Macrophage depletion ameliorates kavalactone damage in the isolated perfused rat liver

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(Received December 15, 2011; Accepted January 17, 2012)

ABSTRACT — Liver toxicity is a side effect observed with some herbal treatments, including Piper methysticum. The possible mechanisms responsible include inflammation subsequent to activation of liver macrophages and oxidative damage. Hepatotoxicity of the pharmacologically active component of Piper methysticum (kavalactones) was tested in isolated, perfused livers from rats which were pretreated with the macrophage intoxicant gadolinium chloride. Perfusions without kavalactones in gadolinium chloride pretreated and untreated livers were included as negative controls. Serial liver lobe biopsies were collected to measure temporal changes in available (reduced) hepatic glutathione. There were no statistically significant changes in reduced glutathione over the course of perfusion in any experimental group. Liver damage was observed using electron microscopy. Hepatic sinusoids displayed extensive damage to the endothelium in kavalactone-perfused, rat livers. This damage was significantly reduced by pre-treatment with gadolinium chloride. Hence liver macrophages may be a factor in liver injury induced by Piper methysticum. Characterisation and modulation of the liver macrophage response may enable the development of strategies to avoid these hepatic side effects.

Key words: Gadolinium chloride, Electron microscopy, Liver, Macrophages, Toxicity

INTRODUCTION

Herbal remedies are often perceived as harmless because they are derived from natural sources and can be effective against a variety of illnesses. However, many herbs do have side effects, and these commonly include hepatotoxicity due to the exposure of the liver following ingestion, and its role in drug metabolism (Pak et al., 2004). The pathology observed includes direct hepatocyte toxicity, sinusoidal endothelial cell damage, immunologically-mediated injury, impairment of bile formation or flow (cholestasis) and mitochondrial injury (Bissell et al., 2001).

Piper methysticum is a perennial plant which has been used for centuries by South Pacific communities for medicinal, social and cultural purposes. Traditionally, the rhizome of the plant is macerated with water or coconut milk to produce a beverage with relaxant and psychoactive properties (Whitton et al., 2003). In the 20th century, it became popular in the Western world as a herbal supplement for anxiety and insomnia.

Whilst whole rhizome extracts and purified preparations of the pharmacologically-active kavalactones such as kavain are generally well tolerated, side effects include dermopathy and central nervous system effects. The most serious adverse reaction observed is hepatotoxicity (Barnes et al., 2007).

Liver macrophages are key cells in the pathogenesis of many forms of hepatic disease (Roberts et al., 2007). For example, sinusoidal damage in alcoholic liver disease is due to activation of liver macrophages (Thurman, 1998). These cells are also implicated in fibrosis, viral hepatitis, steatohepatitis, and activation or rejection of the liver during transplantation (Kolios et al., 2006). In animal studies, depletion of liver macrophages is hepatoprotective during ischemia reperfusion injury (Li et al., 2009), endotoxin treatment (Henrich et al., 2008) and radiotherapy (Du et al., 2010).

Our published experiments with kavain-perfused rat livers demonstrated swollen macrophages containing large...
cytoplasmic vacuoles and phagocytosed material, constriction of sinusoidal blood vessels and retraction of the endothelium compared to controls (Fu et al., 2008). The current studies used the same concentration of kavalactones to test the hypothesis that liver macrophages precipitate kavalactone-induced hepatotoxicity. We also aimed to test whether kavalactones may predispose oxidative stress by affecting levels of available (reduced) hepatic glutathione.

MATERIALS AND METHODS

Chemicals and Reagents

Racemic kavain (95% purity) was purchased from ChromaDex (Santa Ana, CA, USA). Methysticin was purified in our laboratory as previously described (Fu et al., 2009). These kavalactone structures are shown in Fig. 1. Sodium phosphate dibasic was purchased from Astral Scientific (Caringbah, NSW, Australia), sodium nitrite from Bacto Laboratories (Mt Pritchard, NSW, Australia) and glucose was obtained from Ajax chemicals (Taren Point, NSW, Australia). Gadolinium chloride hexahydrate, taurocholic acid (TCA), bovine serum albumin (BSA, Fraction V), L-glutathione reduced and all other chemicals used were of analytical grade and purchased from Sigma (Castle Hill, NSW, Australia). Consumables for electron microscopy were sourced from ProSciTech (Kirwan, Queensland, Australia).

Animals

Male Sprague-Dawley rats weighing between 200-400 g were obtained from the Animal Resources Centre (ARC), Western Australia and housed in the Edward Ford Animal House (A27A), University of Sydney. They were maintained under a 12 hr light-dark cycle with standard laboratory chow and water provided ad libitum. All animal experiments were performed by the Animal Ethics Committee of the University of Sydney.

Isolated perfused rat liver (IPRL) and Liver Lobe Biopsies

This is a non-recovery procedure and rats were anaesthetised with a single intraperitoneal injection of 60 mg/kg sodium pentobarbitone (Hilum, Troy Laboratories, Australia). Isolation and perfusion of rat livers were performed as previously described (Fu et al., 2008).

Liver lobe biopsies were taken at baseline after flushing of erythrocytes but before commencement of perfusion with KH buffer [inferior caudate lobe (ICL)], at 60 min [superior caudate lobe (SCL)] and at 120 min [inferior right lateral lobe (IRLL)] as previously described (Rowe et al., 2011). Each biopsied lobe was cut into thirds longitudinally, which were weighed and recorded. The central third was used for histology, while the lateral thirds were homogenised in 500 μl of cold 100 mM sodium phosphate buffer (pH 7.0) and centrifuged. Twenty microlitres of supernatant was aliquoted and stored at -80°C for protein determination, while the remainder was used immediately to measure reduced glutathione.

The kavalactone concentration selected (43.4 μM) is similar to the EC50 for cytotoxicity in hepatocytes in vitro (50 μM) (Zou et al., 2004) and was used successfully to induce hepatotoxicity in our previous studies (Fu et al., 2008).

Gadolinium chloride hexahydrate treatment

Gadolinium chloride hexahydrate (GdCl3·6H2O) was used to investigate the role of liver macrophages in *Piper methysticum* hepatotoxicity. Gadolinium chloride is known to deplete liver macrophages (Henrich et al., 2008) and the most significant impact occurs after 24 hr (Kara et al., 2009). Accordingly, in this study, intravenous (IV) injections were administered via the tail vein at a dose of 26 mg/kg 24 hr prior to liver perfusion.

Reduced glutathione (GSH) assay

Reduced GSH content in liver samples was assayed using Ellman’s reaction (Ellman, 1959). Liver tissue supernatants were deproteinated by adding an equal volume of 5% w/v sulphosalicylic acid. Precipitated protein was pelleted by centrifugation. The supernatant was removed and diluted 1:10, 1:50 and 1:100 in cold 0.1 M sodium phosphate buffer. In 96 well plates, 180 μl of the supernatant was mixed with 20 μl of 3.7 mM 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB). Absorbance was measured at 415 nm using a BIO-RAD 680 Microplate Reader and Microplate Manager v5.2.1 software. A standard curve was prepared using reduced L-glutathione (Sigma G4251-5 g).

Protein assay

The protein content of liver tissue samples was deter-
mined using the Sigma Bradford Reagent (B 6916) in a 96-well plate assay. Liver tissue supernatants were diluted 1:5, 1:10, 1:50 and 1:100 in cold 0.1 M sodium phosphate buffer. Five microlitres of each sample was plated out in triplicate, and then mixed with 250 μl of Bradford reagent. The plate was incubated at room temperature for 15 minutes. Absorbance was measured at 595 nm using a BIO-RAD 680 Microplate Reader and Microplate Manager v5.2.1. A standard curve was constructed using bovine serum albumin (BSA) in sodium phosphate buffer. Results from other assays were normalised to protein content.

Light and Electron microscopy (EM)
At the conclusion of an IPRL experiment, the liver was perfused with 2% glutaraldehyde in 100 mM sodium cacodylate buffer enriched with 100 mM sucrose (Fu et al., 2008; Wisse et al., 2010). Properly fixed tissue (firm and homogeneous brown) was finely sliced with a razor blade into 1 x 1 x 1 mm tissue blocks and stored in cacodylate/sucrose buffer at 4°C for up to one week. Samples were post-fixed in 1% osmium tetroxide for 1 hr then dehydrated through graded ethanol series. For transmission EM (TEM), 100% ethanol was followed by overnight, room temperature infiltrations with 50:50 ethanol/epon resin and 100% epon resin. Samples were embedded in fresh epon at 60°C overnight. Ultra-thin sections (70-100 nm) were cut with a Leica ultracut UCT ultramicrotome (Leica, Heerbrugg, Switzerland) and collected onto 200-mesh copper grids. Grids were counterstained with 2% uranyl acetate in 50% ethanol then Reynold’s lead citrate for ten minutes each, with washing steps in warm water in between. Images were obtained using a JEOL 1400 transmission electron microscope (Tokyo, Japan) operating at 120 kV.

For scanning EM (SEM), liver samples were prepared as for TEM, up to dehydration through graded ethanol. The tissues were then dried in 100% hexamethyldisilazane for three minutes, mounted on stubs and sputter coated with a thin layer of 20 nm gold or gold/palladium. SEM samples were examined on a Zeiss EVO 30 scanning electron microscope at 15 kV. ImageJ software (version 1.42q) was used to quantify the percentage of gaps in the sinusoidal endothelial cell (SEC) surface of SEM images.

\[
\% \text{ Area of SEC surface occupied by gap} = \left( \frac{\text{Area of Sinusoidal Gap}}{\text{Total area of SEC surface}} \right) \times 100
\]

For each image, the cut-off size for sinusoidal gaps was set at 300 nm to exclude non-pathological changes (Braet et al., 1995). The percentage of the SEC surface occupied by gaps was calculated for fifteen randomly selected images from each liver and averaged. This process was repeated for each IPRL study.

Statistical analysis
The experimental design is described in Table 1. The purpose of these experiments was to compare kavalactone-treated livers with control livers, and to determine the effect of gadolinium chloride pretreatment. The relative effects of individual kavalactones were not investigated. Hence results from kavalactone-treated livers were pooled. All results are expressed as the mean ± standard deviation (SD). Student’s t-test was performed to determine the effects of gadolinium chloride pre-treatment on GSH levels in rats. All other comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. The software used was GraphPad Prism (Version 5). A \(p\)-value of < 0.05 was considered statistically significant.

RESULTS

Soluble protein and reduced glutathione (GSH) content of rat liver
No statistically significant change in soluble protein content in liver biopsies was observed during the 120 min perfusion for any treatment group. Soluble protein values were used to normalise glutathione concentrations between animals. There were no statistically significant changes in reduced glutathione levels over the course of

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perfusion in any experimental group (Fig. 2). Both control and kavalactone-perfused livers in rats that were pretreated with gadolinium chloride had higher GSH levels than non-pretreated livers at all time points. This was statistically significant at baseline and 60 min.

Liver macrophage depletion

Liver macrophages were present in sections from each of the three non-GdCl₃ treated control livers and were not observed in sections from any of the three GdCl₃ treated control livers (data not shown).

Transmission electron microscopy (TEM)

Vacuoles were observed in hepatocytes of perfused livers via light microscopy (data not shown), but not in other non-parenchymal cells. Hepatocytes were further assessed in detail at the ultramicroscopic level by TEM. In kavalactone-perfused livers, vacuoles tended to coalesce and contain membranous structures and electron dense bodies (Fig. 3A - arrows). As observed histologically, these vacuoles often distorted the cell nucleus (Fig. 3B). These vacuoles usually contained debris (Figs. 3B and C - asterices). Some cells contained whorled endoplasmic reticulum surrounding mitochondria (Fig. 3C - circle) and autophagosomes (Fig. 3D).

Two types of vacuoles were present in control liver. There were isolated large vacuoles of similar size to those in seen kavalactone-perfused livers, but these only occasionally contained electron dense structures and almost no debris. The second type were closely grouped vacuoles one to two microns in size which were not seen in kavalactone-perfused livers (data not shown).

Some retraction of sinusoidal endothelium similar to kavalactone-perfused livers was observed. In these cases, the cytoplasmic fenestrated extensions from the endothelial cells started to round up and detach from the microvillous surface of the hepatocytes in the Space of Disse.

Scanning electron microscopy (SEM)

The effect of kavalactones on the rat liver sinusoidal endothelial cell (SEC) surface with and without gadolinium chloride pre-treatment was examined using SEM. Images of control perfusion displayed normal liver fenestrae arranged in sieve plates (Fig. 4A). Following kavalactone perfusion, large gaps developed in the endothelial surface, which exposed the underlying hepatocyte microvilli (Fig. 4B). These were not observed in kavalactone-perfused liver when the rat was pre-treated with gadolinium chloride (Fig. 4C).

Kavalactone treatment of liver resulted in a statistically significant increase in the area of the sinusoidal endothelial surface (SES) occupied by gaps (21.1% ± 5.8% SD n = 4) compared to control perfusions (2.8% ± 0.3% SD n = 3) (p < 0.001). In rats that were pre-treated with gadolinium chloride, the percentage of SES gaps in kavalactone-perfused livers (5.7% ± 3.3% SD n = 4) was similar to that in control-perfused livers (2.8% ± 1.6% SD n = 3).

DISCUSSION

Scanning electron microscopy (SEM) images showed significant damage to the sinusoidal endothelial cell (SEC) surface following kavalactone perfusion compared
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**Fig. 3.** TEM of hepatocytes from kavalactone-perfused liver. Samples were obtained after 120 min of perfusion with Krebs-Henseleit buffer. (A) Arrows show membranous structures and electron dense bodies within vacuoles. (B) Cell nucleus distorted by vacuoles. Asterices show cellular debris. (C) Circle shows whorled endoplasmic reticulum surrounding mitochondria. (D) Autophagosome.

**Fig. 4.** SEM of rat liver sinusoids after 120 min perfusion with KH buffer. (A) Control perfusion. (B) Kavalactone perfusion without gadolinium chloride pretreatment. Sinusoidal endothelial surface (SES) is eroded and underlying microvilli exposed. (C) Kavalactone perfusion following gadolinium chloride pretreatment. Sinusoidal endothelial surface (SES) is protected.
to control perfusions. Damage included gaps in the SEC surface, which may form due to the destruction and/or coalescence of liver fenestrae in sieve plates (McCuskey, 2008) and exposure of the underlying hepatocyte microvilli. Liver fenestrae are important in filtering and controlling the exchange of fluids and solutes between the sinusoidal lumen and the Space of Disse (Braet and Wisse, 2002). The sinusoidal damage observed is similar to that seen clinically in sinusoidal obstruction syndrome (SOS), or veno-occlusive disease (VOD), caused by toxic injury to SECs. Known causes include pyrrolizidine alkaloids from plants such as Senecio species, radiotherapy and preoperative chemotherapy for treatment of colorectal liver metastases (CRLM) (Chen and Huo, 2010; Rubbia-Brandt, 2010). Limited areas of gaps which developed in control perfusions were possibly due to osmolarity problems, cytoskeletal destabilisation or fluctuating perfusion pressure during IPRL or fixation (Wisse et al., 2010).

Gadolinium chloride pre-treatment was effective in depleting the liver macrophage (Kupffer cell) population. Following this treatment, kavalactone-perfused rat livers showed no statistically significant difference in the total percentage of the SEC surface occupied by gaps compared to control-perfused livers. Inactivation and/or depletion of liver macrophages exerts a protective effect against toxins such as alcohol (Adachi et al., 1994) with reduced fatty changes, inflammation and necrosis. Hence the results of this experiment suggest that activation of liver macrophages by kavalactones may play a role in kava hepatotoxicity.

Vacuoles in kava-lactone-treated livers coalesced, similar to vesicles in hepatic steatosis, which is consistent with the hydrophobicity of kavalactones. In steatohepatitis, the lipid vacuoles are most prominent in the centrilobular region with large vacuoles (macrovesicular steatosis) displacing the hepatic nucleus and other organelles to the periphery of cells (Lefkowitch, 2005). EM results (whorled endoplasmic reticulum and autophagosomes) indicate autophagy is occurring in response to kavalactone treatment. Further studies such as immunostaining of kavalactone-perfused liver biopsies for caspase activity or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) would help to determine whether kavalactones induce apoptotic cell death in the IPRL model, as observed in vitro (Tang et al., 2011).

Vacuoles in control perfused liver were morphologically distinct from those in kavalactone-perfused livers. This suggests that vacuolation due to kavalactones is different from vacuolation caused by limitations in the IPRL model. There were no statistically significant changes in reduced glutathione (GSH) levels over the course of perfusion in any experimental group. This is consistent with a previous in vitro study on HepG2 cells which also found no depletion of GSH over 24 hr with 200 μM kavain, 200 μM methysticin or 25 μM yangonin (Tang et al., 2011). These findings suggest that depletion of glutathione is not the primary mechanism by which kavalactones induce hepatic injury. This is further supported by another study which found no significant increase in the ratio of oxidised to reduced glutathione (GSSG/GSH) in HepG2 cells treated with methanolic or acetonic extracts of kava root (Lude et al., 2008). However, a statistically significant increase in this ratio was found in cells treated with a methanolic leaf extract, suggesting that other kava constituents such as pipermethystine (which is present mainly in kava leaves) may deplete available glutathione.

Interestingly, there was a significant increase (p < 0.01) in GSH in rat liver 24 hr after gadolinium chloride pre-treatment. Toxicological studies with gadolinium chloride in rats have not identified any significant clinical effects in male Sprague-Dawley rats with the 26 mg/kg dose used in our studies (Spencer et al., 1997). Possible mechanisms by which gadolinium chloride may stimulate an increase in GSH is via a decrease in sinusoidal GSH efflux, or upregulation of γ-glutamylcysteine synthetase (γGCS) (an enzyme involved in de novo synthesis of GSH) as a response to external stimuli (Yuan and Kaplowitz, 2009).

In conclusion, structural examination of hepatic sinusoids displayed extensive damage to the endothelium in kavain- and methysticin-perfused, rat livers. This damage was significantly reduced by pre-treatment with gadolinium chloride, thus suggesting a role for liver macrophages in Piper methysticum-induced liver injury. Future studies, focused on characterisation of the liver macrophage responses may enable the development of strategies to improve the safety of Piper methysticum.

ACKNOWLEDGMENTS

The authors acknowledge the facilities as well as scientific and technical assistance from staff in the AMMRF (Australian Microscopy & Microanalysis Research Facility) at the University of Sydney. This work was performed using internal funds from the University of Sydney.

REFERENCES


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