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Troxerutin protects the isolated perfused rat liver from a possible lipid peroxidation by coumarin

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Dedicated to Prof. Dr. Götz Harnischfeger on his 65th birthday

Abstract

For more than 40 years coumarin has been successfully used in the therapy of chronic venous insufficiency (CVI). The occurrence of liver injuries is rather rare and happens predominantly when doses are administered which are significantly higher than necessary for therapeutical use. Such effects caused by high coumarin concentrations are reproducible in in vivo experiments in mice or rats and HepG2-cells. In order to characterize the mechanism of liver injuries, the isolated perfused rat liver has been chosen as model. Since liver injuries are quite rare, if coumarin is used in co-medication with troxerutin, a possible protective influence of this flavonoid has been investigated.

In concentrations higher than 4 mmol/l, coumarin alone is effective in the isolated perfused rat liver. Then the release of the enzymes alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) increases and there is a measurable reduction of perfusion flow, oxygen consumption and rate of bile secretion. Additionally, the concentrations of hepatic adenosine triphosphate (ATP) and oxidized and total glutathione (GSSG/GSH) decrease. In the livers of fasting animals, coumarin doubles the concentration of hepatic malondialdehyde (MDA). This effect cannot be detected if troxerutin is added. In general, troxerutin reduces the concentration of all coumarin-metabolites in the perfusate and bile and changes the ratio of the main metabolites, coumarin: 3-hydroxycoumarin: 7-hydroxycoumarin. An analysis of the metabolic steps also shows that the amount of coumarin eliminated via faeces does not stem from absorbed coumarin, because the amount of orally applied coumarin detectable in the bile is less than 1%.

The study demonstrates that troxerutin has hepatoprotective properties and thus protects the liver from a possible lipid peroxidation caused by coumarin. However, it is necessary to point out that these adverse effects caused by coumarin can be detected only in very high concentrations considerably above the regular therapeutical dosage. This allows the conclusion that troxerutin is a beneficial cofactor in coumarin preparations used for the therapy of chronic venous insufficiency.

Keywords: Coumarin; Troxerutin; Cytochrome P450; Lipid peroxidation; Isolated perfused rat liver

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Introduction

The chromenone coumarin is known as a widespread plant-constituent, contained, for example, as most important constituent in Sweet Clover *Melilotus officinalis* (L.) PALL., *M. altissima* THUILL. (Feuer, 1974). In humans, coumarin exhibits anti-inflammatory, oedema-protective and lymphokinetic effects. Clinical studies have shown that a therapy with coumarin reduces all forms of high-protein oedemas, especially lymphoedema, by causing a proliferation of macrophages and to increase the normal lysis of the excess protein (Casley-Smith et al., 1993; Casley-Smith, 1999; Piller, 1993). Additionally, as results of clinical investigations with metastatic forms of malignant melanoma, renal carcinoma and prostatic carcinoma, immunomodulatory and tumouristatic properties have been published (Berkarda et al., 1983; Hardt and Ritschel, 1983; Marshall et al., 1987; Marshall et al., 1990; Piller, 1978; Siegers and Bostelmann, 1993; Thornes et al., 1989; Wall et al., 1993; Weber et al., 1998; Zänker et al., 1984).

The variety of the coumarin metabolism in different species is of great importance (Fentem and Fry, 1992). In mice and humans, the responsible hepatic enzymes have been identified as CYP2A5 and CYP2A6, hydroxylating coumarin on position C7 to umbelliferone. In previous years, some genetic variations of the human cytochrome P450 isozyme have been described which are helpful for understanding the different metabolic rates of some populations (Bourian et al., 1999; Lewis et al., 1999; Oscarson et al., 1999). In contrast to humans and mice, rats metabolize coumarin mainly by hydroxylation on position C3. In in vitro studies using HepG2-cells, coumarin shows cytotoxic effects in concentrations above 1.4 mM (Siegers et al., 1998). In wistar-rats, liver damage is detectable by histology after 9 weeks (female) or 19 weeks (male), if coumarin is administered orally in doses of 64 or 128 mg/kg body weight (Preuss-Ueberschär and Ueberschär, 1988). The alternative metabolic route of 3-hydroxylation is also discussed as a possible pathway in humans with defects in the main route of coumarin-metabolism, creating umbelliferone and its subsequent glucuronidation to 7-hydroxycoumaringlucuronide (Cox et al., 1989; Miles et al., 1990; Rautio et al., 1992). In 1984, an underlying mechanism of liver damage was postulated: In the course of creating 3-hydroxycoumarin, 3,4-coumarinepoxide subsists as a reactive intermediate. In the meantime, new studies show that coumarin is metabolized to 3-hydroxycoumarin without the postulated epoxide (Lake, 1984; Born et al., 1997). Fig. 1 shows the most important pathways for coumarin in humans. Troxerutin, a rutoside derivative, is known as a radical scavenger drug with antioxidant effects, so that a treatment with this flavonoid increases the healing of capillary endothelial defects. In general, several studies

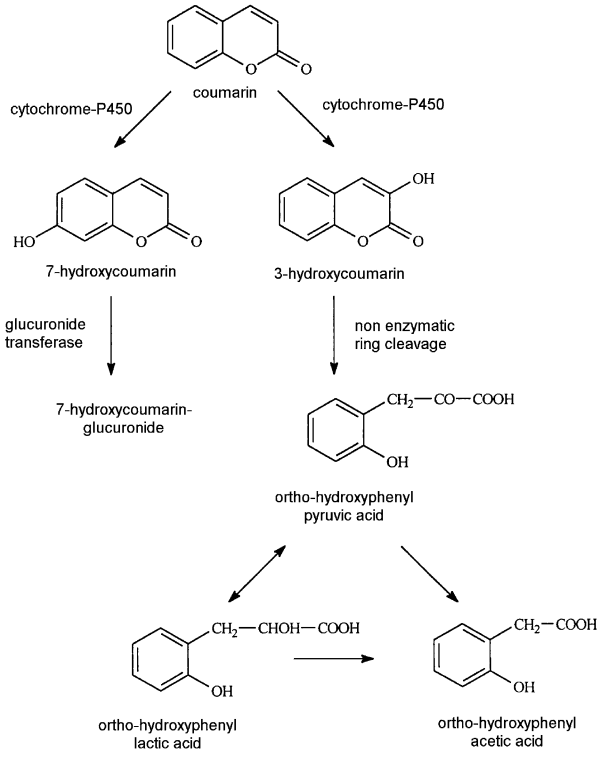

Fig. 1. Metabolic pathway of coumarin in humans and animals.

have shown the beneficial properties of troxerutin for the indication chronic venous insufficiency (CVI), especially in combination with coumarin. Furthermore, hepatic protective effects have been reported for troxerutin (Boisseau et al., 1995; Kiesewetter et al., 1997; Markwardt, 1996; Robak and Gryglewski, 1988).

In the meantime, investigations regarding liver damage have demonstrated that the number of adverse effects observed during a coumarin–troxerutin therapy does not differ from those in patients without this medication (Bruppacher et al., 1997; Freudenstein and Schröder-Bernhardi, 1998). However, as shown in rats, liver damage does occur, if coumarin is applied by itself. Therefore, it is of great interest to analyse the hepatic protective mechanisms of troxerutin for preventing possible coumarin-induced liver injuries in the rat-model. In addition, the main metabolites of coumarin, 3-hydroxycoumarin and 7-hydroxycoumarin, respectively, should be quantified for changes in the metabolism. This can be helpful for understanding the mechanisms of the biological reactions.

Materials and methods

Chemicals

The chemicals were purchased from Aldrich Europe/Janssen Pharmaceuticals, Düsseldorf, Germany (7-

hydroxycoumarin), Boehringer–Mannheim, Mannheim, Germany (reduced glutathione, glutathione-reductase and NADPH), Ceva GmbH, Bad Segeberg, Germany (Nembutal®), E. Merck, Darmstadt, Germany (all substances not specifically attributed to other source), Novartis, Grenzach–Wyhlen, Germany (Liquemin®), Sigma, München, Germany (BSA, sodium taurocholate and thiobarbituric acid) and Synopharma, Hamburg, Germany (butylhydroxytoluol).

Treatment of animals

Male Wistar rats (270–400 g body weight; breeder: Harlan–Winkelmann) were used throughout. The animals were fed with a standard diet (Altromin pellets). When fasting, the rats were deprived of food, not of tap water, for 18–20 h before surgery. In the experiments, the number of animals (and livers, respectively) were usually six per group.

Preparation of livers and liver perfusion

The removal of the liver and its connection to a recirculating perfusion system was performed as previously described (Meijer et al., 1981; Sedlak and Lindsay, 1968; Strubelt et al., 1981). After removal of the liver, the rats died from exsanguination. The albumin-, serum- and hemoglobin-free perfusion medium consisted of 250 ml Krebs–Henseleit buffer, pH 7.4 (118 mM NaCl, 500 mM KCl, 1.1 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.25 mM $CaCl_2$). Sodium taurocholate (32 mg/h) was infused into the perfusate to stimulate bile secretion. The perfusion medium was continuously gassed with carbogen (95% O_2, 5% CO_2) yielding an oxygen partial pressure of about 600 mmHg. Perfusion was performed under conditions of constant pressure (250 mm H_2O) throughout the experiment and the perfusion flow rate was initially regulated at 60 ml/min using a tube clamp. The experiments were started after a 30 min equilibration period (time 0) by adding either 4 mM coumarin or 4.73 mM troxerutin or both to the perfusate. After 120 min, the experiments were stopped. Prior to the experiments it had been shown that this coumarin concentration of 4 mM induces a liver damage. Oxygen consumption of the isolated perfused livers was calculated by measuring the differences in oxygen concentrations between the influent and effluent perfusate using a micro pH/blood gas analyser 1306 (Instrumention Laboratory). The perfusion flow was determined every 30 min by damming the effluent perfusate in a special vial without impairing the perfusion flow and measuring the volume after 20 s. Bile was sampled every 30 min and the rate of bile secretion was calculated in µg/g min. For biochemical determinations, samples of 2 ml were taken from the perfusate every 30 min. Livers were weighed before connecting them to the perfusion system and after completion of the experiments. Finally, the livers were frozen in liquid nitrogen.

Biochemical determinations

The activities of alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH) and lactate dehydrogenase (LDH) were assayed using commercial kits from Sigma Munich (Munich, Germany) and Boehringer–Mannheim (Mannheim, Germany). The methods for determining perfusate enzyme concentrations, malondialdehyde (MDA) in the livers by coupling to thiobarbituric acid, total glutathione (GSH) and oxidized glutathione (GSSG) by the same procedure after blocking GSH with 2-vinylpyridine were carried out according to Brehe and Burch (1976), Griffith (1980) and Siegers et al. (1998). For determination of adenosine triphosphate (ATP), hepatic tissue was frozen immediately in liquid nitrogen and extracts were prepared. ATP was assayed enzymatically using a reagent kit from Sigma Munich (Munich, Germany).

Statistics

Mean values and standard deviations were calculated. The statistical significance of differences between two mean values was calculated by performing Dunnett's *t*-test taking $p \leqslant 0.05$ as a limit of significance (Dunnett, 1964).

Results

In this investigation, the perfused isolated rat liver has been chosen because this model has been established as suitable for in vitro experiments to detect injuring or protecting effects on the liver. In the perfused isolated rat liver, enzymes and physiological parameters are measured as first and sensitive indicators for pathological changes in the organ. Thus also early tendencies such as low effects are detected. In addition, the important influence of nutrition can be shown, if livers are used from animals after fasting. In the perfusate, an increase in concentrations of the cytosolic enzymes alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) indicates a (low) damage of the cell membrane of hepatocytes, whereas higher amounts of glutamate dehydrogenase (GLDH) are also based on a leaking mitochondrial membrane, which is interpreted as a stronger liver damage. A decreasing bile secretion is also an indicator for a reduced liver function. The general influence of fasting on metabolic activities has been well investigated (Diaz Gomez et al., 1975; Dixon

et al., 1960; Krishnan and Stenger, 1966). For detecting an influence of fasting, the measurement of adenosine triphosphate (ATP), oxidized respectively total glutathione (GSH, GSSG) and malondialdehyde (MDA) have been established. The contents of GSH and GSSG decrease and that of MDA increases by lipid peroxidation as a consequence of liver damage. Fasting intensifies these effects.

Effects of coumarin on the isolated perfused rat liver

Before the influence of troxerutin on the effects of coumarin can be investigated, the biological model of the isolated perfused liver has to be adapted for the experiments with coumarin.

In order to define the experimental conditions, characteristic biochemical and physiological parameters are measured with or without fasting and in dependence on an increasing coumarin concentration. These preliminary experiments show that a coumarin concentration of 4 mM in combination with fasting generates a reproducible liver damage as described earlier in the section "Material and Methods." In Tables 1 and 2, the changes of the important biochemical and physiological parameters are listed for a threshold dose of 4 mM coumarin. In general, the mean values for ALT, GLDH and LDH increase during the observational period of 120 min. After 2 h, the values for ALT and LDH of the controls with fasting increase significantly in contrast to the controls without fasting ($p \leqslant 0.05$). The treatment with coumarin changes the ratio of GLDH; in controls, fasting reduces the values in contrast to the coumarin-group, where the fasting yields higher contents. The bile secretion diminishes in all groups during the experiments: At the beginning, the mean value in both coumarin-groups is 1415 µg/g min (normal diet) and 1736 µg/g min (fasting), respectively, and at the end, 13 µg/g min (normal diet) and 80 µg/g min (fasting), respectively. In contrast, the controls start with lower values, 1175 µg/g min (normal diet) and 1308 µg/g min (fasting), respectively, and show a smaller value reduction to 547 µg/g min (normal diet) and 774 µg/g min (fasting), respectively, at the end. In Table 2, the contents of ATP, GSH, GSSG, MDA and the change of liver weights are listed in relation to coumarin-treatment and fasting. All values are measured for 2 h after a liver perfusion. In general, coumarin leads to a reduction of ATP contents and all glutathione contents, especially, in comparison to the controls with normal diet. Increasing effects concerning MDA and liver weight can be detected after treatment with coumarin.

Table 1. Contents of alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH) and bile secretion of the isolated perfused rat liver in dependence of a treatment with coumarin and fasting

Parameter	Period of perfusion (min)	Group			
		Control ($n=8$)	Control with fasting ($n=6$)	Coumarin ($n=6$)	Coumarin with fasting ($n=7$)
ALT	0	0	0	0	0
(U/l perfusate)	30	0	0	0	16±4
	60	8±3	48±9	8±4	32±11
	90	8±4	72±12*	20±11	88±40
	120	20±11	92±16*	216±64*	448±128*
GLDH	0	4.1±0.4	1.0±0.4	4.1±1.7	2.9±1.4
(U/l perfusate)	30	7.0±3.0	3.1±0.5	3.9±0.9	4.9±0.5
	60	11.9±5.1	6.2±0.6	4.5±1.6	6.4±0.6
	90	17.0±6.6	9.8±2.9	6.2±1.6	8.8±2.5
	120	21.6±6.8	14.8±2.0	10.0±2.1	16.1±1.8
LDH	0	0	0	0	0
(U/l perfusate)	30	65±30	410±90	65±35	210±65
	60	135±50	820±125	135±50	445±100
	90	170±60	1130±225	340±85	960±330
	120	275±65	1300±340*	1990±410*	4450±1020*
Bile secretion	0	1175±186	1308±160	1415±247	1736±105
(µg/g min)	30	1121±126	1322±160	1736±260	1695±100
	60	988±80	1135±146	921±41	708±106
	90	748±100	935±133	160±40*	254±74*
	120	547±106	774±93	13±4*	80±32*

Mean values with SD are listed.*Significant differences in comparison to the control ($p \leqslant 0.05$).

Table 2. Contents of adenosine triphosphate (ATP), glutathione (GSH), oxidized glutathione (GSSG), malondialdehyde (MDA) and the change of liver weight of the isolated perfused rat liver in dependence of a treatment with coumarin and fasting after 120 min perfusion

Parameter	Group			
	Control ($n=8$)	Control with fasting ($n=6$)	Coumarin ($n=6$)	Coumarin with fasting ($n=7$)
ATP (μmol/g liver)	2.21 ± 0.13	$1.44 \pm 0.14^{*}$	$0.52 \pm 0.07^{*}$	$0.41 \pm 0.05^{*}$
GSH (μmol/g liver)	5.26 ± 1.21	$1.26 \pm 0.24^{*}$	$0.29 \pm 0.14^{*}$	0.44 ± 0.11*
GSSG (μmol/g liver)	0.159 ± 0.030	$0.060 \pm 0.020^{*}$	$0.011 \pm 0.002^{*}$	$0.011 \pm 0.010^{*}$
MDA (nmol/g liver)	18.6 ± 2.2	$20.2 \pm 0.9^{*}$	$25.4 \pm 2.6^{*}$	$36.2 \pm 4.6^{*}$
Change of liver weight (g)	-0.25 ± 0.22	$+2.18 \pm 0.31$	$+10.16 \pm 0.90$	$+7.53 \pm 0.76$

Mean values with SD are listed.*Significant differences in comparison to the control ($p \leqslant 0.05$).

In conclusion, liver damage can be generated in the isolated perfused rat liver by using a coumarin concentration of 4 mM in combination with fasting.

Hepatic protective influence of troxerutin for coumarin-induced effects

After an effective coumarin concentration of 4 mM was shown in the preliminary experiments with the isolated perfused rat liver, a concentration of 4.73 mM was chosen in the troxerutin experiments. Thus, the ratio between the concentrations of coumarin and troxerutin is equal to the combination of coumarin and troxerutin in the established remedy for chronic venous insufficiency. Taking the first series of experiments for testing the influence of troxerutin into consideration, a study design with four groups was developed: controls, treatment with a 4 mM solution of coumarin, treatment with a 4.73 mM solution of troxerutin and a combination of the solutions of coumarin and troxerutin with similar concentrations; animals in all groups were fasting. The established measurement of enzymes could not be used, because the intensive yellow of troxerutin distorts measurements. Thus, the most important parameters such as MDA and the physiological parameters of bile secretion, oxygen consumption and perfusion flow as well as the change of the liver weight were recorded. In general, troxerutin effects were comparable to the controls such as fasting alone and a treatment with coumarin and fasting. Thus, troxerutin decreases the three physiological parameters bile secretion, oxygen consumption and perfusion flow during the experiments. In comparison to the coumarin-group, the reduction was not as strong; for bile secretion a combination of coumarin and troxerutin in addition to fasting (82 μg/g min) produced comparable results to coumarin and fasting alone (80 μg/g min). At the end of perfusion, there was a significant difference only between controls and a treatment with troxerutin alone ($p \leqslant 0.05$). All results, also for the measurement during the experiments after 30, 60 and 90 min, are presented in Fig. 2. In general, the oxygen consumption decreases during the experimental cycle. Only the controls showed similar values at the first 90 min, whereas the three treatments cycles produced a continuous reduction of oxygen consumption. There is no significant difference between a treatment with coumarin alone and the combination of coumarin and troxerutin. In both cases after 2 h only ca. 40% of the values on beginning of the experiments remained: 2.35 μmol/g min versus 1.04 μmol/g min after the experimental cycle of 2 h by coumarin and 2.42 μmol/g min versus 0.97 μmol/g min after the experimental cycle of 2 h by coumarin and troxerutin (Fig. 3). Also, the perfusion flow during the experimental cycle of 2 h was reduced. The highest reduction can be detected after a pretreatment with troxerutin (from 60.0 to 39.3 ml/min) and a pretreatment with coumarin (from 60.0 to 44.7 ml/min), whereas

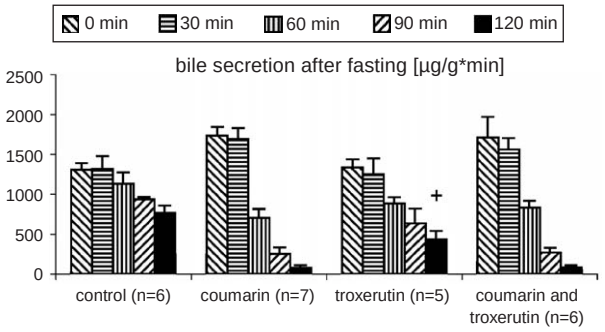

Fig. 2. Diagram of bile secretion after fasting. All values are determined during the experimental cycle of 2 h for every 30 min in dependence of a treatment with coumarin and/or troxerutin of the isolated perfused rat liver. +: there is significant difference between a treatment with troxerutin and the control ($p \leqslant 0.05$).

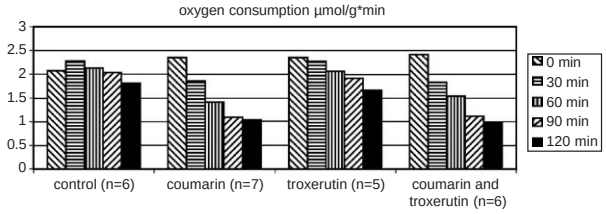

Fig. 3. Diagram of oxygen consumption after fasting. All values are determined during the experimental cycle of 2 h for every 30 min in dependence of a treatment with coumarin and/or troxerutin of the isolated perfused rat liver.

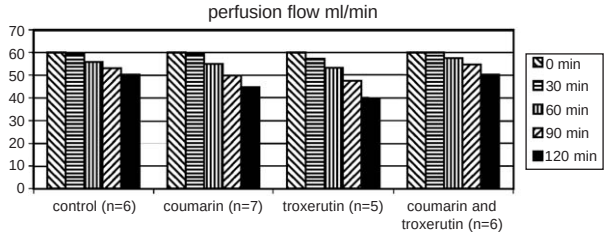

Fig. 4. Diagram of perfusion flow after fasting. All values are determined during the experimental cycle of 2 h for every 30 min in dependence of a treatment with coumarin and/or treatment of the isolated perfused rat liver.

the values of the controls and after a pretreatment with coumarin and troxerutin decreased only from 60.0 to 50.3 ml/min in both cases. For the perfusion flow, all results during the experiments after 0, 30, 60, 90 and 120 min are also presented in Fig. 4. All treatments, coumarin or troxerutin alone or the combination of coumarin and troxerutin increase the weight of the liver significantly in comparison to the controls ($p \leqslant 0.05$). At the end of the experiments (after 120 min) the liver weight increases 2.8-fold (troxerutin 6.11 ± 0.91 g), 3.5-fold (coumarin 7.53 ± 0.76 g) and 4.5-fold (coumarin and troxerutin 9.89 ± 0.44 g) in comparison to the controls without treatment (2.18 ± 0.31 g). The detected changes are not as considerable as for animals which had not fasted. Here, the liver weights of the controls diminished by -0.25 ± 0.22 g and after administering coumarin an increase in 10.16 ± 0.90 g was measured. The results for the change of liver weight during the experiments after fasting are presented in Fig. 5. A lipid peroxidation in consequence of liver damage can be detected by measuring the content of malondialdehyde (MDA). Thus a 4 mM solution of coumarin nearly doubles the amount of MDA which is significant in comparison to the controls (36.2 ± 4.6 nmol/g liver, respectively, 20.2 ± 0.9 nmol/g liver; $p \leqslant 0.05$) as shown in Fig. 6. If applying troxerutin (4.73 mM) or the combination of coumarin (4 mM) and troxerutin (4.73 mM), the resulting contents of MDA are comparable with that of the control (18.4 ± 2.2 nmol/g liver,

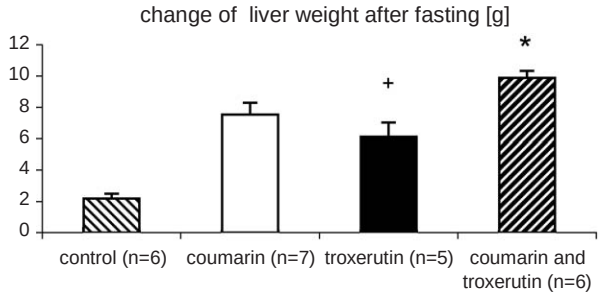

Fig. 5. The change of liver weight of the isolated perfused rat liver by fasting after 120 min perfusion in dependence of a treatment with/without coumarin and/or troxerutin; *: after a treatment with coumarin and troxerutin the liver is significantly heavier than after administering coumarin alone ($p \leqslant 0.01$); +: there is a significant difference between treatment with troxerutin and the control ($p \leqslant 0.05$).

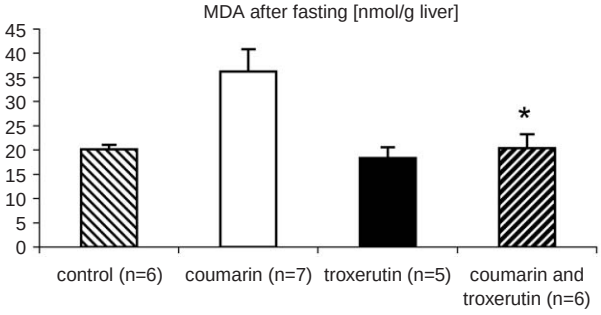

Fig. 6. The content of malondialdehyde (MDA) of the isolated perfused rat liver by fasting after 120 min perfusion in dependence of a treatment with/without coumarin and/or troxerutin; *: the concentration after a coumarin/troxerutin treatment differs significantly from the content after administering coumarin alone ($p \leqslant 0.01$).

respectively, 20.4 ± 2.9 nmol/g liver versus 20.2 ± 0.9 nmol/g liver). Regarding the combination of coumarin and troxerutin, the amount differs significantly from the content by applying coumarin alone ($p \leqslant 0.05$). It is distinct that troxerutin compensates the liver damaging effect of coumarin, as the normal values of the marker MDA indicate.

The metabolism of coumarin in the isolated perfused rat liver after fasting in dependence of treatment with coumarin (4 mM) or a combination of coumarin (4 mM) and troxerutin (4.73 mM)

As described, the amounts of coumarin, free 3-hydroxycoumarin and 7-hydroxycoumarin (because of low concentrations only in the perfusate and not in the collected bile) and after an enzymatic deglucuronidation and desulphation as total 3-hydroxycoumarin or 7-hydroxycoumarin were quantified by HPLC. By using

these values, the content of conjugated metabolites was calculated without a standard deviation. At the end of the experiments, after 120 min in the perfusate, only the amount of coumarin shows no difference between the treatments (2.43 ± 0.08 mmol/l perfusate by coumarin to 2.46 ± 0.05 mmol/l perfusate by the combination of coumarin and troxerutin). For the main metabolites of coumarin, 3-hydroxycoumarin and 7-hydroxycoumarin, however, troxerutin seems to decrease the metabolic activity. Thus, the concentration of free 3-hydroxycoumarin is 1.76 ± 0.44 and 2.86 ± 0.51 µM for the total 3-hydroxycoumarin, resulting in a concentration of 1.10 µM for 3-hydroxycoumaringlucuronid/sulphate in the perfusate, respectively. If troxerutin is administered, the contents also are reduced by more than 60% (0.62 ± 0.28 µM, 1.00 ± 0.35 µM and 0.38 µM). For 7-hydroxycoumarin and its glucuronide/sulphate, concentrations of 0.138 ± 0.020 µM (free 7-hydroxycoumarin), 0.621 ± 0.059 µM (total 7-hydroxycoumarin) and 0.483 µM (7-hydroxycoumaringlucuronid/sulphate) were measured after administering coumarin alone. This demonstrates that troxerutin decreases the amount of the 7-hydroxycoumarin metabolites (0.080 ± 0.029 µM for 7-hydroxycoumarin, 0.411 ± 0.026 µM for total 7-hydroxycoumarin and 0.331 µM for 7-hydroxycoumaringlucuronid/sulphate). The amounts of all metabolites in the perfusate are shown in Fig. 7. Due to the low amount of bile, a differentiation between the free hydroxycoumarins and their conjugates is not possible. Here only the total concentrations of the 3-hydroxycoumarin and 7-hydroxycoumarin after deglucuronidation and desulphation and of coumarin are analysed and presented in Fig. 8. In agreement with the results in the perfusate, the quantities after treatment with coumarin alone are higher than those after the treatment with the combination: concentration of coumarin 4.49 ± 0.53 µM (coumarin alone) to 2.67 ± 0.61 µM (coumarin and troxerutin), concentration of 3-hydroxycoumarin 21.68 ± 4.53 nM (coumarin alone) to 17.72 ± 5.19 nM (coumarin and troxerutin) and concentration of 7-hydroxycoumarin 2.52 ± 0.22 nM (coumarin alone) to 1.93 ± 0.55 nM (coumarin and troxerutin). In contrast with the concentrations of the perfusate, the concentration of coumarin in the bile decreases to 60% after troxerutin is additionally administered. There were also differences in the ratio of the concentrations of the

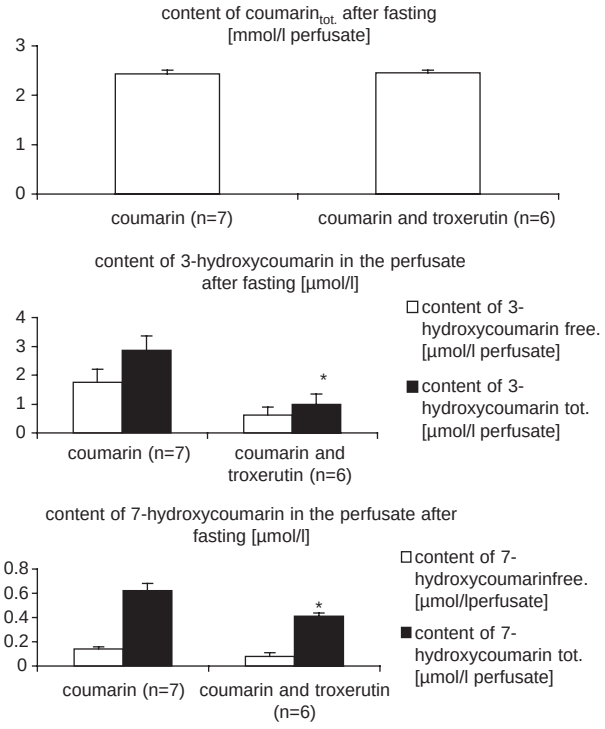

Fig. 7. Coumarin and its main metabolites in the perfusate after 120 min perfusion in dependence of a treatment with 4 mM coumarin and fasting or a combination of 4 mM coumarin, 4.73 mM troxerutin and fasting; 3-hydroxycoumarin and 7-hydroxycoumarin are quantified before (''free'') and after enzymatic deglucuronidation and desulphation (''total''). *: A significant difference is shown for a treatment with coumarin alone and the corresponding group of the combination of coumarin and troxerutin ($p \leqslant 0.01$).

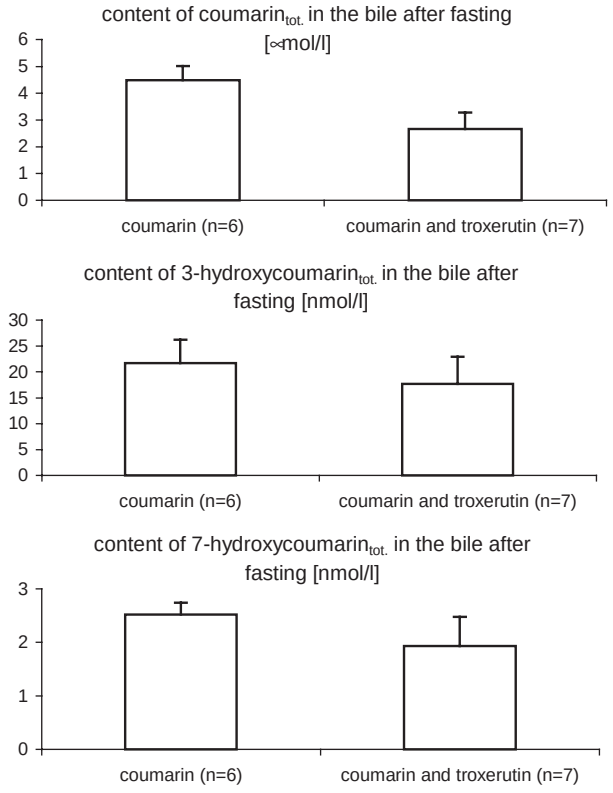

Fig. 8. Coumarin and its main metabolites 3-hydroxycoumarin and 7-hydroxycoumarin in the bile after 120 min perfusion in dependence of a treatment with 4 mM coumarin and fasting or a combination of 4 mM coumarin, 4.73 mM troxerutin and fasting; 3-hydroxycoumarin and 7-hydroxycoumarin were quantified before (''free'') and after enzymatic deglucuronidation and desulphation (''total'').

metabolites. Thus, the ratios for the concentrations of coumarin to 3-hydroxycoumarin to 7-hydroxycoumarin is 1:1.2:0.3 (perfusate/coumarin) and 1:0.4:0.2 (perfusate/coumarin and troxerutin), respectively, in comparison to 1:4.8:0.6 (bile/coumarin) and 1:6.6:0.7 (bile/coumarin and troxerutin), respectively.

Discussion

In this study, damages in the isolated perfused rat liver caused by coumarin could be detected, after effects of coumarin on HepG2-cells had been described (Siegers et al., 1998). Bile secretion, oxygen consumption and perfusion flow are lower after a treatment with coumarin. The increased levels of the cytosolic enzymes ALT and LDH in the perfusate indicate a disorder of the cell-membranes, which lead to a higher permeability for these enzymes. Because the enzymatic level of the mitochondrial enzyme GLDH did not change significantly, the mitochondrial cell membrane was not damaged, which is caused by severe liver damage. Lower concentrations of ATP, oxidized and reduced glutathione at the end of the perfusion are also characteristic symptoms of a liver injury. The implementation of a sufficient biological model, which does not have the disadvantages of cell-cultures with only one type of cells, is the necessary assumption for the investigation of possible protective effects. It is very important to note that for liver damage to occur, a concentration of 4 mM coumarin is required, whereas the usual therapy of chronic venous diseases with coumarin amounts to less than 0.1 percent (0.7 μM as maximal concentration) of coumarin in the plasma (Casley-Smith et al., 1993; Casley-Smith, 1999; Piller 1993). Furthermore, one has to keep the species-specific configuration of cytochromeP450-isozymes in mind, as rat liver does not possess a main coumarin metabolizing cytochromeP450-isozyme such as the human CYP2A6 (Bourian et al., 1999; Fentem and Fry, 1992; Lewis et al., 1999; Oscarson et al., 1999).

In general, fasting is known to influence liver damage by hepatotoxic agents. There are, however, agent-specific effects for diminishing or stimulating a hepatotoxic effect (Jaeger et al., 1974; Strubelt et al., 1981). For coumarin, fasting does not influence the amounts of the measured enzymes such as ALT, ATP, GLDH and LDH. Similar results are detected for the functional parameters of bile secretion, oxygen consumption and perfusion flow. Only the levels of MDA are increased significantly ($p \leqslant 0.05$) by combining coumarin and fasting, which indicates a lipid peroxidation caused by diminished values of glutathione after fasting. It is well known, though, that a reduction of the glutathione concentration to less than 80% of the normal value by fasting leads to liver injuries without the presence of a hepatotoxic agent (Younes and Siegers, 1980; Younes and Siegers, 1981). It is very interesting that when added to coumarin, troxerutin prevents the increase in MDA. Troxerutin protects from a lipid peroxidation as a form of liver injury caused by coumarin and fasting. Troxerutin seems to significantly inhibit the depletion of glutathione produced by coumarin. This postulated mechanism is also important for humans, especially with pharmacogenetic variations of the cytochromeP450-isozyme system. Individuals with defective allele-variants of the CYP2A6, who have a reduced metabolic capacity for the formation of 7-hydroxycoumarin as main metabolite of coumarin, are protected by troxerutin from liver dysfunction caused by possible alternative metabolites of coumarin. The differences in the pattern of metabolites in dependence of the administration of troxerutin seems to have an inhibitory influence on the metabolism of coumarin. Indeed, two different effects can be observed: (I) troxerutin reduces all concentrations of the coumarin-metabolites in the perfusate and bile, (II) the changes in the ratio of the main metabolites coumarin: 3-hydroxycoumarin:7-hydroxycoumarin after administering troxerutin confirm this thesis. For murine hepatic microsomes, in which CYP2A5 as a most important cytochromeP450-isozyme for coumarin hydroxylates the benzopyrone to 7-hydroxycoumarin, troxerutin is described as potent inhibitor for this enzymatic reaction (Tegtmeier et al., 1995). In this study, also comparable effects could be detected in rats. It has been reported that rats eliminate nearly 40% of orally given coumarin via their feces (Fentem and Fry, 1992). This study shows that the majority of coumarin excreted in feces may not be from absorbed coumarin, because only less than 1% of the administered coumarin dosage was detected in the bile. Finally, the changes of liver weight should be discussed in dependence on the different pretreatment. The highest increase in the liver weight after administering coumarin can be understood as reaction of a self-induction by coumarin. This mechanism is well known and has been published for the murine CYP2A5 (Adam et al., 1995). In general, induction-processes lead to an increase in liver weight, also for the coumarin metabolizing enzymes (Emde et al., 1996; Tegtmeier et al., 1993). The lower increase in liver weight after administering coumarin and troxerutin after fasting in comparison to coumarin alone after fasting can be caused by the inhibitory effect of troxerutin for the most important metabolic pathway of coumarin (coumarin is hydroxylated to 7-hydroxycoumarin). If the rats have a normal diet, the induction of enzymes have the highest capacity, so that the highest increase in liver weight results in contrast to fasting.

In conclusion, this study demonstrates that troxerutin protects the liver from possible lipid peroxidation

caused by coumarin. Thereby one needs to observe that these effects of coumarin can be detected only in concentrations considerably higher than the established therapeutical dosage. Consequently, troxerutin is a useful and beneficial completion for coumarin preparations in the therapy of chronic venous insufficiency.

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