum-free culture, fetal calf serum was replaced by human transferrin (5 μg/ml).

4.4. 5-LO activity assay

5-LO activity in intact cells and cell homogenates was determined as described and the formed 5-LO products were analyzed by HPLC (Brungs et al. 1995). In brief, 1 ml aliquots of the cell suspension (2 to 5×10^6 cells) were stimulated for 10 min at 37 °C by arachidonic acid (40 μM) for cell homogenates or arachidonic acid/Ca^{2+}-ionophore A23187 (40 μM and 10 μM, respectively) for intact cells. 5-LO activity is expressed as nanograms of 5-LO metabolites/10^6 cells which includes leukotriene

B_4, the all-*trans* isomers of leukotriene B_4, and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE).

4.5. Western blot

5×10^6 cells were lysed in 50 µl loading buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 5% SDS, 10% mercaptoethanol) and heated for 5 min at 95 °C. After dilution with 50 µl H_2O, the samples were centrifuged. 10 µl of the extract was used and analysed by Western blot (Brungs et al. 1995). In brief, after SDS-PAGE separation of the samples and electroblotting, membranes were blocked with 5% nonfat dry milk in 50 mM Tris/HCl pH 7.4, 100 mM NaCl Tris-buffered saline (TBS) for 1 h at RT. Membranes were washed and then incubated with 5-LO antiserum diluted 1:100 overnight at 4 °C. The membranes were washed three times with TBS and incubated with 1:1000 dilution of alkaline phosphatase (AP)-conjugated immunoglobulin G (Sigma Chemical Co.) for 2 h at RT. After washing with TBS and TBS plus 0.1% NP-40, proteins were visualized with the alkaline phosphatase substrates, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

4.6. RT-PCR analysis

Total RNA was isolated from cells as described (Chomczynski et al. 1987). RT-PCR analysis was performed exactly as described previously using oligodT priming for cDNA synthesis (Härle et al. 1998). The following PCR-primers were used at a concentration of 5 ng/µl: β-actin (24 cycles) 5‘GAGGAGCACCCCGTGCTGCTGA3‘ and 5‘CTAGAAGCATTTGCTGTGGACGATGGAGGGGCC3‘; 5-LO (30 cycles) 5‘ACCATTGAGCAGATCGTGGACACGC3‘ and 5‘GCAGTCCTGCTCTGTGTAGAATGGG3‘. Signal intensities of ethidium bromide stained DNA bands were quantified by densitometry (BioRad Gel Doc 1000 system) and analyzed with the Molecular Analyst program (BioRad).

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