Regulation of diurnal variation of cholesterol 7α-hydroxylase (CYP7A1) activity in healthy subjects

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Running title:

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Summary

Cholesterol 7α-hydroxylase (CYP7A1), the key regulatory enzyme of bile acid synthesis, displays a pronounced diurnal variation. To better understand the regulation of CYP7A1 activity, three day-long examinations were carried out in 12 healthy men. The concentrations of 7α-hydroxycholest-4-en-3-one (C4), a surrogate marker of CYP7A1 activity, bile acids (BA), insulin, glucose, nonesterified fatty acids, triglyceride, and cholesterol were measured in serum in 90-min intervals from 7 AM till 10 PM. To lower and to increase BA concentration during the study, the subjects received cholestyramine and chenodeoxycholic acid, respectively, in two examinations. No drug was used in the control examination. The concentration of C4 showed out pronounced diurnal variation with a peak around 1 PM in most of the subjects. The area under curve (AUC) of C4 concentration was five times higher and three times lower when subjects were treated with cholestyramine and CDCA, respectively. No relationship was found between AUC of C4 and AUC of BA concentration, however, AUC of C4 was positively correlated with that of insulin. Moreover, short-term treatment with cholestyramine resulted in ~10% suppression of glycemia throughout the day. Our results suggest that insulin is involved in regulation of diurnal variation of CYP7A1 activity in humans.

Key words

cholesterol 7α-hydroxylase – bile acid – insulin – glucose – cholestyramine

Introduction

Cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first key regulatory step in the synthesis of bile acids (BA) from cholesterol (Chiang *et al.* 1998, Gilardi *et al.* 2007). The synthesized bile acids are conjugated, exported into bile, stored in the gallbladder and, dependening on metabolic needs, secreted into the intestine to facilitate fat absorption. Approximately 95 % is then reabsorbed and return to the liver. BA returning from the enterohepatic cycle downregulate CYP7A1 activity and they exert such an effect at the level of gene transcription (Ramirez *et al.* 1994). BA bind to nuclear receptor farnesoid X receptor (FXR) and turn on the expression of small heterodimer partner (SHP) which then blocks the CYP7A1 gene transcription. Alternatively, they can downregulate CYP7A1 by non-FXR-dependent pathway (Gilardi *et al.* 2007). Moreover, BA absorbed in the intestine activate synthesis of fibroblast growth factor 19 (FGF19), that signals to the liver through its receptor FGFR4 and synergistically downregulates CYP7A1 gene expression (Inagaki et al. 2005).

CYP7A1 activity varies during the day – the circadian variation of the enzyme activity is well documented in rodents (Noshiro *et al.* 1990) and displays a pronounced circadian variation in humans (Gälman *et al.* 2005). The peak of the enzyme activity in humans is observed shortly after midday, which is rather difficult to reconcile with the fact that at this time the inhibition by BA returning from the enterohepatic cycle should prevail.

Interestingly, it was recently demonstrated that BA seem to directly affect the glucose metabolism through both FXR-independent and FXR-dependent pathways (De Fabiani *et al.* 2003, Ma *et al.* 2006).

Therefore, to better understand the mechanisms behind the regulation of CYP7A1 activity, we measured the diurnal variation of 7α-hydroxycholest-4-en-3-one (C4) concentration, a surrogate marker of CYP7A1 activity in vivo (Axelson *et al.* 1988), and changes of BA, insulin, glucose, nonesterified fatty acids (NEFA), triglyceride, and cholesterol concentration in healthy volunteers. The study was carried out under conditions when BA synthesis was markedly suppressed or increased by short-term treatment with bile acid sequestrant cholestyramine or chenodeoxycholic acid (CDCA), respectively.

Methods

Subjects and study design. Twelve male volunteers (age: 32.1 ± 4.0 years old, BMI: 25.7 ± 4.3 kg/m^2) were included into the study that consisted of three day-long examinations. One of these examinations served as a control examination (without any drug) while the other two examinations studied the effect of short term administration of cholestyramine (Questran®, Bristol-Myers Squibb, Prague, Czech Republic, 16 g/day) and chenodeoxycholic axid (CDCA; Chenofalk®, Dr. Falk Pharma GmbH, Freiburg, Germany, 1 – 1,5 g/day dependent on the weight of the subject). One day before each of these examinations, the first blood sample was drawn at 7 AM (-24 h) and subjects received food for the whole day to standardize their intake before the study. On the day of examination, the first blood sample was drawn again at 7 AM (0 h) and the blood samples were then collected in 90 minute intervals for 15 hours till 10 PM. Again, subjects received food for the whole day and they had to eat at exactly defined intervals (breakfast at 7:15, snack at 9:45, lunch at 12:30, snack at 15:30 and dinner at 17:30). The amount of food was calculated to cover their energy requirements; the diet was relatively low in fat (25 % of energy intake). If the examination included the drug administration, the drugs were given to subjects on the day before the examination and also on the day of the examination. Questran® was given to subjects in two doses on both days – one with breakfast, the other one with dinner. Due to differences in pharmacokinetics, Chenofalk® treatment was started with dinner on day preceding the examination and, on the day of examination, it was given to subjects in two doses at the same time as cholestyramine. The order of the examinations was randomized and they were carried out in three week intervals at a minimum.

The study protocol was approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine and all the participants gave their informed consent.

Biochemistry. Cholesterol and triglyceride (TG) were measured using enzymatic kits from Roche Diagnostics GmbH, Mannheim, Germany, glucose using kits from PLIVA-Lachema Diagnostika, Brno, Czech Republic, nonesterified fatty acids (NEFA) using kits from Wako Chemicals GmbH, Neuss, Germany, and bile acids (BA) using enzymatic kits from Trinity Biotech plc, Bray, Ireland.

Insulin was determined using IRMA kits from Immunotech, Prague, Czech Republic. Concentration of 7α-hydroxycholest-4en-3-one (C4) was determined by HPLC as described by Gälman (Gälman *et al.* 2003) with a modification of C4 extraction procedure (Leníček *et al.* 2008).

Statistics. The differences between examinations were evaluated using ANOVA for repeated measures and if statistically significant differences were detected, a paired t test with Bonferroni correction was used to identify those differences. The differences between -24 h and -0 h were evaluated using a paired t test. The relationship between area under curve (AUC) of C4 and AUC of BA and insulin was tested using simple linear regression analysis.

Results

As expected, C4 concentration as a marker of CYP7A1 activity displayed marked diurnal variation (Fig. 1). The enzyme activity can vary in the order of magnitude within a few hours; the peak was reached around 1 PM in our study in most of the subjects, but, as can be seen, the interindividual variation was rather very high. The one day treatment with cholestyramine resulted in a severalfold increase in fasting C4 concentration on the day of examination and approximately a fivefold increase in the enzyme activity as assessed on the basis of AUC of C4 (Tab. 1, Fig. 2A). Conversely, the treatment with CDCA resulted in a decrease of CYP7A1 fasting concentration and AUC of C4 to approximately one third throughout the day (Tab. 1, Fig. 2A). The BA concentration varied throughout the day with minor peaks reflecting food intake during lunch and dinner (Fig. 2B). The effects of both treatments on serum BA concentration were not so pronounced – there was a decrease in both fasting BA concentration and AUC BA after cholestyramine and, conversely, approximately a 30% increase in AUC of BA after CDCA treatment (Tab. 1, Fig. 2B).

The treatment with both drugs had no effect on both fasting value and AUC of cholesterol, TG, and insulin (Tab. 1, Fig. 1). The fasting concentration of NEFA rose after CDCA treatment but did not differ from the fasting concentration in cholestyramine or control examinations. However, although

the treatment with both drugs had no effect on fasting glucose, the AUC of glucose after cholestyramine was approximately 10% lower than in control experiment ($p < 0.05$) (Tab. 1, Fig 2D). To further analyse the relationship between possible regulatory role that BA and insulin can exert on BA synthesis, the correlation between AUC of BA and AUC of insulin (as a measure of their bioavailability) were correlated to that of AUC of C4. As expected, there was a rather weak but statistically significant negative correlation between AUC BA and AUC C4 when all three examinations in all the subjects were correlated ($r = -0.430$, $p < 0.05$). However, no such relationship could be found when all the treatments were analyzed separately (Tab. 2). On the contrary, there was a strong positive correlation between AUC of insulin and AUC of C4 when subjects were treated with cholestyramine and/or CDCA (Tab. 2). Similar results were obtained when AUC's of BA and insulin obtained in shorter time intervals (7:00 to 11:30, 7:00 to 13:00, 7:00 to 14:30, 7:00 to 16:00) were correlated to AUC C4 (data not shown).

Discussion

In our study we demonstrated that CYP7A1 activity displays a diurnal variation in healthy men with a peak around 1 PM. The treatment with cholestyramine reduces serum BA concentration and severalfold upregulates the CYP7A1 activity and, conversely, the treatment with CDCA increases BA concentration and downregulates its activity. The AUC of C4 as a measure of bile acid synthetic capacity is positively correlated with AUC of insulin on cholestyramine and CDCA, but unexpectedly not with AUC of BA.

Our findings that C4 concentration as a marker of CYP7A1 activity displays a peak around a midday confirms the earlier findings from the study of 5 healthy volunteers (Gälman *et al.* 2005). However, it should be noted that three out of twelve subjects in the study show maximal C4 concentrations early in the morning (Fig. 1) and we should be cautious to generalize our conclusions about the CYP7A1 activity diurnal variation. Based on our data, some of the subjects may have displayed more than one

peak of CYP7A1 activity a day and we cannot even exclude the possibility that in some subjects there may be a peak during the night like in rodents (Noshiro *et al.* 1990).

If we assume that the short-term administration of cholestyramine upregulates CYP7A1 gene expression to a maximum, then the comparison of areas under the C4 curves suggest that, on average, the enzyme activity throughout the day varies around 20 % of the possible maximum and, thus, the enzyme activity is rather suppressed under physiological conditions (Tab. 1, Fig. 2A). On the other hand, it is quite surprising that there is no correlation between AUC of BA and that of C4 (Tab. 2). It may be that the effect of BA on CYP7A1 activity is at its maximum or minimum when the subjects are treated with drugs and this is not connected to concentration of BA anymore. However, the lack of such correlation in the control examination suggests that the effect of other factors on CYP7A1 may prevail. Moreover, given that CYP7A1 activity is rather low for most of the day, it may be difficult to see a further suppresive effect of BA. From the other factors that can override the effect of BA, insulin deserves special attention. We could indeed demonstrate quite a strong positive correlation between AUC of C4 as a measure of CYP7A1 activity and AUC of insulin as a measure of its bio-availability at least when both drugs are used to manipulate the transhepatic flux of BA. Such an observation is in agreement with recent findings that insulin in physiological concentration has a dual effect on CYP7A1 gene expression in human primary hepatocytes (Li *et al.* 2006). First, it induces a very rapid and pronounced activation of CYP7A1 gene expression likely due to the activation of p38 MAPK pathway by insulin (Xu *et al.* 2007). Then, after several hours, it inhibits gene expression through the effect of two insulin-regulated transcriptional factors – forkhead box O1 (FoxO1) and sterol regulatory element binding protein-1c (SREBP-1c) – on transactivation of the CYP7A1 gene (Li *et al.* 2006). Therefore, we can hypothesize that insulin could be the first signal to induce CYP7A1 gene expression at the transition from fasting to postprandial phase. Afterwards, the effect of BA return from enterohepatic cycle, FGF19 signalling, and downregulating effect of insulin should prevail and lower the CYP7A1 activity. Although such a hypothesis fits with our data, we are fully aware of the fact that insulin is only one of many factors that are involved in the complex regulation of transition to the postprandial state. It was shown that the circadian variation of CYP7A1 activity is preserved even in fasting subjects that are allowed to drink only water (Gälman *et al.* 2005) which suggests that the other

mechanisms such as signalling through albumin D-element-binding protein (DBP) (Lavery and Schibler 1993) or through FGF19 (Lundasen *et al.* 2006) may be involved in the regulation of bile acid synthesis during the day.

Our findings that plasma glucose concentration is suppressed after a 24 hour treatment with cholestyramine is in line with the observation that a 6-week-treatment of diabetic patients with cholestyramine improved glycemia and glycated hemoglobin (Garg and Grundy 1994) and with the findings that bile acids may directly inhibit gluconeogenesis by affecting the transcription of its key regulatory enzyme, phosphoenolpyruvate carboxykinase (PEPCK) (De Fabiani *et al.* 2003). The mechanism of this effect is not FXR-dependent. Our data suggest for the first time that the effect of low transhepatic flux of bile acid on gluconeogenesis is immediate and likely does not include the extrahepatic effects because the variations of insulin and NEFA concentrations throughout the day were not affected by cholestyramine.

In conclusion, we observed a positive correlation between the area under the curve of insulinemia and that of 7α-hydroxycholest-4-en-3-one, the surrogate marker of CYP7A1 activity. Our findings support an idea that insulin may play a role in the regulation of bile acid synthesis in humans.

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Table 1

The concentration of cholesterol, triglyceride, glucose, nonesterified fatty acids (NEFA), insulin, bile acids (BA), and 7α-hydroxycholest-4en-3-one (C4) at 7:00 AM on the day before the study (-24 h), At 7:00 AM on the day of the study (0 h) and 15hour area under curve (AUC) of these variables (AUC [0-15 h])

		-24 h	0 h	AUC [0-15 h]
Cholesterol [mmol/l]	C	4.66 ± 0.94	4.60 ± 0.96	66.1 ± 13.5
	Q	4.73 ± 0.76	4.59 ± 0.86	64.4 ± 11.5
	CDCA	4.65 ± 0.91	4.63 ± 0.91	66.8 ± 12.5
Triglyceride [mmol/l]	C	1.64 ± 0.74	1.57 ± 1.03	27.2 ± 16.4
	Q	1.56 ± 0.95	1.76 ± 0.86	27.5 ± 14.9
	CDCA	1.62 ± 0.85	1.61 ± 0.84	28.3 ± 12.2
Glucose [mmol/l]	C	5.10 ± 0.46	5.11 ± 0.45	80.3 ± 6.8 [a]
	Q	5.26 ± 0.63	5.09 ± 0.41	71.5 ± 5.9 [b]
	CDCA	5.45 ± 0.96	4.96 ± 0.59	74.9 ± 9.2 [a]
NEFA [mmol/l]	C	0.37 ± 0.27	0.43 ± 0.15	3.32 ± 0.98
	Q	0.37 ± 0.26	0.55 ± 0.50	3.03 ± 1.69
	CDCA	0.30 ± 0.16	0.50 ± 0.32*	2.31 ± 1.49
Insulin [IU/l]	C	7.8 ± 6.0	6.3 ± 3.7	275 ± 133
	Q	8.0 ± 3.3	9.7 ± 6.4	220 ± 124
	CDCA	9.8 ± 9.5	8.0 ± 5.7	265 ± 141
BA [μmol/l]	C	13.4 ± 6.1	11.0 ± 4.5 [a,b]	186 ± 64 [a]
	Q	11.4 ± 3.8	8.7 ± 2.9* [a]	133 ± 37 [b]
	CDCA	12.6 ± 4.7	13.1 ± 4.0 [b]	242 ± 86 [a]
C4 [μg/l]	C	20.1 ± 22.0	25.3 ± 23.0 [a]	303 ± 168 [a]
	Q	22.7 ± 17.6	86.9 ± 49.8** [b]	1512 ± 784 [b]
	CDCA	30.2 ± 24.9	11.8 ± 13.0** [c]	119 ± 70 [c]

Data are mean ± SD.
C … control experiment, Q … cholestyramine treatment, CDCA … chenodeoxycholic acid treatment
*, ** … p < 0.05, p < 0.01 … -24 hours vs 0 hours using paired t-test
a, b, c … the same letters are assigned to the examinations that do not differ when C, Q, and CDCA are compared using ANOVA for repeated measures (p < 0.05)

Table 2

The values of linear correlation coefficients r between AUC of 7α-hydroxycholest-4-en-3-one (C4)

and AUC of bile acids (BA) and AUC of insulin in subjects in control examination (C), after treatment

with cholestyramine (Q) and chenodeoxycholic acid (CDCA)

	n	AUC of BA	AUC of insulin	
C	12	0.142	0.423	
Q	12	0.113	0.727	**
CDCA	12	0.011	0.729	**

** … p < 0.01

Figure 1

Diurnal variation of 7α-hydroxycholest-4en-3-one (C4) concentration in twelve healthy subjects included into study during control examination (C)
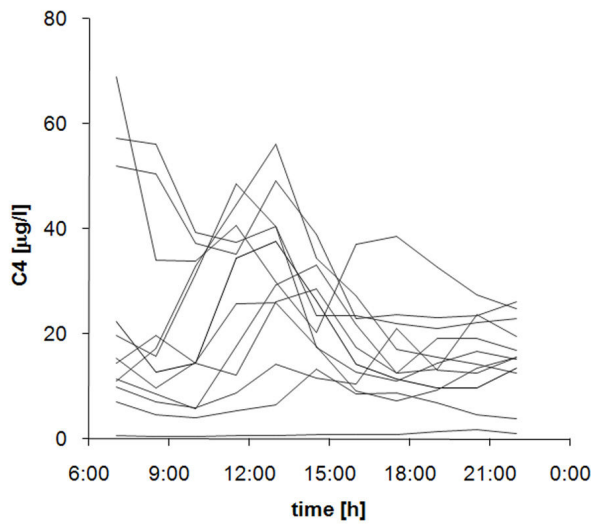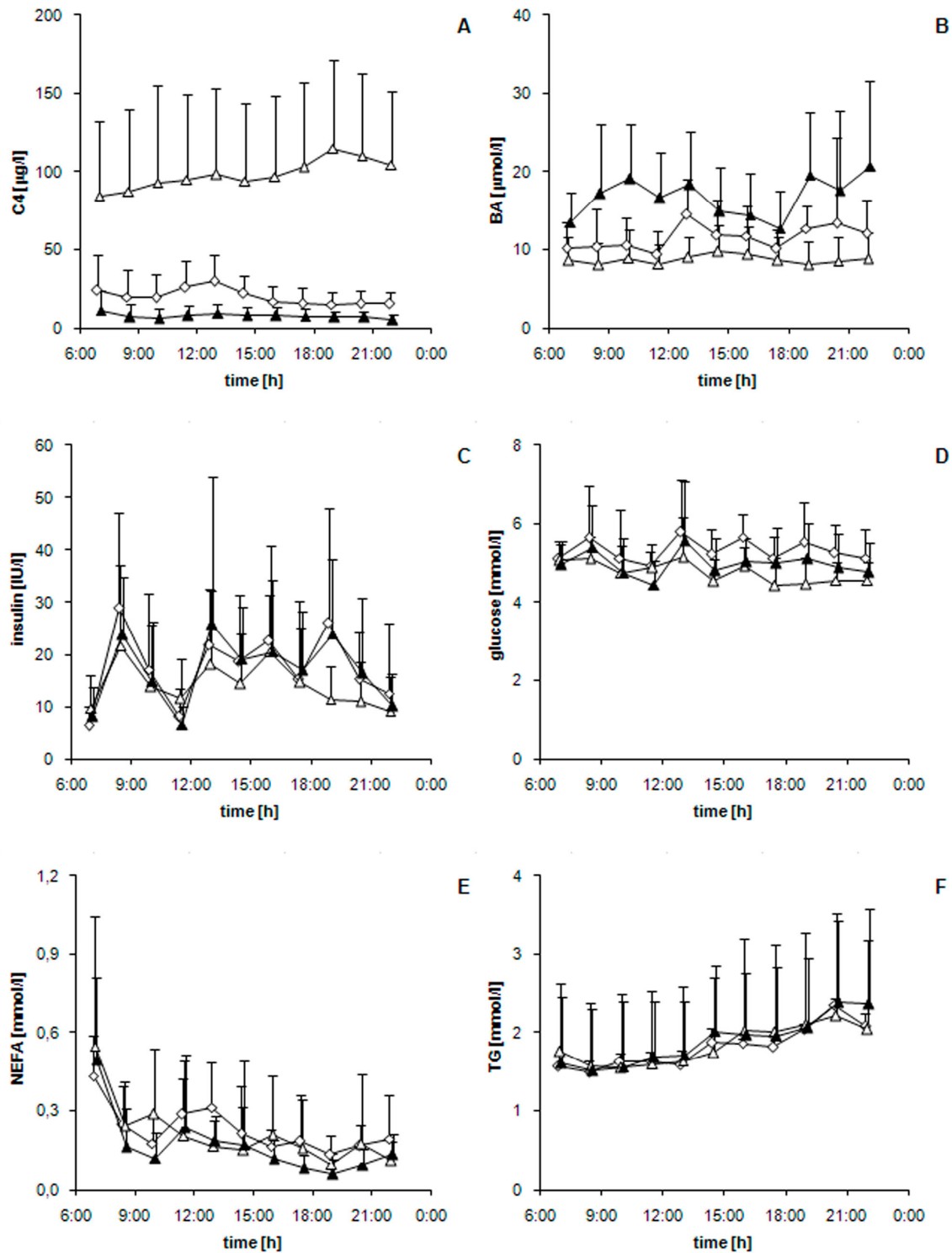
Figure 2

The diurnal variation of concentration of 7α-hydroxycholest-4en-3-one (C4), bile acids (BA), insulin, glucose, nonesterified fatty acids (NEFA), and triglyceride in the control experiment (C), after short treatment with cholestyramine (Q) and after short treatment with chenodeoxycholic acid (CDCA)

Footnote to Figure 2:
◇… control examination (C); △… short treatment with cholestyramine (Q); ▲… short treatment with chenodeoxycholic acid (CDCA)