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RAPID COMMUNICATION

Uptake, Reflux, and Excretion of Bromosulfophthalein in Ischaemia-Reperfusion Injury of Rat Liver

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Summary

The uptake, reflux and excretion of bromosulfophthalein (BSP) were studied on a model of total warm ischaemia for 30 min (group 1) or 60 min (group 2) followed by reperfusion for 45 min in the isolated perfused rat liver of unfasting rats. In group 1, the BSP hepatic uptake was comparable to control livers (30 s ischaemia plus 45 min reperfusion), but was significantly reduced in group 2. The reflux of BSP from liver to perfusate in group 1 and group 2 resulted in the appearance of secondary concentration time peaks of BSP in the reservoir perfusate. This result suggests that ischaemia-reperfusion induced a qualitative change in BSP pharmacokinetics. Excretion of the dye into bile was significantly impaired in group 2 only. The leakage of lactate dehydrogenase into the perfusate was increased moderately in both group 1 and group 2 in comparison to the controls, suggesting a low degree of liver parenchymal injury. In conclusion, the results of this investigation showed that BSP pharmacokinetics were not only undergoing quantitative changes but also a qualitative change in the model of ischaemia-reperfusion injury of the liver obtained from fed rats and may thus serve as a highly sensitive indicator of liver viability.

Key words

Ischaemia – Reperfusion – Liver – Bromosulfophthalein uptake, reflux, excretion

An isolated organ system, such as the perfused liver, is a suitable model for understanding the mechanisms underlying the first phase of liver reperfusion injury (Jaeschke 1991). An excellent characterization of this model, including light and electron microscopy of the liver, has been presented by Bradford *et al.* (1986). The damage was not observed in the liver obtained from fed rats, as assessed by trypan blue uptake and lactate dehydrogenase (LDH) release, but it was readily demonstrated under fasting conditions (Bradford *et al.* 1986). Similarly, Ypones and Strubelt (1988) reported no injury of the liver of fed rats in a hypoxia model produced with nitrogen, when using LDH and glutamic pyruvate transaminase as markers of injury. Although fasting is considered a prerequisite of liver damage, the injury can also be identified in the liver of fed rats. However, scanning electron microscopy is needed to detect ultrastructural alterations in this model (Lemasters *et al.* 1983). This calls for more simple and rapid evaluation of ischaemia-reperfused liver. Bromosulfophthalein (BSP), which has been used as a diagnostic tool for liver dysfunction under various pathophysiological conditions, can be easily and rapidly assayed. This study was therefore designed to examine the pharmacokinetics of BSP in ischaemia-reperfusion injury of the isolated perfused rat liver obtained from fed rats.

BSP was purchased from Sigma (St. Louis, MO). Pentobarbital sodium was from Spofa (Prague, Czech Republic). LDH standard and chemicals used for the determination of LDH (Biolatest) were purchased from Lachema (Brno, Czech Republic). All other chemicals were of the highest purity commercially available.

Male Wistar unfasted rats, 200–300 g, from our breeding station (Dobrá Voda, Slovak Republic), were used. The rats were given pentobarbital sodium

intraperitoneally (50 mg/kg) to induce anaesthesia before surgery. The surgical procedure for the preparation of the rat liver for perfusion was the same as in our previous works (Bezek *et al.* 1990, Kukan *et al.* 1990), whereas the perfusion system was modified according to Ballet *et al.* (1988). During surgery, the livers were perfused using a single-pass mode at 37 °C with approx. 400 ml of haemoglobin-free Krebs-Henseleit bicarbonate buffer at pH 7.4, fortified with glucose (10 mM) and saturated with 95 % oxygen and 5 % carbon dioxide. After surgery, the liver was transferred to a heated porcelain chamber drained into a reservoir.

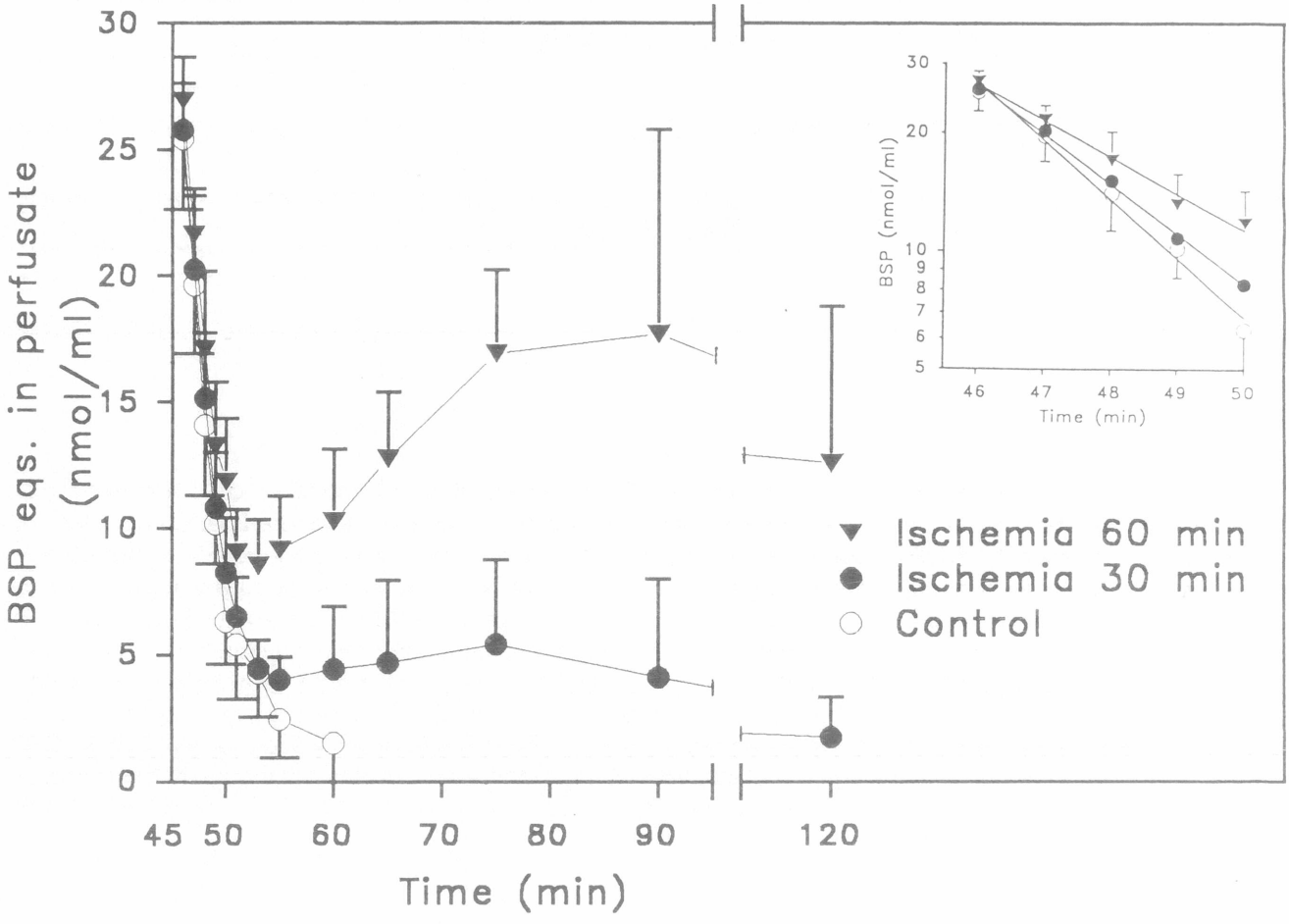

Fig. 1
The effect of ischaemia-reperfusion injury on BSP eqs. concentration in the reservoir perfusate. The decay of BSP during the first 5 min after its introduction into the reservoir is shown in the insert. Each point represents mean ± S.D. from five to six experiments.

Three groups of rat liver were studied. Ischaemia was induced by interrupting the perfusate flow for 30 s in the control livers. In the other two groups, the livers were subjected to ischaemia for either 30 min (group 1) or 60 min (group 2) by stopping the flow and by transferring the livers from the perfusion circuit to a water bath, where they were incubated at 37 °C in a Krebs-Henseleit bicarbonate buffer. All three groups of livers were then reperfused in a recirculating system with the same perfusion medium as given above at constant pressure of 12 cm H_2O, which was maintained by an overflow mechanism (see Fig. 1 of Ballet *et al.* (1988) for details). The volume of the perfusion medium was 200 ml. For the determination of perfusate dynamics steady-state, the portal perfusate flow was measured every 3 min. The portal perfusate flow was not significantly different from controls after 45 min of reperfusion. Information on steady-state bile flow was obtained by collecting bile samples every 5 min into preweighed Eppendorf tubes. Six micromoles of BSP (30 nmol/ml) were introduced after 45 min of reperfusion into the perfusate, thus simulating an intravenous bolus. BSP concentrations in the perfusate and bile were determined in 0.1 N NaOH spectrophotometrically at 580 nm. LDH was measured at 530 nm. Activity was determined using the known content of the standard supplied by the manufacturer. The fractional perfusate BSP disappearance rate (k min^{-1}), was estimated by log-

linear least-squares fit of the first five or four perfusate concentrations. The BSP perfusate disappearance rate (V_{BSP}) was determined by multiplying (k min^{-1}) with the BSP dose introduced into reservoir (in μmol/g liver) (Orzes *et al.* 1985). Thus the obtained value, V_{BSP} = (μmol/min/g liver), reflects the hepatic uptake of BSP. The area under the perfusate concentration-time curve ($AUC_{45\text{-}120\ min}$) for BSP was calculated by the trapezoidal rule. Statistical analysis was carried out by unpaired Student's t-test.

The decay of BSP from the perfusate during the first 5 min after its introduction into the reservoir is shown in the insert to Fig. 1. A relatively low dose of BSP (750 nmol/g liver, providing low initial perfusate concentration, 30 nmol/ml) was selected for this study as the uptake by the liver at this dose was found to be a function of both the affinity of uptake site K_m and the capacity of the uptake system (V_{max}) for BSP (Orzes *et al.* 1985). It should be noted that at a higher dose the uptake of BSP depends on V_{max} but not on K_m. V_{BSP} determined in this study for control livers (Table 1) was nearly identical with the value of the BSP hepatic uptake found in the single-pass perfused rat liver (cf. Table 1 of this study and Table 2, line 5, Orzes *et al.* 1985). As can be seen, V_{BSP} was significantly reduced in group 2, but not in group 1. A decrease in V_{BSP} after 60 min ischaemia indicates that the duration of ischaemia negatively influenced the uptake system for BSP by the liver. An important factor which may contribute to the decreased uptake of BSP is the rate of blood flow through the liver (Goresky 1964). However, the portal perfusate flow was monitored in all experiments, and there were no significant differences in flow after 45 min of reperfusion (data not shown). The impairment of BSP uptake may be due to intrasinusoidal formation of reactive oxygen species which may play a role in ischaemia-reperfusion injury of the liver (Jaeschke 1991). Recently, Kawamoto *et al.* (1995) reported decreased uptake of BSP in the isolated perfused rat liver due to oxygen free radicals generated by the hypoxanthine/xanthine oxidase system.

Table 1
Effect of ischaemia-reperfusion injury of isolated perfused rat liver on bile flow and BSP pharmacokinetics

	Liver weight (g)	Bile flow (μl/min/g)	V_{BSP} (μmol/min/g)	$AUC_{(45\text{-}120\ min)}$ (μmol/ml.min)	Total BSP in bile (μmol)
Control	8.02±0.59	0.77±0.11	0.258±0.042	0.108±0.015	5.05±0.10
1	8.17±0.68	0.40±0.14*	0.218±0.038	0.414±0.260*	4.19±1.64
2	8.36±1.01	0.14±0.06*	0.175±0.024*	1.282±0.485*	1.57±0.90*

*Values are the mean ± S.D. from five to six experiments. * - significantly different from controls $p<0.05$. Co - Ischaemia 30 s plus 45 min reperfusion. 1 - Ischaemia 30 min plus 45 min reperfusion. 2 - Ischaemia 60 min plus 45 min reperfusion.*

Fig. 1 shows the time course of changes in perfusate concentration of BSP. It is evident that secondary concentration time peaks were present in the ischaemic groups of livers. For example, in group 2, the concentration of BSP at 51 min of reperfusion was (mean ± S.D.) 9.1±2.1 nmol/ml and subsequently rose to 16.9±3.3 nmol/ml at 75 min of reperfusion. As far as we know, this aspect of BSP pharmacokinetics after ischaemia-reperfusion injury has not yet been described. In an *in vivo* model of ischaemia-reperfusion injury of the rat liver, Kawamoto *et al.* (1990) reported the plasma BSP concentrations only for the first 13 min after BSP administration. Therefore, it would be interesting to study BSP pharmacokinetics in ischaemia-reperfusion injury of the liver for a longer time period.

Since we assayed the concentrations of BSP spectrophotometrically, the data may represent both BSP and its metabolite(s). The major BSP metabolite, the BSP glutathione conjugate, has not been detected in the perfusate flowing out of the rat liver and it is supposed to be excreted exclusively into the bile (Gumucio *et al.* 1981, Zhao *et al.* 1993). In addition, Yam *et al.* (1976) showed that the BSP glutathione conjugate was taken up by the liver at a faster rate than BSP. It is therefore unlikely that the appearance of secondary concentration time peaks of BSP eqs. in the perfusate was a result of slower uptake of the BSP conjugate. It is rather probable that BSP reflux resulting in the formation of secondary concentration time peaks was due to injured canalicular membranes and/or due to changes in the functional integrity of hepatocyte tight

junctions. The tortuousity (Lemasters *et al.* 1983) and the varicosity of the bile canaliculi (Yasui *et al.* 1994) as well as shrinkage of hepatocytes after ischaemia-reperfusion support this assumption (Lemasters *et al.* 1983).

To determine whether the reflux of BSP from liver to perfusate was due to severe injury of parenchymal cells, the leakage of LDH into the perfusate was measured at 45, 60, 90 and 120 min of reperfusion. The following values were obtained for the corresponding time intervals: 164±83, 201±116, 227±102, 282±92 U/l for group 1 and 392±165, 431±228, 481±223, 623±230 U/l for group 2. Thus the hypothesis that severe liver injury may evoke the appearance of secondary concentration time peaks of BSP can be rejected. In control livers, the release of LDH into the perfusate after 120 min of reperfusion was 198±50 U/l. In the study of Younes and Strubelt (1988) leakage of LDH into the perfusate accounted for approx. 5000 U/l and 400 U/l in the hypoxia model of fasted and fed rats, respectively. $AUC_{(45\text{-}120\ min)}$ for BSP and excretion of BSP into the bile during 75 min after introduction of BSP into the reservoir are given in Table 1. $AUC_{(45\text{-}120\ min)}$ for BSP in group 2 was significantly higher in comparison to both group 1 and the control. Biliary excretion of the dye was significantly impaired only in group 2. The steady-state bile flow was reached after 20–30 min of reperfusion and was reduced significantly in both groups (Table 1). These findings are not unexpected as prolonged hypoxia or ischaemia decreased bile flow and hepatic excretory function *in vivo* (Shorey *et al.* 1969) as well as in the perfused rat liver (Jaeschke *et al.* 1988).

In conclusion, the examination of liver function using BSP pharmacokinetics in ischaemia-reperfusion model of liver injury of non-fasting rats is an appropriate and rapid test for the characterization of this pathophysiological change. The BSP test may be used to advantage in screening, evaluation and testing of new drug candidates with potential protective effects on liver function.

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