REVIEW

Metabolism and actions of conjugated linoleic acids on atherosclerosis-related events in vascular endothelial cells and smooth muscle cells

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Conjugated linoleic acids (CLAs) are biologically highly active lipid compounds that have attracted great scientific interest due to their ability to cause either inhibition of atherosclerotic plaque development or even regression of pre-established atherosclerotic plaques in mice, hamsters and rabbits. The underlying mechanisms of action, however, are only poorly understood. Since cell culture experiments are appropriate to gain insight into the mechanisms of action of a compound, the present review summarizes data from cell culture studies about the metabolism and the actions of CLAs on atherosclerosis-related events in endothelial cells (ECs) and smooth muscle cells (SMCs), which are important cells contributing to atherosclerotic lesion development. Based on these studies, it can be concluded that CLAs exert several beneficial actions including inhibition of inflammatory and vasoactive mediator release from ECs and SMCs, which may help explain the anti-atherogenic effect of CLAs observed *in vivo*. The observation that significant levels of CLA metabolites, which have been reported to have significant biological activities, are well detectable in ECs and SMCs indicates that the anti-atherogenic effects observed with CLAs are presumably mediated not only by CLAs themselves but also by their metabolites.

Keywords:
Atherosclerosis / Conjugated linoleic acid / Endothelial cell / Smooth muscle cell

1 Introduction

Conjugated linoleic acids (CLAs) are a group of positional and geometrical isomers of linoleic acid (LA; C18:2c9c12) characterized by the presence of conjugated double bonds (Fig. 1). To date, 28 isomers of CLAs have been identified with varying geometry (*cis*/*cis*, *trans*/*trans*, *cis*/*trans*, *trans*/*cis*) and positions of the double bonds, which can be found on carbon atoms 6 to 15. CLAs have been shown to occur naturally in food [1]. The most important source of CLAs in the human diet are ruminant-derived products, such as milk, dairy products, and meat [1–4], because CLA isomers are produced in the rumen during microbial biohydrogenation of dietary LA and in tissues through Δ9-desaturation of the rumen-derived *trans*-vaccenic acid (*trans*-11–18:1) [5–7]. The predominant CLA isomer in ruminant-derived products is c9t11-CLA (C18:2c9t11, known as rumenic acid) contributing to more than 90% of total CLAs [2]. In addition to natural foodstuff, dietary CLA supplements, which are available over the counter in supermarkets and drug stores as well as *via* the Internet, can also contribute to CLA intake in humans. CLA supplements are sold as weight loss agents although evidence from most human studies suggests that CLA supplementation does not reduce body weight and body fat or increase fat

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Abbreviations: AA, arachidonic acid; **AP-1,** activator protein-1; **bFGF,** basic fibroblast growth factor; **CLA,** conjugated linoleic acid; **COL,** collagen; **COX,** cyclooxygenase; **cPLA$_2$,** cytosolic phospholipase A$_2$; **EC,** endothelial cell; **ECM,** extracellular matrix; **ET,** endothelin; **ICAM,** intercellular adhesion molecule; **IκB,** inhibitor of NF-κB; **LA,** linoleic acid; **mPGES,** microsomal prostaglandin E-synthase; **NO,** nitric oxide; **PAF,** platelet-activating factor; **PG,** prostaglandin; **PLA$_2$,** phospholipase A$_2$; **PPAR,** peroxisome proliferator-activated receptor; **SMC,** smooth muscle cell; **TF,** tissue factor; **TX,** thromboxane; **VCAM,** vascular cell adhesion molecule; **VEGF,** vascular endothelial growth factor

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free mass [8, 9]. In addition, CLA supplements are considered critically because only few long-term clinical trials, which are necessary to determine the efficacy and safety of CLAs as a functional nutrient in humans, have been performed [10–12]. In contrast to natural foodstuff, dietary supplements have a different CLA isomeric profile. The main difference is the high percentage of t10c12–CLA (C18:2t10c12, up to 50% of total CLAs) in supplements [2], whereas this CLA isomer is only a minor component in dairy products or meat. According to recent studies, average daily intakes of CLAs in middle Europe and the US are estimated to be in the range of 100–400 mg ([13, 14–18]; Table 1). In comparison, CLA intake from dietary CLA supplements marketed for weight loss purposes is markedly higher (2–4 g/day), provided that the manufacturers' recommendations are followed.

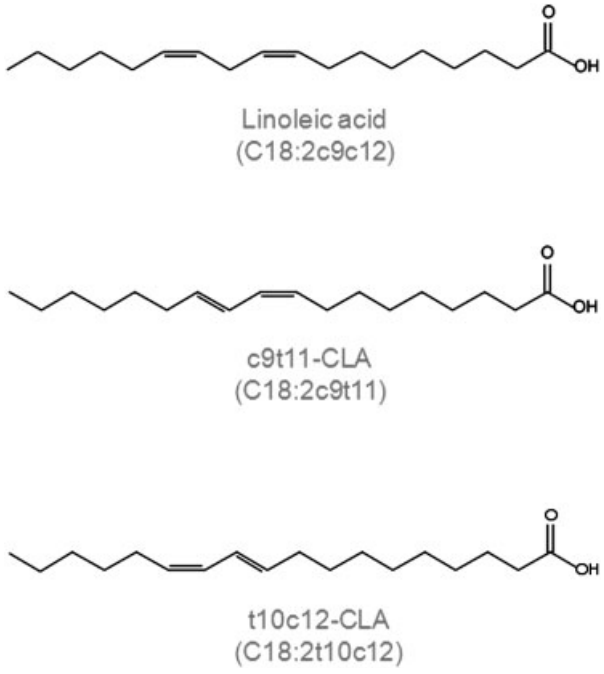

Figure 1. Chemical structures of LA (C18:2c9c12) and two isomers of CLA, C18:2c9t11 and C18:2t10c12.

CLAs have attracted great scientific interest due to several beneficial properties [19–25] including the ability to cause either inhibition of atherosclerotic plaque development or even regression of pre-established atherosclerotic plaques in mice, hamsters, and rabbits [21, 26–32], but the mechanisms underlying these anti-atherogenic effects of CLAs *in vivo* are only partially understood. However, it has been proposed that CLAs may act as ligands and activators of peroxisome proliferator-activated receptors (PPARs) [33–35], which are known to attenuate pro-atherogenic events by inhibiting pro-inflammatory gene expression [36]. Elucidation of the anti-atherogenic effects of CLAs is of great interest, regarding the high prevalence of atherosclerosis in the population of middle Europe and the US [37, 38] and, thus, the need for developing strategies to prevent or treat atherosclerosis.

Although results from cell culture experimentations cannot be directly applied to the situation *in vivo*, cell culture studies are a useful approach to gain insight into the mechanisms of action of a compound. Vascular endothelial cells (ECs) and smooth muscle cells (SMCs) are amongst the most important cells of the arterial wall involved in the development of atherosclerotic plaques [39, 40]. Therefore, ECs and SMCs are appropriate cell culture models when investigating the actions of CLAs in the context of atherosclerosis-related events.

Recent studies in cultured cells (hepatocytes, macrophages, leukemia cells) and tissues of CLA-fed animals indicated that CLA isomers are further metabolized within the cell to fatty acids of different chain lengths possessing

Table 1. Average CLA intake (mg/day) estimated for several countries (adopted from [15])

Country	Method	Daily intake	Estimated isomer	Ref.
European Union	Milk intake	250	C18:2c9t11	[14]
Spain	Milk intake	140	C18:2c9t11	[14]
France	Milk intake	300	C18:2c9t11	[14]
Italy	Milk intake	220	C18:2c9t11	[14]
Greece	Milk intake	150	C18:2c9t11	[14]
Portugal	Milk intake	150	C18:2c9t11	[14]
Portugal	Intake of milk, dairy products and meat	74	Total CLA	[15]
		56	C18:2c9t11	
		9	C18:2t7c9	
Germany	7-day dietary record	350–430	C18:2c9t11	[13]
Sweden	1-day dietary record	160	C18:2c9t11	[16]
USA	Food-frequency questionnaires	93–197	Total CLA	[17]
		72–151	C18:2c9t11	
USA	3-day food duplicates	151–212	Total CLA	[17]
		140–193	C18:2c9t11	
USA	3-day dietary record	104–176	Total CLA	[17]
		79–133	C18:2c9t11	
USA	3-day dietary record	127	C18:2c9t11	[18]

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the characteristic conjugated dienoic structure of CLAs [41–45]. These observations have gained in importance since it has recently been shown that some of these metabolites of CLAs exert potent biological activities [41], indicating that the anti-atherogenic effects observed with CLAs are presumably mediated not only by CLAs themselves but also by their metabolites.

To gain insight into the potential anti-atherogenic mechanisms of action of CLAs, the present review will summarize data from cell culture studies about the metabolism and the actions of CLAs on atherosclerosis-related events in ECs and SMCs.

2 Role of ECs and SMCs in atherogenesis

Both ECs and SMCs are major cellular components of atherosclerotic plaques and, thus, play an important role in the pathogenesis of atherosclerosis [39, 40, 46]. ECs are involved, in particular, in the initial steps of atherosclerosis development [47], whereas SMCs play a dominant role during the progression of atherosclerosis, but also during restenosis after vascular interventions such as coronary angioplasty [40, 48]. The lumen of the arterial wall is covered with a monolayer of ECs, called endothelium, which forms a selectively permeable barrier between the blood and the vascular tissue. Besides the barrier function, the endothelium has numerous other functions. These functions are mediated by the secretion of biologically active substances that control all aspects of the integrity and metabolism of the vascular wall, such as vascular structure and permeability, vascular tone and blood pressure, coagulation and fibrinolysis, and inflammatory response [49, 50]. Together with the underlying sub-endothelial space, the endothelium builds the intima, which is the innermost layer of the vessel wall. In the arterial media, which is separated from the intima by the internal elastic lamina, the contractile SMCs are concentrically arranged in the arterial media and directly linked by cell contacts and connective tissue fibers [40]. This arrangement of SMCs in the arterial wall enables these cells to maintain vessel tone and regulate blood pressure by contraction in response to chemical and mechanical stimuli.

Due to the critical role of the endothelium for vessel wall regulation, atherosclerosis and thrombosis, the major complications of atherosclerosis triggering myocardial infarction and stroke, are initiated, when the endothelium is injured or normal EC function is disturbed, which is called endothelial dysfunction [51–54]. Hence, endothelial dysfunction is considered one of the critical events in the development of atherosclerosis. Endothelial dysfunction is accompanied by an activation of ECs leading to increased adhesion of circulating leukocytes (monocytes, lymphocytes) to the activated endothelium, and, subsequently, transendothelial migration of leukocytes into the sub-endothelial space [51, 55], which is largely responsible for the inflammatory process associated with atherosclerosis [56–58]. Recruitment of circulating leukocytes to the activated endothelium is mediated by inflammatory chemokines such as IL-8 and monocyte chemoattractant protein-1, which are secreted locally in large amounts from activated ECs. Leukocyte adhesion is mediated by inducible cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin, expressed at high levels on the surface of activated ECs. Subsequently, infiltrated monocyte-derived MCs with high phagocytic activity take up lipid-rich lipoprotein particles, which enter the sub-endothelial space due to the impaired barrier function of the activated endothelium. The resulting accumulation of lipids in the vessel wall leads to the first visible atherosclerotic lesions, called fatty streaks.

Similar to ECs, the contractile SMCs are also activated as a response to vascular injury. Activation of contractile SMCs is accompanied by a phenotypic modulation [59, 60], which is characterized by a dramatic change in SMC morphology with a loss of contractile proteins (such as α-actin and SM myosin) and myofilaments and the formation of extensive rough endoplasmic reticulum and a large Golgi complex leading to a greatly increased synthetic, secretory, and proliferative capacity. Hence, this phenotype is called the synthetic or activated one. The synthetic SMCs migrate from the media into the arterial intima, where they proliferate and produce various substances including extracellular matrix (ECM) proteins and inflammatory mediators. The extensive production of ECM proteins including collagen, elastin, glycoproteins, and proteoglycans by activated SMCs and accumulation of ECM proteins in the arterial wall significantly contributes to intimal thickening and, finally, atherosclerotic plaque formation. Collagen, in particular type I collagen, which accounts for 70% of all collagen, is the dominating ECM protein making up to 60% of the total protein content of atherosclerotic plaques [61–63]. Since vascular SMCs are the major source of collagen production within the vessel wall [64], collagen deposition by SMCs is considered to be a hallmark in atherosclerosis development. Activated SMCs, moreover, produce excessive amounts of inflammatory mediators including chemokines, cytokines, and eicosanoids, which contribute to the chronic inflammatory response associated with atherosclerosis [65]. Thus, vascular SMCs are also strongly involved in maintaining the local inflammatory process in the arterial wall, besides activated ECs and infiltrated leukocytes.

3 Metabolism of CLAs in ECs and SMCs

While several studies have been performed with respect to the metabolism of CLAs in cultivated hepatocytes and blood cells as well as tissues of CLA-treated experimental animals only very few studies have addressed the metabolism of CLA

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isomers in cells of the vasculature such as ECs and SMCs [66–68]. Those studies performed with ECs and SMCs revealed that CLA isomers (C18:2c9t11, C18:2t10c12, and C18:2t9t11) are isomer-specifically metabolised to shortened (C16:2) and elongated (C20:2 and C22:2) fatty acids with the characteristic conjugated diene structure of CLAs (Table 2, Fig. 2, Fig. 3). Figure 2A shows a typical Ag^+-high performance liquid chromatograph-diode array detector chromatogram of the CLA region of human aortic ECs treated with 50 μM of C18:2c9t11 and C18:2t10c12 at a detection wavelength of 234 nm. At this detection wavelength of 234 nm, which is nearly specific for the conjugated dienoic structure, it was possible to detect various conjugated metabolites of C18:2c9t11 and C18:2t10c12 such as the β-oxidation products C16:2c7t9 and C16:2t8c10 and the elongation products C20:2c11t13, C20:2t12c14, C22:2c13t15, and C22:2t14c16 in EC and SMC total lipids following treatment with CLA isomers [66, 67]. By comparison of GC-MS chromatograms of different treatments it was possible to identify the CLA metabolites by their mass spectra. Figure 2B shows the partial GC-MS chromatogram (total ion chromatogram) of the C16:2 region of human aortic ECs treated with 50 μM of C18:2c9t11 and C18:2t10c12. Analysis of methyl esters was favoured because synthesis of the 4,4-dimethyloxazoline derivatives for structure elucidation is accompanied with compound loss and artefact formation [66, 67]. As demonstrated in Fig. 3A mass spectra of methyl ester isomers show similar fragmentation patterns but the diene fragments (*m/z* 67, 81) and the molecular ion [M^+] (*e.g.* *m/z* 266, 322, 350) for identification of chain length are of high abundance, therefore, allowing to reliably identify C16:2c7t9, C20:2c11t13 and C22:2c13t15. As illustrated in Fig. 3B, GC-MS analysis was required for correct identification of small amounts of CLA metabolites in EC total lipids study due to co-eluting major fatty acids [67]; *e.g.* the C20:2 metabolites of CLAs, C20:2c11t13 and C20:2t12c14, elute in the region of arachidonic acid (AA).

The conjugated C16:2 isomers detected in total lipids from CLA-treated ECs and SMCs are supposed to originate, at least in part, from chain-shortening of CLAs within the peroxisome [42]. Chain-shortening within the peroxisome serves to provide shortened fatty acids for further β-oxidation within the mitochondrium. The amounts of C16:2c7t9 and C16:2t8c10 found in total lipids of ECs and SMCs treated with 50 μM of C18:2c9t11 and C18:2t10c12, respectively, contributed to about 1.3 to 1.8 g/100 g of total fatty acids [66, 67]. These levels of conjugated C16:2 metabolites are notably since non-conjugated C16:2, which derives from β-oxidation of LA, was not detectable at all in cells treated with LA [66], suggesting that conjugated C16:2 isomers such as C16:2c7t9 and C16:2t8c10 are apparently accumulating in vascular cells treated with CLA isomers. This assumption is strengthened by the observation from others that conjugated β-oxidation products of CLAs were also accumulating in microsomal fractions of hepatic tissues or purified hepatic mitochondria incubated with CLAs [45]. *In vitro* studies measuring mitochondrial respiration following incubation with different fatty acids revealed that the accumulation of conjugated C16:2 in the mitochondrial matrix is due to a slower oxidation rate of CLAs compared with LA [69, 70]. The latter is probably explained by the fact that the oxidation of unsaturated fatty acids requires so-called auxiliary enzymes that work with different efficiencies depending on whether the *cis* or *trans* double bond is even- or odd-positioned [71]. Consequently, differences regarding the oxidation rate occur between fatty acids with different

Table 2. Proportions of conjugated metabolites of CLAs in total lipids of human ECs and SMCs cultured in the presence or absence (control) of 5 or 50 μmol/L of C18:2c9t11 or C18:2t10c12 for 24 h (gram *per* 100 g of total fatty acids)

Treatment	Control	C18:2c9t11		C18:2t10c12	
		5 μM	50 μM	5 μM	50 μM
ECs					
C16:2c7t9	<0.1	0.6±0.1[a)]	1.3±0.1[a)]	<0.1	0.3±0.1
C16:2t8c10	<0.1	<0.1	<0.1	0.4±0.1[a)]	1.8±0.2[a)]
C20:2c11t13	<0.1	0.1±0.1[a)]	1.0±0.1[a)]	<0.1	<0.1
C20:2t12c14	<0.1	<0.1	<0.1	0.2±0.1[a)]	1.0±0.1[a)]
C22:2c13t15	<0.1	0.1±0.1[a)]	0.1±0.1[a)]	<0.1	<0.1
C22:2t14c16	<0.1	<0.1	<0.1	<0.1	<0.1
SMCs					
C16:2c7t9	<0.1	0.4±0.3[a)]	1.5±0.7[a)]	<0.1	0.1±0.1
C16:2t8c10	<0.1	<0.1	<0.1	0.4±0.1[a)]	1.9±0.7[a)]
C20:2c11t13	<0.1	0.2±0.1[a)]	1.1±0.4[a)]	<0.1	<0.1
C20:2t12c14	<0.1	<0.1	<0.1	0.5±0.4[a)]	1.1±0.2[a)]
C22:2c13t15	<0.1	0.1±0.1[a)]	0.1±0.1[a)]	<0.1	<0.1
C22:2t14c16	<0.1	<0.1	<0.1	<0.1	<0.1

Data represent mean±SD of three independent experiments.
a) Significantly different from control, $p<0.05$.

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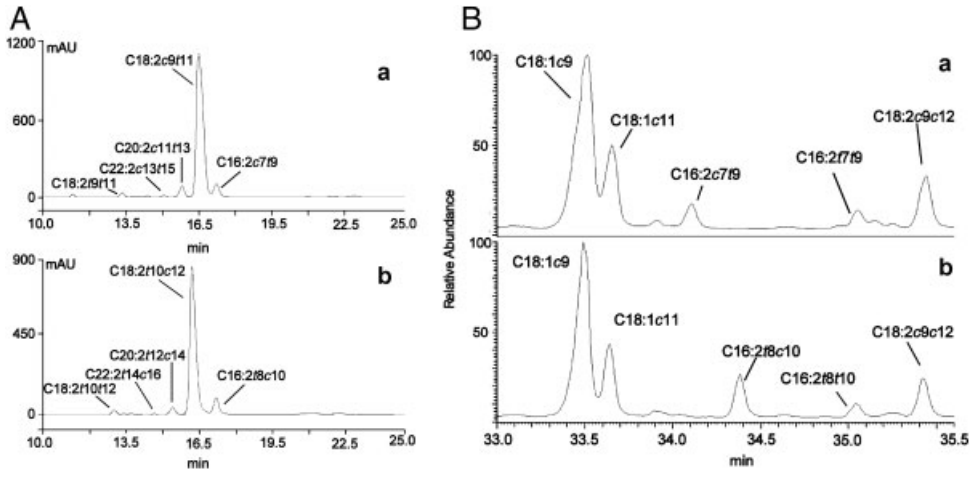

Figure 2. (A) Partial Ag^{+}-HPLC-DAD chromatogram at a detection wavelength of 234 nm of conjugated fatty acids of human aortic ECs treated with 50 μM of either C18:2c9t11 (a) or C18:2t10c12 (b). (B) Partial GC-MS chromatogram (TIC) of CD16:2 region of human aortic ECs treated with 50 μM of either C18:2c9t11 (a) or C18:2t10c12 (b).

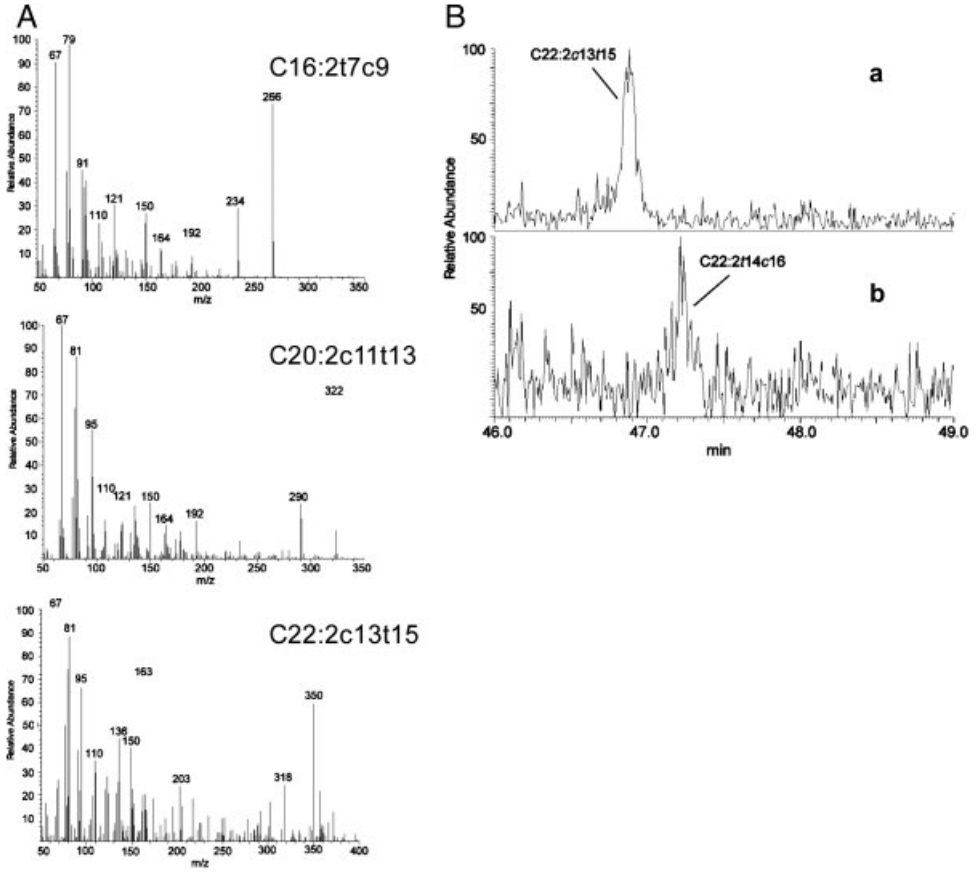

Figure 3. (A) GC-MS spectra of CLA β-oxidation (C16:2t7c9) and elongation products (C20:2c11t13, C22:2c13t15) of human aoritc ECs treated with 50 μM C18:2c9t11. (B) Partial GC-MS chromatogram (extracted ion *m/z* 350) of CD22:2 region of fatty acids of human aortic ECs treated with 50 μM of either C18:2c9t11 (a) or C18:2t10c12.

conjugated double bonds such as C18:2c9t11 and C18:2t10c12; in fact, the very poor availability of *trans*-3-enoyl-CoA, which is formed during β-oxidation of c9t11-CLA, for the auxiliary enzyme Δ3-*cis*-Δ2-*trans*-enoyl-CoA isomerase is responsible for a comparatively slow oxidation of C18:2c9t11, while the t10 double bond of C18:2t10c12 requires no auxiliary enzyme [69]. With respect to the significance of these findings, it has to be pointed out that not only the β-oxidation rate of CLAs themselves is slowed but also the oxidation rates of other fatty acids are altered [69], which might impair energy metabolism and function of vascular cells.

The C20:2 metabolites of CLAs (C20:2c11t13, C20:2t12c14) detected in total lipids from CLA-treated ECs and SMCs are probably derived from enzymatic elongation of CLAs. This has been proposed from others, which have also detected C20:2 fatty acids with conjugated dienoic structure in leukemia cells treated with CLAs and various

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tissues of CLA-fed animals [41, 42]. The generation of C20:2 metabolites of CLAs might be of special biological significance, because a previous study revealed that both, C20:2t12c14 alone, and a mix of C20:2c11t13/t12c14 cause inhibition of heparin-releasable lipoprotein lipase activity and lipid accumulation in 3T3-L1 adipocytes [41]. The C22:2 metabolites of CLAs (C22:2c13t15, C22:2t14c16), which could be reliably identified in EC and SMC total lipids by GC-MS analysis [66, 67] but not in tissues of CLA-fed animals [41, 42], are probably also elongation products of CLA isomers. Those are likely generated from C20:2 metabolites *via* elongation and are indicative of a substantial elongation capacity of vascular cells, which is known from the literature [72, 73]. When compared with the amounts of C16:2 and C20:2 metabolites of CLAs, the amounts of C22:2 metabolites were generally very low in EC and SMC lipids (Table 2). Solely by extraction of the specific m/z 350 trace in the GC-MS analysis we were capable of definitely identifying C22:2c13t15 and C22:2t14c16 in EC and SMC total lipids [66, 67]. Therefore, the physiological relevance of the C22:2 metabolite levels detected with respect to the modulation of EC and SMC function has to be proven. In contrast, typical conjugated desaturation products of CLA isomers such as C20:3 or C20:4, which are produced from CLA isomers by Δ6-desaturation, elongation and further Δ5-desaturation and detectable in hepatic tissues from CLA-fed animals [42, 44], were not found at all in vascular SMCs and ECs treated with CLA isomers [66–68], which is probably due to the very low fatty acid desaturation capacity of vascular SMCs and ECs [72, 73].

The detection of CLA metabolites in tissues from CLA-fed animals and cell cultures treated with CLAs is of importance to explain the biological effects of CLAs, because CLA metabolites were reported to elicit potent biological activities [41, 74, 75]. The observation that significant levels of CLA metabolites are detectable in cells of vascular wall, therefore, indicates that the anti-atherogenic effects observed with CLAs are presumably not only mediated by CLAs themselves but also by their metabolites.

4 Actions of CLAs on atherosclerosis-related events in ECs and SMCs

In contrast to the few reports about the metabolism of CLAs in SMCs and ECs, several reports dealing with the potential of CLAs to modulate inflammatory reactions in vascular cells, in particular in ECs, exist in the literature. These studies are discussed in the following chapters.

4.1 Actions of CLAs on atherosclerosis-related events in ECs

As mentioned above, vascular ECs play a critical role in vessel wall regulation. Therefore, injury to ECs or endothelial dysfunction is a critical event in the development of atherosclerosis. Endothelial dysfunction is accompanied by an increased recruitment of mononuclear cells, a dysregulated release of vasoactive mediators, impaired antioxidant defense mechanisms, and an elevated thrombogenic mediator secretion, which are all important events related to atherosclerosis development. Furthermore, during advanced atherosclerosis neovascularization of atherosclerotic plaques is observed. In the following chapters, evidence is summarized from *in vitro* studies indicating that CLAs are able to modulate some of these atherosclerosis-related events in ECs.

4.1.1 Recruitment of mononuclear cells

Adhesion of mononuclear cells (monocytes, lymphocytes) to the endothelium is a crucial step in the early phase of atherosclerosis [55, 56]. This is evidenced by the fact that inhibition of mononuclear cell adhesion to the endothelium inhibits atherosclerosis development [76]. Although the mechanisms of action underlying the anti-atherogenic effects of CLAs are only partially understood, only few studies have investigated the effects of CLAs on the mechanims involved in leukocyte adhesion [77, 78]. One study using human aortic ECs clearly revealed that CLA isomers, namely C18:2c9t11 and C18:2t10c12, do not modulate the cytokine-stimulated expression of adhesion molecules, monocyte adhesion, and chemokine release [77]. In addition, cytokine-stimulated activation of NF-κB, which is one of the crucial factors for transcriptional induction of adhesion molecules and chemokines because the promoter region of E-selectin, ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 contains multiple binding sites for NF-κB [79–82], was also not reduced by both CLA isomers in that study [77]. The observations in aortic ECs [77] were partially confirmed by a study of another group using umbilical vein ECs in showing that expression of the adhesion molecules VCAM-1 and ICAM-1 were largely unaffected by the same CLA isomers [78]. In spite of the unaltered expression of adhesion molecules, these authors demonstrated that both CLA isomers (C18:2c9t11 and C18:2t10c12) as well as a CLA isomeric mixture containing C18:2c9t11 and C18:2t10c12 inhibit cytokine-induced monocyte binding to umbilical vein ECs [78]. However, using platelet-activating factor (PAF) receptor antagonists and PAF synthesis inhibitors, the authors of that study [78] demonstrated that the CLA isomers and the CLA mix inhibit cytokine-induced monocyte binding to umbilical vein ECs by suppressing production of the pro-inflammatory phospholipid PAF, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine. The conflicting results of these two studies with respect to monocyte adhesion cannot be definitely resolved, but may be explained by the different vascular origin (aorta *versus* umbilical vein) of the ECs used for experimentations. Indeed, it is well established that vascular cells from

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different vascular beds as well as from different sections of the same blood vessel exert differential actions in response to a common stimulus [83, 84]. In addition, differences in the experimental design (time of exposure to fatty acids, type, and concentration of cytokine) but also cell type-specific effects of CLAs, which are well documented in the literature [24, 85, 86], might be causative. Therefore, from these two studies it cannot be unequivocally answered whether the anti-atherogenic effects of CLAs observed in animal feeding experiments are mediated by reduction of leukocyte adhesion to the endothelium. However, the observation from a randomised, placebo-controlled intervention trial that CLA supplementation with two different CLA mixtures does not alter serum concentrations of soluble adhesion molecules in healthy volunteers [87] is supportive of the findings in aortic ECs that CLA isomers, even at unphysiologically high concentrations, have no effect on monocyte adhesion and the mechanisms regulating leukocyte adhesion.

4.1.2 Vasoactive mediator release

The endothelium plays a dual role in the regulation of vessel tone and blood pressure by production and release of both, relaxing [nitric oxide (NO), prostaglandin (PG) I_2] and constricting [endothelin (ET)-1, superoxide anions] factors. In the healthy endothelium, the biological effects of vasorelaxing factors predominate over the effects of vasoconstrictive substances [88], whereas the opposite is the case in the dysfunctional endothelium overlying atherosclerotic plaques. This is strongly suggested by the observation that endothelium-dependent vascular relaxation is markedly impaired in isolated atherosclerotic coronary arteries [89]. In addition, it has been shown that these disturbances in vasoactive mediator release are correlated with the degree of atherosclerosis [90]. Hence, CLAs might exert atheroprotective effects through the modulation of vasoactive mediator release or the restoration of a balanced secretion of both relaxing and constrictive mediators. In this context it is noteworthy that several studies from independent groups demonstrated that CLA isomers such as C18:2c9t11, C18:2t10c12, C18:2t9t11, and C18:2c9c11 as well as CLA isomeric mixtures dose-dependently modulate the release of vasoactive substances including eicosanoids, NO, and the potent vasoconstrictor ET-1 from ECs [91–96]. In Table 3 an overview about studies dealing with the effects of CLAs on production of eicosanoids in ECs is given. This overview clearly shows that CLA isomers and CLA mixtures consistently inhibit both resting and stimulus-induced eicosanoid production in ECs from different vessels (aorta, saphenous vein, umbilical vein) and different species (human and bovine). The reduction of ET-1 release from bovine aortic ECs treated with a 50:50-mixture of C18:2c9t11 and C18:2t10c12 [94] might be of special importance with respect to modulation of atherosclerosis development, since the abnormal vasoconstriction observed during endothelial dysfunction is particularly attributed to increased levels of ET-1 [97]. The pathogenetic relevance of increased ET-1 levels for atherosclerosis is further demonstrated by the observation that ET-1 plasma levels strongly correlate with the carotid intima-media thickness in patients with increased risk to develop atherosclerosis [98]. Hence, this effect of the CLA mixture has to be considered beneficial with respect to maintaining vascular homeostasis, and, therefore, prevention from atherosclerosis. Synthetic PPAR agonists were also shown to suppress ET-1 secretion in cultured ECs, by a mechanism involving inhibition of activator protein-1 (AP-1) pathway [99, 100]. Since CLA isomers cause activation of both PPAR-γ [77, 94] and PPAR-α [94] in aortic ECs, and reduce activation of AP-1 in different cell types [101, 102], it is not unlikely that the inhibitory effect of CLAs on ET-1 release in ECs is mediated by PPAR-dependent repression of AP-1. This, however, remains to be established in ECs.

4.1.3 Antioxidant defense mechanisms

Oxidative damage is considered to be a major factor in the initiation of atherosclerotic lesions in the vascular wall [103, 104] and, therefore, regarded as a causal factor in coronary and other vascular diseases. Hence, the capability of ECs to attenuate oxidative and inflammatory stresses is an important means to protect from atherosclerosis development. The ability of ECs to cope with oxidative stress is largely determined by the cellular antioxidant defense mechanisms, and upregulation of these mechanisms is of fundamental importance in protecting ECs from oxidative damage [105]. Therefore, it might be speculated that CLAs elicit their anti-atherogenic effects by upregulating intrinsic antioxidant enzymes, such as glutathione peroxidases, catalase, and/or superoxide dismutase. In fact, the finding of a previous study that gene expression of the glutathione peroxidase-4 was induced by a CLA mixture containing equal levels of C18:2c9t11 and C18:2t10c12 in ECs [106] is supportive of our assumption. Thus, this effect of CLA on redox enzyme induction may explain, at least in part, the reported beneficial effects of CLA on vascular disease.

The exposition of ECs to oxidative stress might be also prevented by reducing the availability of NO. Although NO is widely considered an athero-protective substance due to its ability to cause vasodilatation and inhibition of platelet aggregation and SMC proliferation, at increased levels, NO may exert several injurious effects to vascular tissues by increasing cellular oxidative stress. For example, NO reacts with superoxide to form peroxynitrite, an extremely potent oxidant, which is a major factor in oxidative-type cellular injury. In fact, the reaction of NO with superoxide is three times faster than dismutation of superoxides catalyzed by superoxide dismutase [107]. Thus, elevated levels of NO can

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Table 3. Overview of studies dealing with the effects of CLAs on resting and stimulus-induced production of eicosanoids in ECs and SMCs

Cell type	Treatment protocol	CLA source	Effective CLA concentration	Effect on eicosanoid release	Ref.
HSVEC	Resting: 72 h treatment with CLAs, last 3 h labelling with ^{14}C-AA (1 μCi)	CLA mix (50:50 C18:2c9t11 and C18:2t10c12) CLA isomers (C18:2c9t11, C18:2t10c12)	50 μM (C18:2c9t11, C18:2t10c12) 100 μM (CLA mix)	↓ PGF_{2a} (CLA mix, C18:2c9t11, C18:2t10c12)	[96]
	Stimulated: 72 h treatment with CLAs, last 3 h labelling with ^{14}C-AA (1 μCi)+30 min treatment with calcium ionophore A23187 (3 μM)	CLA mix (50:50 C18:2c9t11 and C18:2t10c12) CLA isomers (C18:2c9t11, C18:2t10c12)	50 μM (C18:2c9t11, C18:2t10c12) 100 μM (CLA mix)	↓ PGI_2, ↓ TXB_2, ↓ PGF_{2a}, ↓ PGE_2, ↓ PGD_2 (CLA mix, C18:2c9t11) ↑PGI_2, ↑TXB_2, ↑PGF_{2a}, ↑PGE_2, ↑PGD_2 (C18:2t10c12)	
HAEC	Resting: 24 h treatment with CLAs	CLA isomers (C18:2c9t11, C18:2t10c12)	5 and/or 50 μM	↓ 6-ketoPGF$_{1\alpha}$, ↓ TXB_2, ↓ PGE_2 (C18:2c9t11, C18:2t10c12)	[93]
BAEC	Resting: treatment with CLA mix (10 μg/mL) for 3 or 24 h	CLA mix	35 μM	↓ 6-ketoPGF$_{1\alpha}$ (CLA mix)	[92]
	Stimulated: Pre-treatment with CLA mix (10 μg/mL) for 3 or 24 h, followed by incubation with 5 μM bradykinin for 3 h	CLA mix	35 μM	↓ 6-ketoPGF$_{1\alpha}$ (CLA mix)	
HUVEC	Stimulated: Overnight treatment with IL-1β (100 U/mL), then treatment with CLAs, after 5 min addition of thrombin (0.2 U/mL) for 15 min	CLA isomers (C18:2c9t11, C18:2t10c12, C18:2c9c11, C18:2t9t11)	5.5 μM (C18:2c9t11) 2.6 μM (C18:2t10c12)	↓ 6-ketoPGF$_{1\alpha}$ (C18:2c9t11, C18:2t10c12) ineffective up to 60 μM (C18:2c9c11, C18:2t9t11)	[95]
HCASMC	Resting: 24 h treatment with CLAs	CLA isomers (C18:2c9t11, C18:2t10c12)	5 and 50 μM (C18:2c9t11, C18:2t10c12)	↓ PGE2, ↓ 6-ketoPGF1α (C18:2c9t11, C18:2t10c12)	[145, 146]
	Stimulated: 24 h treatment with CLAs and TNF-α (10 ng/mL)	CLA isomers (C18:2c9t11, C18:2t10c12)	5 and 50 μM (C18:2c9t11, C18:2t10c12)	↓ PGE2, ↓ 6-ketoPGF$_{1\alpha}$ (C18:2c9t11, C18:2t10c12)	
HASMC	Resting: 24 h treatment with CLAs	CLA isomers (C18:2c9t11, C18:2t10c12)	5 and 50 μM (C18:2c9t11, C18:2t10c12)	↓ PGE2, ↓ 6-ketoPGF$_{1\alpha}$ (C18:2c9t11, C18:2t10c12)	[146]
	Stimulated: 24 h treatment with CLAs and TNF-α-basal cytokine-stimulated	CLA isomers (C18:2c9t11, C18:2t10c12)	5 and 50 μM (C18:2c9t11, C18:2t10c12)	↓ PGE2, ↓ 6-ketoPGF$_{1\alpha}$ (C18:2c9t11, C18:2t10c12)	

BAEC, bovine aortic ECs; HAEC, human aortic ECs; HASMC, human aortic SMCs; HCASMC, human coronary artery SMC; HSVEC, human saphenous vein ECs; HUVEC, human umbilical vein ECs.

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lead to oxidative stress *via* formation of peroxynitrite. High levels of NO sufficient to induce cell injury may be produced by inducible nitric oxide synthase in response to cytokine stimulation [108]. Since it has been demonstrated that CLAs (isomers and mixtures) cause inhibition of stimulus-induced expression of inducible nitric oxide synthase in several cell lines including macrophages and mesangial cells [35, 102, 109, 110], it is possible that CLAs exert similar effects in ECs. This, however, remains to be established in future experiments.

4.1.4 Thrombogenic mediator secretion

It has been suggested that CLAs possess anti-thrombotic properties, which is based on the observation that CLA isomers, such as C18:2c9t11, C18:2t10c12, and C18:2t9t11, and CLA mixtures inhibit platelet aggregation in *in vitro* aggregation experiments performed with either platelet suspensions or whole blood [111–113]. These effects of CLAs have been suggested to be mediated by decreasing the formation of the pro-aggregatory thromboxane (TX) A_2 from AA by platelets [111–113]. Although these studies clearly demonstrate that platelet aggregation is modulated by direct effects of CLAs on platelet metabolism, it cannot be ruled out that platelet aggregation and thrombus formation are inhibited by CLAs by modulating EC function. It is well known that one important function of the endothelium is to maintain an anti-thrombogenic blood–tissue interface by regulating the secretion of both, hemostatic (*e.g.* tissue factor (TF), PAF, plasminogen activator inhibitor-1) and fibrinolytic (*e.g.* tissue-plasminogen activator, thrombomodulin) factors. Hence, it is not surprising that during endothelial dysfunction, the secretion of these factors is dysregulated, leading to increased levels of hemostatic factors such as PAF and TF and reduced levels of fibrinolytic factors, which favours coagulation processes and thrombus formation [114]. Therefore, the finding that two CLA isomers (C18:2c9t11, C18:2t10c12) as well as a CLA isomeric mix were shown to inhibit EC production of PAF, which has stimulatory effects on platelet activation, provides indication that CLAs may be capable of exhibiting anti-thrombotic effects by modulating EC function [78]. Interestingly, CLAs were also demonstrated to attenuate TF expression in cultured macrophages [115]. Since TF is also released from ECs in significant amounts, in particular from ECs overlying atherosclerotic plaques [114], it might be interesting to study in future experiments whether TF secretion is also attenuated by CLAs in ECs. Besides suppressing EC release of hemostatic factors, CLAs might also exert anti-thrombotic effects by modulating EC metabolism of AA and thereby formation of the pro-aggregatory TXA_2. This assumption is indeed supported by the observation that C18:2c9t11 and C18:2t10c12 reduce AA levels, inhibit cyclooxygenase (COX) activity and reduce the release of TXB_2, the stable marker of TXA_2, in aortic ECs [93].

4.1.5 Neovascularization

Neovascularization is the process of generating new blood vessels mediated by progenitor cells and/or ECs leading initially to tube formation and subsequently to a stabilized neovascular channel [116]. Angiogenesis, the predominant form of neovascularization in atherosclerosis, is mediated by ECs sprouting from the adventitial vasa vasorum, leading to new capillaries, which are called neovessels [117, 118]. Angiogenesis is induced by several angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and TNF-α, all of which are present at increased levels in atherosclerotic plaques [119–122]. Neovascularization occurs when the tunica intima thickens, and oxygen diffusion is impaired. Since the intima thickness increases with plaque growth, plaque neovasculature is found in most human atherosclerotic plaques [123]. Neovessels may also serve as an important route for leukocyte recruitment and infiltration into atherosclerotic plaques [124]. This is based on the observation that expression of VCAM-1, ICAM-1, and E-selectin are two- to threefold higher on neovessels than on arterial luminal endothelium [125, 126]. As a result, neovascularization in the arterial wall is associated with inflammation and lipid deposition, and thereby favours plaque development [116], whereas inhibition of angiogenesis reduces accumulation of macrophages in atherosclerotic plaques and counteracts atherogenesis [116].

Angiogenesis is not only involved in atherosclerotic plaque growth, but also plays an important role in cancer development, because it is required for tumour growth and survival [127–130]. CLAs are well known to be potent anti-carcinogenic compounds [20, 131, 132]. Increasing evidence indicates that CLA isomers, including C18:2c9t11 and C18:2t10c12, inhibit carcinogenesis through their ability to suppress angiogenic growth factors such as VEGF and bFGF [133–137] as well as by inhibiting the expression of the high-affinity VEGF receptor, fetal liver kinase-1 [134, 138], whose activation is critically required for the formation of new vessels [139]. Moon *et al.* [137] not only demonstrated that CLAs decrease bFGF-induced EC proliferation and DNA synthesis *in vitro*, but also found a potent inhibitory effect of CLAs on embryonic vasculogenesis and bFGF-induced angiogenesis *in vivo*, which indicates the *in vivo* relevance of these findings. Due to the strong similarities between angiogenesis in atherosclerotic plaques and angiogenesis in tumours [140], it is not unlikely that CLAs might also elicit their anti-atherogenic effects by inhibiting angiogenesis of ECs from adventitial vaso vasorum.

4.2 Actions of CLAs on atherosclerosis-related events in SMCs

Activated SMCs are important contributors to atherosclerotic plaque development and the main cellular

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components of atherosclerotic plaques [141]. Although events associated with phenotypic modulation (activation) of vascular SMCs, such as SMC migration and proliferation, secretion of inflammatory mediators, recruitment of mononuclear cells, and deposition of ECM proteins are critical steps in atherosclerosis, only few studies have addressed the impact of CLA isomers or CLA mixtures on either of these processes. These studies are discussed in the following chapters.

4.2.1 Inflammatory mediator secretion

Important inflammatory mediators secreted by SMCs are eicosanoids, which is a collective term for PGs, TXs, leukotrienes, and hydroxyeicosatetraenoic acids. Eicosanoids can be formed from different polyunsaturated 20-carbon fatty acids, from which AA is the most prominent. Therefore, AA-derived eicosanoids are the most common and biologically most important. PGI_2 and PGE_2 are the two major eicosanoids formed by vascular SMCs [142], and basal levels contribute to vascular homeostasis under normal conditions. However, during the inflammatory process associated with atherogenesis, vascular SMCs become activated [141, 143], leading to a pronounced elevation in the secretion of PGI_2 and PGE_2. The resulting excessive levels of PGs promote vascular dysfunction and, thereby, atherosclerotic plaque development [144].

Although several studies exist in the literature demonstrating an inhibitory effect of CLAs (both, isomers and mixtures) on eicosanoid production [93, 109, 110], only two studies have investigated the CLA effects on eicosanoid production in vascular SMCs [145, 146]. According to these studies, CLA isomers (C18:2c9t11, C18:2t10c12) are capable of attenuating the secretion of eicosanoids (PGI_2 and PGE_2) in vascular SMCs [145, 146], like in ECs. Table 2 gives an overview about studies dealing with the effects of CLAs on resting and stimulus-induced production of eicosanoids in ECs and SMCs. In quiescent SMCs, mechanistic studies revealed that the reduced secretion of PGI_2 and PGE_2 from SMCs is largely the result of a reduction of the cellular AA pool [145, 146]. The latter is explained by the fact that CLA isomers compete with other fatty acids such as LA and AA for the incorporation into membrane phospholipids. In addition, CLA isomeres interfere with the production of AA from LA [146, 147], which also results in a reduced AA pool and, subsequently, reduced eicosanoid production. It is worth mentioning that the reduction of AA levels in SMC lipids was already observed at quite low concentrations of C18:2c9t11 and C18:2t10c12 of about 5 μM [146], indicating that both CLA isomers effectively displace AA from membrane phospholipids and/or inhibit Δ5- and Δ6-desaturation of LA in SMCs. This concentration is in the range of the plasma concentration of C18:2c9t11 achieved in men consuming a CLA-rich diet (9.6 to 18 μM according to [148, 149]). It is also not too far away from the plasma concentration of C18:2t10c12, which has been reported to be 1.2 μM in men [150]. Therefore, these findings indicate that the effects observed in cultivated SMCs might also occur *in vivo*.

AA becomes available for PG synthesis only after it is released from phospholipid moieties. Although release of AA can be mediated by several phospholipases and lipases, it appears that phospholipase A_2 (PLA_2) plays a critical role in this process, which catalyses the release of AA from membrane phospholipids by hydrolysing the ester bonds at the *sn*-2 position of membrane phospholipids, where AA is mostly bound [151]. Studies in macrophages and ECs demonstrated that CLAs reduce the activity of PLA_2 [93, 152]. Thus, it is not unlikely that reduction of eicosanoid release from SMCs also involves inhibition of PLA_2 activity, which however has not been studied yet. Moreover, since CLA isomers are direct inhibitors of COX enzymes [74, 75], the rate-limiting enzymes for prostanoid synthesis from AA, inhibition of these enzymes by CLA isomers in vascular SMCs might also contribute to the observed reduction of PGI_2 and PGE_2 release from SMCs. Whether inhibition of COX activities is mediated by the CLA isomers themselves or their metabolites is unclear, because it has been shown that desaturation products of CLA isomers are also capable of inhibiting COX activities [74]. This, however, deserves further investigations.

Mechanistic studies in activated SMCs revealed that the reduction of cytokine-stimulated PGI_2 and PGE_2 release by C18:2c9t11 and C18:2t10c12 is mediated by a PPAR-γ-dependent inhibition of the NF-κB-pathway [145]. NF-κB is a central regulator of not only the expression of adhesion molecules and chemokines but also of enzymes involved in the synthesis of eicosanoids from AA, such as cytosolic phospholipase A_2 ($cPLA_2$), COX-2, and microsomal PGE synthase (mPGES) [80]. This explains why increased expression of COX-2, mPGES, and $cPLA_2$, which are co-localized to the endoplasmic reticulum and nuclear envelope [153, 154] by NF-κB activators, such as cytokines, results in the excessive formation of eicosanoids [155]. Hence, the observation that C18:2c9t11 and C18:2t10c12 caused a marked inhibition of cytokine-induced expression of $cPLA_2$, COX-2, and mPGES in vascular SMCs probably largely explains the reduction of PGI_2 and PGE_2 release from activated SMCs. Inhibition of NF-κB activation and, subsequently, reduced expression of genes involved in prostanoid synthesis as well as other inflammatory genes has also been demonstrated to be causative for the reduced expression of inflammatory genes in vascular SMCs treated with pharmacological PPAR-γ ligands [156–159]. This indicates that CLA isomers have similar properties as pharmacological PPAR-γ ligands with respect to modulating eicosanoid release from activated SMCs. Because excessive formation of inflammatory mediators by SMCs contributes to atherosclerotic plaque development, the findings in SMCs suggest that the anti-inflammatory action of CLA is, at least partially, responsible for the anti-atherogenic effects of CLA observed *in vivo*.

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4.2.2 Recruitment of mononuclear cells

As a response to vacular injury, adhesion molecules and chemokines are upregulated not only in ECs but also in activated SMCs [160, 161]. At the molecular level, transcriptional upregulation of adhesion molecules and chemokines in SMCs is mediated by dissociation of NF-κB from its inhibitor proteins (IκBs) in the cytosol and translocation of the active NF-κB into the nucleus where it binds to specific DNA sequences in the promoter region of adhesion molecules and chemokines [79, 80]. Increased adhesion molecule expression and chemokine release by activated SMCs also contributes to the recruitment of mononuclear cells into sites of vascular injury, and thereby to the chronic inflammatory process associated with atherosclerosis [160, 161]. There is only one study available in the literature investigating the effect of CLAs on adhesion molecular expression and leukocyte adhesion in SMCs [162]. This study revealed that a 50:50 mixture of C18:2c9t11 and C18:2t10c12 but not the individual CLA isomers inhibit cytokine-induced expression of the adhesion molecules ICAM-1 and VCAM-1 in vascular SMCs [162]. In addition, investigations in vascular SMCs by our own group demonstrated that CLA isomers, C18:2c9t11 and C18:2t10c12, significantly attenuate the TNF-α-induced release of the chemokine ''regulated on activation normal T-cell expressed and secreted'' (Ringseis *et al.*, unpublished results), which is, like ICAM-1 and VCAM-1, transcriptionally regulated by NF-κB [163]. Since C18:2c9t11 and C18:2t10c12 were demonstrated to attenuate TNF-α-induced DNA binding of NF-κB in SMCs [145, 164], it is very likely that the reduced ''regulated on activation normal T-cell expressed and secreted'' release by CLAs is due to inhibition of NF-κB. The inhibitory effect of CLA isomers on NF-κB activation in SMCs is at least partially due to inhibition of IκBα phosphorylation, as observed in a recent study of our group [164]. According to recent reports inhibition of phosphorylation of IκB by CLAs is mediated by blocking IκB kinase- and Akt-, a serine/threonine kinase, signalling [165–167]. Although the reason for the lack of effect of the individual CLA isomers on adhesion molecule expression remains unresolved from the above-mentioned study [162], the observed attenuation of adhesion molecule expression and chemokine release by the CLA mixture and the CLA isomers, respectively, might be of relevance when trying to explain the anti-atherogenic effects of CLA *in vivo*.

4.2.3 ECM protein production

Production of collagen, which is the predominating ECM protein in advanced atherosclerotic lesions, by vascular SMCs is considered to be a hallmark in atherosclerosis development since it significantly contributes to intimal thickening and, finally, atherosclerotic plaque formation [62]. In spite of its great relevance for atherosclerosis development, only one study has investigated the potential

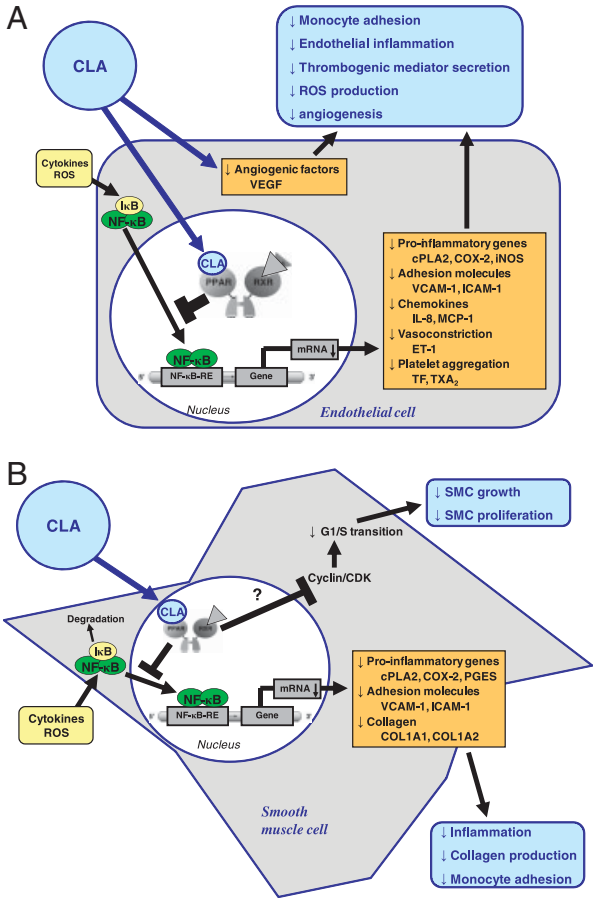

Figure 4. Illustration of the effects of CLAs on functional properties of ECs (A) and SMCs (B). Through the activation of PPARs CLAs are capable of inhibiting NF-κB regulated pro-inflammatory gene transcription leading to reduced monocyte adhesion, endothelial inflammation, collagen production, and inflammatory mediator secretion. Whether CLAs also inhibit SMC growth and proliferation by blocking G_1/S cell cycle transition through induction of cyclin-dependent kinase inhibitors remains to be established.

of CLAs to modulate ECM protein production [164]. According to this study, the two most frequently studied CLA isomers, C18:2c9t11 and C18:2t10c12, are capable of inhibiting the production of collagen by activated SMCs [164]. As a mechanism of action, PPAR-γ-mediated inhibition of NF-κB by CLA isomers has been suggested [164]. This suggestion is based on the finding that the inhibitory effect of CLA isomers on collagen production and NF-κB activation was attenuated by selectively antagonizing PPAR-γ activity and on the knowledge that different agents, such as oxidized LDL, stimulate SMC collagen production *via* activation of NF-κB [168, 169]. Correspondingly, inhibition of NF-κB by either parthenolide or the antioxidant (−)-epigallocatechin-3-gallate causes abrogation of stimulus-induced collagen production *in vitro* [170]. Similarly, SMC collagen production is also inhibited by the PPAR-γ activator

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15-d-PGJ2 [171], which causes attenuation of NF-κB too [172]. Stimulation of collagen production *via* activation of NF-κB is explained by the fact that the promoter of the collagen (COL)1A2 gene, which encodes the α2 chain of type I collagen, contains at least two putative NF-κB-binding sites [173]. 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 [174, 175], which are major factors inducing the phosphorylation of the inhibitors of NF-κB and subsequent translocation of NF-κB to the nucleus [176].

A further mechanism contributing to the reduced SMC collagen production by CLA isomers might be the above-mentioned inhibition of AA metabolism by COX and lipoxygenase enzymes to biologically active eicosanoids [145, 146], because these metabolites are supposed to be mediators of pathological fibrotic conditions increasing the formation of collagen by stimulating pro-fibrotic factors such as TGF-β1 [177]. Thus, future studies have to clarify whether inhibition of AA metabolism, besides PPAR-γ activation, might also contribute to the reduced SMC collagen formation in response to C18:2c9t11 and C18:2t10c12. Nevertheless, due to the fundamental role of SMC collagen production for atherosclerosis development, the lowering of SMC collagen production by CLAs might explain, at least in part, the anti-atherogenic effects of CLAs.

4.2.4 SMC migration and proliferation

As mentioned above, no studies are available in the literature addressing the potential of CLAs to influence SMC migration and proliferation, which are critical steps in atherosclerosis development. However, because SMC migration and proliferation precedes SMC production of ECM proteins, which is efficiently inhibited by C18:2c9t11 and C18:2t10c12 [164], it would be interesting to know whether CLA isomers inhibit SMC migration and proliferation as well. From studies with synthetic ligands of PPAR-α and PPAR-γ it is well established that these agents are efficacious at inhibiting SMC proliferation by blocking G1/S cell cycle transition through induction of cyclin-dependent kinase inhibitors [178–180], thereby, leading to SMC growth inhibition and reduced neointima formation [178]. Considering that CLA isomers, such as C18:2c9t11 and C18:2t10c12, are ligands and activators of PPAR-α and PPAR-γ, and CLAs exert PPAR-dependent actions in SMCs [145, 164], inhibition of SMC proliferation by CLAs would be not unexpected. Indeed, both CLA isomers and CLA mixtures were demonstrated to inhibit proliferation of numerous cell types including cancer and non-cancer cells, such as ECs [181–186]. Furthermore, investigations into the mechanisms involved revealed that inhibition of cell proliferation is mediated by blocking cell cycle progression at the G1/S transition [181–184]. Therefore, future studies should investigate the potential of CLAs to inhibit SMC migration and proliferation, which might explain inhibition of atherosclerotic plaque formation by CLAs.

5 Conclusions

Based on data from *in vitro* studies about the actions of CLAs on atherosclerosis-related events in ECs and SMCs, it can be concluded that CLAs exert several beneficial actions, which may help explain the anti-atherogenic effect of CLAs observed in animal models of experimental atherosclerosis. As summarized in Figs. 4A and B, these beneficial actions of CLAs include inhibition of mononuclear cell adhesion to ECs and SMCs, attenuation of EC and SMC inflammatory mediator secretion, upregulation of intrinsic antioxidant defense mechanisms in ECs, reduction of EC thrombogenic mediator secretion, and inhibition of SMC collagen production. Furthermore, there are strong indications to support the assumption that CLAs exert inhibitory effects on neovessel formation in atherosclerotic plaques. Moreover, based on the observation that CLAs elicit inhibitory effects on the proliferation capacity of numerous cell types, inhibition of SMC migration and proliferation by CLAs is also not unlikely, but remains to be established. The observation that significant levels of CLA metabolites, which have been reported to have strong biological activities, are well detectable in ECs and SMCs indicates that the anti-atherogenic effects observed with CLAs are presumably mediated not only by CLAs themselves but also by their metabolites. Regarding the extent of formation of CLA metabolites, there might be differences between individuals depending on the expression and activity of key genes involved in the principal metabolic pathways of CLAs (β-oxidation, elongation, desaturation). Since genetic variations are responsible for alterations in expression and activity of such genes, future studies have to clarify whether the formation of CLA metabolites differs between individuals with different genetic background, *i.e.* in carriers *versus* non-carriers of common single nucleotide polymorphisms in key enzymes involved in these metabolic pathways.

Although isomer-specific effects of CLAs are well documented in the literature [85, 86], there is only little evidence for isomer-specific effects of CLAs in ECs and SMCs. The majority of data from studies dealing with the actions of CLAs in ECs and SMCs suggest that the actions of CLAs on atherosclerosis-related events are largely independent of structural differences (position and geometry of the double bonds) between the individual CLA isomers. This suggestion is also in accordance with observations from *in vivo* experiments with rabbits and hamsters, where different CLA isomers and CLA mixtures seem to have similar effects on atherosclerosis development [21, 28–31]. Nevertheless, in mouse models of atherosclerosis there is evidence for opposing effects of C18:2c9t11 and C18:2t10c12 on atherosclerotic plaque formation [32], namely, the C18:2c9t11 isomer significantly reduced the cross-sectional lesion area

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of the aortic root, whereas the C18:2t10c12 isomer significantly increased lesion area compared with control. Moreover, *en face* examination of the aorta of C18:2t10c12-fed mice revealed an increased lesion area in specific regions of the vessel and suggested that the C18:2t10c12 isomer induced a pro-oxidative state [32]. Due to these pro-atherogenic effects observed with C18:2t10c12, but also other detrimental effects of C18:2t10c12 reported in the literature (lipid peroxidation, decrease in insulin sensitivity, lowering of HDL cholesterol) [187–193], the intake of dietary CLA supplements, which usually have a high content of C18:2t10c12, should generally be considered critically.

Although there is convincing evidence that CLAs (isomers or mixtures) exert anti-atherogenic effects in animal models [26, 27], the preventive and therapeutic potential of CLAs on inflammatory diseases including atherosclerosis in humans remains to be determined [27]. Most of the human studies reported no effect of supplementation with CLAs (isomers or mixtures) on surrogate markers of atherosclerosis (inflammatory parameters, plasma lipid profile) [194–197], and some human studies even reported detrimental effects on risk factors associated with atherosclerosis [187, 193, 198, 199]. It has been suggested [27] that differences in purity, content of CLA isomers, and co-existence of other fatty acids in CLA supplements and/or differences in the CLA dose are responsible for the inconsistent data on the effects of CLAs on atherosclerosis development in humans. In addition, in several study designs human subjects were not limited in diet (*i.e.* dietary fat intake) and/or physical activities. Furthermore, some of the inconsistencies between human studies are probably also explained by a different health status of the study collectives, *e.g.* it is not unexpected that there may be divergent effects of CLAs in obese or diabetic subjects compared with the normal-weight or healthy subjects. Moreover, differences in the gender of the subjects and/or gene–diet interactions might also contribute to these inconsistencies. Therefore, further human studies are needed to investigate the effectiveness and safety of CLA supplementation and to elucidate these confounding factors.

The authors have declared no conflict of interest.

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