Feedback inhibition of apolipoprotein A-I synthesis by its propeptide in hepatocyte cell culture and in the cell-free system

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Abstract

Addition of chemically synthesized proapolipoprotein A-I hexapeptides in a concentration of 10^-4 mol/l in the medium of primary rat hepatocyte cell cultures decreased net apolipoprotein A-I synthesis by up to 13%. The inhibition was dose dependent and slightly different for the three peptides with differing amino acid sequences. The synthesis of albumin remained unaltered. In a reticulocyte cell-free system the three propeptides, in a concentration of 10^-4 mol/l, inhibited apolipoprotein synthesis by 42%, 43%, and 48%, respectively. Total protein synthesis was inhibited by 19%, 22%, and 24% only. All three propeptides in concentrations of 10^-7 to 10^-4 mol/l inhibited apo A-I synthesis in a dose dependent manner, indicating that the identical C-terminal sequence (Phe-Trp-Gln-Gln) is responsible for the inhibitory effect. The synthesis of albumin was not inhibited. The proalbumin hexapeptide (Arg-Gly-Val-Phe-Arg-Arg) at a concentration of 10^-3 mol/l inhibited albumin synthesis by 63% whereas incorporation into apoA-I was inhibited by only 14%. Amino acids in concentrations equimolar to the propeptides had no effect on the incorporation into total protein, apoA-I, and rat serum albumin. These results suggest that the synthesis of apolipoprotein A-I is regulated by a feedback mechanism with its propeptide as inhibitor.

Keywords: apolipoproteins; apo A-I; high density lipoproteins; chemical synthesis; lipoprotein metabolism; liver; propeptides; albumin

Introduction

A high serum level of HDL lowers the risk of myocardial infarction. The predominant protein associated with HDL is apoA-I which is synthesized in hepatocytes and enterocytes [1-3]. A high serum level of apoA-I lowers the risk of myocardial infarction [4, 5]. Although apoA-I plays a central role in the regulation of lipid metabolism and thus in prevention of myocardial infarction, little information is available on the regulation of its synthesis. ApoA-I is synthesized as a 267 amino acid precursor protein, preproapoA-I [6-13]. The presegment is 18 amino acids long and cotranslationally cleaved off, resulting in proapoA-I [11-16]. ProapoA-I is the precursor of the mature apoA-I, differing from apoA-I by a hexapeptide extension at the N-terminal end [14]. For rat apoA-I three slightly different propeptides have been described: Trp-Glu-Phe-Trp-Gln-Gln (WFQWQQ) [8], Ser-Glu-Phe-Trp-Gln-Gln (WFQWQQ) [15], and Trp-Asp-Phe-Trp-Gln-Gln (WFQWQQ) [16]. The amino acid sequence of the human prosegment has been shown to be Arg-His-Phe-Trp-Gln-Gln (RHFWQQ) [8, 11, 17]. The apo A-I prosegment differs from propeptides of other serum proteins in two respects: it does not terminate with paired basic amino acids [6] and its processing is extracellular rather than intracellular [3, 8, 14-20]. The hexapeptide is slowly split off in plasma by...
a Ca\(^{2+}/\)Mg\(^{2+}\) dependent protease, located on the surface of plasma lipoprotein particles and endothelial cells [14]. The bone morphogenetic protein-I has been identified as enzyme that cleaves human apolipoprotein A-I [21]. The procollagen C-proteinase enhancer is involved in the proteolytic processing of proapoA-I [22]. Several possible functions of the propeptide extension have been discussed. It may be involved in the formation of the tertiary structure of apoA-I or it may facilitate transport through or out of the cell [23, 24]. In addition, the propeptide itself may have a biological function and be involved in the regulation of apoA-I synthesis, as has been described for other propeptides such as albumin [25] and procollagen [26-31]. All three rat propeptides were synthesized chemically. In order to test the hypothesis that the released propeptide is involved in the regulation of apoA-I synthesis, we studied the effect of the chemically produced hexapeptides on the synthesis of apoA-I in primary rat cell cultures and in a cell-free system. To evaluate the specificity of the inhibition we also studied the effect of the propeptide of albumin on albumin and on apoA-I synthesis.

Materials and methods

Chemical synthesis of peptides

The hexapeptides of proapoA-I and of proalbumin were synthesized conventionally, beginning from the C-terminal end by stepwise N-terminal peptide chain elongation (Orpegen, Heidelberg, Germany). Details of the synthesis of the hexapeptides have been published previously [32]. Purity of the peptides was confirmed by thin-layer chromatography, HPLC, amino acid analysis and mass spectrometry [33]. Quality control of synthetic peptides was kindly performed by W.D. Lehmann, DKFZ Heidelberg, Germany.

Purification of apolipoprotein A-I and antiserum preparation

HDL was isolated from plasma of male Wistar rats by density gradient centrifugation according to [34] as described previously [35]. After centrifugation for 20 min at 1000 g at 4°C, the supernatant was submitted to a density gradient of KBr and NaCl and centrifuged for 20 min at 3000 g at 4°C. Aliquots of 4 ml of the pooled HDL fractions were pipetted into polyallomer tubes. A discontinuous gradient was formed by carefully layering 3 ml of a NaCl/KBr solution with a density of 1.0191,23 and centrifuged at 280.000 g at 20°C for 24 h. Subsequently HDL was extensively dialysed against PBS +0.01 % EDTA at pH 7.4. After extraction with ether/ethanol apoA-I was isolated by gel chromatography on a Sepharyl S-200 column and subsequently submitted to isoelectrofocusing at the pH range between 5.5 and 5.7. Peak 3 of the Sepharyl S-200 column showed one band at pH 5.6, indicating that apoA-I was pure (Figure 1). This band was eluted with 5 M urea, dialysed, and lyophilised and subsequently used for production of specific antiserum.

Primary hepatocyte cell cultures

Hepatocytes were isolated by perfusion of rat liver with collagenase [39]. Viability was estimated by Trypan blue staining. Hepatocytes were suspended to a concentration of about 10^6 cells per ml in Williams medium containing 10% calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Two hrs later medium was exchanged by serum free medium [40, 41]. With the change of medium after 24 hrs of incubation the peptides of apoA-I were added in concentrations of 10^{-7} to 10^{-4} mol/l. Control cultures were incubated with peptide free medium or with the albumin hexapeptide in concentrations of 10^{-5} to 10^{-3} mol/l. Twenty four hrs later medium was withdrawn and centrifuged for 10 min at 3000 g. Subsequently total protein, apoA-I and RSA concentrations were measured in the supernatant [42]. Each measurement of the proteins was done in duplicate. The mean value was calculated from six different cell cultures. The synthesis rate of apoA-I and albumin was related to total protein in the supernatant (ng/mg) and expressed in percent.

Cell-free protein synthesis

Total rat liver RNA was isolated by extraction with 7 M guanidine-HCL and centrifugation on a CsCl gradient. Subsequently mRNA was isolated by chromatography on oligo (dT)-cellulose [42]. 1H- leucine was incorporated into protein using a cell-free lysate from rabbit reticulocytes (NEN) with 1.5 µg of the isolated m-RNA as described [25, 43]. The reaction mixture was incubated with hexapeptide concentrations from 10^{-8} to 10^{-4} mol/l for 50 min at 37°C.

Measurement of total protein, apolipoprotein A-I, and albumin

Total protein was measured in the supernatant of hepatocyte cell cultures and in the cell-free system by the Bio-rad assay with BSA as standard according to [44]. Total protein synthesis was measured as hot trichloroacetic acid insoluble radioactivity [45]. ApoA-I and albumin were measured in the supernatant by Elisa using a modified commercially available kit (Avidin alkaline phosphatase staining, Sigma) [46] by precipitation with specific antiserum.

Statistical analysis

Results are expressed as mean ± SD of a number of
determinations as indicated. The data were compared by Student’s t test. Differences were considered to be statistically significant when \( p < 0.05 \).

**Results**

Addition of WEFWQQ, WDFWQQ, and SEFWQQ to the cell culture, in concentrations of \( 10^{-7} \) to \( 10^{-4} \) mol/l, inhibited net increase of apoA-I dose dependent (Table 1). Compared to the control culture a peptide concentration of \( 10^{-4} \) mol/l inhibited apoA-I synthesis to \( 87 \pm 4.9\% \), \( 90 \pm 1.9\% \), and \( 94 \pm 3.0\% \) (n = 6). WEFWQQ inhibited albumin synthesis only slightly, and not in dose dependent manner. The other two peptides did not affect albumin synthesis at all. For control of specificity the influence of the proalbumin hexapeptide Arg-Gly-Val-Phe-Arg-Arg (RGVFRR) was determined on the synthesis of apoA-I, rat serum albumin, and total protein in concentrations of the peptide from \( 10^{-5} \) to \( 10^{-3} \) mol/l (n = 1). Proapo A-I synthesis was not influenced whereas rat serum albumin synthesis was inhibited in dose dependent manner. At a concentration of \( 10^{-3} \) mol/l RGVFRR albumin synthesis was inhibited to 77\%, confirming previous results in isolated hepatocytes [25].

**Table 1** Influence of the pro-peptides on apolipoprotein A-I and albumin-synthesis in primary hepatocyte cell cultures.

<table>
<thead>
<tr>
<th>Peptide conc. in medium</th>
<th>ng Apo A-I/mg total protein ± SD</th>
<th>% of control ± SD</th>
<th>µg Albumin/mg total protein ± SD</th>
<th>% of control ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WEFWQQ (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>118.0 ± 13.1</td>
<td>100.0</td>
<td>26.7 ± 7.9</td>
<td>100.0</td>
</tr>
<tr>
<td>( 10^{-7} ) M</td>
<td>112.9 ± 11.5</td>
<td>95.7 ± 4.3</td>
<td>25.0 ± 6.7</td>
<td>93.6 ± 2.4</td>
</tr>
<tr>
<td>( 10^{-6} ) M</td>
<td>109.0 ± 15.4</td>
<td>92.4 ± 5.8</td>
<td>24.3 ± 4.5</td>
<td>91.0 ± 1.7</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>103.8 ± 15.1</td>
<td>88.0 ± 6.1</td>
<td>25.6 ± 5.5</td>
<td>95.9 ± 2.1</td>
</tr>
<tr>
<td>( 10^{-4} ) M</td>
<td>102.4 ± 13.8</td>
<td>86.8 ± 4.9</td>
<td>25.2 ± 5.6</td>
<td>94.4 ± 2.1</td>
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<tr>
<td><strong>WDFWQQ (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>113.4 ± 14.7</td>
<td>100.0</td>
<td>24.7 ± 2.9</td>
<td>100.0</td>
</tr>
<tr>
<td>( 10^{-7} ) M</td>
<td>111.2 ± 15.5</td>
<td>97.9 ± 1.3</td>
<td>26.6 ± 3.1</td>
<td>107.7 ± 2.1</td>
</tr>
<tr>
<td>( 10^{-6} ) M</td>
<td>107.9 ± 15.1</td>
<td>95.0 ± 2.4</td>
<td>26.2 ± 4.0</td>
<td>106.1 ± 1.8</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>108.2 ± 12.6</td>
<td>95.5 ± 2.9</td>
<td>26.2 ± 3.8</td>
<td>106.1 ± 2.0</td>
</tr>
<tr>
<td>( 10^{-4} ) M</td>
<td>102.0 ± 10.0</td>
<td>90.4 ± 1.9</td>
<td>25.8 ± 3.5</td>
<td>104.7 ± 1.4</td>
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<td><strong>SEFWQQ (n = 4)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>117.8 ± 5.2</td>
<td>100.0</td>
<td>24.0 ± 2.0</td>
<td>100.0</td>
</tr>
<tr>
<td>( 10^{-7} ) M</td>
<td>119.1 ± 5.4</td>
<td>100.9 ± 1.5</td>
<td>23.2 ± 2.0</td>
<td>97.1 ± 6.6</td>
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<tr>
<td>( 10^{-6} ) M</td>
<td>114.6 ± 6.1</td>
<td>97.3 ± 1.2</td>
<td>23.9 ± 2.0</td>
<td>99.7 ± 4.2</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>112.6 ± 2.7</td>
<td>95.7 ± 2.7</td>
<td>23.3 ± 1.6</td>
<td>97.4 ± 5.8</td>
</tr>
<tr>
<td>( 10^{-4} ) M</td>
<td>110.7 ± 1.9</td>
<td>94.0 ± 3.0</td>
<td>23.6 ± 2.1</td>
<td>98.1 ± 6.2</td>
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<tr>
<td><strong>RGVFRR albumin-hexapeptide (n = 1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>125.3</td>
<td>100.0</td>
<td>31.0</td>
<td>100.0</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>124.9</td>
<td>99.7</td>
<td>29.6</td>
<td>95.5</td>
</tr>
<tr>
<td>( 10^{-4} ) M</td>
<td>124.1</td>
<td>99.0</td>
<td>27.2</td>
<td>87.7</td>
</tr>
<tr>
<td>( 10^{-3} ) M</td>
<td>123.0</td>
<td>98.2</td>
<td>23.8</td>
<td>76.8</td>
</tr>
</tbody>
</table>

Addition of WEFWQQ, WDFWQQ, and SEFWQQ to the cell-free system in concentrations of \( 10^{-8} \) to \( 10^{-4} \) mol/l inhibited incorporation of \(^3\)H-leucine into apoA-I dose dependently (Figure 2). A peptide concentration of \( 10^{-4} \) mol/l inhibited incorporation to \( 57 ± 8\% \), \( 52 ± 5\% \), and \( 58 ± 3\% \) compared to the control (n = 7). Incorporation into total protein was less effected. A peptide concentration of \( 10^{-4} \) mol/l inhibited incorporation to \( 76 ± 5\% \), \( 78 ± 2\% \), and \( 81 ± 3\% \) only. Incorporation into albumin was not inhibited by the apoA-I propeptides. Amino acids in equimolar concentrations to the propeptides had no effect on the incorporation into total protein, apoA-I and rat serum albumin.

The proalbumin hexapeptide in concentrations from \( 10^{-6} \) to \( 10^{-3} \) mol/l dose dependently inhibited albumin synthesis (n = 4). A concentration of \( 10^{-3} \) mol/l inhibited incorporation into albumin to \( 36 ± 8\% \) of pretreatment value, confirming previous results [25], whereas incorporation into apoA-I was inhibited to \( 86 ± 3\% \) only. The total protein synthesis was inhibited to \( 67 ± 4\% \). The inhibition of total protein- and albumin synthesis was also significantly different compared to the synthesis of apoA-I by addition of the albumin propeptide (RGVFRR). The inhibition of total protein- and apo AI synthesis was highly significantly different compared to albumin synthesis and the inhibition of apoAI synthesis was significantly stronger than the inhibition of total protein synthesis.

**Discussion**

A high level of HDL in serum reduces the risk of...
Figure 2abcd Influence the propeptides of apoA-I and albumin on the synthesis of apoA-I, total protein, and albumin in percent of the control (without peptides); *(p < 0.05).

Atherosclerosis and myocardial infarction [4]. The major protein associated with high density lipoproteins is apoA-I and the apoB/apoA-I ratio is superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction [47, 48]. ApoA-I is synthesized in the liver as preproprotein. It undergoes co-translational proteolytic processing to proapoA-I which is secreted. Subsequently the propeptide is split off extracellularly
Three slightly different propeptides of apoA-I have been identified. However, the COOH-terminal amino acids sequences of all three rat propeptides and also of the human propeptide are identical, indicating that these structures are essential for its function. Apo A-I synthesis is strongly inhibited by all three peptides in the cell free system. In primary hepatocyte cell cultures apoA-I synthesis is although inhibited, but to a lesser degree. This may be due to a limited uptake or degradation of the peptides by the cells. The weaker inhibitory effect of SEFWQQ in the cell culture compared to the other two peptides may also be explained by a lower uptake rate of this peptide by the hepatocytes.

Albumin synthesis is not inhibited by the apoA-I propeptides, indicating a specific feedback inhibition of apoA-I synthesis by its propeptide. The specificity of this inhibition is further supported by the fact that the albumin propeptide inhibits the synthesis of albumin but not of apoA-I.

Proteolytic processing of proproteins can lead to biological active peptides, like insulin. For other peptides, released from proproteins, such as albumin and procollagen no definite biological functions have been demonstrated. An extensive review of structures, processing, and possible functions of propeptides are discussed by [54]. Interestingly, the synthesis of albumin and of procollagen type III are inhibited by their specific propeptides [25-31]. Not only proteins which are synthesized via proproteins can be inhibited on the translational level. The key enzymes for the biosynthesis of polyamines, ornithine decarboxylase and S-adenosylmethionine decarboxylase are specifically inhibited by the polyamines spermine and spermidine [55]. We could demonstrate also that the synthesis of apolipoprotein A-I is specifically inhibited by its propeptide at the translational level. This is in agreement with the fact that the level of apolipoprotein A-I mRNA is primarily regulated post-transcriptionally through a higher stability or increased mRNA half-life, as has been reported [56]. Peptides with a low molecular weight can inhibit protein synthesis by binding to mRNA [57, 58]. Whether the same mechanism is responsible for the inhibition of apoA-I synthesis by its propeptide remains to be investigated.

Many drugs have been developed to reduce the risk of atherosclerosis and myocardial infarction in patients with elevated cholesterol. The majority of these drugs reduce the LDL level. Only one drug on the market acts to increase the HDL level. Although this drug, torcetrapib, a cholesteryl ester transfer protein inhibitor, increases HDL cholesterol levels, it is not associated with a significant decrease in progression of coronary atherosclerosis [59]. However, this finding does not exclude a beneficial effect of increased HDL. The lack of efficacy may be related to the mechanism of action of torcetrapib or molecule specific effects [59]. Furthermore this drug was associated with an increase in systolic blood pressure [59-61]. Therefore, it might well be that a drug stimulating apolipoprotein A-I synthesis and thus increasing HDL level by another mechanism, leads to a decrease in coronary events. Another approach to stimulating HDL may be with apolipoprotein A-I mimetic peptides which emulate many of the atheroprotective biological functions attributed to HDL [62, 63]. If the observed inhibition by its propeptide is of physiological relevance in the synthesis of apoA-I then a modified peptide, competing with the propeptide, may be able to increases the rate of synthesis of apoA-I. Such a drug would be easier to produce than a more complex apolipoprotein A-I mimetic peptide. In addition to its atheroprotective function a high level of apolipoprotein A-I may also be helpful in the prevention of Alzheimer’s disease by modulation of Abeta aggregation [64].

Conflicts of interest
Authors declare no conflicts of interest.

Acknowledgments
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Abbreviations
ApoA-I: apolipoprotein A-I; HDL: high density lipoproteins; LDL: low density lipoproteins; RSA: rat serum albumin

References


