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The Influence of Old Age and Poloxamer-407 on the Hepatic Disposition of Diazepam in the Isolated Perfused Rat Liver

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Abstract

Background and Purpose—The normal liver sinusoidal endothelium is thin and punctuated with fenestrations 50–200 nm in diameter that filter endobiotics and xenobiotics. Defenestration of the liver sinusoidal endothelium in old age and after pre-treatment with poloxamer-407 (P407) has been shown to prevent the transfer of small chylomicrons across the liver sinusoidal endothelium. The aim of this study was to investigate the impact of liver sinusoidal endothelium fenestrations on the hepatic uptake of the highly protein-bound drug diazepam. We hypothesized that defenestration will reduce the hepatic extraction of drugs which are highly bound to albumin.

Methodology—The isolated perfused rat liver (IPRL) model and multiple indicator dilution technique were used to investigate the effect of fenestrations in the liver sinusoidal endothelium on the hepatic disposition of diazepam in old and young rats, and in young rats treated with P407 or vehicle. A bolus dose of ¹⁴C-diazepam and non-extracted tracers (³H-sucrose and Evans blue) was injected into the portal vein. The single-pass recovery of diazepam and markers and the apparent volume of distribution were determined.

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Disclosure Statement

The authors declare to have no conflicts of interest in the authorship or publication of this work.

Results—Scanning electron microscopy confirmed reduced porosity of the liver sinusoidal endothelial cells in P407-treated rats and old rats compared to young and control rats. The fractional recovery of diazepam was significantly increased in P407-treated rats compared to controls (0.20 ± 0.16 , $n = 12$, P407; 0.08 ± 0.05 , $n = 8$, controls; $p = 0.0029$), and in old rats compared to young rats (0.15 ± 0.03 , $n = 11$, old; 0.10 ± 0.02 , $n = 11$, young; $p = 0.0004$) following a single pass.

Conclusion—Defenestration due to age-related pseudocapillarization and treatment with P407 resulted in reduced hepatic extraction of diazepam after a single pass through the IPRL. These results highlight the importance of the liver sinusoidal endothelium in the ultrafiltration of highly protein-bound drugs, and may also provide an additional mechanism for reduced hepatic clearance of diazepam in conditions associated with defenestration.

Keywords

Diazepam; Fenestrae; Hepatic clearance; Liver sinusoidal endothelial cell; Pseudocapillarization

Introduction

Hepatic clearance is influenced by substrate delivery to the liver parenchymal cells and by the inherent metabolic capacity of hepatocytes. Substrate delivery is dependent on parameters such as blood flow, substrate binding to plasma proteins and transfer of substances from the blood into the space of Disse via specialized cells called liver sinusoidal endothelial cells (LSEC). The sinusoidal endothelium is a thin fenestrated endothelium lacking a basal lamina and punctuated with fenestrations of 50–200 nm in diameter grouped together in clusters known as liver sieve plates [1–4]. The fenestrated endothelium appears to function as an ultrafiltration system allowing entry of plasma proteins and smaller substances such as small lipoproteins (50–100 nm in diameter) [5, 6] into the space of Disse while blocking the passage of larger substances like chylomicrons (1,000 nm in diameter) and liposomal drug formulations [2, 7, 8].

Normal aging is associated with changes in LSEC known as pseudocapillarization [9] that include loss of fenestrations, thickening of the endothelium, peri-sinusoidal collagen deposition and basal lamina formation [9–11]. These changes are similar to the capillarization of LSEC that occurs in liver cirrhosis [12, 13]. Capillarization has been shown to have a dramatic impact on the hepatic disposition of substances such as albumin, indocyanine green [14] and propranolol [15]. Pseudocapillarization of the liver sinusoid has been demonstrated with healthy aging in rats, mice, humans and baboons [9, 10, 16, 17]. Interestingly, it was recently reported that use of a serotonin agonist can reverse age-related pseudocapillarization of the liver sinusoid in mice [18].

The role of the liver sinusoidal endothelium as a physical barrier to substrate delivery has recently been demonstrated in animal models. Defenestration of the liver sinusoidal endothelium from age-related pseudocapillarization or from pre-treatment with poloxamer-407 (P407), a synthetic surfactant causing defenestration and massive hyperlipidemia [19], prevented the transfer of small chylomicrons across the liver sinusoidal endothelium to the space of Disse [6]. More recently, it was shown that P407-mediated

defenestration and age-related pseudocapillarization resulted in reduced hepatic extraction of the water-soluble substrate paracetamol [20, 21]. While the rate of hepatic uptake of some highly protein-bound drugs is limited by protein binding, other highly bound drugs undergo extensive extraction by the liver. Although the healthy sinusoidal endothelium is unlikely to pose a barrier to most substrates, including drugs, structural changes following aging or cirrhosis may reduce the hepatic extraction of drugs that undergo flow-limited metabolism [22]. Similarly, clearance of albumin-bound drugs may be impeded by reduced access of the drug to the space of Disse via a defenestrated endothelium. Thus, it is likely that the effect of defenestration of the LSEC on hepatic drug clearance would be proportional to the degree of protein binding [5]. In reducing the access of drug-albumin complexes to hepatocytes, defenestration may have further impacts on hepatic clearance of highly protein-bound drugs by reducing the facilitated uptake of drug-protein complexes at hepatocyte cell membranes [23–25].

The aim of this study was to investigate the role of the fenestrated liver sinusoidal endothelium in regulating the hepatic uptake of diazepam, a drug that is highly bound to albumin in plasma [26], in old rats with age-related pseudocapillarization and in an acute rat model of sinusoidal defenestration, i.e. treatment with P407.

Materials and Methods

Materials

Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (Sydney, N.S.W., Australia). ^3H -Sucrose (50 μCi) was obtained from Bio Scientific P/L (Sydney). ^{14}C -diazepam (56.0 mCi/mmol) was purchased from Amersham (Sydney). Evans blue was obtained from Crown Scientific (Sydney). Carbogen (95% O_2 /5% CO_2) gas was obtained from BOC Gases (North Ryde, N.S.W., Australia). P407 was obtained from BASF Australia Ltd (Sydney).

Animals

Male Fisher 344 rats (aged 2–6 months, weight 193–340 g) were obtained from the Animal Research Center (Perth, W.A., Australia). From this cohort, rats were randomly assigned to ‘treatment’ or ‘control’ groups whereby treatment rats received an intraperitoneal injection of P407 (1 g/kg) or vehicle 24 h prior to the experiment. Rats treated with P407 were used as a model for the defenestration that is also seen in the aging sinusoidal endothelium [19]. A second cohort of young (aged 4–6 months, weight 370–490 g) and old adult (aged 24–26 months, weight 310–430 g) Fisher 344 male rats was obtained from the National Institute of Aging (Bethesda, Md., USA). All animals were allowed free access to water and were fed ad libitum with commercial rat pellets. This study was approved by the Royal North Shore Hospital Animal Care and Ethics Committee and the University of Sydney Animal Ethics Committee.

Multiple Indicator Dilution Experiments

The multiple indicator dilution (MID) technique [27] was employed to determine the hepatic disposition of diazepam and has been described previously [28, 29]. ^3H -sucrose served as a

marker of the extracellular space and Evans blue as a marker of albumin [30]. Briefly, rats were anesthetized with an injection of ketamine (75 mg/kg i.p.; Parnell Laboratories Pty Ltd, Sydney) and xylazine (10 mg/kg; Troy Laboratories Pty Ltd, Sydney). After performing a midline laparotomy incision, the portal vein was cannulated with an 18-gauge intravenous cannula (BD Medical, Sydney). The thoracic inferior vena cava was then cannulated with a 12-cm-long polyethylene tubing (i.d. 1.4 mm/o.d. 1.9 mm). The liver was perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mmol/l glucose and 2% BSA. The buffer was saturated with 95% O₂/5% CO₂ throughout the experiment. Flow rate was maintained at approximately 1–1.5 ml/min/g of liver using a Minipuls Pump System (John Morris Scientific, Sydney). Viability of the liver throughout the experiment was assessed by macroscopic appearance, portal venous pressure and scanning electron microscopic assessment of the endothelium.

A bolus dose containing ¹⁴C-diazepam (0.07 µCi), ³H-sucrose (0.07 µCi) and Evans blue (0.33 mg) in Krebs-Henseleit bicarbonate buffer (100 µl) enriched with 2% BSA was injected into the liver via the portal vein. Outflow samples (n = 72) were collected at 2-second intervals with a Universal Fraction Collector (Extech Equipment, Boronia, Vic., Australia). An aliquot (100 µl) of each outflow sample was then analyzed for ³H- and ¹⁴C-related radioactivities using a liquid scintillation counter (1500 Tri-Carb Liquid Scintillation Analyzer, Packard Instrument Co., Sydney). Evans blue intensity was analyzed spectrophotometrically in a UV-visible spectrophotometer at 620 nm (Sunrise Tecan, Clontarf, Qld., Australia).

Assessing the Effects of P407

The effect of P407 pre-treatment was confirmed by analysis of blood samples for lipid concentrations and examination of the liver sinusoids using electron microscopy. Blood samples were taken from the inferior vena cava from 4 control and 6 P407-treated animals. Total cholesterol and total triglyceride levels were determined by an accredited National Association of Testing Authorities hospital laboratory.

Scanning Electron Microscopy

Upon completion of the MID experiments, each liver was perfusion fixed with 3% glutaraldehyde/2.5% paraformaldehyde in 0.1 mol/l sodium cacodylate buffer (0.06 mol/l sucrose, 2 mmol/l CaCl₂). Six randomly selected blocks from each animal were post-fixed with 1% osmium tetroxide and dehydrated through a series of ethanol gradients to 100% before dehydration with hexamethyl disilazane (Sigma Aldrich, Sydney) for 3 min. The samples were mounted on stubs and sputter coated with platinum [20, 31, 32]. Livers were examined using a JEOL 6380 scanning electron microscope (JEOL Ltd, Tokyo, Japan).

Pharmacokinetic Modeling

Dose-normalized outflow time-activity curves were constructed and outflow concentrations were expressed as the fraction of the dose per milliliter of outflow. Recovery was estimated from the area under the curve (AUC) and flow (Q). It should be noted that recovery is equal to (1 – E_H), where E_H is the hepatic extraction ratio, which is the fraction of a substrate that is eliminated by the liver.

The mean transit time was calculated from the ratio of the area under the first moment of the curve and AUC. Both were determined using the rectangular rule because the outflow was collected for the entire time period rather than at discrete time points [7]. The apparent volume of distribution of markers was calculated from the mean transit time corrected for time zero (T_0) and the flow rate (Q , ml/s). T_0 was estimated from the first appearance of ^3H and ^{14}C radioactivity above background and represents the transit time of solutes through the non-hepatic parts of the isolated perfused rat liver (IPFR) system (such as tubing) [27, 33].

Statistics

Results are expressed as means $\pm$ SD. Comparison of the data was performed using Student's *t* test and considered significant at $p < 0.05$.

Results

Figure 1 illustrates dose-normalized outflow curves for representative control (fig. 1a), P407-treated (fig. 1b), young (fig. 1c) and old (fig. 1d) rats. The mean AUC of diazepam was significantly increased in P407 rats compared to controls (1.13 ± 0.96 , P407; 0.46 ± 0.30 , control; $p = 0.04$), as well as in old rats compared to young rats (0.70 ± 0.17 , old; 0.45 ± 0.13 , young; $p = 0.001$). No significant change in the AUC was observed for the two markers sucrose and Evans blue for either group.

Following a single pass through the IPRL we observed an increase in the fractional recovery of diazepam in P407-treated rats compared to controls (0.20 ± 0.16 , P407; 0.08 ± 0.05 , control; $p = 0.03$). The recoveries of the non-extracted tracers Evans blue and sucrose were not significantly different (table 1). We observed a significant increase in the ratio of the recovery of diazepam to both sucrose and Evans blue with P407 treatment indicating reduced hepatic uptake of diazepam in P407-treated rats. Interestingly, there was a trend towards an increase in the apparent volume of distribution of diazepam and sucrose in P407-treated rats compared to controls (table 2). There was no significant difference in the ratio of apparent volume of distribution of diazepam to sucrose or in the ratio of diazepam to Evans blue.

The study observed a significant increase in the fractional recovery of diazepam in old rats after a single pass through the liver (0.15 ± 0.03 , old; 0.10 ± 0.02 , young; $p = 0.0004$), while the recoveries of the tracers sucrose and Evans blue were unchanged (table 3). The ratios of recovery of diazepam to sucrose and diazepam to Evans blue were both significantly increased in old rats. Consistent with the findings in P407-pretreated rats, we observed an increase in the apparent volume of distribution of diazepam in old compared to young rats (0.86 ± 0.28 , old; 0.54 ± 0.20 , young; $p = 0.006$). There was also an age-related increase in the hepatic volume of distribution of sucrose in old rats, and this may explain why the ratios of apparent volume of distribution of diazepam to sucrose, and diazepam to Evans blue did not differ significantly between old and young rats (table 4).

Scanning electron microscopy of the liver sinusoid was performed for control and P407-treated rats, as well as for young and old rats with representative images shown in figure 2.

The sinusoidal endothelium of the P407-treated (fig. 2a) and old (fig. 2c) rats showed a reduction in the porosity and number of fenestrations compared to control (fig. 2b) and young (fig. 2d) rats, respectively.

The impact of P407 treatment was further confirmed by analysis of blood lipid concentrations in P407-treated (n = 6) and control (n = 4) rats. P407-treated rats had a significantly higher total cholesterol concentration (1.6 ± 0.1 mmol/l, control; 56.3 ± 48.8 mmol/l, P407; $p = 0.04$) and total triglyceride concentration than control rats (1.7 ± 0.7 mmol/l, control; 49.5 ± 35.3 mmol/l, P407; $p = 0.02$). There was no significant difference in the alanine aminotransferase activity between control and P407 rats (45.5 ± 4.7 U/l, control; 49.7 ± 20.2 U/l, P407; $p = 0.64$).

Discussion

In this study, we investigated the role of the fenestrated sinusoidal endothelium in the hepatic disposition of the highly protein-bound drug diazepam. We found that the hepatic extraction of diazepam following a single pass through the IPRL was significantly reduced in rats with defenestration due to age-related pseudocapillarization and in rats with P407-mediated defenestration. Defenestration of the sinusoidal endothelium in old and P407-treated rats, but not young or control rats, was confirmed through scanning electron microscopy.

It has been suggested that defenestration may impede the clearance of protein-bound drugs, and that the effect of defenestration of the LSEC on hepatic drug clearance would be proportional to the degree of protein binding [5]. Defenestration associated with cirrhosis has been associated with impaired uptake of solutes across the thickened and defenestrated endothelium [34], with exclusion of albumin-bound indocyanine green from the space of Disse in humans [14], and with impaired transfer and clearance of albumin-bound lignocaine [12] and propranolol [35] in rats.

Consistent with this theory, in this study defenestration was associated with increased recovery, and therefore reduced hepatic extraction, of diazepam of approximately 50% following a single pass through the IPRL. In further support of this, we recently reported that the hepatic extraction of the highly water-soluble, minimally protein-bound substrate paracetamol was reduced by approximately 10% in P407-treated rats [20] and in old rats with age-related pseudocapillarization [21]. We observed a hepatic extraction ratio of approximately 0.8 and 0.85 for P407-treated and old rats, respectively, compared to 0.92 and 0.91 for control and young rats, respectively. This was slightly higher than the 0.65 reported by Igari et al. [53] (1984) in the rat liver, and much higher than the 0.12 reported in cats [36] and the 0.013–0.033 reported in man [37]. However, it is consistent with the 0.16 availability reported in the IPRL by Miyauchi et al. [38]. However, it is also important to note that the clearance of diazepam varies greatly depending on whether data were obtained using in vivo or in vitro methods [39]. Some of this variability may be due to the effects of the protein-albumin complex on uptake at the hepatocyte in vivo [23–25]. Despite this variability, we observed an effect of defenestration due to age-related pseudocapillarization and P407 treatment, and this has important implications for the dosing of diazepam in older adults.

Impaired transfer due to defenestration may also be a potential mechanism for the increase in the elimination half-life of diazepam in old age [40, 41], which may also be a factor in the enhanced sensitivity of the elderly to the sedative effects of these drugs [42], in addition to the age-related pharmacodynamic changes [43, 44]. In addition, the decrease in diazepam clearance in liver disease reflects an impairment in drug extraction by the liver [45], which in part may be secondary to LSEC defenestration. However, it is also important to recognize that the reduction in drug metabolism enzymes with age may also contribute to the reduced clearance, at least in old rats [46].

We did observe an increase in the apparent volume of distribution of diazepam in both P407-treated rats compared to controls, as well as in old rats compared to young rats. This could be due to an age-related increase in the sucrose volume [47], changes in the vasculature in old age [48] or age-related alterations in body composition [42], such as an increased incidence of fatty liver [49] resulting in a larger space for diazepam to distribute into. We do recognize that estimates of volume of distribution do become inaccurate as the extraction increases using the MID method. However, it has been reported that the volume of distribution of diazepam is increased by approximately 50% in cirrhotic patients compared to controls [45].

In this study, we used the gold standard for hepatic drug disposition in the IPRL: the MID method. Combined with the use of the non-extracted internal controls (sucrose and Evans blue), we were able to confirm that the reduced hepatic extraction of diazepam in P407-treated rats was representative of the age-related change in the single-pass recovery of diazepam in the IPRL. Scanning electron microscopy confirmed defenestration in both groups. However, we acknowledge several limitations of this study. Evans blue is the marker of the albumin space and not the vascular space, although it is used as a surrogate for the vascular space [30]. The small but significant increase in the Evans blue volume of distribution may reflect the small increase in extra-cellular (sucrose) volume with age [47], indicating that pseudocapillarization of the aged endothelium does not impede the distribution of sucrose into the extracellular space [6]. Similarly, capillarization in cirrhotic livers does not impede the distribution of sucrose [50]. The relatively high standard deviations obtained for diazepam disposition are likely to be the result of between-subject variability. While in humans much of the variability in diazepam pharmacokinetics has been shown to occur due to genetic polymorphism [51], this is not likely to be the case in inbred rats used in this study. There may have been contamination of the outflow curves by the metabolites of diazepam, which were unable to be quantified at the tracer diazepam concentrations used in this study. However, the high extraction ratios and early peaks of the MID curves mean that this is unlikely to have influenced the AUC results. Furthermore, it has been reported that the metabolites or metabolic byproducts of radiolabelled diazepam contribute negligibly to the total radioactivity in the hepatic outflow in the IPRL [52].

Conclusion

The results suggest that albumin-bound drugs such as diazepam are transferred from blood to the space of Disse by ultrafiltration through fenestrations and that this is reduced in rats with defenestration of the liver sinusoidal endothelium due to old age and P407 treatment.

Furthermore, these results suggest that it is likely that the transfer of protein-bound drugs across the hepatic sinusoidal endothelium may be affected by age-related pseudocapillarization and capillarization due to cirrhosis, which will impact on hepatic clearance and toxicity. Reduced transfer of diazepam across the LSEC, resulting in reduced hepatic clearance, may be a potential mechanism for the increased sensitivity of older people and people with liver cirrhosis to the effects of diazepam, in addition to the age-related pharmacodynamic changes.

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Fig. 1. Representative dose-normalized outflow curves for control (a), P407-treated (b), young (c) and old (d) rats for diazepam and tracers following a single pass through the IPRL.

Fig. 2.

Representative scanning electron micrographs of hepatic sinusoids of control (a), P407-treated (b), young (c) and old (d) rats. The sinusoidal endothelium of the control and young animals is perforated with fenestrations in clusters ('sieve plates'). The sinusoidal endothelium of the P407-treated and old rats has very few fenestrations.

Table 1

Fractional recoveries of diazepam and tracers (Evans blue and sucrose) after a single pass through the IPRL of control and P407-treated rats (means $\pm$ SD)

	Control (n = 8)	P407 (n = 12)	p value
Fractional recovery			
Diazepam	0.08 $\pm$ 0.05	0.20 $\pm$ 0.16	0.029
Evans blue	0.92 $\pm$ 0.19	1.05 $\pm$ 0.18	0.142
Sucrose	0.69 $\pm$ 0.07	0.81 $\pm$ 0.24	0.112
Ratio of recovery			
Diazepam/sucrose	0.12 $\pm$ 0.08	0.25 $\pm$ 0.17	0.040
Diazepam/Evans blue	0.10 $\pm$ 0.06	0.20 $\pm$ 0.15	0.043
Sucrose/Evans blue	0.80 $\pm$ 0.27	0.79 $\pm$ 0.25	0.978

Table 2

Hepatic apparent volumes of distribution after bolus injection of tracer amounts of ^{14}C -diazepam into the IPRL of control and P407-treated rats (means $\pm$ SD)

	Control (n = 8)	P407 (n =12)	p value
Apparent volume of distribution			
Diazepam, mg/l	1.26 $\pm$ 0.33	1.51 $\pm$ 0.25	0.097
Evans blue, mg/l	0.27 $\pm$ 0.08	0.29 $\pm$ 0.10	0.745
Sucrose, mg/l	0.30 $\pm$ 0.07	0.36 $\pm$ 0.04	0.059
Ratio of apparent volume of distribution			
Diazepam/sucrose	4.21 $\pm$ 0.86	4.26 $\pm$ 0.80	0.891
Diazepam/Evans blue	4.93 $\pm$ 1.78	5.83 $\pm$ 2.07	0.318
Sucrose/Evans blue	1.17 $\pm$ 0.35	1.42 $\pm$ 0.58	0.250

Table 3

Fractional recoveries of diazepam and tracers (Evans blue and sucrose) after a single pass through the IPRL of young and old rats (means $\pm$ SD)

	Young (n = 11)	Old (n = 11)	p value
Fractional recovery			
Diazepam	0.10 $\pm$ 0.02	0.15 $\pm$ 0.03	0.0004
Evans blue	0.99 $\pm$ 0.12	0.99 $\pm$ 0.14	0.913
Sucrose	0.76 $\pm$ 0.07	0.80 $\pm$ 0.04	0.108
Ratio of recovery			
Diazepam/sucrose	0.13 $\pm$ 0.03	0.19 $\pm$ 0.04	0.0003
Diazepam/Evans blue	0.10 $\pm$ 0.03	0.15 $\pm$ 0.03	0.0002
Sucrose/Evans blue	0.77 $\pm$ 0.12	0.82 $\pm$ 0.10	0.308

Table 4

Apparent hepatic volumes of distribution after bolus injection of tracer amounts of diazepam, Evans blue and sucrose into the IPRL of young and old rats (means $\pm$ SD)

	Young (n = 11)	Old (n = 11)	p ^I
Apparent volume of distribution			
Diazepam, mg/l	0.54 $\pm$ 0.20	0.86 $\pm$ 0.28	0.006
Evans blue, mg/l	0.25 $\pm$ 0.10	0.30 $\pm$ 0.11	0.253
Sucrose, mg/l	0.24 $\pm$ 0.06	0.32 $\pm$ 0.06	0.009
Ratio of apparent volume of distribution			
Diazepam/sucrose	2.29 $\pm$ 0.98	2.72 $\pm$ 0.82	0.281
Diazepam/Evans blue	2.71 $\pm$ 2.08	3.12 $\pm$ 1.07	0.563
Sucrose/Evans blue	1.10 $\pm$ 0.36	1.16 $\pm$ 0.34	0.685

^I Comparing young vs. old.