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Troglitazone but not conjugated linoleic acid reduces gene expression and activity of matrix-metalloproteinases-2 and -9 in PMA-differentiated THP-1 macrophages

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Abstract

Gene expression and activity of matrix-metalloproteinases (MMP)-2 and -9 in macrophages are reduced through peroxisome proliferator-activated receptor γ (PPARγ)-dependent inhibition of NF-κB. Since conjugated linoleic acids (CLAs) are PPARγ ligands and known to inhibit NF-κB via PPARγ, we studied whether CLA isomers are capable of reducing gene expression and gelatinolytic activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages, which has not yet been investigated. Incubation of PMA-differentiated THP-1 cells with either *c*9*t*11-CLA, *t*10*c*12-CLA or linoleic acid (LA), as a reference fatty acid, resulted in a significant incorporation of the respective fatty acids into total cell lipids relative to control cells ($P<.05$). Treatment of PMA-differentiated THP-1 cells with 10 and 20 μmol/L troglitazone but not with 10 or 100 μmol/L *c*9*t*11-CLA, *t*10*c*12-CLA or LA reduced relative mRNA concentrations and activity of MMP-2 and MMP-9 compared to control cells ($P<.05$). DNA-binding activity of NF-κB and PPARγ and mRNA expression of the NF-κB target gene $cPLA_2$ were not influenced by treatment with CLA. In contrast, treatment of PMA-differentiated THP-1 cells with troglitazone significantly increased transactivation of PPARγ and decreased DNA-binding activity of NF-κB and relative mRNA concentration of $cPLA_2$ relative to control cells ($P<.05$). In conclusion, the present study revealed that CLA isomers, in contrast to troglitazone, did not reduce gene expression and activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages, which is probably explained by the observation that CLA isomers neither activated PPARγ nor reduced DNA-binding activity of NF-κB. This suggests that CLA isomers are ineffective in MMP-associated extracellular matrix degradation which is thought to contribute to the progression and rupture of advanced atherosclerotic plaques.

Keywords: Conjugated linoleic acid; Peroxisome proliferator-activated receptor γ; Matrix-metalloproteinase; NF-κB; Macrophages; Atherosclerosis

1. Introduction

Most of the acute clinical manifestations of atherosclerosis such as myocardial infarction and stroke are due to the rupture of advanced atherosclerotic plaques. Activated macrophages within the inflamed atherosclerotic plaques play a key role in inducing plaque rupture by secreting matrix-metalloproteinases (MMPs) that degrade the extracellular matrix and therefore contribute to destabilization of atherosclerotic plaques [1–3]. MMP-9 (gelatinase B) and MMP-2 (gelatinase A) are predominant MMPs secreted by activated inflammatory macrophages [4,5], and elevated blood levels of these MMPs in patients with coronary artery disease clearly indicate their important role in the atherosclerotic process [6,7]. Therefore, factors that regulate the activation of MMPs are potential therapeutic targets for stabilizing rupture-prone atherosclerotic plaques.

Recent studies demonstrated that peroxisome proliferator-activated receptor γ (PPARγ) plays a critical role in the regulation of MMP activity in human monocytes and macrophages as well as vascular smooth muscle cells. Activation of PPARγ in response to different PPARγ ligands resulted in a decreased phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 expression and gelatinolytic activity in macrophages [8] and a decreased MMP-9 secretion in

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vascular cells [9–11]. PPARγ-mediated inhibition of proinflammatory transcription factors such as NF-κB, which plays an essential role in the regulation of MMP-9 and MMP-2 and other inflammatory responses [12,13], largely constitutes the mechanistic basis for this effect [11,14]. In addition, attenuation of inflammatory processes by pharmacological PPARγ ligands also contributes to the cardiovascular benefits of this class of drugs.

We and others have shown that conjugated linoleic acids (CLA), a group of conjugated isomers of linoleic acid (LA) naturally occurring in food such as milk and meat of ruminants, are capable of inhibiting NF-κB DNA-binding activity and proinflammatory gene transcription in macrophages and smooth muscle cells through activation of PPARγ [15,16]. In addition, CLAs have been demonstrated to exert potent anti-atherogenic actions in animal models of experimental atherosclerosis [17,18]. Although the underlying mechanisms of action are only poorly understood, PPARγ-dependent repression of pro-inflammatory gene expression has been proposed [18]. However, whether CLAs are capable of affecting gene expression and gelatinolytic activity of MMPs has not yet been investigated.

Therefore, the present study aimed to explore the effect of the CLA isomers on gene expression and gelatinolytic activity of MMP-2 and MMP-9 in PMA-treated THP-1 monocytic cells. THP-1 monocytic cells can be induced to differentiate into macrophages following treatment with PMA. During PMA-induced monocyte–macrophage differentiation, which is accompanied by up-regulation of MMP-9 and MMP-2 [5,19,20], cells become flat and adhere to the surface of cell culture plates and exhibit striking morphological similarities to macrophages [21]. Thus, PMA-differentiated THP-1 cells have been widely used as a representative macrophage cell line and used as an investigative model of atherosclerosis in vitro [22]. Since the activity of MMPs is at least in part controlled by a family of endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs), we also investigated the effect of CLA on TIMP-1 and TIMP-2, which are also synthesized by macrophages and are effective in binding to the catalytic site and inhibiting the active forms of MMPs [23]. To further elucidate the potential mechanisms of action of CLA on MMP and TIMP expression we also determined the DNA-binding activity of NF-κB, which has been demonstrated to be an important transcriptional regulator of MMP and TIMP genes [12,13,24]. In addition, we determined the mRNA expression of the NF-κB target gene cytosolic phospholipase A_2 ($cPLA_2$) which was recently shown to be down-regulated by CLA isomers in vascular smooth muscle cells [16]. Due to the transrepression activity of PPARγ activation on NF-κB we also analyzed the PPARγ DNA-binding activity and the mRNA expression of the PPARγ target gene CD36 in response to treatment with CLA isomers. As isomers, *c*9*t*11-CLA, which contributes to more than 90% of total CLA in natural foods [25], and *t*10*c*12-CLA, which is one of the main isomers in chemically produced CLA mixtures, were used. As reference substances, LA, which is frequently used when investigating the biological effects of CLA [26,27], and troglitazone, a pharmacological PPARγ ligand, were used.

2. Methods and materials

2.1. Materials and reagents

*c*9*t*11-CLA (≥96% pure) and *t*10*c*12-CLA (≥98% pure) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). LA (≥99% pure), PMA and troglitazone were obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Cell culture and treatments

The human monocytic THP-1 cells were obtained from the American Type Culture Collection (ATCC)/LGC Promochem (Wesel, Germany). THP-1 monocytes were cultured according to the ATCC protocol for propagation in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.05 mmol/L 2-mercaptoethanol and 1% penicillin/streptomycin. For the induction of monocyte–macrophage differentiation, cells were seeded (5×10^5 cells/24 wells) in RPMI1640 medium with 50 ng/ml PMA for 72 h as described previously [26]. After differentiation, nonattached cells were removed by aspiration, and the adherent cells were washed with RPMI1640 medium three times. Afterwards, PMA-differentiated THP-1 cells were treated with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L troglitazone for 6 and/or 24 h. PMA-differentiated THP-1 cells treated without fatty acids or troglitazone were used as control. Incubation media containing fatty acids were prepared by diluting the fatty acid stock solutions (100 mmol/L fatty acid in ethanol) or the troglitazone stock solution (20 mmol/L in DMSO) to the concentrations indicated. Vehicle controls contained 0.1% ethanol or 0.1% DMSO. No differences were observed between vehicle controls and non-vehicle controls in either experiment. For all experiments only cells between Passages 5 and 20 were used.

2.3. Cell viability

The viability of cells after treatment with fatty acids and troglitazone was examined by the MTT assay [28].

2.4. Fatty acid analysis of macrophage total lipids

After treatment of PMA-differentiated macrophages with fatty acids as indicated above, cells were washed with PBS and total lipids were extracted with hexane/isopropanol (3:2 v/v). The lipid extracts were dried under nitrogen, transmethylated with trimethylsulfonium hydroxide, and fatty acid methyl esters (FAME) were separated by GC as described previously in detail [29].

2.5. Reverse transcription-polymerase chain reaction

After treatment of cells as indicated above, the total RNA of the cells was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA synthesis and relative quantification of mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2, CD36 and $cPLA_2$ compared to the housekeeping gene glyceraldehyde-3-phosphat dehydrogenase (GAPDH) were determined by real-time detection polymerase chain reaction (PCR) as described previously [30]. Relative quantification was performed using the △△Ct method [31]. Ct values of target genes and the housekeeping gene were obtained using Rotorgene Software 5.0. Relative expression ratios are expressed as fold changes of mRNA abundance compared to control cells. The effect of PMA-induced THP-1 monocyte–macrophage differentiation on gene expression of MMPs and TIMPs was also analyzed by semi-quantitative reverse transcription-PCR (RT-PCR) as described recently in detail [16]. The number of PCR cycles was determined in preliminary experiments ensuring that relative quantification of mRNA expression was performed within the linear range of amplification of each PCR product. Sequences of gene-specific primers, obtained from Operon (Köln, Germany) were as follows (forward, reverse): GAPDH (5′-GAC CAC AGT CCA TGC CAT CAC-3′, 5′-TCC ACC ACC CTG TTG CTG TAG-3′), MMP-2 (5′-GGC CCT GTC ACT CCT GAG AT-3′, 5′-GGC ATC CAG GTT ATC GGG GA-3′), MMP-9 (5′-CAA CAT CAC CTA TTG GAT CC-3′, 5′-TGG GTG TAG AGT CTC TCG CT-3′), TIMP-1 (5′-AAT TCC GAC CTC GTC ATC AG-3′, 5′-TGC AGT TTT CCA GCA ATG AG-3′), TIMP-2 (5′-TGA TCC ACA CAC GTT GGT CT-3′, 5′-TTT GAG TTG CTT GCA GGA TG-3′), cPLA2 (5′-GAG CTG ATG TTT GCA GAT TGG GTT G-3′, 5′-GTC ACT CAA AGG AGA CAG TGG ATA AGA-3′), CD36 (5′-GGT GTG GTG ATG TTT GTT GC-3′, 5′-CAG GGC CTA GGA TTT GTT GA-3′).

Table 1
Fatty acid composition of total lipids of PMA-differentiated THP-1 macrophages cultured in the absence (control) or presence of 100 μmol/L of *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 24 h[a]

Treatment	Control	*c*9*t*11-CLA	*t*10*c*12-CLA	LA
	g/100 g total FAME			
Fatty acid				
C14:0	2.6±0.1	1.9 ±0.1*	2.1±0.1*	1.9±0.1*
C16:0	31.5±0.4	23.2±0.9*	26.4±0.4*	22.7±0.5*
C16:1	4.2±0.1	3.3±0.1*	3.0±0.1*	2.6±0.1*
C18:0	13.9±0.1	10.3±0.6*	11.2±0.3*	9.7±0.1*
C18:1	26.3±0.2	20.3±0.4*	19.2±0.2*	16.2±0.3*
C18:2 (n-6)	4.8±0.5	4.6±0.1	3.9±0.1	31.9±0.1*
C18:2*c*9*t*11	<0.1	23.4±0.9*	0.1±0.1	0.1±0.1
C18:2*t*10*c*12	<0.1	<0.1	19.3±0.8*	<0.1
C20:4 (n-6)	4.2±0.1	4.1±0.3	4.9±0.2	5.0±0.1*
C20:5 (n-3)	2.7±0.1	1.4±0.1*	1.8±0.1*	1.3±0.1*
C22:4 (n-6)	0.7±0.1	0.7±0.1	0.5±0.1	0.6±0.1
C22:6 (n-3)	3.9±0.1	1.7±0.3*	2.7±0.1*	2.4±0.1*

[a] Values are means±S.D. (n=2).
* Significantly different from control, P<.05.

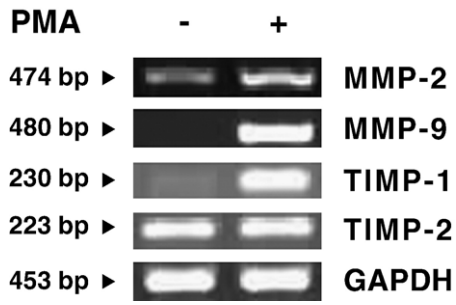

Fig. 1. Relative mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in differentiated and undifferentiated THP-1 cells. Monocyte–macrophage differentiation was induced by treatment of THP-1 cells with 50 ng/ml PMA for 72 h. After incubation, total RNA was extracted, 1.2 μg total RNA reverse transcribed and cDNA was subjected to semiquantitative RT-PCR using gene-specific primers as described in Methods and Materials. Representative images of PCR product gel electrophoresis following RT-PCR analysis are shown for one independent experiment.

2.6. Gelatinolytic zymography

For determination of gelatinolytic activity, cells were treated as indicated above, except that serum-free medium was used. After treatment, culture medium was collected and concentrated (10×) using Vivaspin 500 concentrators (Vivascience, Stonehouse, UK). The gelatinolytic activities of secreted active and pro-form of MMP-2 and -9, respectively, in the concentrated culture medium were analyzed by zymography using 10% polyacrylamide/sodium dodecyl sulphate (SDS) gels containing 0.1% (w/v) gelatin as described by Hawkes et al. [32]. In brief, 30 μl of concentrated culture medium was diluted with 15 μl of 3× nonreducing sample buffer (30% wt/vol glycerol, 187.5 mM Tris-base, 6.9% SDS, 0.15% bromphenol blue, pH 6.8), incubated at 45°C for 15 min, and each lane was loaded with 20 μl of the sample. After electrophoresis, gels were washed two times in 200 ml of 2.5% Triton X-100 at room temperature for 15 min and incubated in development buffer (0.05 M Tris-HCl, 5 mM $CaCl_2$, 0.03% Triton X-100, pH 8.8) for another 15 min at room temperature, followed by an overnight incubation at 37°C in the same buffer to allow digestion of the gelatin substrate. After digestion, gels were rinsed briefly with water, fixed with 200 ml of 40% ethanol and 10% glacial acetic acid for 30 min at room temperature, stained for 2 h with 0.116% Coomassie blue (Sigma) in 25% ethanol and 8% acetic acid, and destained in a solution of 25% ethanol and 8% acetic acid. Gelatinolytic activity was detected as clear bands against the blue-stained background. Gelatinolytic bands were size calibrated with a prestained protein ladder (PAGE Ruler, Fermentas, St. Leon-Rot,

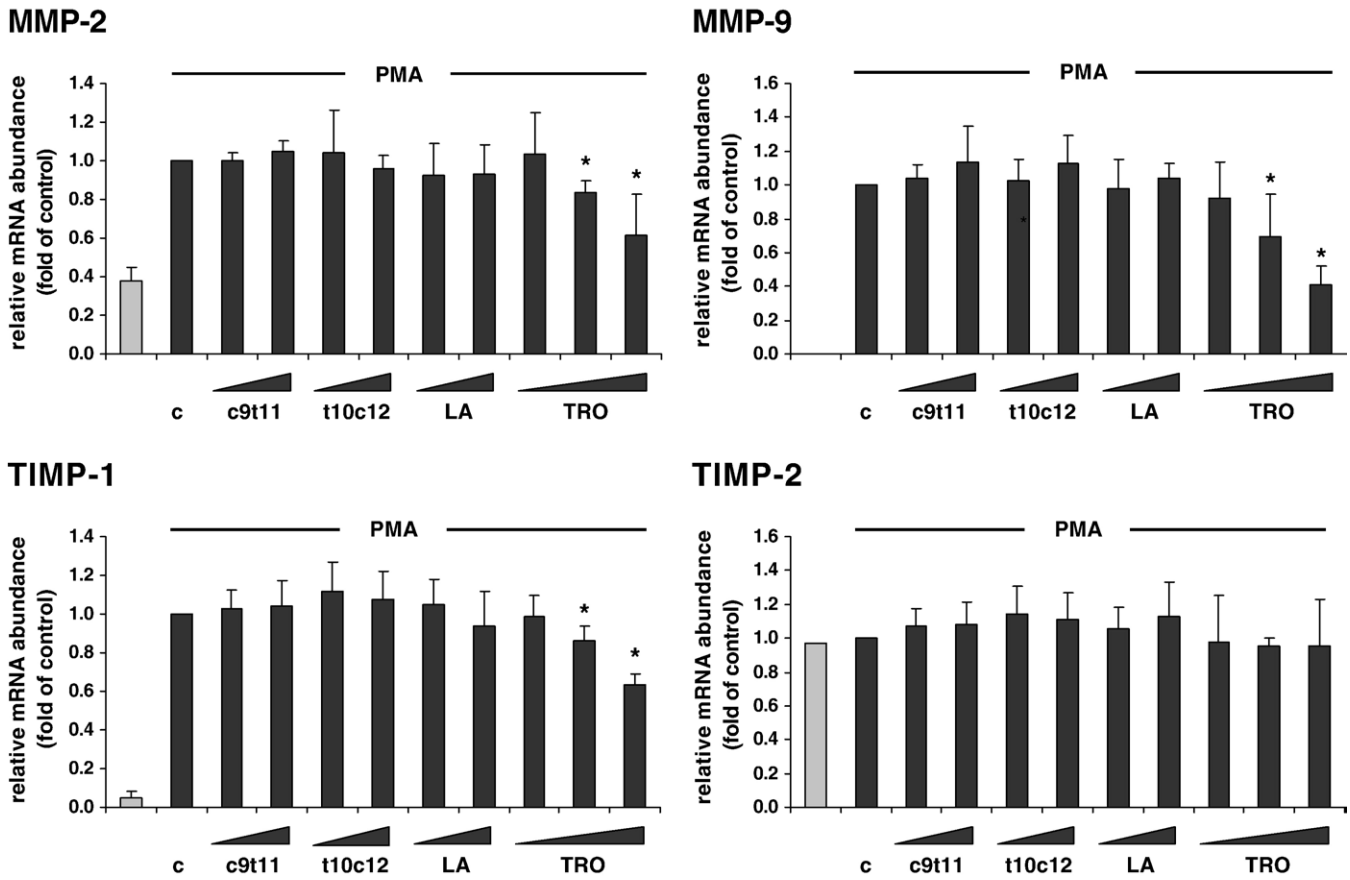

Fig. 2. Effect of 24-h treatment of PMA-differentiated THP-1 macrophages with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L troglitazone (TRO) relative to vehicle control (c) on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. Grey bars represent the value of undifferentiated THP-1 monocytes. After incubation, total RNA was extracted, 1.2 μg total RNA reverse transcribed and cDNA was subjected to real-time RT-PCR using gene-specific primers as described in Methods and Materials. Data represent the mean±S.D. of at least three independent experiments measured in quadruplicate and are expressed as fold of mRNA abundance of vehicle control (=1.0±0).

Germany). Gels were photographed and the intensity of the bands determined by densitometric analysis using Gel-Pro Analyzer software (Intas, Upland, CA, USA).

2.7. DNA-binding activity of NF-κB and PPARγ

For the measurement of PPARγ and NF-κB DNA-binding activities, nuclear extracts were prepared from PMA-differentiated THP-1 macrophages treated as indicated above with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Protein concentrations in the nuclear extracts were determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. PPARγ and NF-κB transactivities in the nuclear extracts were determined by the transcription factor assays TransAM PPARγ Kit and TransAM NF-κB p50 Kit (both from Active Motif), respectively, according to the manufacturer's protocol. For the measurement of PPARγ and NF-κB transactivities 20 μg of nuclear protein was used in each assay.

2.8. Statistical analysis

For statistical analysis, data were subjected to nonparametric analysis by Friedman test using the Minitab Statistical Software (Minitab, State College, PA, USA). A nonparametric test was used because data of most parameters did not show a normal distribution as evidenced by Shapiro–Wilk test. Differences between means of treatment and control of $P<.05$ were considered significant.

3. Results

3.1. Effects of treatment with PMA on morphology and phenotype of THP-1 cells

THP-1 monocytes treated without PMA for 72 h were round in shape and did not adhere to the plastic surface of cell culture plates. In contrast, THP-1 cells treated with PMA for 72 h became flat and amoeboid in shape, and adhered to the surface of cell culture plates, which is characteristic of the differentiation of monocytes to macrophages.

3.2. Effects of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on viability of PMA-differentiated THP-1 cells

Treatment with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L troglitazone for 24 h had no effect on the viability of PMA-differentiated THP-1 cells compared to control treatment. The average cell viability after treatment with 100 μmol/L of *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L troglitazone was in the range of 94% and 98% (mean for three independent experiments) of vehicle control cells (=100%).

3.3. Effects of fatty acid treatment on fatty acid composition of PMA-differentiated THP-1 cell total lipids

Incubation of PMA-differentiated THP-1 cells with either *c*9*t*11-CLA, *t*10*c*12-CLA or LA resulted in a significant incorporation of the respective fatty acids into total cell lipids relative to control cells ($P<.05$; Table 1). The incorporation of *c*9*t*11-CLA, *t*10*c*12-CLA or LA was accompanied by a concomitant decrease in the proportions of the saturated fatty acids C14:0, C16:0 and C18:0, and the monounsaturated fatty acids C16:1 and C18:1 relative to control cells ($P<.05$). Treatment with CLA isomers further decreased the proportions of C20:5 (n-3) and C22:6 (n-3) relative to control cells ($P<.05$). Treatment with LA increased the proportions of C20:4 (n-6) compared to control cells ($P<.05$).

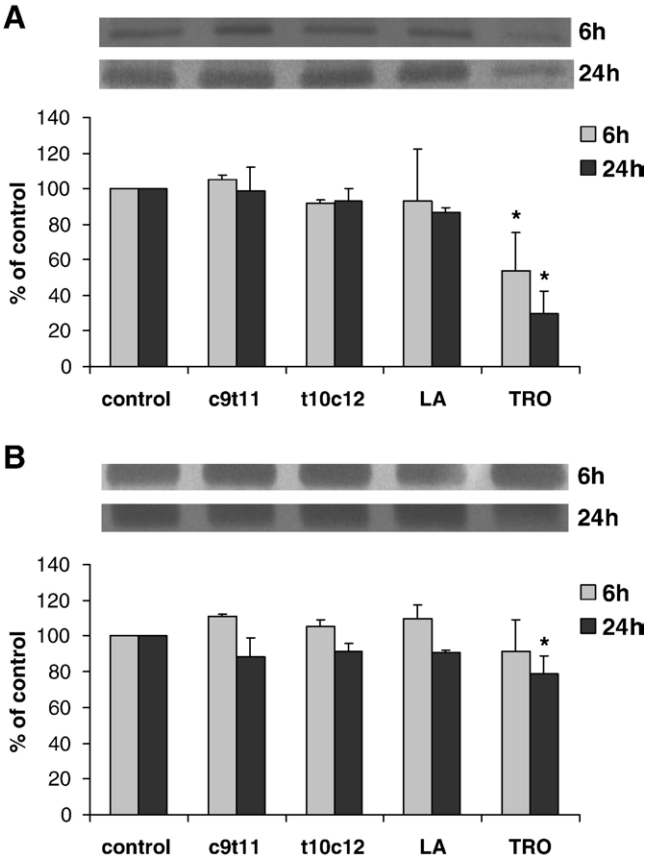

Fig. 3. Effect of treatment (6 h, 24 h) with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on secretion of MMP-2 and MMP-9 in PMA-differentiated THP-1 macrophages. After incubation, culture medium was collected, concentrated and assayed for secretion and activation of MMP-2 and MMP-9 by gelatin zymography as described in Methods and Materials. Representative images of gelatin zymography and densitometric analysis of MMP activity are shown for active 66-kDa MMP-2 (A) and active 82-kDa MMP-9 (B). Data from densitometric analysis represent the mean±S.D. of three independent experiments and are expressed as percentage of MMP activity of control (=100±0%).

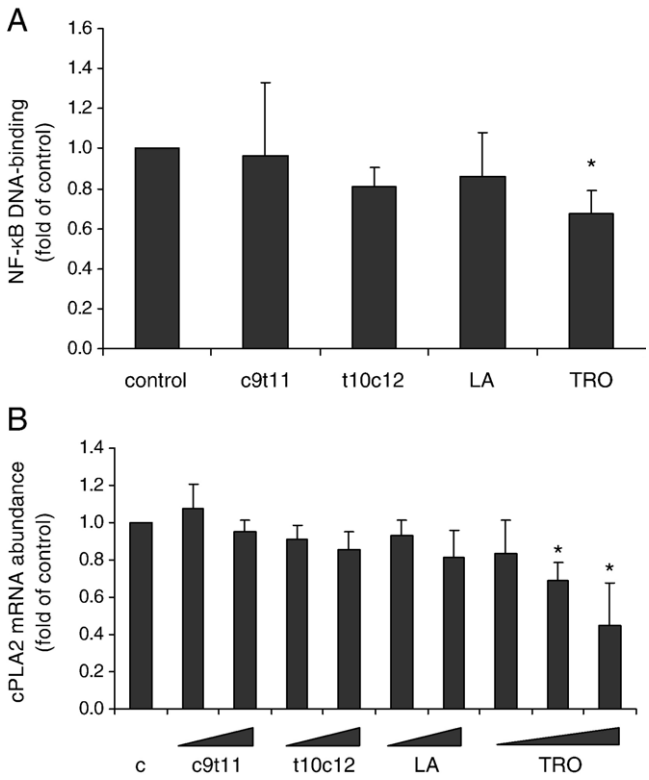

Fig. 4. Effect of 24-h treatment with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on DNA-binding activity of NF-κB (A) and effect of 24-h treatment with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L TRO relative to vehicle control (c) on mRNA expression of the NF-κB target gene $cPLA_2$ (B) in PMA-differentiated THP-1 macrophages. After treatment, nuclear extracts and total RNA were prepared and binding of NF-κB subunit p50 to the NF-κB consensus binding sequence and relative mRNA concentration of $cPLA_2$ were determined by an ELISA-based assay and real-time detection PCR, respectively, as described in Methods and Materials. Data represent the mean± S.D. of three independent experiments and are expressed as fold of vehicle control (=1.0±0).

3.4. Effect of treatment with PMA on mRNA concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 in THP-1 cells

As demonstrated in Fig. 1, relative mRNA concentrations of MMP-2, MMP-9 and TIMP-1 were markedly increased in THP-1 cells treated with PMA for 72 h compared to cells treated without PMA. The most pronounced increase in the relative mRNA concentration was observed for MMP-9 and

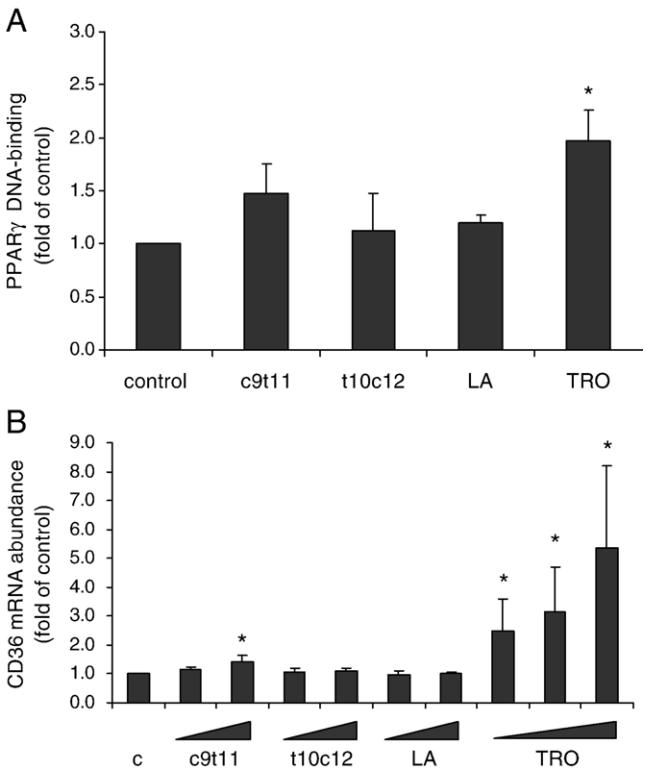

Fig. 5. Effect of 24-h treatment with 100 μmol/L of either *c9t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on DNA-binding activity of PPARγ (A) and effect of 24-h treatment with 10 and 100 μmol/L of either *c9t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L TRO relative to vehicle control (c) on mRNA expression of the PPARγ target gene CD36 (B) in PMA-differentiated THP-1 macrophages. After treatment, nuclear extracts and total RNA were prepared and binding of PPARγ to the PPAR response element and relative mRNA concentration of CD36 were determined by an ELISA-based assay and real-time detection PCR, respectively, as described in Methods and Materials. Data represent the mean±S.D. of three independent experiments and are expressed as fold of PPARγ DNA-binding activity of vehicle control (=1.0±0).

TIMP-1, which were not or only barely detectable in THP-1 cells treated without PMA. The relative mRNA concentration of TIMP-2 was not different between THP-1 cells treated with or without PMA.

3.5. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in PMA-differentiated THP-1 cells

As shown in Fig. 2, treatment of PMA-differentiated THP-1 cells with either 10 or 100 μmol/L *c9t*11-CLA, *t*10*c*12-CLA or LA for 24 h did not alter relative mRNA concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 compared to control cells. Treatment of PMA-differentiated THP-1 cells with troglitazone for 24 h dose-dependently reduced relative mRNA concentrations of MMP-2, MMP-9 and TIMP-1 compared to control cells ($P<.05$), whereas the relative mRNA concentration of TIMP-2 was not influenced by troglitazone compared to control treatment.

3.6. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on the activity of MMP-2 and MMP-9 in PMA-differentiated THP-1 cells

Treatment with 100 μmol/L *c9t*11-CLA, *t*10*c*12-CLA or LA for 6 or 24 h did not influence the gelatinolytic activities of secreted active MMP-2 (66 kDa) and active MMP-9 (82 kDa) in PMA-differentiated THP-1 cells (Fig. 3). However, treatment with 20 μmol/L troglitazone for 24 h reduced the gelatinolytic activities of secreted active MMP-2 and active MMP-9 in PMA-differentiated THP-1 macrophages ($P<.05$). The decreased activity of active MMP-2 in response to troglitazone was even observed after 6 h of treatment.

3.7. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on NF-κB DNA-binding activity and cPLA$_2$ mRNA expression in PMA-differentiated THP-1 cells

The DNA-binding activity of NF-κB was not influenced by treatment with 100 μmol/L *c9t*11-CLA, *t*10*c*12-CLA or LA but was significantly reduced by treatment with 20 μmol/L troglitazone in PMA-differentiated THP-1 cells compared to control cells ($P<.05$; Fig. 4A). The relative mRNA concentration of the NF-κB target gene cPLA$_2$ was dose-dependently reduced by treatment of PMA-differentiated THP-1 cells with 10 and 20 μmol/L troglitazone ($P<.05$), but not by CLA isomers or LA relative to control cells (Fig. 4B).

3.8. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on PPARγ DNA-binding activity and CD36 mRNA expression in PMA-differentiated THP-1 cells

The DNA-binding activity of PPARγ was not influenced by treatment with 100 μmol/L *t*10*c*12-CLA or LA in PMA-differentiated THP-1 cells relative to control cells (Fig. 5A). Treatment with 100 μmol/L *c9t*11-CLA tended to increase PPARγ DNA-binding activity in PMA-differentiated THP-1 cells ($P<.15$), whereas treatment with 20 μmol/L troglitazone significantly increased PPARγ DNA-binding activity in PMA-differentiated THP-1 cells compared to control cells ($P<.05$). The relative mRNA concentration of the PPARγ target gene CD36 was increased by treatment of PMA-differentiated THP-1 cells with 100 μmol/L *c9t*11-CLA and 1, 10 and 20 μmol/L troglitazone ($P<0.05$), but not by *t*10*c*12-CLA or LA relative to control cells (Fig. 5B).

4. Discussion

Several cell culture studies revealed that MMP expression and activity in macrophages and other cells of the vascular wall are reduced through PPARγ-dependent inhibition of NF-κB [11,14,33], which is a major regulator of MMP gene expression and activity [12,13,24,34]. Since CLAs are PPARγ ligands and known to inhibit NF-κB-mediated expression of pro-inflammatory genes via PPARγ in various

cell lines [15,16], we studied whether CLA isomers are capable of affecting gene expression and gelatinolytic activity of MMPs in macrophages which have not yet been investigated. As a model system we used the well-established PMA-differentiated THP-1 macrophage cell model, which is widely used to investigate the effects of compounds on macrophage MMP gene expression and activity [20,35,36]. In addition, several studies dealing with CLA have used the THP-1 macrophage cell model [18,26,37].

The present study clearly demonstrated that CLA isomers did not reduce PMA-induced gene expression and gelatinolytic activity of MMP-2 and MMP-9 in the THP-1 macrophage cell model. In addition, gene expression of TIMP-1 and TIMP-2, which are critical for the regulation of MMP activity in macrophages [23,38], was not altered either by treatment with CLA isomers. In contrast, the synthetic PPARγ-ligand troglitazone significantly reduced gene expression and activity of both MMPs which is consistent with findings from recent studies [11,14]. In addition, troglitazone significantly increased PPARγ transactivation and reduced DNA binding of NF-κB which probably constitutes the mechanistic basis for the reduced gene expression and activity of MMPs in response to troglitazone as evidenced in recent studies [11,14]. Consistent with the reduced NF-κB DNA-binding activity in response to treatment of THP-1 macrophages with troglitazone is the observed dose-dependent decrease in the mRNA expression of $cPLA_2$ which is an inflammatory gene known to be regulated by NF-κB. Since neither of the CLA isomers had any effect on PPARγ transactivation, NF-κB DNA-binding activity and mRNA expression of $cPLA_2$, we suggest that this failure is responsible for the unaltered gene expression and activity of MMP-2 and MMP-9 in PMA-differentiated THP-1 macrophages. These findings indicate that pharmacological and natural PPARγ ligands obviously differ with respect to modulation of gene expression and activity of MMPs in THP-1 macrophages. However, these findings do not exclude the possibility that CLA isomers might exert inhibitory effects on other inflammatory genes such as cyclooxygenase (COX)-2 in THP-1 cells. Although it has been demonstrated that CLA reduces COX-2 gene expression and prostaglandin E_2 release via inhibition of NF-κB in vascular smooth muscle cells and RAW264.7 macrophages [16,39,40], it has also been shown that CLA reduces COX-2 levels and prostaglandin biosynthesis in MCF-7 breast cancer cells independently of NF-κB by antagonization of the AP-1 pathway [41]. In addition, findings from Li et al. [39,40] using RAW264.7 macrophages also suggested that *t*10*c*12-CLA potentially inhibits alternative signaling pathways in addition to the NF-κB pathway, e.g., signaling by the mitogen-activated protein kinase family such as extracellular signal-related kinase or c-jun NH_2-terminal kinase, which also contributes to the anti-inflammatory effects of CLA.

With respect to the reason underlying the lack of effect of CLA isomers on the parameters addressed in the present study, we are confident of sufficient treatment (incubation time, CLA concentration) and incorporation of the fatty acids into the cells as demonstrated by markedly increased concentrations of CLA isomers in THP-1 cell total lipids following treatment with CLA. CLA concentrations in THP-1 cell total lipids were similar to those found in other cell types treated with CLA isomers [16,42] and in the range of those causing significant PPARγ transactivation in various cell types [16,27]. We also suggest that failure of treatment with CLA isomers is not due to a low expression of PPARγ, because the PPARγ subtype is abundantly expressed in THP-1 cells [18] and markedly up-regulated during PMA-induced monocyte–macrophage differentiation [43]. One reason possibly explaining the failure of treatment with CLA might be the fact that CLA isomers are comparatively weak PPARγ ligands due to a low binding affinity and, therefore, cause only a weak PPARγ transactivation. In contrast, troglitazone is a synthetic compound known to have a high affinity for PPARγ as evidenced by ligand-binding assays [44]. The latter was also evidenced in the present study by the use of a PPARγ transactivation assay which revealed a marked response by troglitazone but only a weak, but not significant, response by *c*9*t*11-CLA and no response at all by *t*10*c*12-CLA. The relative mRNA level of PPARγ, however, was not affected by treatment with either troglitazone or fatty acids (data not shown) which is consistent with previous reports [18,26]. In contrast, the relative mRNA level of the PPARγ target gene CD36 was not only markedly increased by troglitazone but also by *c*9*t*11-CLA by about 1.5-fold, whereas *t*10*c*12-CLA and LA had no effect. This finding concurs with our observation that *c*9*t*11-CLA but not *t*10*c*12-CLA slightly increased PPARγ DNA-binding activity indicating that at least *c*9*t*11-CLA causes PPARγ transactivation. The observed increase in CD36 mRNA concentration by *c*9*t*11-CLA is consistent with findings from a recent study also using THP-1 macrophages [26]. However, in the study of Weldon et al. [26] increased mRNA concentrations of CD36 were also observed in response to *t*10*c*12-CLA. We cannot explain this discrepancy between our study and the study of Weldon et al. [26] at the moment, but differences in the cell culture conditions applied might be causative. One important difference between our study and the study of Weldon et al. [26] is the use of serum-free medium during ligand treatment of THP-1 cells in the latter study. This might have reduced the competition and interference of the fatty acids added to the medium, e.g., CLA or LA, with those normally found in serum and, therefore, might have increased the ligand availability for PPARγ. Nevertheless, a study using RAW264.7 macrophages also revealed that the relative mRNA concentration of CD36 was only elevated in response to *c*9*t*11-CLA but not to *t*10*c*12-CLA [15], which is therefore largely confirmatory of what we have found herein using THP-1 macrophages. LA, which was used as a reference fatty acid, did not increase PPARγ transactivation and CD36 mRNA level either in THP-1 cells which is probably also

explained by its comparatively low binding affinity for PPARγ [45]. The lower binding affinity of CLA isomers for PPARγ compared to troglitazone might be of decisive importance in the THP-1 macrophage cell model used herein, because other PPAR subtypes such as PPARα and PPARβ/δ are also highly expressed in THP-1 cells [43] and CLA binds to and activates all PPAR subtypes with similar efficiency [46,47]. Therefore, it might be speculated that the low PPARγ activation by CLA in THP-1 cells is due to a competition of the various PPAR subtypes for binding of CLA isomers to their ligand-binding domains. Thus, the effect of CLA on PPARγ would be expected to be higher in a cell type expressing predominantly the PPARγ subtype. Indeed, a recent study [15] demonstrated that several CLA isomers, amongst others *c*9*t*11-CLA and *t*10*c*12-CLA, caused a pronounced activation of PPARγ in RAW264.7 macrophage cells which predominantly express PPARγ, whereas neither PPARα nor PPARβ/δ was detectable. In addition, the aforementioned study [15] revealed that CLA-induced PPARγ transactivation was accompanied by a concomitant decrease in the production of pro-inflammatory products such as nitric oxide, interleukin-6 and tumor necrosis factor-α which is indicative of the potential of CLA isomers to mediate negative regulation of pro-inflammatory transcription factors such as NF-κB or AP-1 in macrophages via PPARγ. Although we cannot definitely rule out the reason for the lack of effect of CLA on MMP expression and activity in THP-1 cells, we are convinced that the macrophage cell model used herein is an appropriate cell model in view of elucidating the anti-atherogenic actions of CLA as observed in vivo [17,18]. THP-1 macrophages, in contrast to RAW264.7 macrophages, may better reflect the in vivo situation because primary monocyte-derived macrophages also express detectable levels of PPARα and PPARβ/γ besides PPARγ [11].

Pathological studies have shown that rupture-prone regions of atherosclerotic plaques are frequently infiltrated with a large number of monocyte-derived macrophages [48]. These plaque-associated macrophages produce large quantities of MMPs, particularly MMP-9 and MMP-2 [5,49], which participate in extracellular matrix degradation and destabilization of plaques, thereby promoting acute cardiovascular events such as myocardial infarction and stroke which are typical late-stage events of atherosclerosis. Because CLA failed to influence macrophage MMP secretion and activity in the present study we suggest that CLA may exert its anti-atherogenic actions by other mechanisms than those addressed in the present study or during earlier stages of atherosclerosis. For instance, several independent studies revealed that CLA exerts lipid-lowering actions (e.g., total and LDL cholesterol) in different animal models and humans subjects [50–53], suggesting that typical atherogenic risk factors are influenced by CLA in a beneficial manner. In addition, CLA has also been shown to have a beneficial effect on insulin sensitivity [54] and glucose homeostasis, e.g., it normalizes an impaired glucose tolerance [46], thereby modulating major metabolic disorders predisposing to atherosclerosis.

In conclusion, the present study revealed that CLA isomers, in contrast to troglitazone, did not reduce gene expression and activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages. The lack of effect of CLA isomers is probably explained by the observation that CLA isomers neither activated PPARγ nor reduced DNA-binding activity of NF-κB, which is an important regulator of MMP gene expression [12,13,24,34]. In contrast, the inhibitory effect of the pharmacological PPARγ ligand troglitazone on gene expression and gelatinolytic activity of MMP-2 and -9 was accompanied by an increased PPARγ transactivation and a reduced DNA-binding activity of NF-κB. This suggests that CLA isomers are ineffective in MMP-associated extracellular matrix degradation which is thought to contribute to the progression and rupture of advanced atherosclerotic plaques in the late stage of atherosclerosis.

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