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Short communication

Insulin suppresses apolipoprotein(a) synthesis by primary cultures of cynomolgus monkey hepatocytes

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Summary Raised plasma lipoprotein(a) (lp(a)) concentrations have been reported in patients with Type I (insulin-dependent) diabetes mellitus, which were lowered by insulin therapy. To investigate the biochemical background of these changes, we studied the effect of insulin on apolipoprotein(a) (apo(a)) synthesis and mRNA levels in primary cultures of cynomolgus monkey hepatocytes. Low concentrations of insulin (10 nmol/l) had a small but significant decreasing effect ($p < 0.046$) on apolipoprotein(a) secretion (–16 %). Maximum inhibition (–33 %) was obtained after incubation for 72 h with 1000 nmol/l insulin. Apolipoprotein B-100 secretion was 30 %–36 % decreased when using 10–1000 nmol/l and no change was observed for the secretion of apolipoprotein A-1 and albumin which were measured as control proteins. Steady state apolipoprotein(a) mRNA concentrations paralleled the decrease in apolipoprotein(a) synthesis (–29 % after incubating the cells for 48 h with 100 nmol/l insulin) indicating that the decreased synthesis is regulated at the (post)-transcriptional level. Concentrations of apolipoprotein B-100 and apolipoprotein A-1 mRNA were not changed after incubation with insulin. We conclude that high concentrations of insulin suppress apolipoprotein(a) synthesis in monkey hepatocytes at the (post)-transcriptional level. These data may provide an explanation for the increased plasma concentrations of lipoprotein(a) as found in patients with insulin dependent diabetes mellitus. [Diabetologia (1999) 42: 41–44]

Keywords Apolipoprotein(a), insulin, cynomolgus monkey hepatocytes.

Lipoprotein(a) (lp(a)) is a lipoprotein particle in which apolipoprotein(a) is covalently linked to low density lipoprotein (LDL) via a disulphide bridge. It plays an important part in atherogenesis and high plasma concentrations of lp(a) are considered to be a risk factor for vascular diseases [1].

Diabetic patients are at high risk of developing cardiovascular disease. In addition to other deviations in lipid metabolism, abnormal concentrations of lp(a) could contribute to the increased risk in these patients. It has been shown that lp(a) concentrations are increased in patients with Type I (insulin-dependent) diabetes mellitus, especially in those with microalbuminuria or poor metabolic control [2, 3]. In contrast, in well-controlled Type I diabetic patients no increase in lp(a) plasma concentrations were observed [4].

Several studies have shown that insulin therapy associated with improved glycaemic control resulted in decreased lp(a) plasma concentrations (by about 30 %) in patients with Type I diabetes [2, 3]. No effect on plasma lp(a) concentrations after improved glycaemic control was, however, found in patients with Type II (non-insulin-dependent) diabetes [5] and the reports on a possible association between lp(a) and insulin concentrations in healthy subjects remain controversial. Furthermore, it is not clear whether insulin has a role in the regulation of lp(a) plasma concentra-

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Abbreviations: Lp(a), Lipoprotein(a); apo(a), apolipoprotein(a).

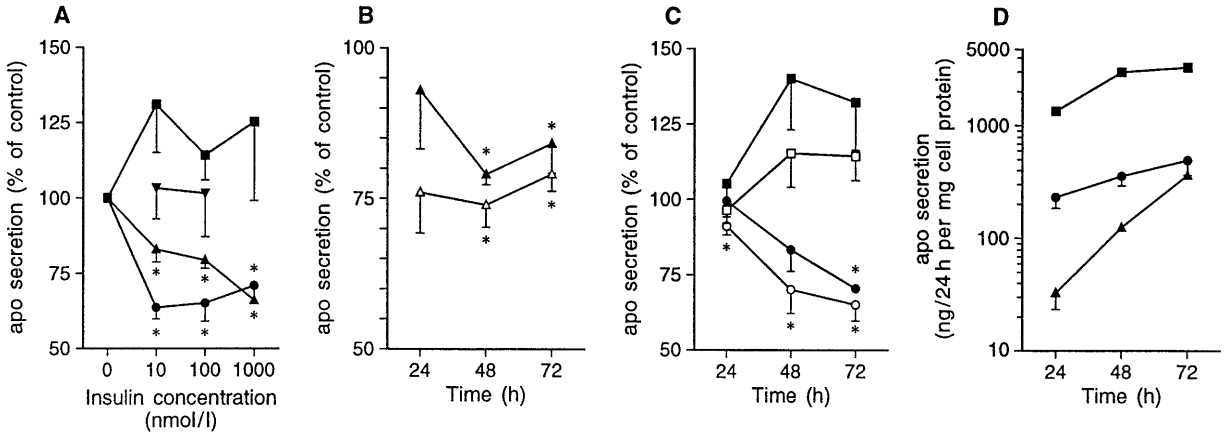

Fig. 1 A, B. Dose- and time-dependency of the effect of insulin on apolipoprotein synthesis. After a 24 h attachment period, hepatocytes were cultured for 24, 48 or 72 h without or with different concentrations of insulin. Medium was renewed every 24 h. Data are expressed as a percentage of control. Values are means ± SEM of duplicate incubations of hepatocytes from four independent hepatocyte isolations. **A** Dose effect of insulin on secretion of apo(a) (▲), apo B-100 (●), apo A-l (■) and albumin (▼) after 72 h of culture. **B** and **C** Time effect of 10 nmol/l (closed symbols) or 100 nmol/l (open symbols) insulin on secretion of apo(a) (**B**) and apo B-100 and apo A-l (**C**). **D** Apo (a), apo B-100 and apo A-l production in control medium in time. Albumin synthesis rate was 32 ± 9 (control) and 29 ± 7 μg/24 h per mg cell protein (means ± SEM, $n = 3$) for the incubations with 10 and 100 nmol/l insulin.
* Indicates a significant difference ($p < 0.05$) between control and treated cells at the same time point

tions by influencing its synthesis or catabolism. Therefore, we have assessed the effect of insulin on apolipoprotein(a) (apo(a)) synthesis in cultured cynomolgus monkey hepatocytes.

Materials and methods

Simian hepatocyte isolation and culture. Simian hepatocytes were isolated from livers of both male and female monkeys. The monkeys were 1.5 to 3 years old and obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The monkeys were bred at this institute and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The monkeys were fed Primate Diet G.O. (Hope Farms B.V., Woerden, The Netherlands) ad libitum with one or two pieces of additional fruit per day and were fasted overnight before killing. The isolation and culture procedure was done as described previously [6, 7]. After a 24-h attachment period, the incubations without or with insulin (Actrapid Penfill 1.5, 100 U/ml, Novo Nordisk Pharmaceutique S.A., Chartres, France) were started in RPMI 1640 medium, containing 10 μg/ml transferrin 0.1 μmol/l Cu^{2+}, 0.3 nmol/l Se^{4+}, 50 pmol/l Zn^{2+}, 10 μg/ml glucagon, 50 ng/ml epidermal growth factor (EGF), 10 μU/ml growth hormone, 100 mU/ml prolactin, 5 μg/ml linoleic acid, 20 μg/ml bovine serum albumin, 50 nmol/l dexamethasone (all from Sigma, St. Louis, Mo., USA) and supplemented with 10% heat inactivated fetal calf serum (Boehringer Mannheim, Mannheim, Germany), 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin. The medium was renewed every 24 h.

Apo(a) and other protein measurements. Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AS, Umeå, Sweden) [7]. Concentrations of apo A-l, apo B-100 and albumin in the medium were measured as described previously [6, 7] and were normalized for cell protein in the dishes.

RNA isolation and hybridization. After incubation of the hepatocytes for 48 h with 100 nmol/l insulin or control medium, total RNA was isolated and mRNA concentrations of apo(a), apo B-100, apo A-1, actin, GAPDH and 18S ribosomal RNA were assessed by northern-blot hybridization. Isolation of RNA, probes, labelling conditions and hybridization were done as described previously [6, 7]. A kringle IV synthetic double-stranded probe of 75 nucleotides, with the sense sequence: GGGAATTCGAACCTGCCAAGCTTGGTCATCTATGACACCACACTCGCATAGTCGGACCCCAGAATAAAGC TTGGG, was used to detect apo(a) mRNA(s). This probe was labelled by the random primer method according to Megaprime DNA labelling systems (Amersham Life Science, Bucks., UK).

Statistical analysis. Statistical significance of differences was calculated by Student's *t*-test for paired data with $p < 0.05$ as the level of significance.

Results

Insulin decreases apo(a) secretion. Addition of increasing amounts of insulin to the medium for 72 h resulted in a dose-dependent decrease of apo(a) secretion (Fig. 1) at a concentration of 10 nmol/l insulin (–16%) ($p < 0.046$), whereas a suppression of 33% was reached at 1000 nmol/l ($p < 0.047$). Secretion of apo B-100 was decreased by 30–36% at different insulin concentrations and apo A-l secretion tended to be higher compared with the control, but this was not significant (Fig. 1). Albumin secretion was determined as control protein, since it is the most abundantly synthesized protein in hepatocytes. No effect on the albumin secretion by the cynomolgus monkey hepatocytes was observed.

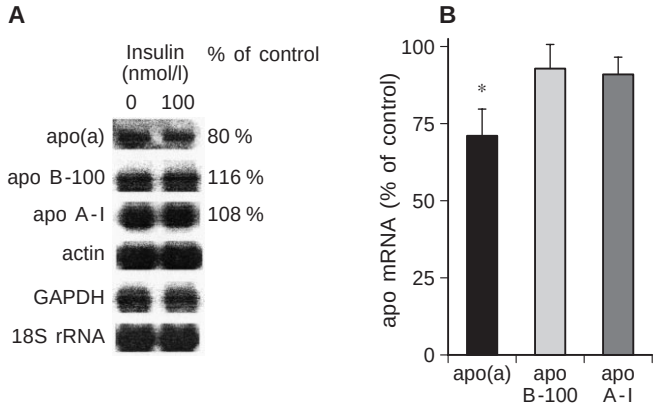

Fig. 2. **A** Effect of insulin on apo(a) mRNA, apo B-100 and apo A-1 concentrations. **A** Phosphor Imager Scan of a representative Northern blot. **B** Histogram of the effect of insulin on apo(a), apo B-100 and apo A-1 mRNA. Values are means ± SEM of experiments with hepatocytes of four independent cultures.
* Indicates a significant difference ($p < 0.05$) between control and treated cells at the same time point

To assess the time-course of inhibition, cells were exposed to 10 and 100 nmol/l insulin for different lengths of time between 24 and 96 h of culture. No significant change in apo(a) secretion was seen after the first 24 h incubation period. Maximum suppression of apo(a) secretion, when using 10 nmol/l or 100 nmol/l of insulin, was reached after 48 h of incubation (–21 % and –26 %, respectively) (Fig. 1).

Secretion of apo B-100 was only decreased after 72 h of incubation with 10 nmol/l insulin, whereas 100 nmol/l insulin decreased apo B-100 secretion during all incubation periods (Fig. 1). Secretion of apo A-1 did not change significantly during any of the incubation periods (Fig. 1).

Insulin suppresses apo(a) mRNA concentrations. To assess at what the level of regulation is insulin inhibits apo(a) synthesis, apo(a) mRNA concentrations were determined (Fig. 2). Actin and GAPDH mRNA and 18S rRNA, which were not affected by insulin, were used as internal standards. Northern blot analysis showed that apo(a) mRNA was decreased (–29 %), after incubation for 48 h with 100 nmol/l insulin. In the same culture experiments the apo B-100 and apo A-l mRNA concentrations did not change significantly compared with the control.

Discussion

In this study we showed that low concentrations of insulin decreased apo(a) synthesis by a direct effect on cynomolgus monkey hepatocytes. Suppression was regulated at the (post)-transcriptional level, since apo(a) mRNA concentrations were decreased to the same extent as the apo(a) protein synthesis. Synthesis of apo B-100 was also suppressed but its mRNA concentration was not affected by insulin. The synthesis of apo A-l and albumin, measured as controls, did not change significantly under the influence of the hormone.

This study provides direct evidence that hepatic apo(a) synthesis is influenced by relevant (10 nmol/l) insulin concentrations, considering the fact that hepatocytes degrade insulin extensively and that the half-life of insulin, at least in primary rat hepatocyte cultures, is between 4 and 5 h. Therefore, we also used relatively high initial insulin concentrations (100 nmol/l, 1000 nmol/l). Secretion of apo(a) and mRNA concentrations were suppressed by about 20–35 % when the primary hepatocytes were incubated with insulin. Although the effect was moderate, it was consistently seen in all four independent cultures, irrespective of the apo(a) phenotype which differed between combinations of S2 and S4 in the monkeys. Moreover, similar decreases in plasma lp(a) concentrations (about 30–35 %) were found in in vivo studies, in which Type I diabetic patients with a poor metabolic control were treated with insulin [2, 3], suggesting that the primary hepatocytes reflect the in vivo situation well.

In contrast, no such effect was observed in Type II diabetic patients treated with insulin resulting in improved metabolic control [5]. The reason for this is not fully clear but differences in the metabolic state between Type I and Type II diabetes could be responsible, since patients with Type II diabetes show insulin resistance in combination with high basal insulin concentrations which could, in part, account for the controversial data on lp(a) in diabetes mellitus. Further, it should be noted that different studies are often difficult to compare, since variables such as phenotype, insufficient sample sizes of studies and the stage of diabetic complications could influence the outcome of the investigations [8]. For instance, microalbuminuria and kidney diseases are known to increase lp(a) concentrations [9].

By measuring plasma lp(a) no distinction can be made between an effect of insulin on the lp(a) synthesis and its removal from the circulation. Our results indicate that the regulation by insulin takes place at the apo(a) synthesis level, since apo(a) production by the hepatocytes was decreased. In addition, apo(a) mRNA concentrations were suppressed to a similar extent as the protein suggesting transcriptional regulation. This contention is supported by a preliminary report that apo(a) transcriptional activity was decreased by insulin, as shown by promoter-reporter studies with 1.4 kb of the 5′ prime flanking region of the apo(a) gene in HepG2 cells [10].

In conclusion, we found that 10 nmol/l of insulin decreases apo(a) synthesis in primary cultures of cynomolgus monkey hepatocytes by suppression of

mRNA concentrations. These data could provide an explanation for the increased plasma lp(a) concentrations found in patients with poorly controlled insulin-dependent diabetes and its return to normal after treatment with insulin.

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