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ORIGINAL CONTRIBUTION

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Conjugated linoleic acid isomers inhibit platelet-derived growth factor-induced NF-κB transactivation and collagen formation in human vascular smooth muscle cells

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Abstract *Background* Atherosclerosis is characterized by extensive thickening of the arterial intima partially resulting from deposition of collagen by vascular smooth muscle cells (SMCs). Polyunsaturated fatty acids stimulate collagen formation through NF-κB activation. *Aim of the study* The present study aimed to explore the effect of conjugated linoleic acids (CLAs) which are known to inhibit NF-κB activation on collagen formation by SMCs. *Methods* Vascular SMCs were cultured with 50 μmol/l of CLA isomers (c9t11-CLA, t10c12-CLA) or linoleic acid (LA) and analysed for collagen formation and NF-κB p50 transactivation. *Results* Treatment with CLA isomers but not LA significantly reduced PDGF-stimulated [^{3}H] proline incorporation into cell layer protein of SMCs without altering cell proliferation. Simultaneous treatment with the PPARγ inhibitor T0070907 abrogated this effect. Treatment of SMCs with c9t11-CLA and t10c12-CLA significantly reduced PDGF-induced NF-κB p50 activation. *Conclusions* CLA isomers inhibit PDGF-stimulated collagen production by vascular SMCs, which is considered to be a hallmark of atherosclerosis, in a PPARγ-dependent manner. Whether inhibition of the NF-κB-pathway is of significance for the reduction of collagen formation by CLA isomers needs further investigation.

Key words conjugated linoleic acid – atherosclerosis – collagen formation – vascular smooth muscle cells – PPARγ

Introduction

Atherosclerosis is characterized by an extensive thickening of the arterial intima, which is the result of the deposition of collagen and other extracellular matrix proteins, lipids, and minerals ultimately leading to the formation of atherosclerotic plaques. The major component of these atherosclerotic plaques is collagen representing up to 60% of the total protein present [16]. Recent studies demonstrated that polyunsaturated fatty acids stimulate collagen formation in fibroblasts through the activation of the redox-sensitive transcription factor nuclear factor-kappaB (NF-κB) and by altering the expression of NF-κB pathway-specific genes and pro-inflammatory target genes such as chemokines and cytokines [12, 14]. Correspondingly, inhibition of NF-κB by parthenolide completely blocked the polyunsaturated fatty acid-induced collagen formation in fibroblasts [14]. Studies in vascular smooth muscle cells (SMC) also implicated a redox-sensitive pathway as the principle mechanism by which oxidized LDL stimulates collagen formation [15].

Conjugated linoleic acids (CLA), a naturally occurring group of positional and geometric isomers of linoleic acid (LA), were demonstrated to exert

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potent anti-atherogenic actions in animal models of experimental atherosclerosis without exact knowledge about the underlying mechanisms [18, 35, 36]. It has been demonstrated that treatment with CLA inhibits stimulus-induced NF-κB activation in various cell types including vascular SMCs via a peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanism [7, 28, 40], suggesting that the anti-inflammatory action of CLA is at least partially involved in the atheroprotective properties of CLA. However, whether the inhibitory action of CLA on stimulus-induced NF-κB activation is also of significance with respect to collagen formation is currently unknown. To our knowledge studies investigating the effect of CLA on collagen formation are not available from the literature.

Therefore, since collagen deposition is considered to be a hallmark of atherosclerosis and in order of elucidating the anti-atherogenic actions of CLA, the present study aimed to explore the effect of CLA isomers on collagen formation in vascular SMCs. Vascular SMCs are an appropriate cell culture model in this context because this cell type is the major source of extracellular protein production within the vessel wall [2]. Since during atherosclerosis development mitogenic signals but also cytokines such as TNFα promote phenotypic modulation of SMCs which leads to SMC migration into the intima and extensive production of collagen [5], the modulatory potential of CLA on collagen formation was studied in the presence of the mitogen platelet-derived growth factor (PDGF). In order of elucidating the effect of CLA on NF-κB-pathway activation we measured DNA-binding of NF-κB subunit p50 (NF-κB p50), and IκBα phosphorylation. In order to address whether PPARγ-signalling is involved in the potential modulation of collagen formation by CLA, we performed the experiments in the presence and absence of T0070907, a potent and selective antagonist of human PPARγ. Since differential effects of a common stimulus on growth or functional properties of vascular SMCs cultured from different vascular beds as well as from different sections of a common blood vessel are well established [31, 41], we used vascular SMCs from two different blood vessels (aorta and coronary artery). As isomers *cis*-9,*trans*-11-CLA (c9t11-CLA), which contributes to more than 90% of total CLA in natural foods such as milk, dairy products, and meat of ruminants [32], and *trans*-10,*cis*-12-CLA (t10c12-CLA), which is one of the main isomers in chemically produced CLA mixtures, were used. LA was used as reference fatty acid. The concentration of fatty acids (50 μmol/l) chosen for cell experiments was largely based on observations from previous cell culture studies demonstrating significant accumulation of CLA in cell lipids and/or induction of potent biological activities [10, 28, 30, 37, 39].

Methods

Chemicals

CLA isomers, c9t11-CLA (≥96% pure) and t10c12-CLA (≥98% pure), were obtained from Cayman Chemical (Ann Arbor, MI, USA). LA (≥99% pure) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Preparation of the stock solutions of fatty acids and of the test media was performed as described previously [10]. In order to avoid oxidation of fatty acids during preparation of stock solutions conversion of fatty acids into their sodium salts was performed after the vials had been thoroughly flushed with nitrogen and under protection from direct light. In addition, fatty acid sodium salts were kept on ice until their addition to the culture medium. Tritium-labelled proline ([2,3-^{3}H] proline) was purchased from Amersham (Buckinghamshire, UK). MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), PDGF-AB, BSA (bovine serum albumin), and TNFα, were purchased from Sigma-Aldrich. Trichloracetate (TCA) was purchased from Fluka (Buchs, Switzerland). The PPARγ antagonist T0070907 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

Human aortic smooth muscle cells, from a 35-year-old female donor, and human coronary artery smooth muscle cells, from a 40-year-old male caucasian donor, were obtained from PromoCell (Heidelberg, Germany) and cultured in SMC growth medium 2 containing 5% fetal calf serum, 0.5 μg/l epidermal growth factor, 2.0 μg/l basic fibroblast growth factor, 5 mg/l insulin, 50 mg/l gentamicinsulfate and 50 μg/l amphotericin B (all from PromoCell). Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, TNS was added to prevent enzymatic damage to the cells. Only cells from passages 3–8 were used for experiments.

Cell viability

Cell viability after treatment of HAoSMCs and HCASMCs with 50 μmol/l of either c9t11-CLA, t10c12-CLA or LA was examined by the MTT assay [38].

Cell proliferation assay

Cell proliferation was assessed using the Biotrak cell proliferation ELISA system (Amersham, Buckinghamshire, UK) and performed according to the manufacturers protocol. In brief, cells were seeded in 96-well microtitre plates and, after reaching 70–80%

confluence, incubated for 24 hours with labelling medium [100 μM BrdU (5-bromo-2′-deoxyuridine BrdU)] and 50 μmol/l of fatty acids (c9t11-CLA, t10c12-CLA or LA) in the presence of PDGF (10 ng/ml). Cells incubated with labelling medium and PDGF were used as stimulated controls. Cells incubated with labelling medium alone were used as unstimulated controls. At the end of the labelling period, cells were fixed and DNA denatured using a fixative, which was followed by a 30 minutes blocking step. After removing the blocking buffer cells were subsequently incubated with peroxidase-labelled anti-BrdU for 90 minutes at room temperature. The immune complex was detected by the subsequent reaction with TMB and reading the resultant colour at 450 nm in a microtitre plate spectrophotometer.

Collagen formation assay

To assess the effect of treatment on collagen synthesis, we examined the incorporation of [^{3}H] proline into TCA-precipitable proteins according to the method of Dubey et al. [9]. For each experiment SMCs were cultured to 70–80% confluence in 24-well plates and treated with PDGF and fatty acids (c9t11-CLA, t10c12-CLA or LA) for 24 hours. For experiments using the PPARγ antagonist T0070907, treatment of cells with 1 μmol/l of T0070907 was performed simultaneously. Cells treated with PDGF only (10 ng/ml) were used as controls ("stimulated controls"). Cells treated without PDGF and without fatty acids were used as "unstimulated controls". After incubation medium was changed to fresh medium and cells were labelled with [2,3-^{3}H] proline (10 μCi/well) for 24 hours. Afterwards medium was removed, the cell layer was washed twice with PBS, and the cell layer scraped of in ice-cold 10% TCA. Precipitated protein was collected by centrifugation at 14,000 g for 20 minutes. The cell layer precipitate was solubilized in 500 μL of 0.3 N NaOH/0.1% SDS at 37°C for 1 hour, and transferred to scintillation vials. Scintillation fluid (Rotiszint eco plus, Carl Roth, Karlsruhe, Germany) was added, and radioactivity determined in a liquid scintillation counter (Tri-Carb 2100TR, Packard, Meriden, CT, USA).

NF-κB p50 DNA-binding activity

For the measurement of NF-κB p50 transactivity, cells were seeded in 25 cm^2 tissue culture flasks and incubated with fatty acids (c9t11-CLA, t10c12-CLA) in the presence or absence of TNFα (10 ng/ml) or PDGF (10 ng/ml) for 8 hours. Additionally, simultaneous incubations with 1 μmol/l of the PPARγ inhibitor T0070907 were also performed. Afterwards nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol and protein concentrations were determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. NF-κB transactivity in the nuclear extracts was determined by the Transcription Factor assay TransAM^{TM} NF-κB p50 (Active Motif).

Immunoblot analysis of phosphorylated form of IκBα (p-IκBα)

For immunoblotting, coronary artery SMCs were plated in 6-well plates and cultured as described above. Cytoplasmic fractions of cells were obtained using the Nuclear Extract Kit from Active Motif according to the manufacturer's protocol, and protein concentrations determined by the BCA assay. Equal amounts of cytoplasmic protein (45 μg) were electrophoresed by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Loading of equal amounts of protein in each line was verified by Panceau S staining. The membranes were blocked overnight at 4°C in 5% Blotto (5% skim milk in phosphate-buffered saline containing 0.1% Tween), and then incubated with a mouse monoclonal anti-p-IκBα primary antibody (Active Motif) for 2 hours at room temperature. Membranes were washed with 1% Blotto, and incubated with a HRP conjugated secondary antibody anti-mouse IgG (Amersham Biosciences) for 1 hour at room temperature. Afterwards blots were washed again, and bands corresponding to p-IκBα visualized by chemiluminescence (ECL Plus, Amersham Biosciences). The signal intensities were detected with bio-Imaging system (Biostep) and quantified using TotalLab TL100-Quick Start analysis software (nonlinear dynamics).

Statistical analysis

Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). For statistically significant F values, individual means of the treatment groups were compared by Tukey's test. Means were considered significantly different for $P < 0.05$. Significant effects are denoted with superscript letters. Bars marked with different superscript letters significantly differ.

Results

Treatment with CLA isomers has no effect on the viability of vascular SMCs

Viability of aortic and coronary SMCs was not affected by treatment with 50 μmol/l of c9t11-CLA, t10c12-CLA or LA for 21 hours followed by a 3 hours

treatment with TNFα (10 ng/ml) in the presence of the same fatty acids relative to unstimulated controls; cell viabilities were between 93 and 106% for aortic SMCs and 92 and 105% for coronary artery SMCs, respectively, relative to unstimulated controls (=100%).

Treatment with CLA isomers has no effect on mitogen-induced proliferation of vascular SMCs

Incubation with the mitogen PDGF significantly stimulated proliferation of aortic and coronary artery SMCs compared to unstimulated controls ($P < 0.05$; Fig. 1). However, treatment of both SMC types with 50 μmol/l of either c9t11-CLA, t10c12-CLA or LA did not modulate PDGF-stimulated cell proliferation.

Treatment with CLA isomers reduces mitogen-induced [^{3}H] proline incorporation into cell layer protein of vascular SMCs in a PPARγ-dependent manner

[^{3}H] proline incorporation into the cell layer protein of aortic and coronary artery SMCs was markedly induced by treatment with PDGF when compared to unstimulated controls ($P < 0.05$; Fig. 2A, B). Treat-

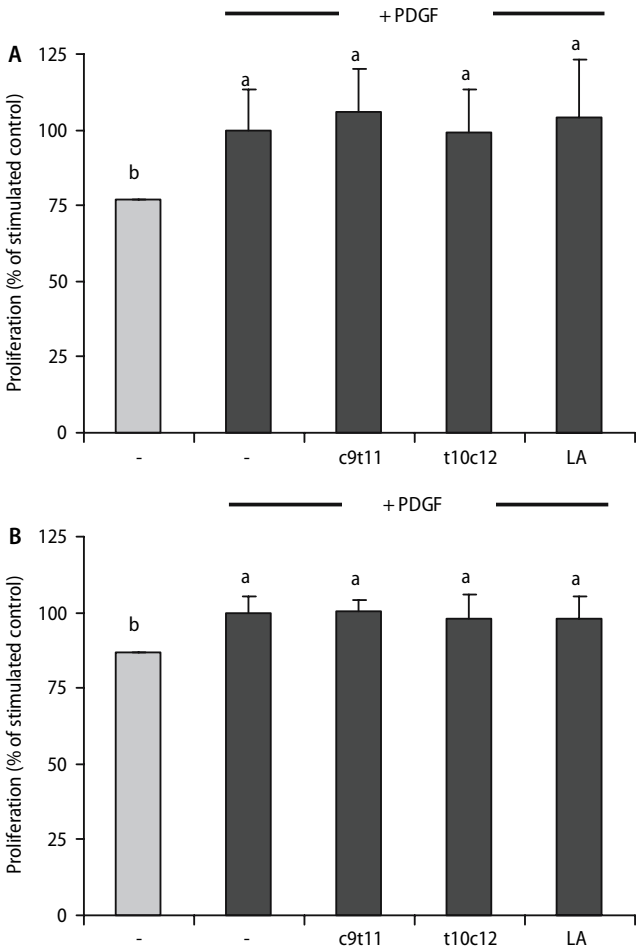

Fig. 1 Effect of treatment for 24 hours with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) on PDGF (10 ng/ml)-induced proliferation of aortic SMCs (**A**) and coronary artery SMCs (**B**). Cells treated with PDGF only for 24 hours were used as controls (stimulated controls). Cells treated with medium only for 24 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of cell proliferation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$)

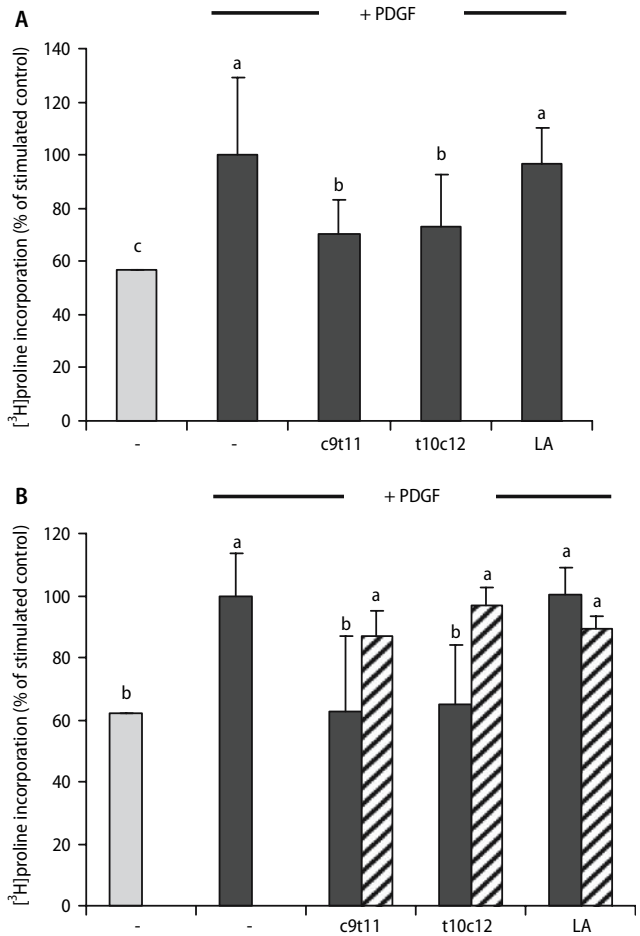

Fig. 2 Effect of treatment for 24 hours with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) on PDGF (10 ng/ml)-induced [^{3}H] proline incorporation into the cell layer protein of aortic SMCs (**A**) and coronary artery SMCs (**B**). Effect of simultaneous treatment with 1 μmol/l of the PPARγ antagonist T0070907 is also shown (*shaded bars*). Cells treated with PDGF only for 24 hours were used as controls (stimulated controls). Cells treated with medium only for 24 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of [^{3}H] proline incorporation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$)

ment with 50 μmol/l of either c9t11-CLA or t10c12-CLA significantly reduced PDGF-stimulated [^{3}H] proline incorporation into cell layer protein of aortic and coronary artery SMCs compared to stimulated controls ($P < 0.05$), whereas treatment with 50 μmol/l of LA revealed no effect.

Simultaneous treatment of coronary artery SMCs with 1 μmol/l of the PPARγ inhibitor T0070907 abrogated the inhibitory effect of c9t11-CLA and t10c12-CLA on PDGF-stimulated [^{3}H] proline incorporation into the cell layer protein (Fig. 2B).

Treatment with t10c12-CLA reduces TNFα-induced NF-κB p50 transactivation and treatment with c9t11-CLA and t10c12-CLA reduces PDGF-induced NF-κB p50 transactivation in vascular SMCs

Treatment of coronary artery SMCs with TNFα or PDGF markedly induced DNA-binding of NF-κB p50 compared to unstimulated control treatment ($P < 0.05$; Fig. 3A and B). Incubation of cells with 50 μmol/l of t10c12-CLA significantly reduced TNFα-induced DNA-binding of NF-κB p50 relative to stimulated controls ($P < 0.05$), whereas c9t11-CLA had no effect. Simultaneous treatment of coronary artery SMCs with the PPARγ inhibitor T0070907 completely abolished the inhibitory effect of t10c12-CLA on TNFα-stimulated DNA-binding of NF-κB p50. In addition, treatment of cells with 50 μmol/l of c9t11-CLA and t10c12-CLA but not with LA significantly reduced PDGF-induced DNA-binding of NF-κB p50 relative to stimulated controls ($P < 0.05$). Co-treatment of coronary artery SMCs with the PPARγ inhibitor T0070907 only partially abrogated the inhibitory effect of c9t11-CLA and t10c12-CLA on PDGF-stimulated DNA-binding of NF-κB p50.

Furthermore, immunoblot analysis revealed that treatment of coronary artery SMCs with 50 μmol/l of c9t11-CLA and t10c12-CLA but not with LA inhibited PDGF-induced protein concentration of p-IκBα (Fig. 3C).

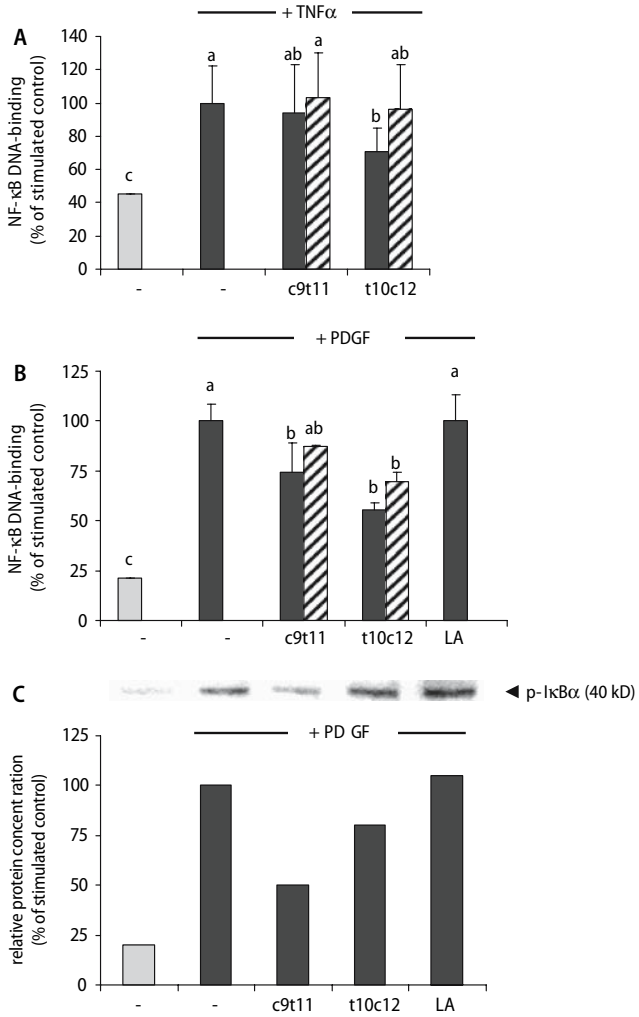

Fig. 3 Inhibitory effect of CLA isomers on stimulus-induced NF-κB activation. **A–B** Effect of treatment with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) for 8 hours on TNFα (**A**)- and PDGF (**B**)-induced transactivation of the NF-κB subunit p50 in coronary artery SMCs. Effect of simultaneous treatment with 1 μmol/l of the PPARγ antagonist T0070907 is also shown (*shaded bars*). Cells treated with TNFα (10 ng/ml) or PDGF (10 ng/ml) alone for 8 hours were used as controls (stimulated controls). Cells treated with medium only for 8 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of NF-κB p50 transactivation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$). **C** Effect of treatment with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) for 4 hours on PDGF (10 ng/ml)-induced protein concentration of phosphorylated form of IκBα (p-IκBα) in coronary artery SMCs. Representative immunoblot and corresponding densitometric analysis of bands specific to p-IκBα are shown for one independent experiment.

Discussion

CLA are well-known to exert potent anti-atherogenic actions in animal models of atherosclerosis [18, 35, 36]. However, due to the rather descriptive nature of these in vivo-studies the mechanisms of action underlying the anti-atherogenic properties of CLA are only partially understood. Based on cell culture experiments, which are appropriate to provide a more mechanistic inside, some mechanisms of action of CLA such as activation of PPARγ-signalling and inhibition of proinflammatory mediator release have already been proposed [7, 28, 40]. In an attempt to address further potentially novel anti-atherogenic mechanisms of action we focused on collagen formation by vascular SMCs which is considered to be a hallmark in atherosclerosis development since it

significantly contributes to intimal thickening and, finally, atherosclerotic plaque formation [16]. Following a cell-based approach we used vascular SMCs from human aorta and coronary arteries as a suitable model for studying the modulatory potential of CLA on this critical step in atherosclerosis and investigated the effect of two different CLA isomers on collagen production using [^{3}H] proline incorporation. Since the major synthetic destination of proline is collagen, the incorporation of proline into newly synthesized protein provides a reliable index of collagen synthesis [19]. Herein, we could demonstrate for the first time that PDGF-stimulated collagen production by vascular SMCs was markedly inhibited in the presence of the CLA isomers c9t11-CLA and t10c12-CLA. This finding possibly explains, at least in part, the observation from in vivo-studies that CLA reduces atherosclerotic plaque development using animal models of atherosclerosis [18, 35, 36]. In contrast, treatment with LA as a reference fatty acid had no effect on PDGF-stimulated [^{3}H] proline incorporation. This indicates that the inhibitory effect of CLA on collagen production by vascular SMCs is dependent on the specific conjugated dienoic structure of CLA.

Considering that the amounts of total and collagenous protein largely depend on cell growth and cell number, we also investigated the effects of treatment on cell kinetics and cell viability. Effects of treatment with CLA isomers on cell kinetics (proliferation) were determined by estimating cell proliferation from incorporation of 5-bromo-2′-deoxyuridine as a marker of DNA replication. There was no significant difference in cell proliferation between SMCs treated with CLA isomers, LA or PDGF only, confirming that the decreased collagen synthesis by treatment with CLA was not due to changes in cell proliferation or cell number. The finding that the proliferative response of coronary artery SMCs to PDGF stimulation was rather weak is largely explained by the presence of serum containing several factors stimulating cell proliferation during incubation. However, we decided to perform these experiments in the presence of serum because it represents a more physiological situation for the SMCs than the absence of serum. Nevertheless, the proliferative response to PDGF was sufficient to study potential alterations in cell proliferation by CLA. To exclude the possibility that the reduction in [^{3}H] proline incorporation was due to cell death, an MTT assay was performed. CLA isomers as well as LA had no effect on cell viability, thus excluding cytotoxicity as a cause for the reduction in the amounts of collagen by CLA.

Vascular injury as the initial event of atherosclerosis is correlated with nuclear localization of NF-κB. In its latent form, NF-κB exists in the cytosol of unstimulated cells bound to the inhibitory proteins IκBs. Upon activation by cytokines, mitogens or oxidative stress, NF-κB dissociates from the IκBs and translocates to the nucleus where it regulates the expression of genes involved in inflammation [8]. It has been demonstrated that n-6 and n-3 polyunsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid stimulate collagen formation in fibroblasts through the activation of the NF-κB-pathway and NF-κB target gene expression [12, 14]. This is probably explained by the fact that the promoter of the COL1A2 gene which encodes the α2 chain of type I collagen contains at least 2 putative NF-κB binding sites [4]. In addition, the COL1A1 gene which encodes the α1 chain of type I collagen is probably also induced by NF-κB, because both COL1A1 and COL1A2 are highly sensitive to reactive oxygen species (ROS) [26, 27], which are major factors inducing the phosphorylation of IκB and subsequent translocation of NF-κB [1]. Thus, we hypothesized that inhibition of NF-κB transactivation by CLA would inhibit collagen formation in vascular SMCs. Inhibition of stimulus-induced NF-κB transactivation by CLA has been observed in several cell culture models [7, 40]. Similarly, our NF-κB transactivation experiments in the present study revealed that c9t11-CLA and t10c12-CLA inhibit PDGF-induced DNA-binding activity of NF-κB p50 in vascular SMCs. In addition, t10c12-CLA also inhibited TNFα-induced DNA-binding activity of NF-κB p50. We have no explanation that c9t11-CLA was capable of reducing transactivation of NF-κB when stimulated with PDGF but not when stimulated with TNFα. However, since we have recently shown that both CLA isomers decreased transactivation of the p65 subunit of NF-κB [28], which forms transcriptionally active heterodimers with the p50 subunit, we suggest that c9t11-CLA is also a potent inhibitor of NF-κB transactivation in vascular SMCs. Supportive of this assumption is the observation of our recent study, that both CLA isomers strongly reduced TNFα-induced mRNA concentrations of NF-κB target genes cyclooxygenase-2 and cytosolic phospholipase A_2 in vascular SMCs [28]. Although, we did not study the effect of CLA on collagen production during inhibition of the NF-κB-pathway, the present results suggest that NF-κB inhibition is possibly involved in the inhibitory effect of CLA on collagen production. Future experiments using selective inhibitors of the NF-κB-pathway like parthenolide, which blocks polyubiquitination and degradation of the IκB complex, should clarify this question.

To further investigate the molecular mechanism underlying the inhibition of NF-κB transactivation by CLA in vascular SMCs, we determined relative protein concentrations of the phosphorylated form of IκBα, p-

IκBα. IκBα phosphorylation in response to different stimuli (mitogens, cytokines, and ROS) results in the degradation of IκBα and subsequent release of activated NF-κB, which translocates into the nucleus and increases transcription of NF-κB target genes. Hence, inhibition of IκBα phosphorylation results in a reduced DNA-binding of activated NF-κB. Thus, the finding from the present study that both CLA isomers, in particular c9t11-CLA, decreased PDGF-induced protein concentrations of p-IκBα indicates that the inhibitory effect of CLA on NF-κB activation is at least partially due to inhibition of IκBα phosphorylation. Similar findings have also been reported from other groups using different experimental models [13, 22, 24]. According to these reports inhibition of phosphorylation of IκBα by CLA is mediated by blocking IκB kinase (IKK)- and Akt-, a serine/threonine kinase, signalling [13, 22, 24]. Future studies have to show whether these signalling pathways are also blocked by CLA in vascular SMCs.

Since recent studies demonstrated that PPARγ agonists inhibit collagen synthesis from myofibroblasts, activated hepatic stellate cells (HSC), and vascular SMCs [3, 11, 42], we further investigated the effect of CLA isomers on SMC collagen formation in the presence of the synthetic PPARγ antagonist T0070907. The present study clearly demonstrated that the inhibitory effect of CLA isomers on collagen formation could be abrogated by T0070907 indicating that PPARγ-signalling is indeed involved in the inhibitory effect of CLA on SMC collagen formation. Inhibition of collagen synthesis by PPARγ activators from myofibroblasts, HSCs, and vascular SMCs has been shown to involve interruption of TGF-β signalling [3, 11, 42] by directly interfering with the Smad3-signalling pathway [11]. TGF-β is a potent stimulator of collagen production through the induction of connective tissue growth factor (CTGF) gene expression and PDGF production, and both, CTGF and PDGF, are potent stimulators of collagen production. Since it has been further demonstrated that the suppression of TGF-β-signalling and gene expression of CTGF is mediated through reducing oxidative stress [42], and NF-κB has been described as the primary mediator of oxidative stress it appears to be possible that the reduced collagen production by CLA in vascular SMCs is due to the observed PPARγ-dependent inhibition of NF-κB. In line with this assumption is the finding that inhibition of NF-κB activity by the antioxidant (-)-epigallocatechin-3-gallate in activated HSCs was accompanied by the interruption of TGF-β signal transduction and a reduced collagen production [5]. Moreover, the PPARγ activator 15-d-PGJ2, which reduced TGF-β-induced CTGF expression and collagen formation in human aortic SMCs [11], was also reported to inhibit NF-κB [33]. In addition, direct inhibition of NF-κB transactivation by PPARγ activation [22] without involving TGF-β signalling, might also be causative for the reduction of collagen biosynthesis by CLA, because the COL1A2 gene is a NF-κB target gene as mentioned above. Nevertheless, since we did not address the exact molecular link between the CLA-mediated inhibition of NF-κB and SMC collagen synthesis herein and corresponding studies dealing with vascular SMCs are not available from the literature further research is required to support our hypothesis.

Due to the essential role of PPARγ for inhibition of collagen formation [11] it was not surprising that LA, which is a relatively weak activator of PPARγ compared with CLA, did not reduce collagen formation from vascular SMCs. However, another reason partially explaining the lack of effect of LA on collagen formation might be that CLA, in contrast to LA, markedly reduces membrane concentrations of arachidonic acid due to displacement of arachidonic acid from membrane phospholipids and inhibits metabolism of arachidonic acid by COX and lipoxygenase enzymes to biologically active metabolites (prostaglandins, thromboxans, leukotrienes) in vascular SMCs and various other cell types [10, 17, 28, 29]. Because these metabolites are supposed to be mediators of pathological fibrotic conditions increasing the formation of collagen by stimulating profibrotic factors such as TGF-β1 [21], antagonism of specific eicosanoid receptors or selective inhibition of COX-2 resulted in attenuation of fibrosis under different pathological conditions concomitant with a decrease in TGF-β1 [20, 25, 34]. Thus, we propose that inhibition of arachidonic acid metabolism by CLA beside PPARγ activation might also contribute to the reduced collagen formation by CLA and might largely explain the differential action of CLA compared to LA in this cell system.

Similar observations regarding the effect of CLA on collagen formation and NF-κB target gene expression in aortic and coronary artery SMCs suggest that the effects of CLA on collagen production are largely independent of the vascular location of SMCs (aorta vs. coronary artery) which is in contrast to findings from other studies showing that various stimuli such as adenosine or 2-hydroxyestradiol displayed differential, even opposing, effects on growth or functional properties of SMCs from coronary arteries compared to SMCs from the aorta [31, 41]. However, this discrepancy might be explained by the fact that uptake and concentrations of CLA are similar in SMCs from different vascular locations after treatment with CLA as observed in a recent study of our group [29], whereas the effects of adenosine on SMC function largely depend on the distribution pattern of membrane-associated receptor subtypes (e.g. adenosine

receptors) which vary considerably between SMCs from the coronary artery and the aorta [31].

In summary, the present study revealed for the first time that the CLA isomers c9t11-CLA and t10c12-CLA, but not LA, inhibited PDGF-stimulated collagen production by vascular SMCs. Moreover, the study showed that simultaneous treatment with a PPARγ antagonist abrogated the inhibitory effect of CLA isomers on SMC collagen formation, indicating that PPARγ-signalling might be involved in the mediation of this cellular effect of CLA. This might also explain why LA which is only a weak PPARγ activator had no effect on SMC collagen formation. Since activation of the NF-κB-pathway has been implicated in the stimulatory effect of oxidized LDL and n-6 and n-3 polyunsaturated fatty acids on collagen production in vascular SMCs and fibroblasts, respectively, the observation that treatment with CLA causes inhibition of the NF-κB-pathway suggests that this anti-inflammatory effect is possibly involved in the inhibition of collagen production by CLA in vascular SMCs. However, direct proof for the involvement of NF-κB inhibition in the reduced collagen formation has to be given. Deposition of collagen and other extracellular matrix proteins in the vessel wall largely contributes to the formation of atherosclerotic plaques, ultimately leading to acute clinical manifestations of atherosclerosis such as myocardial infarction and stroke. Thus, the reduced collagen production by vascular SMCs treated with CLA might explain, at least in part, the observation from in vivo-studies that CLA reduces atherosclerotic plaque development using animal models of atherosclerosis [18, 35, 36]. In conclusion, the present results provide insight into a new mechanism of action of CLA relating the anti-inflammatory effect of CLA to inhibition of collagen formation from vascular SMCs.

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