Biochemical Pharmacology, Vol. 44, No. 8, pp. 1683–1686, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Effect of lysosomotropic agents on the taurocholate-stimulated biliary excretion of horseradish peroxidase

(*Received* 16 *April* 1992; *accepted* 10 *July* 1992)

Abstract—The effects of the lysosomotropic agents chloroquine and leupeptin on the taurocholate-stimulated biliary excretion of horseradish peroxidase (HRP) was studied in bile fistula rats. HRP (0.5 mg/100 g body wt) was injected into the portal vein during taurocholate (0.4 μmol/min/100 g body wt) or saline infusion. HRP appeared in bile showing both an early (approx. 5 min) and a late (approx. 25 min) excretion peak. The late peak, which represented about 95% of the total HRP excreted, is due to transcellular vesicular transport. The early peak is mainly due to paracellular leakage although a rapid vesicular transport also contributes. Taurocholate infusion significantly increased the biliary output of HRP (both peaks) and of the endogenous lysosomal enzyme acid phosphatase. Pretreatment with chloroquine or leupeptin inhibited the taurocholate-stimulated late excretion of HRP into bile, without affecting its early excretion. The lysosomotropic agents did not affect the biliary excretion of bile salts but significantly inhibited the taurocholate-stimulated biliary excretion of acid phosphatase. The results are consistent with a role of lysosomes in the taurocholate-stimulated major transcellular vesicular transport of HRP into bile.

Horseradish peroxidase (HRP)*, a glycoprotein of approximately 40,000 mol wt, is used as a marker of both paracellular and transcellular (vesicular) biliary access [1]. HRP, after entering hepatocytes via fluid phase endocytosis, is excreted intact in bile following a direct transcellular vesicular pathway [2], although most of the protein is transported to lysosomes for degradation [3]. It is known that proteins and other compounds sequestered in hepatocyte lysosomes can undergo biliary excretion [4–8]. Nevertheless, a significant lysosomal contribution to the amount of HRP appearing intact (enzymatically active) in bile seems unlikely, at least under normal conditions [2, 3].

Recent studies have reported that the transcytotic entry of HRP into bile can be stimulated by the physiological bile salt taurocholate (TC), whereas the keto-bile salt taurodehydrocholate fails in producing such a stimulus [9, 10]. However, the mechanisms implicated in this process are unknown. In this connection, we have suggested previously that TC (but not dehydrocholate) stimulates the release of endogenous proteins into bile exclusively through a lysosomal pathway [11–13].

Therefore, to provide biochemical information on a possible role for lysosomes in the TC-stimulated biliary HRP excretion, we studied the effects of the lysosomotropic agents chloroquine (Cq) and leupeptin (Leu) in bile fistula rats.

Materials and Methods

Adult male Wistar rats weighing 280–360 g were used throughout. Before the experiments, animals were maintained on a standard laboratory diet in a constant temperature environment (25°) and under a constant 12 hr-light/12 hr-dark cycle. The animals were anaesthetized with sodium pentobarbital (5 mg/100 g body wt, i.p.) and thus were maintained throughout the experiments. The common bile duct and the femoral vein were cannulated with polyethylene catheters (PE-10, Intramedic, U.S.A., and PC-40, Rivero y Cia., Argentina, respectively). The rectal temperature was kept at 37.0° with a heating lamp. Bile was collected in all rats for 10 min (basal period). Then, TC dissolved in saline (0.9% NaCl, pH 7.4) or saline alone was infused until the end of the experiment. Forty minutes after initiation of the infusions, when bile salt excretion had reached new stable values, HRP (0.5 mg/100 g body wt) dissolved in 0.3 mL of saline was injected over a 15-sec interval into the portal vein. Bile collection was continued for 80 min. At the end of the experiments, the rats were killed by exsanguination and the livers were removed and weighed.

In other groups of rats, Cq (5 mg/100 g body wt, i.p.) or Leu (2 mg/100 g body wt, i.v.) was injected 1 hr before beginning the bile collection. Such doses of lysosomotropic agents have been reported to inhibit the biliary excretion of proteins (or protein metabolites) via lysosomes [4–6]. After the basal period of bile collection, TC infusion and HRP administration were performed as described above. Saline-infused groups were not included since it has been proven that lysosomotropic agents produce no effect on the biliary HRP excretion during basal bile salt excretion [3].

The volume of bile was determined by weight assuming a density of 1.0 g/mL. Bile salts were measured with 3α-hydroxysteroid dehydrogenase [14]. HRP was determined spectrophotometrically by measuring the rate of oxidation of 4-aminoantipyrine at 510 nm [15]. Acid phosphatase (EC 3.1.3.2) was determined by Babson *et al.* [16] using a commercial kit (FAcP-cinetic, Wiener Laboratory, Argentina). One unit of activity corresponded to the hydrolysis of 1 μmol α-naphthylphosphate/min at 37°.

Significant differences were assessed by Student's *t*-test; a difference was considered significant at the $P < 0.05$ level.

TC, HRP (type II), Cq, Leu and 3α-hydroxysteroid dehydrogenase were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest grade commercially available.

Results and Discussion

Figure 1 shows that the biliary excretion of bile salts and lysosomal marker acid phosphatase was stimulated markedly during TC infusion in untreated rats. Lysosomotropic agents (Cq, Leu) did not affect bile salt excretion, but significantly inhibited the TC-stimulated biliary acid phosphatase excretion. These results are consistent with a TC-stimulated biliary discharge of lysosomes, which can be abolished not only by Cq as reported [12], but also by Leu.

In Fig. 2, it can be seen that after intraportal injection

* Abbreviations: HRP, horseradish peroxidase; TC, taurocholate; Cq, chloroquine; and Leu, leupeptin.

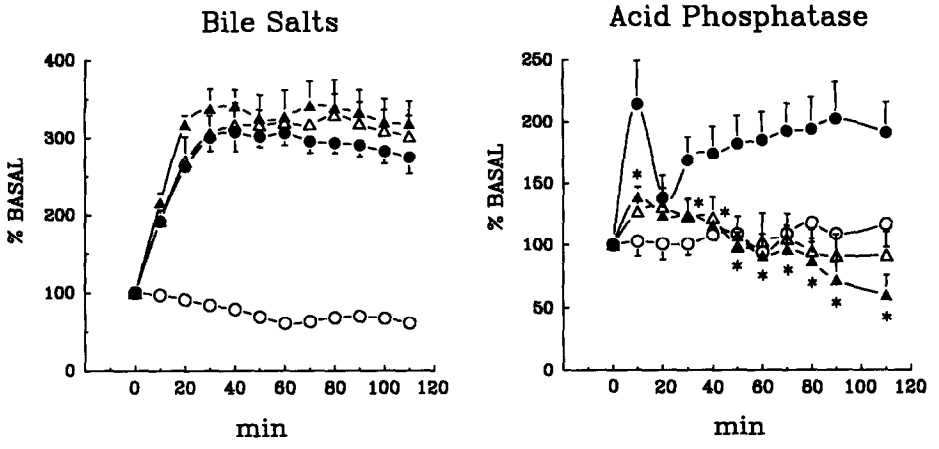

Fig. 1. Effects of lysosomotropic agents on the TC-stimulated biliary excretion of bile salts and acid phosphatase. The results are expressed as percent of basal values (plotted at time zero) and represent means ± SEM. For the TC-infused group, the basal values were: bile salts 55.18 ± 2.76 nmol/min/g liver and acid phosphatase 40.4 ± 3.1 μU/ming/g liver. Cq (5 mg/100 g body wt, i.p.) or Leu (2 mg/100 g body wt, i.v.), injected 1 hr before beginning the bile collection, did not affect the basal biliary excretions significantly. Saline or TC (0.4 μmol/min/100 g body wt) was infused from time zero to the end of the experiments. TC induced a statistically significant increase of bile salt and acid phosphatase excretion in comparison with saline ($P < 0.05$). An asterisk (*) indicates a significant difference of both Cq + TC and Leu + TC in comparison with TC ($P < 0.05$). Key: (○—○) saline (N = 5); (●—●) TC (N = 6); (△—△) Cq + TC (N = 5); and (▲—▲) Leu + TC (N = 5).

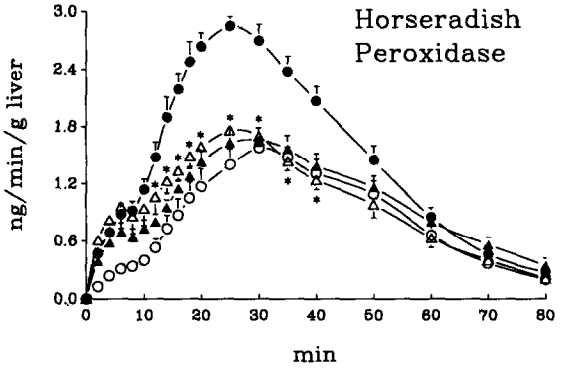

Fig. 2. Effects of lysosomotropic agents on TC-stimulated biliary HRP excretion. HRP (0.5 mg/100 g body wt) was injected into the portal vein 40 min after beginning TC or saline infusion (see Fig. 1). Values are means ± SEM. TC induced a statistically significant increase of biliary HRP excretion in comparison with saline ($P < 0.05$). An asterisk (*) indicates a significant difference of both Cq + TC and Leu + TC in comparison with TC ($P < 0.05$). Key: (○—○) saline (N = 5); (●—●) TC (N = 6); (△—△) Cq + TC (N = 5); and (▲—▲) Leu + TC (N = 5).

HRP appeared in bile showing an early and a late excretion peak.

The early appearance of HRP in bile is due to movement across tight junctions [1], although a small contribution of a rapid microtubule-independent vesicular pathway has also been demonstrated [9]. Thus, the first peak of HRP in bile may be indicative of paracellular permeability. Present (Fig. 2 and Table 1) as well as previous results [9, 10] indicate that TC increases the first HRP excretion peak. Moreover, our data indicate that lysosome inhibitors did not affect the TC-stimulated early excretion of HRP.

Table 1. Effects of lysosomotropic agents on TC-stimulated cumulative biliary HRP excretion

	First peak (ng/g liver)	Second peak (ng/g liver)
Saline (5)	2.25 ± 0.74	61.39 ± 5.43
TC (6)	5.93 ± 0.67*	104.45 ± 6.32*
Cq + TC (5)	6.51 ± 1.36*	68.28 ± 5.88†
Leu + TC (5)	4.61 ± 0.69*	72.72 ± 6.80†

The first peak was defined as 0–8 min and the second peak as 8–80 min. For details, see Fig. 2 and text. Values are means ± SEM; the number of determinations is given in parentheses.

* Significantly different from saline ($P < 0.05$).

† Significantly different from TC ($P < 0.05$).

The late appearance of HRP in bile (under basal conditions) is indicative of the well-defined direct transcellular vesicular pathway, which transports intact HRP into bile [2]. Figure 2 shows that TC, in agreement with previous reports [9, 10], increased the late entry of HRP into bile. Since TC does not affect HRP hepatic uptake, it has been suggested that the bile salt increases HRP excretion by stimulating the excretory phase of the transcellular vesicular pathway [10]. At first glance, one would expect that TC stimulates the transport of HRP-containing vesicles to canalicular membrane and their biliary discharge by the direct (non-lysosomal) pathway. Nevertheless, the possibility of a lysosomal pathway for the TC-stimulated HRP excretion should be taken into account since TC, as previously suggested [11, 12], can induce the lysosome-mediated, but not the direct access of protein into bile. The results presented in Fig. 2 and Table 1 seem to support such an assumption. In fact,

lysosomotropic agents Cq and Leu abolished the TC-induced increase in the late HRP excretion. It is important to note that both Cq and Leu did not decrease HRP excretion to below saline-group values, in accordance with Mori *et al.* [3] who reported that lysosomotropic agents do not affect the biliary excretion of the protein during basal bile salt excretion. Since, under such a condition, HRP arrives into bile through a direct route, an effect of lysosome inhibitors on this pathway seems unlikely. Actually, it has been reported [4–6, 12, 13, 17] that Cq and Leu inhibit specifically the lysosomal release into bile of proteins, without affecting their direct (non-lysosomal) excretion. Therefore, a significant contribution of the direct pathway to the TC-stimulated HRP excretion can be excluded.

Additionally, the results of Mori *et al.* [3] also allow one to discard any effect of lysosomotropic agents on the HRP hepatic uptake mechanism. This was expected, since lysosomotropic agents do not alter the hepatic uptake of fluid phase markers [18–20].

It has been reported that under normal conditions no enzymatically active catabolites of HRP are secreted into bile [21], which indicates that biliary HRP enzymic activity represents intact (not metabolized) protein. Thus, HRP sequestered into lysosomes cannot be excreted in bile before a complete loss of its enzymic activity. However, our data suggest that TC stimulates the biliary excretion of intact HRP through the lysosomal pathway. We think that during TC administration HRP-containing lysosomes may be rapidly discharged in the canaliculus not allowing HRP degradation. This view is in very good agreement with Lenzen *et al.* [22], who also suggested a biliary excretion of enzymatically active HRP through lysosomes under stimulated conditions (i.e. glucagon choleresis).

Finally, Cq [7-chloro-4-(4-diethylamino-1-methylbutyl-amino)quinoline] and Leu (acetyl-Leu-Leu-Arg-al), drugs with different chemical structures, block lysosomes by two different ways: Cq, by raising intralysosomal pH [4], and Leu by inhibiting lysosomal proteases [5]. Therefore, the possibility of similar unspecific effects of different drugs interfering with the analysis of the data seems unlikely.

In conclusion, our data indicate that the TC-stimulated major transcellular vesicular transport of HRP into bile involves the passage through lysosomes. In addition, this finding, taken together with previous data [11–13], is consistent with the existence of a lysosomal pathway for biliary protein excretion, which can be regulated by the physiological bile salt TC.

Acknowledgements—This work was supported by a Research Grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. We thank Dr. Emilio A. Rodríguez Garay and Dr. Marcelo G. Roma for their critical review of the manuscript.

Instituto de Fisiología Experimental
Consejo Nacional de Investigaciones Científicas y Técnicas
Universidad Nacional de Rosario
Rosario, Argentina

RAÚL A. MARINELLI*
GUILLERMO L. PEÑALVA

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* Corresponding author: Dr. Raúl A. Marinelli, Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 570, 2000 Rosario, Argentina. Tel. (041) 390017/20, Ext. 200; FAX 0054 (041) 255574.

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