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Phospholipiduria in 2-bromoethanamine-induced renal papillary necrosis

N. T. K. THANH[1]*, D. K. OBATOMI[2] and P. H. BACH[3]

[1] Pharmaceutical Sciences Research Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, UK. e-mail: nguyetkt@aston.ac.uk

[2] Life Sciences Department, Faculty of Science and Health, University of East London, Romford Road, London, E15 4LZ, UK

[3] Khaya Lami House, Castle Road, Horsell, Woking, Surrey, GU21 4EU, UK

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The possibility that phospholipid excretion in urine might be a marker of renal papillary necrosis (RPN) induced by 2-bromoethanamine (BEA) was investigated in male Wistar rats following a single i.p. injection in time course and dose–response experiments. Urinary and serum creatinine as well as electrolytes were also measured in parallel to histological examination of tissues. A single i.p. injection of BEA caused a characteristic dose-related change in urinary phospholipids, notably sphingomyelin (SPM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) excretion on the first day of treatment. At a lower dose of 30 mg kg^{-1} BEA, there was no alteration in the levels of the phospholipids; however, significant increases in the excretion of SPM, PC and PE at the 90 and 240 mg kg^{-1} dose level were observed. There was an early increase in the three phospholipids irrespective of whether the excretion is expressed in units per hour excretion or units per milligram creatinine. The increased excretion of SPM and PC remained over 4 days while PE levels returned to normal after day 1. On day 1, urinary flow rate and creatinine were also increased significantly while there was a significant fall in the levels of some electrolytes (Na^+, K^+, Cl^-). Parallel histological examination also confirmed the presence of RPN at the two higher doses (90, 240 mg kg^{-1}) of BEA. Other measurements such as blood urea nitrogen and the levels of Ca^{2+} and Mg^{2+} in blood were unaffected at all dose levels of BEA. These results demonstrate the potential of specific urinary phospholipids as diagnostic bio-markers for early renal injury associated with RPN and may provide an important improvement in the non-invasive approach to the therapeutic management of renal diseases, and potentially prevent further degenerative changes.

Keywords: 2-bromoethanamine, renal papillary necrosis, urinary phospholipids, diuresis, creatinine.

Introduction

Necrosis of renal papilla has been associated with the excessive consumption of single or mixed analgesic drugs for almost 40 years (Bach and Thanh 1998) and *has posed* a significant and costly health problem. While the mechanism of the renal damage remains unclear, early asymptomatic renal papillary necrosis (RPN) often eludes correct diagnosis until end-stage renal disease becomes apparent.

A variety of compounds including BEA have been well studied as model papillotoxins in animals (Bach 1981, Bach and Bridges 1985, Bach and Gregg

*Corresponding author: N T K Thanh, Pharmaceutical Sciences Research Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK.

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Lipid extraction and separation of phospholipids by HPLTC

Aliquots of urine samples were extracted with chloroform/methanol (2:1, v/v) as previously described (Thanh *et al.* 1997). The PLs were separated by the solvent system methanol/chloroform/*n*-propanol/methyl acetate/43 mM KCl (10:25:25:25:9, v/v) (Schmitz *et al.* 1984). Samples were applied to the plate 10 mm from the edge by a Camag Linomat IV applicator with a 5 mm band width, and 4 mm between bands. The sample was applied at $0.2\,\mu l\,s^{-1}$ with nitrogen (BOC Gases Ltd, London, UK) pressure set at 4.5 bar. The Linomat sprays samples in the form of bands onto the HPTLC silica gel (Merck Ltd, Dorset, UK).

Lipids on the plate were developed in a Camag (Camag, Muttenz, Switzerland) horizontal developing chamber. In this chamber, an HPTLC plate was developed from both opposing sides towards the middle. This permitted the number of samples to be doubled as compared with development in a tank.

A Desaga CD 60 densitometer with the method for validation software version 4.3 (Desaga GmbH, Heidelberg, Germany) was used for the densitometric evaluation. The scanner was linked via an RS 232 interface to a personal computer, from which all commands were passed to the scanner. The scanner transmitted all measurement data in digital form to the computer to create chromatograms. Integration, calibration and calculation of the results were then performed. Urinary excretion of lipids was expressed either as units per 16 h or units per mg creatinine for comparison.

Analytical procedures

Serum and urinary creatinine levels were determined using the appropriate kits (Sigma Ltd, Poole, UK). The method of assay was based on the Jaffe reaction and was performed as previously described (Thanh 1998). The concentrations of Na^+, K^+, Cl^- in the urine were determined as previously described (Obatomi *et al.* 1992).

Histopathology

Histopathology was used to detect the type, site and magnitude of the renal damage. The prepared sections were stained in haematoxylin–eosin to reveal general cell structure from which the site of the lesion such as necrosis, focal hyperplasia or swelling could be detected.

All data are presented as mean ±SEM (standard error of the mean). Differences between treated animals and control were evaluated statistically by using ANOVA, with statistical significance defined as $P < 0.05$.

Results

There were no clinical signs of toxicity such as weight loss following administration of BEA even at the high dose ($240\,mg\,kg^{-1}$) during the 4-day period of the experiment (data not shown).

A significant increase ($P < 0.01$) in SPM, PC, and PE was observed at doses of 90 and $240\,mg\,kg^{-1}$ of BEA on day 1 (figure 1). No lesions were observed in animals treated with a dose of $30\,mg\,kg^{-1}$ BEA. Throughout the 4-day period, the medium and high doses of BEA produced similar increases in urinary lipid excretion. For TPL, the pattern of response is the same as that of the individual lipids.

Figure 2 demonstrates the daily changes (16 h urine collection) in urinary phospholipid excretion following a single i.p. injection of BEA ($240\,mg\,kg^{-1}$) over 4 days. The overall pattern of excretion of the phospholipids follows similar trends irrespective of the method of expression (either per 16 h collection or per milligram creatinine). BEA produced a significant increase in the selected phospholipids assayed. SPM and PC excretion (when expressed either per hour or per milligram creatinine) rose significantly on days 1 and 2 followed by a return to control level on day 3 and a further significant rise on day 4. However, PE showed a significant increase ($P < 0.001$) only on day 1 and subsequently the levels remained within normal values. Excretion of urinary lipids in the control showed no significant variation.

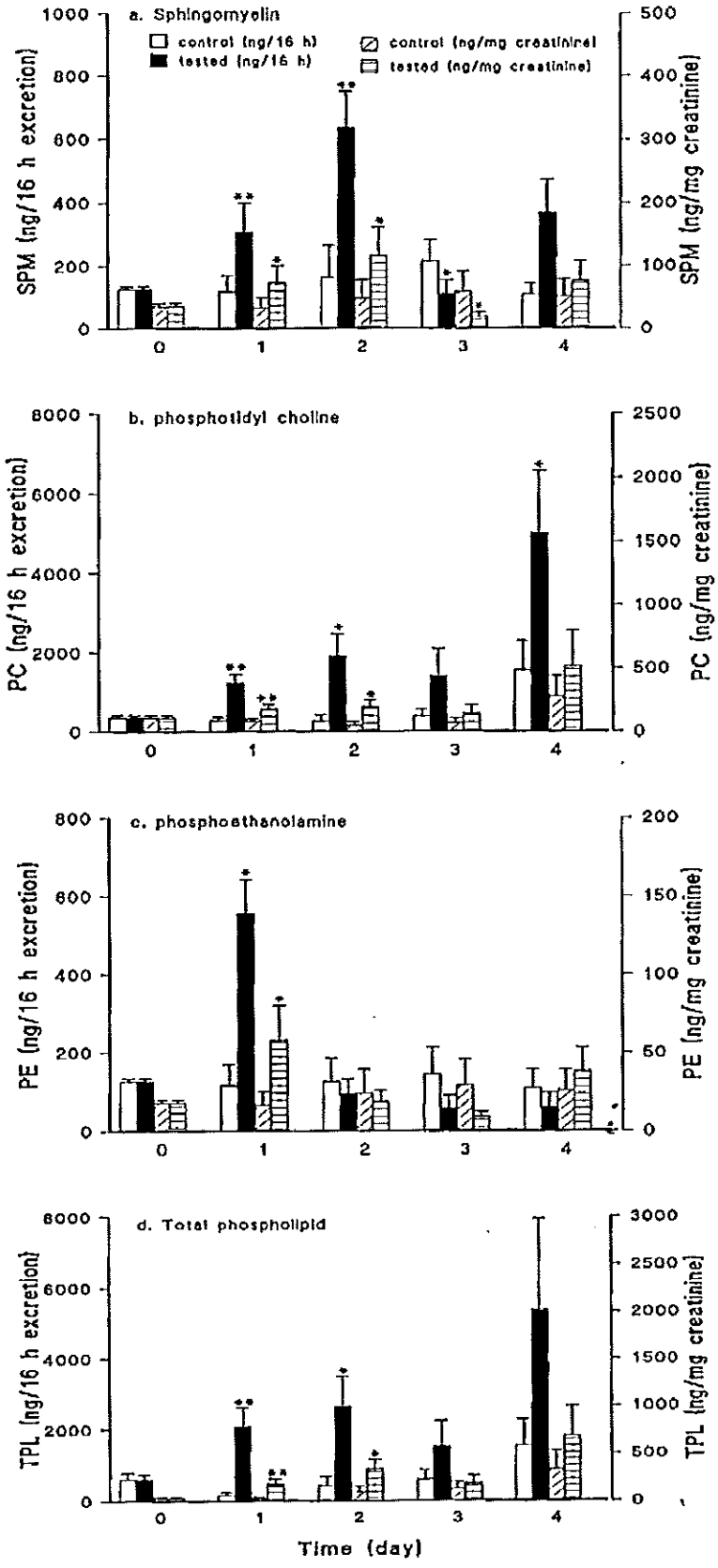

Figure 2. Daily changes in the urinary excretion of SPM, PC, PE and TPL over 4 days following a single dose of BEA, 240 mg kg^{-1}. Urine samples were collected over 16 h each day. Values are presented either relative to flow rate or to creatinine concentration and as mean ± SEM, $n = 6$. As compared with the control, *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's t-test).

marker for the segment-specific effect of chemicals such as BEA, since urinary phospholipid mainly originates from the medulla and papilla (Bach *et al.* 1991, Bach and Thanh 1998).

At present, the evidence of histopathological examination is the means of confirming the presence of RPN. As such, we have carried out histopatological assessment in the present study. The data obtained revealed lipid accumulation in both the medullary interstitial cells and the endothelial cells of the *vasa recta* on day 4 (by which time papillary necrosis had developed) after BEA treatment. Furthermore, there were also markedly increased quantities of lipid in the cells of the collecting ducts and the covering epithelia.

Acute exposure to lower doses of BEA did not produce any toxic effect whereas higher doses (90 and 240 mg kg^{-1}) produce a dose dependent toxicity. A significant increase of urinary phospholipid excretion following administration of BEA was seen as early as day 1 for SPM at the high dose and for PC and PE at the medium and high doses. The greater excretion of phospholipids may reflect the increased turnover of cells in the tubular epithelium and the sloughing of cells into the urine in response to the toxic action of BEA. The data support previous findings that histochemical renal lipid accumulation in RPN (Bach *et al.* 1991) was accompanied by lipidosis in the urine. The increase in urinary phospholipid excretion might be due to the extent of the phospholipidosis observed in the renal medulla and papilla. The observation made in this study is quite similar to the effect obtained with the aminoglycoside antibiotics therapy, which induced a marked urinary excretion of phospholipids in both rat (Josepovitz *et al.* 1986, Fashola *et al.* 1999, Thanh *et al.* 2001) and man (Ibrahim *et al.* 1989, Saunders *et al.* 1997), although this drug affected the renal cortical section (the proximal tubule) of the kidney. Our finding is most significant because it is the first time that significant changes in urinary SPM, PC, PE and TPL have been observed in the rat as early as 24 h after a single nephrotoxic dose of BEA.

BEA produced significant dose- or time-related changes in urine volume, creatinine and electrolytes during the treatment period in agreement with previous findings. As demonstrated previously (Bach and Thanh 1998), elevations of urine volume (diuresis) have been shown to be a feature of BEA.toxicity. There is a general assumption that animals increase their urinary flow due to marked reduction in the percentage of filtering juxtaglomerular nephrons in order to protect against BEA-induced RPN (Bach and Thanh 1998).

Because of the diuretic effect of BEA the expression of the excretion of phospholipids per 16 h was thought to be misleading, thus a secondary baseline was chosen by which to normalize the values against creatinine. Interestingly, the expression of excretion of phospholipids per milligram creatinine produced a similar pattern of results with expression per 16 h. This shows that the increase in phospholipids is not due to the diuretic effect of BEA and the increase observed is unambiguous expression of RPN, which had developed in the animals. Creatinine is synthesized in the kidney, liver and pancreas (Ibrahim *et al.* 1994) and the amount produced is generally proportional to body muscle mass. In the absence of renal disease, the excretion rate of creatinine in an individual is constant. We have observed that such a significant change in urinary creatinine in this study confirms a renal disease (RPN). It will therefore not serve as the best parameter with which to normalize phospholipid excretion in RPN. It is therefore best to express our data per h excretion.

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