Conjugated Linoleic Acids Lower the Release of Eicosanoids and Nitric Oxide from Human Aortic Endothelial Cells

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ABSTRACT The objective of this study was to determine the effects of cis-9, trans-11 and trans-10, cis-12 CLA on the release of vasoactive eicosanoids and nitric oxide (NO) in human aortic endothelial cells. Experiments were conducted in which cells were incubated with these fatty acids, and the concentrations of various eicosanoids [6-keto prostaglandin (PG) F1α, as a stable product of PGI2, thromboxane (TX) B2 as a stable product of TXA2, and PGE2] and NO in the medium were determined. Cells treated with 50 μmol/L of either cis-9, trans-11 or trans-10, cis-12 CLA released less of all of the eicosanoids and NO than control cells treated with medium alone (P < 0.05). The ratio between the amounts of 6-keto-PGF1α and that of TXB2 released did not differ between control cells and cells treated with either CLA isomer. Moreover, cells treated with 50 μmol/L of cis-9, trans-11 or trans-10, cis-12 CLA had a lower amount of arachidonic acid in their phosphatidylethanolamine fraction and a lower mRNA concentration and activity of secretory phospholipase A2 than control cells (P < 0.05). These data suggest that eicosanoid formation was impaired by a reduced availability of arachidonic acid for the cyclooxygenase pathway. In conclusion, this study shows that cis-9, trans-11-CLA and trans-10-12-CLA influence the release of various eicosanoids and NO from human aortic endothelial cells. The effects observed in this study might be important because eicosanoids and NO released from endothelial cells are involved in the regulation of vessel tone and platelet aggregation. The results of the present study suggest that both CLA isomers had unfavorable effects on endothelial function.


KEY WORDS: ● conjugated linoleic acids ● endothelial cell ● eicosanoids ● nitric oxide

The endothelium plays an important role in the regulation of vasomotor tone, platelet aggregation and smooth muscle cell proliferation and migration. The main vasorelaxing factor produced by endothelial cells is nitric oxide (NO), which is synthesized by endothelium nitric oxide synthase (eNOS), a heme-containing oxidase. NO formation can be activated by several factors such as physical stimuli, hormones, various cytokines or factors formed during blood coagulation such as thrombin (1). NO also inhibits aggregation of platelets and proliferation of smooth muscle cells. Prostacyclin (PGI2) and thromboxane A2 (TXA2), which are components of the eicosanoid family, are formed in endothelial cells from arachidonic acid. Arachidonic acid is a normal component of the phospholipids of endothelial cell membranes and derives mainly from Δ5- and Δ6-desaturation of linoleic acid (2). It becomes available for eicosanoid synthesis only after it is released from phospholipid moieties by the action of phospholipase A2 (PLA2). Arachidonic acid released from phospholipids is converted to prostaglandin (PG) H2 by cyclooxygenases (COX-1 and COX-2). COX-1 is constitutively expressed, whereas the expression of COX-2 can be markedly enhanced upon cell activation such as in an inflammatory response (3). PGE2 is further enzymatically transformed to several prostaglandins such as prostaglandins D2, E2, F2, I2 and TXA2. Prostaglandins play an extraordinarily important role in vascular homeostasis. PGI2, through activation of the IP receptor present in smooth muscle cells and platelets, causes vasodilatation and inhibits platelet aggregation (4). TXA2, on the contrary, has vasoconstrictory and proaggregating effects (5). PGE2 is a much less potent vasodilator than PGI2. In view of their counteracting effects, the balance between PGI2 and TXA2 is important for the maintenance of vascular homeostasis. A decrease in this ratio is associated with pathophysiologic conditions such as thrombosis and ischemia (6,7).

It has been well established that the function of endothelial cells, particularly their production of eicosanoids, can be altered by modulating their membrane fatty acid composition, particularly the relative amounts of arachidonic acid and eicosapentaenoic acid (8). Several studies showed that conjugated linoleic acids (CLA), a group of positional and geometric isomers of linoleic acid characterized by the presence of conjugated double bonds, reduce the formation of eicosanoids...
in various animal cells and tissues (9–12). Recently, it was shown that CLA also inhibits the expression of inducible nitric oxide synthase (iNOS) and NO production in murine macrophages (13). Less information is available to date about the effects of CLA on the production of eicosanoids and NO in endothelial cells. The aim of this study was therefore to investigate the effects of two different isomers of CLA, cis-9, trans-11 CLA and trans-10, cis-12 CLA on the formation of vasoactive eicosanoids and NO in human aortic endothelial cells, which were chosen as a model. The cis-9, trans-11 CLA was considered because it is the predominant CLA isomer in milk and dairy products; >80% of CLA present in milk exists as the cis-9, trans-11 isomer (14). Trans-10, cis-12 CLA is present in milk and most other foods only in trace amounts, but it is of particular interest because it is more active than cis-9, trans-11 CLA in several respects (15,16). The mechanism by which CLA inhibits the formation of eicosanoids in various cell types is not yet fully understood. Some studies suggest that inhibition of eicosanoid formation by CLA is caused through a reduced arachidonic acid concentration in membrane phospholipids (7,10); others suggest that it is due to an inhibition of PGH synthase (17) or COX-2 (13,18,19). To investigate possible mechanisms involved in altered production of eicosanoids by CLA isomers in endothelial cells, we proposed to determine the amounts of arachidonic acid in endothelial phospholipids as well as gene expression or activities of enzymes involved in the formation of eicosanoids (Δ5, Δ6-desaturase, secretory PLA2, COX-1, COX-2). We studied gene expression of eNOS as the key enzyme in the production of NO.

MATERIALS AND METHODS

Materials. Cis-9, trans-11 CLA (≥96% pure) and trans-10, cis-12 CLA (≥98% pure) isomers were obtained from Cayman Chemicals (Ann Arbor, MI). Endothelial cell basal medium MV (ECBM) with SupplementPack, Hepes buffered salt solution (Hepes-BSS), trypsin-EDTA (0.025% trypsin and 0.01% EDTA) and trypsin neutralizing solution (TNS) containing 0.05% trypsin inhibitor and 0.1% bovine serum albumin (BSA), were purchased from PromoCell (Heidelberg, Germany). HBSS and gentamicin were purchased from Invitrogen (Karlsruhe, Germany). HBSS was evaporated under nitrogen. Individual lipid fractions of the extracts were separated by a solid-phase extraction method as described by Suzuki et al. (21) with modifications. Lipids were transmethylated with trimethylsilylum nitrogen (22). FAME were separated by GC (23).

Release of eicosanoids from human aortic endothelial cells. After the 24-h incubation with CLA isomers and the 1-h incubation in HBSS, cells were centrifuged (2400 x g for 3 min) and the supernatants were assayed for the eicosanoids 6-keto PGF1α, PGE2 and TXB2, using EIA-kits (No. 515211, 514010 and 519031, Cayman Chemicals). 6-keto PGF1α and TXB2 were determined as measures of the unstable PGI2 and TXA2.

Activity of secretory PLA2 (sPLA2) in human aortic endothelial cells. The sPLA2 activity was determined in the cell incubation medium after 24 h of treatment with CLA isomers and in the supernatant after an additional 1-h incubation in HBSS by a sPLA2 Assay Kit (No. 765001, Cayman Chemicals).

Release of NO from human aortic endothelial cells. As an index of the NO concentration, nitrate and nitrite concentrations were determined in the cell incubation medium after 24 h of treatment with CLA isomers and in the supernatant after the additional 1-h incubation in HBSS by a Nitrate/Nitrite Colorimetric Assay Kit (No. 780001, Cayman Chemicals).

mRNA expression of the enzymes Δ5-desaturase, Δ6-desaturase, secretory PLA2, COX-1, and COX-2. cDNAs were determined in the cells 24 h after treatment with CLA isomers and the 1-h HBSS incubation. Total RNA of the cells was isolated using the Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol; 1.2 µg total RNA was used for the cdNA synthesis at 42°C for 1 h. Cell mRNA expressions of Δ5-desaturase, Δ6-desaturase, eNOS and COX-2 were estimated by quantitative real-time RT-PCR using a MJ Research Opticon system (Biorne Diagnostik GmbH, Oldendorf, Germany). cdNA was amplified in a 15-µL reaction containing 2 µL RT-mixture, Brilliant SYBRGreen QPCR Master Mix (Stratagene, Amsterdam, The Netherlands) and specific primers (Table 1). The primers for Δ5-desaturase, Δ5-desaturase, COX-1 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Metabion (Martinsried, Germany).

Activity of secretory PLA2 was estimated by real-time RT-PCR using the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). cdNA was amplified in a 15-µL reaction containing 2 µL RT-mixture, 5 µL of Taq DNA polymerase (Promega, Mannheim, Germany), 0.375 µL 10X SYBRGreen 1 (Sigma) and group V sPLA2-specific primers obtained from Carl Roth (Karlsruhe, Germany). After an initial denaturation step (10 min, 95°C), PCR was carried out for 30–35 cycles. Each cycle comprised denaturation for 30 s at 95°C, annealing at primer-specific temperature (Table 1), elongation for 1 min at 72°C and a final extension for 7 min at 72°C. Cell mRNA expression of group V sPLA2 was estimated by real-time RT-PCR using the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). cdNA was amplified in a 15-µL reaction containing 2 µL RT-mixture, 5 µL of Taq DNA polymerase (Promega, Mannheim, Germany), 0.375 µL 10X SYBRGreen 1 (Sigma) and group V sPLA2-specific primers obtained from Carl Roth (Karlsruhe, Germany). After an initial denaturation step (120 s, 95°C), PCR was carried out for 40–50 cycles, each cycle comprising denaturation for 30 s at 95°C, annealing for 30 s at 59°C and elongation for 40 s at 72°C, followed by a final extension for 7 min at 72°C. Fluorescence intensity in all real-time PCR probes was measured at the end of the extension step and related to GAPDH.

mRNA expression of COX-2 was estimated by semiquantitative RT-PCR using a mastercycler (Eppendorf AG, Hamburg, Germany). The reaction was conducted in 20 µL of PCR buffer containing 2 µL RT-mixture, 5 µL BioTherm DNA Polymerase in 10X reaction buffer, 10 mmol/L dNTP and COX-2 specific primers (Table 1) obtained from Invitrogen. The initial denaturation step (120 s, 95°C) was followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, elongation for 1 min at 72°C and a final extension step for 7 min at 72°C. The PCR products were stained with ethidium bromide at a concentration of 100 mmol/L. For the preparation of the test media, aliquots of these stock solutions were used. The solvent was evaporated under nitrogen and the fatty acids were converted to their sodium salts by adding equimolar amounts of sodium hydroxide solution. The solution containing the fatty acid salts was added to the growth medium to obtain the final concentrations.

Lipid analysis. The total cellular lipids were extracted with 1 mL hexane/isopropanol (3:2 v/v). The lipid extracts were dried under nitrogen. Individual lipid fractions of the extracts were separated by a solid-phase extraction method as described by Suzuki et al. (21) with modifications. Lipids were transmethylated with trimethylsilylum
Cell viability was 96%.

Cell count after incubation with medium alone was 2.45 ± 0.47 (means ± SD, n = 6).

**RESULTS**

**Cell growth and viability.** The growth and viability of the cells were not affected by incubation with either CLA isomer. Cell count after incubation with medium alone was 2.45 ± 0.47 × 10^6/flask; after incubation with 5 and 50 μmol/L of cis-9, trans-11 CLA, the cell counts were 2.44 ± 0.24, 2.85 ± 0.49, 2.55 ± 0.39 and 2.48 ± 0.35 × 10^6/flask (means ± SD, n = 6), respectively. Cell viability was 96 ± 3% for control cells, 95 ± 1 and 98 ± 1% for cells treated with 5 and 50 μmol/L of cis-9, trans-11 CLA and 95 ± 2 and 97 ± 2% for cells treated with 5 and 50 μmol/L of trans-10, cis-12 CLA (n = 6). The protein levels also did not differ in control cells (0.50 ± 0.14 mg/10^6 cells), cells treated with 5 and 50 μmol/L of cis-9, trans-11 CLA (0.48 ± 0.11 and 0.55 ± 0.20 mg/10^6 cells, respectively) and cells treated with 5 and 50 μmol/L of trans-10, cis-12 CLA (0.52 ± 0.14 and 0.63 ± 0.21 mg/10^6 cells, respectively) (n = 6).

**Incorporation of CLA isomers into human aortic endothelial cell lipids.** The incorporation of both CLA isomers into endothelial cell lipids was dependent upon their concentrations in the medium (Table 2). Concentrations of either CLA isomer in endothelial cell lipids were much higher after incubation with 50 μmol/L of either CLA isomer than after incubation with 5 μmol/L. After incubation of cells with 5 μmol/L of both CLA isomers, the concentrations of either CLA isomer were similar in neutral lipids, PC and PE; after incubation with 50 μmol/L of both CLA isomers, the concentrations of either CLA isomer were significantly higher in PC than in PE or neutral lipids.

**Concentrations of arachidonic acid in phospholipids of human aortic endothelial cells.** The concentration of arachidonic acid in PE was generally much higher in PC than in PE or neutral lipids. The effect was similar for both CLA isomers. The concentration of arachidonic acid in PE was generally much higher in PC than in PE or neutral lipids.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cis-9, trans-11 CLA</th>
<th>Trans-10, cis-12 CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μmol/L</td>
<td>Control</td>
<td>5 μmol/L</td>
</tr>
<tr>
<td>g/100 g total fatty acids</td>
<td>2.5 ± 1.2b</td>
<td>27.0 ± 7.2a</td>
</tr>
<tr>
<td>g/100 g total fatty acids</td>
<td>14.3 ± 10.6a</td>
<td>2.0 ± 1.1b</td>
</tr>
<tr>
<td>g/100 g total fatty acids</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

1 Values are means ± sd, n = 6.
2 Means in a row with unlike letters differ, P < 0.05.
Cells treated with 50 μmol/L of CLA had a significantly lower relative mRNA concentration of 6-desaturase than control cells; cells treated with 50 μmol/L of cis-9, trans-11 CLA or trans-10, cis-12 CLA tended (P < 0.10) to have a lower mRNA concentration of that enzyme than control cells (Fig. 2). Cells treated with 5 μmol/L of cis-9, trans-11 CLA or trans-10, cis-12 CLA did not differ from control cells in their relative mRNA of 6-desaturase.

Relative mRNA concentrations of Δ5- and Δ6-desaturase. Relative mRNA concentration of Δ5-desaturase did not differ between control cells and cells treated with 5 or 50 μmol/L of cis-9, trans-11 CLA or trans-10, cis-12 CLA (data not shown). Cells treated with 50 μmol/L of trans-10, cis-12 CLA had a significantly lower relative mRNA concentration of Δ6-desaturase than control cells; cells treated with 50 μmol/L of cis-9, trans-11 CLA tended (P < 0.10) to have a lower mRNA concentration of that enzyme than control cells (Fig. 2). Cells treated with 5 μmol/L of cis-9, trans-11 CLA or trans-10, cis-12 CLA did not differ from control cells in their relative mRNA of Δ6-desaturase.

Release of 6-keto-PGF₁α, TXB₂, and PGE₂ from endothelial cells. Cells treated with 5 μmol/L of cis-9, trans-11 CLA did not differ in the release of 6-keto-PGF₁α, TXB₂ and PGE₂ from control cells (Figs. 3 and 4). Cells treated with 5 μmol/L of trans-10, cis-12 CLA released less 6-keto-PGF₁α into the medium than control cells but did not differ from control cells in the release of TXB₂ and PGE₂ into the medium. Cells treated with 50 μmol/L of either cis-9, trans-11 CLA or trans-10, cis-12 CLA released less of all of the eicosanoids than control cells. Cells treated with 50 μmol/L of cis-9, trans-11 CLA did not differ from those treated with 50 μmol/L of trans-10, cis-12 CLA in the release of 6-keto-PGF₁α, TXB₂ and PGE₂. The ratio between the amount of 6-keto-PGF₁α and that of TXB₂ released did not differ between control cells (0.92 ± 0.40), cells treated with 5 and 50 μmol/L of cis-9, trans-11 CLA (0.82 ± 0.30 and 1.05 ± 0.51, respectively) and cells treated with 5 and 50 μmol/L of trans-10, cis-12 CLA (0.60 ± 0.35 and 0.86 ± 0.11) (means ± sd, n = 6).

Relative mRNA concentration and activity of sPLA₂. Cells treated with 5 μmol/L of either cis-9, trans-11 CLA or trans-10, cis-12 CLA did not differ from control cells in the relative mRNA concentration of sPLA₂, whereas cells treated with 50 μmol/L of either cis-9, trans-11 CLA or trans-10, cis-12 CLA had a lower relative mRNA concentration of that enzyme.
zyme than control cells (Fig. 5A). Cells that were treated for 24 h with 5 or 50 μmol/L of cis-9, trans-11 CLA had a significantly lower activity of sPLA₂ than control cells; cells treated with 5 μmol/L of trans-10, cis-12 CLA did not differ from control cells in the activity of sPLA₂; cells treated with 50 μmol/L of trans-10, cis-12 CLA tended to have lower activity of sPLA₂ (P < 0.10) compared with control cells (Fig. 5B).

Relative mRNA concentrations of COX-1 and COX-2. Cells treated with 5 μmol/L of cis-9, trans-11 CLA had a higher relative mRNA concentration of COX-1 than control cells; cells treated with 50 μmol/L of cis-9, trans-11 CLA, 5 or 50 μmol/L of trans-10, cis-12 CLA did not differ from control cells in the relative mRNA concentration of that enzyme (Fig. 6). Cells treated with 50 μmol/L of cis-9, trans-11 CLA had a lower mRNA concentration of COX-2 than control cells; in cells treated with 50 μmol/L of trans-10, cis-12 CLA, the mRNA concentration of that enzyme tended to be lower (P < 0.10) than in control cells. Cells treated with 5 μmol/L of cis-9, trans-11 CLA or trans-10, cis-12 CLA did not differ from control cells in the relative mRNA concentration of COX-2.

Release of NO from endothelial cells. Endothelial cells treated with 5 or 50 μmol/L of cis-9, trans-11 CLA released less NO during a 1-h incubation in HBSS than control cells (Fig. 7). Cells treated with 50 μmol/L of trans-10, cis-12 CLA also released less NO than control cells; the release of NO did not differ between cells treated with 5 μmol/L of trans-10, cis-12 CLA and control cells.

Relative mRNA concentrations of eNOS. Relative mRNA concentration of eNOS did not differ between control cells and cells treated with 5 or 50 μmol/L of cis-9, trans-11 or trans-10, cis-12 CLA (data not shown).

FIGURE 5 Relative mRNA concentration (A) and activity (B) of secretory phospholipase A₂ in human aortic endothelial cells that were incubated in media without CLA (control) or with 5 or 50 μmol/L of cis-9, trans-11 (c9,11) or trans-10, cis-12 (t10,c12) CLA. Values are means ± SD, n = 6. Means with different letters differ, P < 0.05.

DISCUSSION

This study was conducted to investigate the effect of two isomers of CLA on the release of NO and vasoactive eicosanoids and NO in human aortic endothelial cells. Two different concentrations of both CLA isomers were used in the incubation experiments. Incubating cells with media containing 5 μmol/L of CLA for 24 h resulted in moderate concentrations of CLA in cell lipids, up to 3 g/100 g of total fatty acids. Incubating cells with media containing 50 μmol/L resulted in very high concentrations of CLA in cell lipids, up to 35 g/100 g of total fatty acids. This shows that the concentration of CLA isomers in endothelial cells can be extremely elevated, while the cells have normal morphological appearance and growth characteristics. This opens the possibility of studying the influence of high concentrations of CLA on various endothelial cell functional properties. These concentra-
reduced the release of PGI$_2$, PGE$_2$ and TXA$_2$. Two other production of eicosanoids. Reduced activity of sPLA$_2$ together with resting cells.

endothelial cells, such as in our study, which was performed formation of various eicosanoids in resting or activated human endothelial cells, such as in our study, which was performed with resting cells. The results of our study suggest that reduced formation of eicosanoids by CLA might be the result of a diminished availability of arachidonic acid, which is rate limiting in the production of eicosanoids. Reduced activity of sPLA$_2$ together with a lower concentration of arachidonic acid in PE, the major substrate of PLAl, might reduce the concentration of arachidonic acid available for cyclooxygenase reaction. Lower concentrations of arachidonic acid in PE of endothelial cells treated with the CLA isomers might have been caused by reduced activity of 16-desaturase, the rate-limiting enzyme in the formation of arachidonic acid from linoleic acid. For technical reasons, we were not able to determine the activity of that enzyme; however, we assume that the reduced gene expression in endothelial cells treated with CLA was associated with reduced activity of that enzyme. Previous studies in cells and animals also showed that various CLA isomers suppress 16-desaturation of linoleic acid, leading to lower concentrations of arachidonic acid in cell or tissue lipids (16,27,28). The finding that trans-10, cis-12 CLA caused a stronger inhibition of 16-desaturase gene expression than cis-9, trans-11 CLA, whereas the concentrations of arachidonic acid in PE did not differ after treatment with both CLA isomers, is unexplained.

The application of semiquantitative RT-PCR showed that high concentrations of cis-9, trans-11 CLA reduced gene expression of the inducible enzyme COX-2, and high concentrations of trans-10, cis-12 tended to do so. Reduced gene expression of that enzyme by various CLA isomers was also observed in recent studies in murine macrophages and hepatoma cells (18,19). It was suggested that decreased gene expression of COX-2 in macrophages treated with CLA is caused by activation of peroxisome proliferator-activated receptor $\gamma$ (19). Some studies found a correlation between the expression of COX and the formation of eicosanoids in cells treated with CLA (13,18,19). Our study showed that cis-9, trans-11 and trans-10, cis-12 CLA do not suppress gene expression of COX-1. However, the possibility exists that CLA or elongated and desaturated products from CLA such as conjugated eicosatetraenoic act as antagonists for COX, thereby reducing available enzyme for arachidonic acid (29).

Our study is the first to demonstrate that treatment of endothelial cells with cis-9, trans-11 or trans-10, cis-12 CLA lowers the release of NO. To determine possible reasons for this, we examined gene expression of eNOS, the principal enzyme of NO synthesis. Because gene expression of eNOS was not influenced by cis-9, trans-11 or trans-10, cis-12 CLA,

LITERATURE CITED


