0022-3565/98/2863-1122$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 286, No. 3
Printed in U.S.A.

JPET 286:1122–1128, 1998

Inhibition of Cholesterol Biosynthesis in Primary Cultured Rat Hepatocytes by Artichoke (*Cynara scolymus* L.) Extracts[1]

ROLF GEBHARDT[2]

Physiologisch-chemisches Institut der Universität, D-72076 Tübingen, Germany

Accepted for publication April 6, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

High-dose aqueous extracts from artichoke leaves were found to inhibit cholesterol biosynthesis from ^{14}C-acetate in primary cultured rat hepatocytes in a concentration-dependent biphasic manner with moderate inhibition (approximately 20%) between 0.007 and 0.1 mg/ml and more strong inhibition at 1 mg/ml. Cytotoxic effects detected by lactate dehydrogenase leakage and the 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide-assay were restricted to higher concentrations. Replacement of ^{14}C-acetate by ^{14}C-mevalonate largely omitted the inhibiting effect of artichoke extracts indicating an inhibition at the level of hydroxymethylglutaryl-CoA-reductase. However, no direct inhibition of this enzyme could be detected and no other enzymic steps later in the biosynthetic pathway for cholesterol seemed to be affected. Instead, inhibition was found to occur in a time-dependent manner, to last for several hours even after washing out the extracts by fresh medium and to be fully reversible within 20 hr after removal of the extracts. In addition, the stimulation of HMGCoA-reductase activity by insulin was efficiently blocked by the extracts, although other insulin-dependent phenomena, such as increased lactate production, were not influenced. These results suggest an indirect modulation of hydroxymethylglutaryl-CoA-reductase activity as the most likely inhibitory mechanism of the artichoke extracts. Screening of several known constituents of artichoke extracts revealed that cynaroside and particularly its aglycone luteolin were mainly responsible for inhibition, whereas chlorogenic acid was much less effective and caffeic acid, cynarin and other dicaffeoylquinic acids were without significant influence. Indeed, luteolin also efficiently blocked the insulin effect on cholesterol biosynthesis. In conclusion, these results demonstrate that artichoke extracts may inhibit hepatic cholesterol biosynthesis in an indirect but efficient manner and, thus, may contribute via this action to the recently confirmed hypolipidemic influence of this phytopharmacon in man.

High-dose extracts from artichoke leaves are traditionally used for the treatment of dyspeptic disorders (Anonymous, 1988; Ernst, 1995). It is commonly assumed that a pronounced choleretic action of the artichoke extracts, demonstrated recently in a clinical double-blind study (Kirchhoff *et al.*, 1994), is the most relevant reason for this therapy. However, there are several other interesting beneficial effects of this phytopharmacon (Fintelmann, 1996). Among these, a pronounced antioxidative capacity was reported recently (Gebhardt, 1997a), although a moderate hypolipidemic influence of artichoke extracts has already been known for many years (Hammerl and Pichler, 1959; Hammerl *et al.*, 1973; Lietti, 1977; Wojcicki *et al.*, 1981). The latter was attributed mainly to the dicaffeoylquinic acid isomere, cynarin (Eberhardt, 1973; Fröhlich and Zigler, 1973; Mars and Brambilla, 1974; Montini *et al.*, 1975; Pristautz, 1975; Wojcicki, 1976). Although the lipid lowering influence of artichoke extracts seems to be established beyond doubt (Gebhardt, 1995a; Wegener and Schmidt, 1995; Fintelmann, 1996), most studies on cynarin required high doses of this compound, to show significant effects, and, thus, were not fully convincing. Moreover, no mechanistic concepts emerged from these studies with respect to the assumed hypolipidemic action of cynarin and/or other compounds present in the artichoke extracts.

Recently, primary cultures of rat hepatocytes have been shown to provide a valuable *in vitro* system for elucidating the mechanisms of action of other phytopharmaca as demonstrated extensively in the case of garlic extracts (Gebhardt, 1993, 1995b; Gebhardt *et al.*, 1994b; Gebhardt and Beck, 1996). We have tested the hypothesis that artichoke extracts might directly influence (*i.e.*, inhibit) hepatic cholesterol biosynthesis using such primary cultures. Furthermore, first attempts were made to elucidate the possible mechanism of the inhibitory action and to compare the relative potency of several known components of artichoke extracts for inhibition.

Received for publication December 8, 1997.
[1] This work was supported by Sertürner Arzneimittel GmbH, Gütersloh, Germany.
[2] Current address: Universitätsklinikum, Institut für Biochemie, Liebigstr. 16, D-04103 Leipzig, Germany.

ABBREVIATIONS: HMGCoA-reductase, hydroxymethylglutaryl-CoA-reductase (EC 1.1.1.34); HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase (EC 1.1.1.27); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide; SI-TLC, silver-ion thin-layer chromatography.

Materials and Methods

Materials. Extracts of artichoke leaves (Hepar-SL forte) were obtained from Sertürner Arzneimittel GmbH (Gütersloh, Germany). Mevastatin was a kind gift from Dr. H. J. M. Kempen (TNO, Leiden, The Netherlands). Collagenase was provided by Knoll AG (Ludwigshafen, Germany), and radiolabeled compounds were purchased from Amersham Buchler (Braunschweig, Germany). The Extrelut^{R}20-columns were from Merck (Darmstadt, Germany). All other chemicals were from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Daisenhofen, Germany).

Animals. Male Sprague-Dawley rats (220–280 g) were used as hepatocyte donors. They were kept in a controlled 12-hr light-and-dark cycle on a standardized diet of Alma H 1003 (Botzenhardt, Kempten, Germany) and tap water *ad libitum*.

Isolation and cultivation of hepatocytes. Rat hepatocytes were isolated by the two-step collagenase technique described in detail elsewhere (Gebhardt *et al.*, 1990). Viability of the cells averaging to 91.8 ± 3.1% was routinely checked by staining with trypan blue. The isolated hepatocytes were suspended in Williams E medium containing 10% newborn calf serum, 2 mM glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml) and 10^{-7} M dexamethasone (Gebhardt, 1991). They were seeded in 1 ml of culture medium at a density of 1.25×10^5 cells/cm^2 into six-well dishes (Greiner, Germany) precoated with a thin film of collagen (Gebhardt and Jung, 1982) and were incubated at 37°C, 90% humidity and 7% CO_2 (Gebhardt *et al.*, 1994a). From 2 hr on, serum-free medium was used.

Preparation of artichoke extracts. Aqueous extracts of artichoke leaves were prepared as described (Gebhardt, 1997a). Briefly, 100 mg artichoke extract powder (Supra-Sern, Ch.-B.: 70.615, 70.612, 70.839, 70.788, 70.789 and 71.009, all containing 80% native extract) were mixed with 8 ml Williams medium E and gently shaken for 20 min at room temperature. Thereafter, the mixture was centrifuged to remove insoluble material and the supernatant was sterilized by filtration. This stock solution was further diluted with Williams medium E as indicated in figures and tables.

Incubation of the primary cultures with artichoke extracts or purified artichoke-derived compounds. Two hours after inoculation of the hepatocyte cultures the medium was removed and fresh medium was added containing [^{14}C]acetate [18.5 KBq/ml (0.5 μCi/ml)] and the compounds to be tested in the appropriate dilutions. After incubation at 37°C for additional 2 hr the medium was removed and the cell-layer was washed twice with saline and scraped into 2 ml of distilled H_2O. The cells were homogenized by sonication [20 sec, grade 3, (Gebhardt, 1991)]. In several experiments [^{14}C]acetate was replaced by [^{14}C]mevalonate [9 KBq/ml (0.24 μCi/mL)].

In experiments designed to reveal the time dependence of the exposure to the artichoke extracts the above mentioned incubation protocol was abandoned. Instead, the artichoke extracts were present in the medium during a preincubation period, followed by a second preincubation of variable length using fresh medium without the extracts and, finally, by the 2-hr incubation in the presence of [^{14}C]acetate. Control cultures, in this case, received as much medium changes as the test cultures.

Alternatively, for determination of the time course of inhibition, hepatocytes were incubated with the extracts in the presence of [^{14}C]acetate for shorter intervals up to 120 min. Acetate incorporation in this case was expressed as percent of control incubations during the same incubation period.

In some experiments, hepatocytes were exposed to the extracts for 2 hr and then cultured in fresh medium containing 1 μM insulin overnight for 16 hr. Subsequently, the cultures were incubated in the presence of [^{14}C]acetate for 2 hr, to compare the capacity for acetate incorporation. Alternatively, HMGCoA reductase activity was directly determined after the stimulation with insulin.

Determination of acetate incorporation into and separation of nonsaponifiable neutral lipids. The incorporation of [^{14}C]acetate into nonsaponifiable neutral lipids was determined after saponification of the homogenates with 0.5 M KOH in EtOH and subsequent efficient separation on Extrelut-columns (large-pore kieselgur) according to Pill *et al.* (1985) as described (Gebhardt, 1991, 1993). The neutral lipophilic nonsaponifiable substances were eluted with n-heptane (Gebhardt, 1991; Aufenanger *et al.*, 1986). The precursors such as [^{14}C]acetate or [^{14}C]mevalonate are retained on the column to more than 99%. For measurements of incorporation the eluate was collected directly in scintillation vials and measured in the scintillation counter after addition of 10 ml of Ultima Gold (Packard, Merident, CT). The yield of the elution step (93 ± 2%) and the recovery were determined as described (Gebhardt, 1993).

Determination of the sterol composition of the nonsaponifiable neutral lipid fraction. For determination of the newly synthesized sterol pattern the eluate was evaporated to dryness in a vacuum concentrator (Desaga, Heidelberg, Germany), taken up in 50 μl chloroform and applied to SI-TLC plates (silica gel 60F 254, Merck, Darmstadt, Germany) which were impregnated with silver nitrate and activated as described by Aufenanger *et al.* (1986). They were developed only once using a mixture of n-heptane/ethyl acetate (2:1, vol/vol) on a total length of 16 cm according to Aufenanger *et al.* (1986) and Pill *et al.* (1987). The amount of radioactivity was determined by scraping stripes of 2 mm into vials for scintillation counting. This technique provided quite similar results as radiochromatigraphic scanning of the TLC plates as determined in separate validation experiments. With [^{14}C]mevalonate a similar protocol was used. Alternatively, the sterol pattern was determined by HPLC on ODS Ultrasphere (0.46 × 25 cm) in a mobile phase of methanol/ethanol/water (86:10:4) according to Mayer *et al.* (1991) with collecting fractions from the column and detection of radioactivity by scintillation counting.

Determination of cytotoxicity and other analytical procedures. Cytotoxicity of the compounds tested was determined by means of the LDH leakage assay or the MTT assay as described (Gebhardt, 1997a). HMGCoA-reductase activity was determined in isolated microsomes as described (Shapiro *et al.*, 1974). Lactate was determined according to Gutmann and Wahlefeld (1974). Protein was determined following the procedure of Lowry *et al.* (1951).

Statistical evaluation. The data were evaluated statistically using Student's *t* test or analysis of variance. Data are given as means ± S.D.

Results

To avoid unspecific effects of artichoke extracts on the inhibition of cholesterol biosynthesis, we have directly determined the cytotoxic potential of these extracts in primary rat hepatocytes using MTT assay (table 1) and the LDH leakage assay (not shown). Both assays, in close accord, indicate that

TABLE 1
Cytotoxicity of artichoke extracts on cultured rat hepatocytes assessed with the MTT assay

Artichoke Extracts Conc. (mg/ml)	Absorbance (A_{550nm}-A_{660nm})[a]	
	2 hr	24 hr
None	396 ± 20[b]	348 ± 17
0.1	388 ± 18	354 ± 21
0.5	392 ± 24	349 ± 23
1.0	378 ± 23	341 ± 18
2.0	384 ± 19	309 ± 12[c]
4.0	365 ± 17[c]	226 ± 28[d]

[a] Determined during an incubation period of 2 or 24 hr in the presence of the test material followed by 1-hr incubation with MTT as described in "Materials and Methods."
[b] Values represent means ± S.D. of triplicate or quadruplicate (controls) determinations in three independent experiments.
[c] Statistically different from controls, $P < .01$.
[d] Statistically different from controls, $P < .001$.

cytotoxicity started at concentrations above 1 mg/ml within a period of 24 hr. At 10 mg/ml more than 80% of the hepatocytes were dead as judged from leakage of LDH into the culture medium (not shown). Therefore, the exact half-maximal effective cytotoxic concentrations could not be determined, because of the limited solubility of the compounds.

Incorporation of ^{14}C-acetate into nonsaponifiable lipids in cultured hepatocytes was inhibited by artichoke extracts in a biphasic manner (fig. 1). Between 0.007 and 0.1 mg/ml a slight inhibition was noted, amounting to about 20% of total incorporation. Above 0.1 mg/ml inhibition was reinforced, leading to a stronger, but still partial block of incorporation (approx. 65%) at 1 mg/ml and above (fig. 1). Chromatographic analysis using silver-ion thin layer chromatography revealed that more than 93% of the radioactivity incorporated into the fraction of nonsaponifiable neutral lipids, could be attributed to cholesterol even at high concentrations of the extracts (fig. 2). In particular, no accumulation of sterol precursors was noted under these conditions. Similar findings were obtained, when the pattern of sterol precursors was analyzed by HPLC (not shown). These results indicate that artichoke extracts do not seem to inhibit enzymatic steps involved in the complex conversion of squalen to cholesterol as was found for aqueous extracts of garlic (Gebhardt, 1991, 1993).

However, replacement of ^{14}C-acetate by ^{14}C-mevalonate largely omitted the inhibiting effect of artichoke extracts on the incorporation of these labeled precursors into nonsaponifiable lipids even at high extract concentrations (table 2). Similarly, mevastatin, a known HMGCoA-reductase inhibitor, also inhibited the incorporation of ^{14}C-acetate much more than that of ^{14}C-mevalonate. Thus, it is most likely that the inhibition exerted by the artichoke extracts is effective at the level of HMGCoA-reductase. However, when added directly to enzymatic assays for HMGCoA-reductase, artichoke extracts, in contrast to mevastatin, did not directly inhibit HMGCoA-reductase activity in isolated microsomes (table 3). This effect was seen up to the highest concentration of the extracts used.

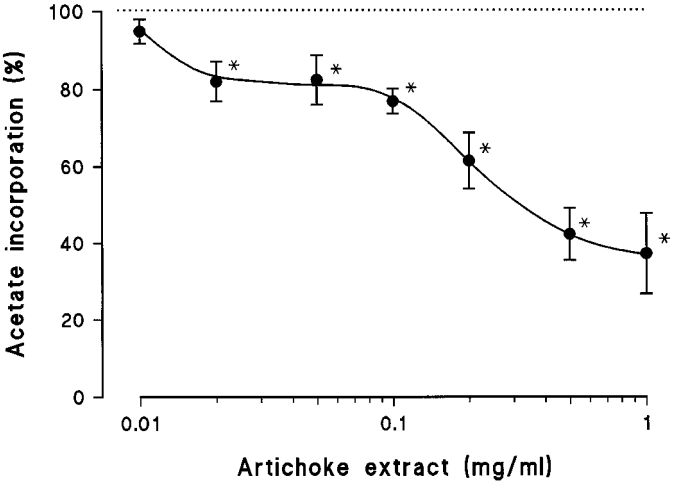

Fig. 1. Inhibition of the incorporation of ^{14}C-acetate into nonsaponifiable lipids by artichoke extracts in rat hepatocytes. Incorporation of ^{14}C-acetate into nonsaponifiable lipids was determined during a 2-hr period in controls and in the presence of different concentrations of artichoke extracts as described in "Materials and Methods." Data represent means ± S.D. of six independent experiments. *Statistically significant from controls: $P < .01$.

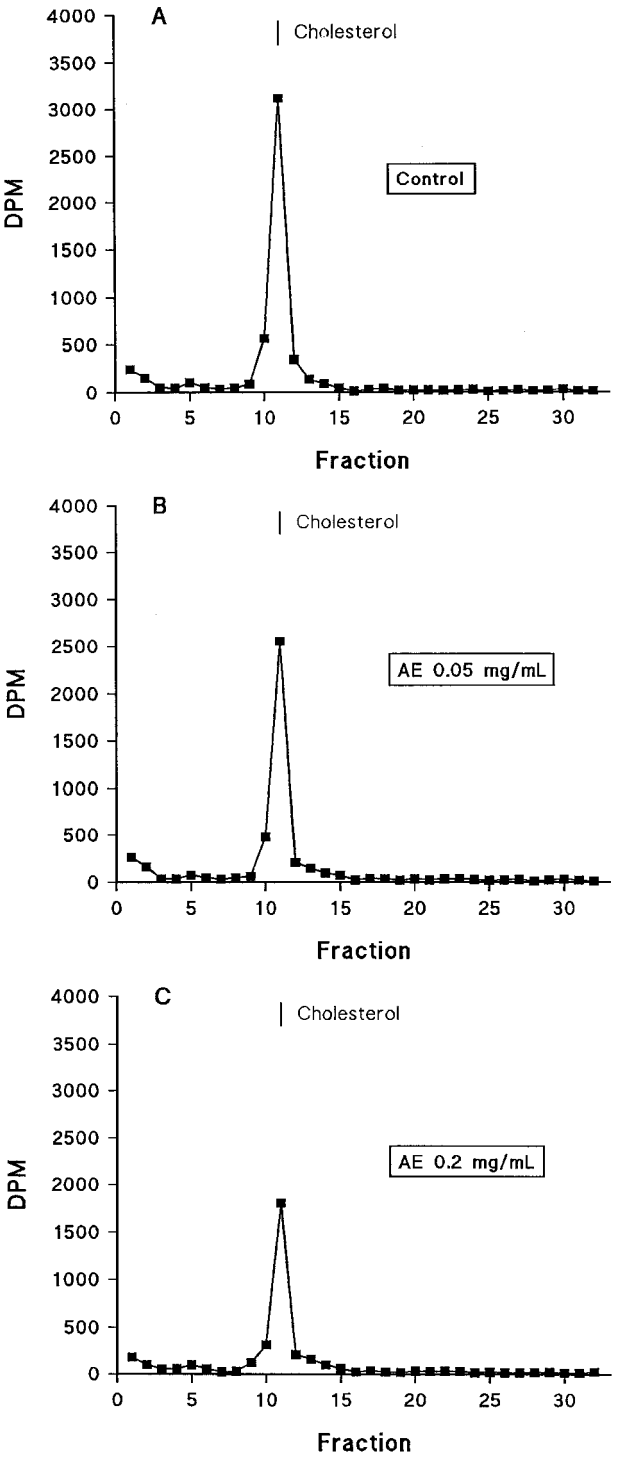

Fig. 2. Profile of sterol compounds produced by cultured hepatocytes in the absence (A) or presence (B and C) of artichoke extracts. Incorporation of ^{14}C-acetate into sterol compounds (cholesterol and cholesterol precursors) was determined by SI-TLC as described in "Materials and Methods." Concentrations of artichoke extracts were 0.2 mg/ml (B) and 0.1 mg/ml (C). The peak of radioactivity eluted in fraction 11 corresponds to cholesterol.

Most interestingly, the inhibition of ^{14}C-acetate incorporation into nonsaponifiable lipids occurred already within a few minutes and was completed after 30 to 40 min (fig. 3). After a preincubation of at least half an hour with the artichoke extracts inhibition lasted for several hours even after switch-

TABLE 2

Inhibition of the biosynthesis of nonsaponifiable neutral lipids from ^{14}C-acetate or ^{14}C-mevalonate by artichoke extracts and mevastatin in rat hepatocytes

Material Tested	Concentration	Incorporation of Radioactivity into Nonsaponifiable Lipids (dpm/μg Protein)			
		^{14}C-acetate	% Inhib.	^{14}C-mevalonate	% Inhib.
None		7.1 ± 0.3		6.3 ± 0.4	
Artichoke extract	1000	2.7 ± 0.2	62[a]	6.0 ± 0.4	5
	100	5.7 ± 0.1	20[b]	6.2 ± 0.3	0
	20	6.5 ± 0.2	9[b]	6.4 ± 0.3	0
Mevastatin	100	0.6 ± 0.1	91[a]	5.3 ± 0.2	16
	30	1.5 ± 0.1	79[a]	5.5 ± 0.2	12

AE-1 and AE-2: artichoke extracts at concentrations of 100 and 20 μg/ml, respectively. Values represent means ± S.D. of three independent experiments.
[a] Statistically different from controls: P < .001.
[b] Statistically different from controls: P < .01.

TABLE 3

Inhibition of HMGCoA-reductase by artichoke extracts and mevastatin

Material Tested	Concentration (μg/ml)	HMGCoA-Reductase Activity[a] (pmol/min/mg Protein)	
			%
None		238 ± 21[b]	100
Artichoke extract	1000	221 ± 23	93
	100	227 ± 24	96
	20	236 ± 17	99
Mevastatin	100	12 ± 4[c]	5
	30	33 ± 5[c]	14

[a] HMGCoA-reductase activity in microsomes prepared from hepatocytes was measured in the presence of the materials shown.
[b] Values represent means ± S.D. of three determinations.
[c] Statistically different from controls: P < .001.

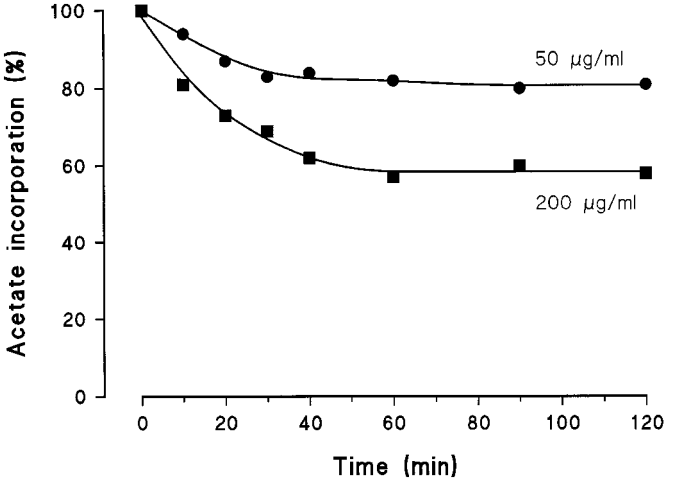

Fig. 3. Time course of the inhibition of acetate incorporation by artichoke extracts. Extracts (50 and 200 μg/ml) were added together with ^{14}C-acetate and incorporation into sterol compounds was determined by SI-TLC as described in "Materials and Methods." Incorporation was expressed as percent of control cultures incubated for the same period of time in the absence of artichoke extracts.

TABLE 4

Time dependence of the inhibition of the biosynthesis of non-saponifiable neutral lipids from ^{14}C-acetate by artichoke extracts

Concentration of AE (μg/ml)	Preincubation Time (hr)[a]		Inhibition (%)[b]
	With AE	Without AE	
100	0.5	0	18 ± 4[c,d]
100	0.5	2	17 ± 3[d]
50	1	0	16 ± 4[d]
50	1	2	11 ± 2[e]
100	1	0	21 ± 5[d]
100	1	2	22 ± 6[d]
100	1	8	16 ± 5[d]
100	1	20	3 ± 2

[a] Acetate incorporation was determined at the end of the total preincubation period during the usual 2-hr incubation described in "Materials and Methods."
[b] Inhibition was expressed as percent of time matched controls.
[c] Values represent means ± S.D. of triplicate determinations in three independent experiments.
[d] Statistically different from controls, P < .01.
[e] Statistically different from controls, P < .05.

TABLE 5

Influence of artichoke extracts on insulin-dependent stimulation of ^{14}C-acetate incorporation into non-saponifiable neutral lipids and lactate formation

Cultivation[a]	Incorporation of ^{14}C-Acetate into Nonsaponifiable Lipids (dpm/μg Protein)[b]		Lactate Production (μmol/mg Protein)[c]
		% Inhib.[d]	
No addition	5.3 ± 0.7[e]		0.56 ± 0.03
Artichoke extract[f]	3.8 ± 0.6	28[g]	0.62 ± 0.05
Insulin[h]	15.9 ± 1.4		1.96 ± 0.11
Insulin + artichoke extract	8.2 ± 0.8	48[i]	1.87 ± 0.15
Luteolin[j]	4.5 ± 0.4	16	0.52 ± 0.04
Insulin + luteolin	5.1 ± 0.5	68[i]	1.55 ± 0.16[g]

[a] Cultivation between 2 and 20 hr after seeding was performed in the presence or absence of the compounds listed below.
[b] Acetate incorporation was performed in fresh medium in the presence of ^{14}C-acetate without further additions for 2 hr.
[c] Lactate was determined at the end of the cultivation period.
[d] Inhibition by artichoke extract in relation to respective control.
[e] Values represent means ± S.D. of triplicate determinations from three independent experiments.
[f] 0.1 mg/ml.
[g] Statistically different from respective control: P < .01.
[h] 0.1 μM.
[i] Statistically different from respective control: P < .001.
[j] 0.01 mg/ml.

ing to extract-free medium as shown in table 4. However, within a period of 20 hr in extract-free medium inhibition was fully reversible (table 4). Similar effects were seen even at low concentrations of the artichoke extracts, although the extent of inhibition decreased more rapidly in this case.

Furthermore, insulin stimulation of acetate incorporation was efficiently reduced by artichoke extracts (table 5). Other effects of insulin, such as the stimulation of lactate production, were not affected (table 5). Direct assays of HMGCoA-reductase at the end of the cultivation with insulin revealed a reduction of enzyme activity from 314 ± 36 pmol/min/mg protein in the presence of insulin to 172 ± 24 pmol/min/mg protein in the presence of insulin and artichoke extracts. In the absence of insulin HMGCoA-reductase activity amounted to 124 ± 27 and 87 ± 16 pmol/min/mg protein in the absence or presence of the extracts, respectively. The parallel changes in enzyme activity and acetate incorporation indicate that the reduction of HMGCoA-reductase activity by the artichoke

extracts might be responsible for the selective effect on acetate incorporation.

When several purified compounds known to be present in artichoke extracts, such as caffeic acid, chlorogenic acid, cynarin (1,5-dicaffeoyl quinic acid), cynaroside and luteolin, were tested for their ability to inhibit ^{14}C-acetate incorporation into nonsaponifiable lipids different results were obtained (table 6). Caffeic acid and cynarin did not significantly inhibit at all, although chlorogenic acid reached 15% inhibition at 100 μg/ml. Additional experiments showed that other dicaffeoylquinic acids also did not inhibit in this concentration range (not shown). In contrast, cynaroside and its aglycone luteolin at 100 μg/ml inhibited by 20 and 60%, respectively (table 6). At 50 μg/ml luteolin still inhibited by more than 50%. When luteolin was present at 10 μg/ml during 18 hr of incubation inhibition amounted to 16% (table 5). However, in the presence of insulin the same concentration largely blocked the increase in cholesterol biosynthesis and resulted in an overall inhibition of 68% (table 5). Higher concentrations did not result in stronger inhibition. Neither cell damage nor an equally potent reduction of insulin-stimulated lactate production was found under these conditions. Additional viability assays revealed that all compounds were not cytotoxic at the concentrations used.

Discussion

The standardized extract of artichoke leaves (HEPAR SL forte) reduced cholesterol biosynthesis in cultured rat hepatocytes in a biphasic manner. At low concentrations a partial inhibition (approximately 20%) was noted, although at higher concentrations (more than 0.1 mg/ml) inhibition increased until almost 65% of cholesterol biosynthesis was blocked at 1 mg/ml. Because cytotoxic effects were noted exclusively above this concentration, it appears unlikely that the strong inhibition at high concentrations is due to an irreversible deterioration of the hepatocytes or of this metabolic pathway. Indeed, the cells were found to perform undisturbed urea synthesis even at a concentration of 2 mg/ml of the artichoke extracts (Gebhardt R, unpublished results). Thus, different components of artichoke extracts with different profiles of interaction with cholesterol biosynthesis seem to be responsible for this biphasic inhibition (see below).

With respect to therapeutically significant doses, the lower concentration range characterized by the plateau inhibition seems to be of the greatest importance, because it is unlikely that higher serum concentrations are reached even after consumption of high-dosed artichoke preparations. The partial inhibition observed in this range as well as at higher concentrations seems to occur at the level of HMGCoA-reductase as suggested by the absence of inhibition when radiolabeled acetate was replaced by mevalonate, but could not be attributed to a direct inhibition of this enzyme as obvious from comparison with mevastatin, a known inhibitor of this enzyme (Endo *et al.*, 1976). Instead, an indirect modulation of HMGCoA-reductase activity by artichoke extracts is more likely in view of our results. An interference with acetate uptake into hepatocytes could be excluded (Gebhardt R, unpublished results).

At present, there is not much information about the mechanism of this indirect influence. However, it is very interesting that the effect of the artichoke extracts occurs in a time-dependent manner reaching a steady-state level of inhibition after 30 to 40 min. The inhibitory effect is not limited to the actual period of incubation, but is seen even hours later, when the extracts were washed out with fresh medium long before. Nevertheless, the process is fully reversible within 20 hr. These results suggest that some regulatory mechanism of HMGCoA-reductase is influenced in a long-lasting manner. This influence could possibly involve 1) inhibition of activating mechanisms and/or 2) stimulation of inactivating mechanisms of the enzyme (fig. 4). Additional evidence for such a mechanism is provided by the fact that artichoke extracts effectively blocked insulin-dependent stimulation of HMG-CoA-reductase without affecting insulin effects in general.

In this context, it is interesting to note that in the case of garlic a powerful indirect inhibition via interference with the phosphorylation/dephosphorylation control of HMGCoA-reductase has been found (Gebhardt, 1995b, 1997b). However, this interference is restricted to garlic-derived organosulfur compounds (Gebhardt, 1995b; Gebhardt and Beck, 1996). Preliminary experiments showed that 1) similar mechanisms were not affected and 2) protein content was not reduced by the artichoke extracts (Gebhardt R, unpublished results).

As obvious from the analysis of the sterol pattern produced by cultured hepatocytes in the presence of artichoke extracts, no influence at later enzymatic steps of the cholesterol biosynthetic pathway was found. Again, this is different from

TABLE 6
Inhibition of the biosynthesis of non-saponifiable neutral lipids from ^{14}C-acetate by purified compounds present in artichoke extracts

Material Tested	Inhibition (%)[a]	
	50 μg/ml	100 μg/ml
Caffeic acid	3.0 ± 1.6 (3)[b]	2.8 ± 0.6 (3)
Chlorogenic acid	10.3 ± 2.3 (3)[c]	15.0 ± 5.6 (3)[c]
Cynarin	1.8 ± 0.9 (3)	2.6 ± 0.4 (3)
Cynaroside	19.2 ± 3.8 (4)[d]	22.2 ± 4.2 (5)[d]
Luteolin	51.3 ± 4.7 (3)[d]	62.8 ± 5.2 (3)[d]

[a] Inhibition is expressed as percent controls.
[b] Values represent means ± S.D. of triplicate determinations from several independent experiments (number in parentheses).
[c] Statistically different from controls, $P < .05$.
[d] Statistically different from controls, $P < .01$.

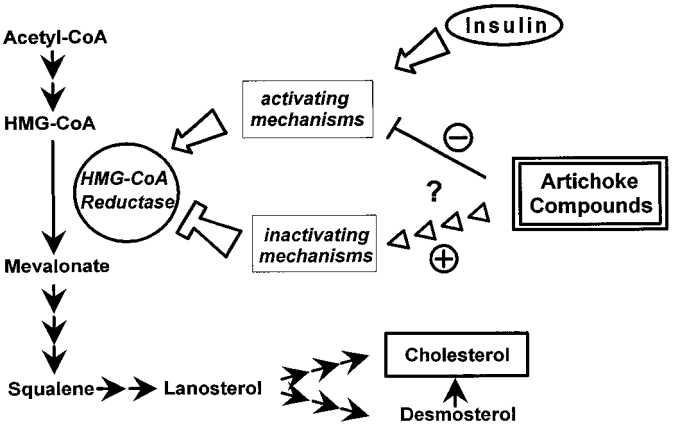

Fig. 4. Schematic depiction of the pathway for cholesterol biosynthesis illustrating the presumable mode of action of artichoke compounds. The scheme gives a simplified overview of cholesterol biosynthesis from acetyl-CoA with individual arrows not always meant to indicate single enzymatic steps. As indicated artichoke components may indirectly reduce the activity of HMGCoA-reductase via inhibition of activating regulatory mechanisms and/or stimulation of inactivating regulatory mechanisms.

the influence of garlic extracts where multiple sites of interactions are observed, including inhibition of lanosterol 14α-demethylase (Gebhardt, 1993; Gebhardt *et al.*, 1994b), which eventually result in a different type of indirect inhibition by precursors of lanosterol (Gebhardt, 1993, 1997b), similar to what has been hypothesized for other inhibitors of lanosterol 14α-demethylase (Gibbons *et al.*, 1980; Trzaskos *et al.*, 1987; Mayer *et al.*, 1991). Consequently, at least in hepatocytes, no accumulation of sterol precursors should occur in response to artichoke extracts. This is important, because several cholesterol precursors such as desmosterol are well known for adverse effects in man (Goh *et al.*, 1989).

Concerning the active principle, the assay of several compounds known to occur in artichoke extracts revealed that luteolin seems to represent the active ingredient. Cynaroside (luteolin-7-O-glucoside) and to some extent chlorogenic acid may also participate in inhibition, although much less effective, and may explain the biphasic response curve shown in figure 1. Indeed, the effect of luteolin and of the other compounds seemed monophasic (Gebhardt R, unpublished results). Although the content of chlorogenic acid in the artichoke extracts (ranging from 0.5 to 1.5%) was approximately four times as high as that of the flavonoids (luteolin and cynarosid) the latter compounds seem to be responsible for the inhibitory influence of the extracts, particularly, because additional luteolin may be liberated from cynaroside under physiological conditions.

Luteolin not only exerted the highest inhibitory potency, but also efficiently blocked the stimulation of cholesterol biosynthesis by insulin. This effect that was quite sensitive further emphasizes the indirect nature of the inhibition. Whether the known property of luteolin to inhibit protein kinases (Jinsart *et al.*, 1992) is part of this mechanism is currently investigated. Interestingly, the reduction of insulin-stimulated lactate production by luteolin was low compared to the effect on cholesterol biosynthesis and was much less than reported by Suolinna *et al.* (1975) for Ehrlich ascites tumor cells.

However, the results clearly demonstrate that cynarin which was considered as the active principle for a long time (Fröhlich and Zigler, 1973; Mars and Brambilla, 1974; Montini *et al.*, 1975; Pristautz, 1975; Wojcicki, 1976, 1978), is completely inactive with respect to the inhibition of hepatic cholesterol biosynthesis. The same seems to be true for the naturally occurring dicaffeoylquinic acids (Gebhardt R, unpublished results). Thus, effects ascribed to these compounds *in vivo* must be due to alternative interactions affecting blood lipid and cholesterol levels.

In conclusion, our results indicate that artichoke extracts may reduce hepatic cholesterol biosynthesis in a physiologically favorable manner, *i.e.*, by indirect inhibition that might avoid problems known to occur with strong direct inhibitors of HMGCoA-reductase during long-term administration. Because artichoke extracts may also enhance biliary cholesterol excretion as a result of the choleretic influence (Kirchhoff *et al.*, 1994), both mechanisms may contribute to the clinically known reduction of blood cholesterol levels.

Acknowledgments

The excellent technical assistance of Mrs. Martina Fausel and Mrs. Andrea Hanika is gratefully acknowledged.

References

Anonymous (Korrektur 1.9.1990) Monographie Cynarae folium, Artischockenblätter. BGA-Aufbereitungsmonographie, Bundesanzeiger 6.7.1988.
Aufenanger J, Pill J, Schmidt FH and Stegmeier K (1986) The effects of BM 15.766, an inhibitor of 7-dehydrocholesterol -$\triangle^7$-reductase, on cholesterol biosynthesis in primary rat hepatocytes. *Biochem Pharmacol* **35:**911–916.
Eberhardt G (1973) Untersuchungen über die Wirkung von Cynarin bei Leberzellverfettung. *Z Gastroenterol* **11:**183–186.
Endo A, Kuroda M and Tanzawa K (1976) Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. *FEBS Lett* **72:**323–328.
Ernst E (1995) Die Artischocke: eine Heilpflanze mit Geschichte und Zukunftsperspektive. *Naturamed* **10:**1–4.
Fintelmann V (1996) Antidyspeptische und lipidsenkende Wirkungen von Artischockenblätterextrakt. Ergebnisse von Hepar-SL-forte an 553 Patienten. *Z Allg Med* **72:**48–57.
Fröhlich E and Zigler W (1973) Über die lipidsenkende Wirkung von Cynarin. *Subsidia Med* **25:**5–12.
Gebhardt R (1991) Inhibition of cholesterol biosynthesis by a water-soluble garlic extract in primary cultures of rat hepatocytes. *Arzneim-Forsch/Drug Res* **41:**800–804.
Gebhardt R (1993) Multiple inhibitory effects of garlic extracts on cholesterol biosynthesis in hepatocytes. *Lipids* **28:**613–619.
Gebhardt R (1995a) Artischockenblätterextrakt: In-vitro Nachweis einer Hemmwirkung auf die Cholesterin-Biosynthese. *Med Welt* **46:**348–350.
Gebhardt R (1995b) Amplification of palmitate-induced inhibition of cholesterol biosynthesis in cultured rat hepatocytes by garlic-derived organosulfur compounds. *Phytomedicine* **2:**29–34.
Gebhardt R (1997a) Antioxidative and protective properties of extracts from leaves of the artichoke (*Cynara scolymus L.*) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. *Toxicol Appl Pharmacol* **144:**279–286.
Gebhardt R (1997b) Garlic: The key to sophisticated lowering of hepatocellular lipid. *Nutrition* **44:**379–380.
Gebhardt R and Jung W (1982) Biliary secretion of sodium fluorescein in primary cultures of adult rat hepatocytes and its stimulation by nicotinamide. *J Cell Sci* **56:**233–244.
Gebhardt R and Beck H (1996) Differential effects of garlic-derived organosulfides on cholesterol biosynthesis in primary rat hepatocyte cultures. *Lipids* **31:**1269–1276.
Gebhardt R, Fitzke H, Fausel M, Eisenmann-Tappe I and Mecke D (1990) Influence of hormones and drugs on glutathione-S-transferase levels in primary cultures of adult rat hepatocytes. *Cell Biol Toxicol* **6:**365–378.
Gebhardt R, Alber J, Wegner H and Mecke D (1994a) Different drug metabolizing capacities in cultured periportal and pericentral hepatocytes. *Biochem Pharmacol* **48:**761–766.
Gebhardt R, Beck H and Wagner KG (1994b) Inhibition of cholesterol biosynthesis by allicin and ajoene in rat hepatocytes and HepG2 cells. *Biochim Biophys Acta* **1213:**57–62.
Gibbons GF, Pullinger CR, Chen HW, Cavenee WK and Kandutsch AA (1980) Regulation of cholesterol biosynthesis in cultured cells by probable natural precursor sterols. *J Biol Chem* **255:**395–400.
Goh EH, Colles SM and Otte KD (1989) HPLC analysis of desmosterol, 7-dehydrocholesterol, and cholesterol. *Lipids* **24:**652–655.
Gutmann I and Wahlefeld AW (1974) L-(+)-Lactat. Bestimmung mit Lactat-Dehydrogenase und NAD, in *Methoden der Enzymatischen Analyse* Vol II, 3rd ed (Bergmeyer U ed) pp 1510–1514, Verlag Chemie, Weinheim.
Hammerl H and Pichler O (1959) Untersuchungen über den Einfluß eines Artischockenextraktes auf die Serumlipide in Hinblick auf die Arteriosklerosoprophylaxe. *Wiener Med Wochenschr* **109:**853–855.
Hammerl H, Kindler K, Kränzl C, Nebosis G, Pichler O and Studlar M (1973) Über den Einfluß von Cynarin auf Hyperlipidämien unter besonderer Berücksichtigung des Typs II (Hypercholesterinämie). *Wiener Med Wochenschr* **41:**601–605.
Jinsart W, Ternai B and Polya GM (1992) Inhibition of rat liver cyclic AMP-dependent protein kinase by flavonoids. *Biol Chem Hoppe-Seyler* **373:**205–211.
Kirchhoff R, Beckers CH, Kirchhoff GM, Trinczek-Gärtner H, Petrowitz O and Reimann H-J (1994) Increase in choleresis by means of artichoke extract. Results of a randomised placebo-controlled double-blind study. *Phytomedicine* **1:**107–115.
Lietti A (1977) Choleretic and cholesterol lowering properties of two artichoke extracts. *Fitoterapia* **484:**153–158.
Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.
Mars G and Brambilla G (1974) Wirkung von 1,5-Dicaffeoylchinasäure (Cynarin) auf die Hypertriglyceridämie im fortgeschrittenen Alter. *Med Welt* **25:**1572–1574.
Mayer RJ, Adams JL, Bossard MJ and Berkhout TA (1991) Effects of a novel lanosterol 14α-demethylase inhibitor on the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in Hep G2 cells. *J Biol Chem* **266:**20070–20078.
Montini M, Levoni P, Ongaro A and Pagani G (1975) Kontrollierte Anwendung von Cynarin in der Behandlung hyperlipämischer Syndrome. *Arzneimittel-Forsch* **25:**1311–1314.
Pill J, Johne G, Stegmeier K, Frey A, Frey B and Schmidt FH (1985) New, rapid method for the separation of labelled sterols synthesized by cell cultures by means of Extrelut. *Fresenius Z Anal Chem* **322:**512–513.
Pill J, Aufenanger J, Stegmeier K, Schmidt FH and Müller D (1987) Thin-layer chromatography of radioactively labelled cholesterol and precursors from biological material. *Fresenius Z Anal Chem* **327:**558–560.

Pristautz H (1975) Cynarin in der modernen Hyperlipämiebehandlung. *Wien Med Wschr* **125:**705–709.
Shapiro DJ, Nordstrom JL, Mitchelen JJ, Rodwell JW and Schimke RT (1974) Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. *Biochim Biophys Acta* **370:**369–377.
Suolinna EM, Buchsbaum RN and Racker E (1975) The effect of flavonoids on aerobic glycolysis and growth of tumor cells. *Cancer Res* **35:**1865–1872.
Trzaskos JM, Favata MF, Fischer RT and Stam SH (1987) In situ accumulation of 3ß-hydroxylanost-8-en-32-aldehyde in hepatocyte cultures. *J Biol Chem* **262:** 12261–12268.
Wegener T and Schmidt M (1995) Artischockenblätterextrakt-Lipidsenkung auf pflanzlicher Basis. *Ärztez Naturheilverfahren* **36:**378–389.
Wojcicki J (1976) Effect of 1,5-dicaffeoylquinic acid on ethanol-induced hypertriglyceridemia. *Arzneimittelforschung* **26:**2047–2048.
Wojcicki J (1978) Effect of 1,5-dicaffeoylquinic acid (cynarine) on cholesterol levels in serum and liver of acute ethanol-treated rats. *Drug Alcohol Depend* **3:**143–145.
Wojcicki J, Samochowiec L and Kosmider K (1981) Influence of an extract from artichoke (Cynara scolymus L.) on the level of lipids in serum of aged men. *Herba Pol* **27:**265–268.

Send reprint requests to: Prof. Dr. Rolf Gebhardt, University of Leipzig, Institute of Biochemistry, Liebigstr. 16, D-04103 Leipzig, Germany.