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# Fatty acids differentially modify the expression of urokinase type plasminogen activator receptor in monocytes

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ABSTRACT

The urokinase plasminogen activator system with its receptor uPAR contributes to the migratory potential of macrophages, a key event in atherosclerosis. We here investigated whether free fatty acids (FFA) modify the expression for uPAR in the PMA-differentiated human monocyte/macrophage-like cell line U937. Two hundred micromolar palmitate induced a threefold increase of the uPAR mRNA expression. Although the mono- and polyunsaturated fatty acids oleate and linoleate also stimulated uPAR expression, oleate had a significantly lower effect than palmitate. The observed effects were time and dose dependent. Inhibition of PKC-and ERK-pathways resulted in a strong down-regulation of basal uPAR expression whereas the FFA induced up-regulation remained unchanged. In contrast, FFA induced uPAR up-regulation was abolished by the specific inhibition of p38 MAPK. In conclusion we demonstrate that uPAR expression in human monocytes/macrophages is differentially stimulated by FFA. These effects are partially mediated by the p38 MAP-kinase signaling pathway.



One of the key events of initiating atherosclerotic plaque formation is attachment of circulating blood monocytes to the vessel wall and its reactive activation [1]. Following damage of the endothelium, monocytes are recruited to the sub-endothelial space. For cell migration different mechanisms are required: chemotaxis, phenotypic cell changes, cell adhesion and de-adhesion and remodeling of the extracellular matrix.

The uPA/uPAR-system is one of the best known proteolytic enzyme systems and plays an important role in mediating cell migration [2]. Components of the system are the serine protease zymogen plasminogen, the active protease plasmin, the activators uPA and tPA, the cellular receptor uPAR and the inhibitors PAI-1 and PAI-2 [3].

The urokinase type plasminogen activator receptor (uPAR) plays a central role within the cascade and was first described as the specific receptor for uPA in 1985 [4,5]. In contrast to early thoughts the receptor is not only the binding site for urokinase type plasminogen activator and numbers of ligands have been identified. Furthermore it is able to directly activate intracellular signaling [6].

In a non-proteolytic fashion uPAR can also mediate cellular adhesion, differentiation, proliferation and migration [6]. Only some of these functions require the presence of uPA. uPAR can bind several integrins [7] and can also bind directly to the matrix protein vitronectin [8]. Other extracellular ligands are the chemokine high molecular weight kininogen (Hka) [9], and UPARAP [10]. uPAR can be cleaved in the D1D2 linker region and the remaining D2D3 fragment has direct chemotactic activities [11].

The significance of uPAR in the pathogenesis of atherosclerosis has been demonstrated previously. In human coronary and aortic vessels it could be demonstrated that uPAR content increases progressively with the severity of atherosclerosis [12]. Increased expression of uPAR furthermore affects monocyte adhesion in acute myocardial infarction, a consequence of atherosclerosis [13].

The characteristics of the metabolic syndrome include clustered metabolic abnormalities on the basis of insulin resistance. Hyperglycemia, hyperinsulinemia and elevated (plasma) FFA are some of the features. The metabolic syndrome is strongly associated with Diabetes mellitus and cardiovascular disease morbidity and mortality [14]. Specifically nutritive fatty acids are well known to modify insulin resistance and cardiovascular risk [15,16]. Thus saturated fat appears to be associated with a higher cardiovascular compared to unsaturated fatty acids [16].

It has not been clarified whether FFA can modulate uPAR expression in macrophages. In the present study we assessed the effects of various FFA on the uPAR expression at mRNA levels. We investigated the intracellular signal transduction pathways of

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FFA induced uPAR expression in monocyte derived macrophages using U937 cells.

## Materials and methods

*Cell culture.* U937 cells were grown in suspension in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/l $HCO_3$ and 10 mM Hepes supplemented with 5.5 mM glucose, 1 mM sodium pyruvate, 50 mg/ml penicillin and streptomycin and 10% fetal calf serum (FCS) at 37 °C in a humidified (5% $CO_2$, 95% air) atmosphere.

Adding 100 nM phorbol myristate acetate (PMA) for 24 h, prior to stimulation experiments, induced differentiation into macrophage-like cells [5,17]. Stimulation was performed in standard medium, containing 10% FFA free FCS. Relevance of PKC, ERK1/2 and p38 MAPK signaling was analyzed by specific inhibitors as mentioned in Results.

Bovine serum albumin (BSA) bound fatty acids were prepared in 10 mM concentration as described previously [18].

The nonesterified fatty acids concentration was verified using enzymatic measurement.

The molar ratio of fatty acids to BSA in the 10 mM stock solution was 6:1. The fatty acids stock solutions were diluted in cell culture medium prior to experiments to obtain the final FFA concentrations. The final concentration of BSA was 0.5%.

*Real time RT-PCR.* Total RNA was extracted using Trizol reagent according to manufactures protocol (Invitrogen, Karlsruhe, Germany). A total of 1 μg RNA was transcribed to cDNA by using M-MuLV RT and random hexamers in a 10 μl reaction (Roche Diagnostics, Mannheim, Germany). The cDNA derived from 100 ng RNA was used for real time PCR amplification in a 20 μl reaction containing 2.5 mmol/l MgCl2, 0.2 mmol/l dNTPs, 0.2 μmol/l of each primer and 1.25 U Taq polymerase (Invitrogen, Karlsruhe, Germany). The uPAR and α-tubulin real time PCR analysis was performed with the fluorescent reagent SYBR Green I (BMA, Rockland, USA) and the Rotorgene 200 thermal cycling system (LTF, Wasserburg, Germany). For all amplifications intron spanning oligonucleotides (primer) have been created to avoid genomic DNA amplification. Primer sequences used were as follows: CTGGAGCTGGTGGAGAAAAG and TAACGGCTTCGGGAATAGG for uPAR; ACCTGTCACCCCGACTCAAC and GGTCTTGTCACTGGGCATCT for α-tubulin. Annealing temperature was 56 °C. Each sample was measured in triplicate. Amplification of α-tubulin cDNA in every sample was used as internal standard to correct for cDNA input and integrity. Results have been calculated as the quotient of uPAR/α-tubulin mRNA.

The correctness of the amplification products was confirmed by sequencing (Megabase).

*Statistics.* Results are reported as means ± SD and were analyzed by nonparametric or parametric tests according to the distribution of the variables, which was calculated following Kolmogorov–Smirnov. Two-sided $p < 0.05$ was regarded as representing significance.

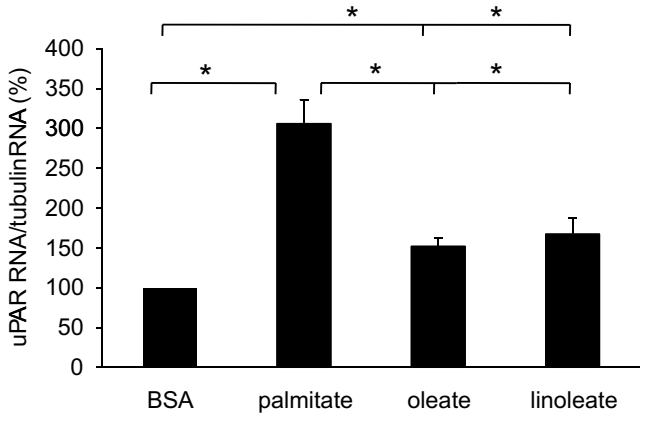


**Fig. 1.** FFA stimulate uPAR gene expression. In differentiated U937 cells, the addition of 200 μM FFA induces uPAR expression significantly. Palmitate has the strongest effects and is significantly more potent than oleate or linolenate.

## Results

### *FFA differentially stimulate uPAR expression*

To test whether uPAR gene expression is regulated by different FFA we incubated differentiated U937 cells with 200 μM palmitate, oleate and linoleate for 48 h.

Palmitate was able to stimulate the uPAR expression to 306 ± 30% ($p = 0.002$) compared to the incubation with fatty acid free BSA (set as 100%). Oleate and linoleate increased the expression up to 151.9% (±11%) and 167% (±20.5%). The uPAR elevation was significantly lower with oleate incubation compared to palmitate ($p = 0.015$) (Fig. 1).

### *The palmitate induced uPAR up-regulation is dose and time dependent*

To clarify whether the observed effects are dose dependent, different amounts of palmitate were used to stimulate uPAR. We tested 25, 50, 100 and 200 μM palmitate with an incubation time of 48 h. The addition of 100 μM palmitate was sufficient to stimulate uPAR mRNA expression (Fig. 2A).

To evaluate the time dependency of the observed effects 200 μM palmitate was used as stimulus for various durations. There was no detectable change in uPAR mRNA expression at 4, 8, or 12 h (data not shown). As early as 24 h we observed a significant increase of the uPAR transcript (62 ± 12.5%), that increased with the duration of incubation up to 322 ± 29% compared to BSA control incubation (Fig. 2B).

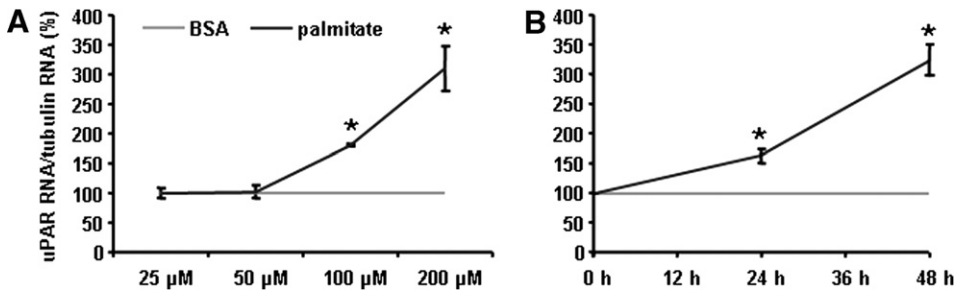


**Fig. 2.** FFA mediated uPAR expression is dose- and time dependent. U937 cells were incubated with increasing concentrations of palmitate for 48 h (A) and 200 μM palmitate for different times as indicated (B). Data are expressed as means with SEM from three independent experiments, performed in duplicates. $^{*}p < 0.05$.

*The FFA stimulated uPAR expression is independent of PKC signaling*

Since uPAR is known to be regulated by PKC [19] and FFA are known to activate this kinase [20] we investigated whether the FFA induced uPAR expression is mediated by this signaling cascade.

PMA pre-treated cells were incubated with palmitate alone, palmitate together with the inhibitor (10 μM) or palmitate together with the solvent DMSO (0.1%). To chemically inhibit PKC signaling we used GFX which is known to inhibit several PKC isoforms, including PKCα, PKCβ1, PKCδ, PKCε as well as PKCζ [21].

The addition of the inhibitor lead to a marked down-regulation of the basal uPAR mRNA (3.6 ± 0.65%). However FFA mediated uPAR stimulation was still detectable and furthermore, the fold change of uPAR stimulation remained unchanged (Fig. 3A).

*FFA stimulation of uPAR is independent of ERK1/2*

Growth and proliferation of various cell types are commonly associated with ERK activation. These processes can also be regulated/mediated by uPAR.

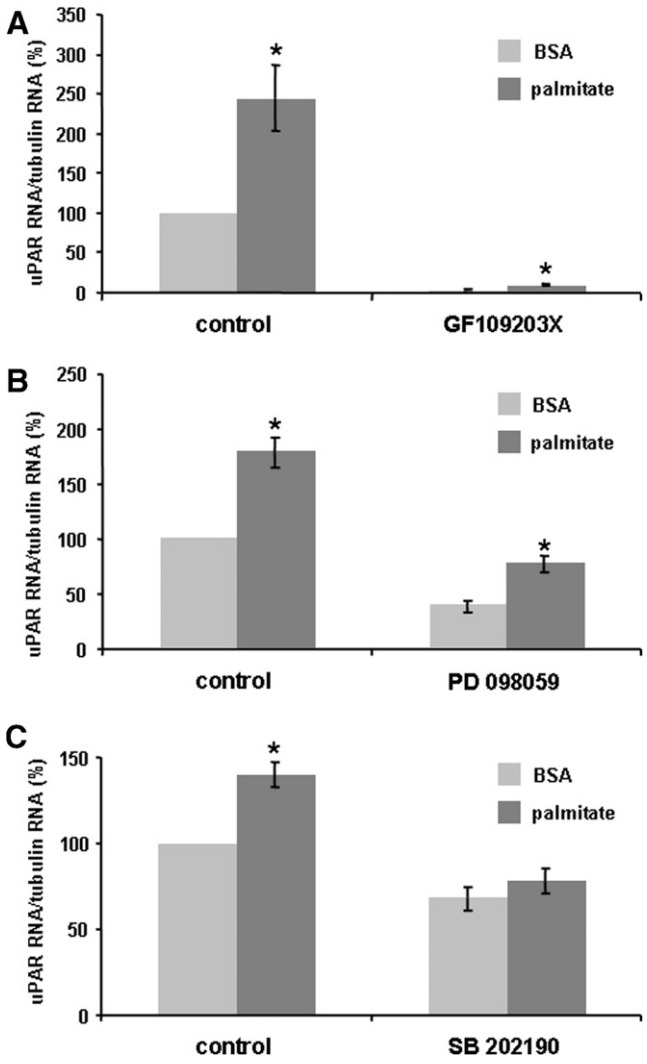


**Fig. 3.** The effects of FFAs on uPAR expression are p38 MAPK mediated, but independent of PKC or Erk1/2 Cells were incubated for 48 h with 200 mM palmitate in the presence of specific inhibitors for PKC (A), Erk (B) or p38 MAPK (C). Data are expressed as means ± SEM of three independent experiments. $^{*}p < 0.05$ for palmitate vs. BSA in the respective group.

To test the hypothesis, that FFA stimulated uPAR activation requires intact ERK signaling we performed experiments, where differentiated U937 cells were incubated in parallel with 10 μM of the ERK inhibitor PD 098059 and 200 μM palmitate.

Basal uPAR mRNA expression depends on intact ERK signaling (Fig. 3B). The addition of PD to BSA treated cells can significantly inhibit uPAR expression (−62 ± 5%). The ability to stimulate uPAR by addition of palmitate was maintained, even if ERK signaling was suppressed. In fact, the magnitude of FFA stimulated uPAR expression was similar between these groups (~80% vs. 100%). This strongly suggests that the FFA effect is independent of ERK activation.

*p38 MAPK regulates FFA induced uPAR expression*

The p38 mitogen-activated protein kinase (MAPK) plays an essential role in the normal inflammatory response; FFA can also cause an activation of inflammatory processes. To test whether FFA stimulated uPAR expression might be linked to p38 MAPK signaling, we specifically blocked this pathway by parallel incubation with 10 μM SB 202190, a specific p38 MAPK inhibitor.

FFA induced uPAR expression was completely blunted, when p38 MAPK pathway was inhibited, suggesting an important role for that kinase in mediating FFA induced effects on uPAR expression.

## Discussion

The current study demonstrates that FFA induce uPAR gene expression in vitro. The plasminogen activator system with its receptor uPAR has been shown to play an important role in the regulation of adhesion, migration, differentiation and proliferation in various cell types [6]. It has been shown that uPAR not only acts as a proteinase receptor for uPA but also activates intracellular signaling pathways.

We investigated a saturated fatty acid, a mono-unsaturated fatty acid, and a polyunsaturated fatty acid and found all three tested FFA were able to induce the uPAR expression significantly. We demonstrated that these effects are time and dose dependent. Furthermore, the effect was dependent on the grade of saturation of the fatty acid.

Dietary factors play an important role in atherogenesis. Despite convincing clinical evidence that fatty acids affect cardiovascular risk, the biological effects of different fatty acids, their role in disease risk and their mechanisms of action are not completely understood. One putative mechanism by which FFA might affect atherosclerosis is the regulation of uPAR expression in macrophagic cells, which has never been studied to date.

Others have shown that a diet rich in MUFA (rich in olive oil) was able to reduce ICAM-1-expression in human peripheral mononuclear cells significantly and furthermore is able to reduce the expression of macrophage-associated adhesion molecule CD11b [22]. CD11b, also known as Mac1, a leukocyte $\alpha_M\beta_2$-integrin receptor that binds fibrinogen, can interact with uPAR [6].

Inflammatory processes play a role in the initiation of atherosclerosis. FFA can induce inflammation. These mechanisms involve increased ROS production [23] which in turn stimulates signaling cascades including PKC and MAPK leading to translocation of transcription factors; NFκB, AP-1 and SP-1 are known to play major roles. In a subsequent vicious cycle the expression of genes, including cytokines is induce. Some of the cytokines are able to induce uPAR expression in other cell types.

To determine the signaling pathways involved in uPAR induction by FFA, differentiated U937 cells exposed to 200 μM palmitate were simultaneous incubated with either GF109203X (a PKC inhibitor), PD 098059 (a MEK inhibitor), or SB 202190, a specific inhibitor of p38 MAPK. GF109203X and PD were able to suppress the

basal uPAR expression significantly. This result confirms the importance of PKC- and MEK-signaling in the regulation of uPAR expression. However, the inhibitors did not significantly block the FFA induced uPAR expression. Although PKC and MEK are known to be activated by FFA and to play a role in promoting several proatherogenetic mechanisms [20] these results strongly suggest that PKC and MEK are not involved in the FFA induced activation of uPAR transcription.

Among the MAP-kinase subtypes, ERKs are activated in response to mitogen or growth factor stimulation, whereas c-Jun N-terminal kinase and p38 MAPK are stimulated during cellular stress conditions. FFA can activate p38 MAPK [24] and the regulation of uPAR through p38 MAPK pathway has been described in a PC3MLN4 prostate cancer cell line, where this pathway mediated in part hypoxia induced uPAR stimulation [25]. In agreement with these reports we observe significant effects of p38 MAPK on uPAR regulation.

P38 is known to be stress inducible. Known inducers are besides hyperglycemia, oxidative stress, osmotic stress, ROS (reactive oxygen species), proinflammatory cytokines and others [26]. It has been demonstrated that elevation of plasma FFA can induce states of inflammation and induce ROS production [23].

Whether palmitate induced MAPK activation itself or other metabolites, cytokines or NO cause these effects remain to be elucidated.

In summary, we demonstrate that specific FFA affect the expression of the adhesion molecule uPAR differentially and in a p38 MAPK dependent manner.

Different epidemiological studies demonstrate beneficial effects of a Mediterranean diet, which is rich in oleic acid, on cardiovascular risks [27,28].

FFA can modulate vascular function and dysfunction and therefore regulate endothelial activity. Underlying mechanisms hereby involve the release of NO, prostaglandins, leukotrienes as well as adhesion molecules, often promoted by activation of nuclear factor κB by reactive oxygen species [29].

Tsimikas assessed proinflammatory potential of LDLs isolated from a Greek population, which was rich in oleic acids. These LDL, when exposed to oxidative stress, promoted less monocyte chemotaxis and reduced monocyte adhesion. They demonstrated a strong negative correlation between oleic acid LDL content and monocyte adhesion [30]. In the same study they demonstrated that the supplementation of linoleic acids can influence the proinflammatory properties of minimally oxidized LDL.

Our data suggest an addition link between fat metabolism and development of atherosclerosis at the level of macrophagic adhesion molecules and might partially elucidate the molecular basis of the clinically observed different effects of specific nutritive fatty acids on atherosclerosis risk.

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