Free Radical Research, September 2011; 45(9): 1000–1012

Phenolic antioxidants tert-butyl-bisphenol and vitamin E decrease oxidative stress and enhance vascular function in an animal model of rhabdomyolysis yet do not improve acute renal dysfunction

HYUN BO KIM[1], ANU SHANU[1,2], SARAH WOOD[1,2], SARAH N. PARRY[1,2], MICHAEL COLLET[1], AISLING MCMAHON[3] & PAUL K. WITTING[1]

[1]*Discipline of Pathology, Redox Biology Group, The University of Sydney, Sydney, NSW 2006, Australia,* [2]*Vascular Biology, and* [3]*Biogerontology Groups, ANZAC Research Institute, Concord Repatriation General Hospital, Concord, NSW 2139, Australia*

(Received date: 13 March 2011; Accepted date: 18 May 2011)

Abstract

Rhabdomyolysis (RM) caused by severe burn releases extracellular myoglobin (Mb) that accumulates in the kidney. Extracellular Mb is a pro-oxidant. This study tested whether supplementation with *tert*-butyl-bisphenol (BP) or vitamin E (Vit E, as α-tocopherol) at 0.12% w/w in the diet inhibits acute renal failure (ARF) in an animal model of RM. After RM-induction in rats, creatinine clearance decreased ($p < 0.01$), proteinuria increased ($p < 0.001$) and renal-tubule damage was detected. Accompanying ARF, biomarkers of oxidative stress (lipid oxidation and hemeoxygenase-1 (HO-1) gene and protein activity) increased in the kidney ($p < 0.05$). Supplemented BP or Vit E decreased lipid oxidation ($p < 0.05$) and HO-1 gene/activity and restored aortic cyclic guanylyl monophosphate in control animals ($p < 0.001$), yet ARF was unaffected. Antioxidant supplementation inhibited oxidative stress, yet was unable to ameliorate ARF in this animal model indicating that oxidative stress in kidney and vascular cells may not be causally related to renal dysfunction elicited by RM.

Keywords: *Antioxidant, oxidative stress, myoglobinuria, burns, polyphenol, acute renal failure*

Abbreviations: *ARF, acute renal failure; ARE, antioxidant response element; BP, tert-butyl-bisphenol; CE, cholesteryl esters; CEO(O)H, cholecterylester hydroperoxides and hydroxides; cGMP, cyclic guanylyl monophosphate; GFR, glomerular flow rate; HO-1, hemeoxygenase-1; •NO, nitric oxide; NF-κB, nuclear factor kappa Beta; Mb, myoglobin; TNF, tumour necrosis factor; Vit E, vitamin E.*

Introduction

Acute renal failure (ARF) is a manifestation of severe burns—its incidence varies between 1–39% of patients [1,2]. This low clinical frequency, however, is offset by an ~ 85% mortality rate [3,4]. One factor affecting kidney function in burns patients is burn thickness. Full thickness burns penetrate tissue, damage muscle or bone and can lead to muscle necrosis and the release of myoglobn (Mb) into the blood [5,6]. Accumulation of extracellular Mb in the kidneys can lead to ARF [7,8] and this may play a role in mortality associated with these clinical sequelae in burns patients.

The pathogenesis of Mb-induced/mediated renal failure is still debated. The process of RM results in both the initiation of an endotoxin cascade [8] and volume depletion, which can lead to ischemia/reperfusion injury in the kidneys. In addition, Mb can scavenge endothelial nitric oxide (•NO) [9], which promotes vasoconstriction by limiting •NO bioavailability [10]. Also Mb can cause tubular obstruction through cast formation [8]. Together these actions decrease renal perfusion and cause renal insufficiency [8,11]. Despite these observations, it is presently not clear whether the tubular casts are the result of renal failure or causally related to the pathology [8].

Correspondence: Dr Paul Witting, Discipline of Pathology, The University of Sydney, NSW, 2006 Australia. Tel: 61-2-9114-0524. Fax: 61-2-9351-2429. Email: p.witting@sydney.edu.au

ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.590137

The detection of urinary cross-linked Mb formed through Mb peroxidase activity strongly supports the notion that pro-oxidative Mb is present in the kidney during RM [3]. Exposure of kidney epithelial cells to pro-oxidant Mb enhances oxidative stress and promotes dysfunction and this may be related to increases in cellular iron [12,13]. However, adjunctive therapies aimed at inhibiting renal oxidative damage during RM are not routinely employed in the clinical management of burns.

We recently demonstrated that the low-molecular weight polyphenol 3,3',5,5'-tetra-*tert*-butyl-biphenyl-4,4'-diol (BP) protected cultured kidney epithelial cells from Mb-mediated damage [13]. Synthetic BP was designed through a structure-function study [14] and is characterized by a lower redox potential than vitamin E (Vit E) [15]. Vit E is a natural antioxidant that has eight analogues with α-tocopherol (α-TOH) exhibiting the greatest biological activity [16]. Similar to the synthetic polyphenol BP, Vit E protects cell membranes from oxidation [17] and this activity has proven beneficial in some animal models where oxidative damage is purported to play a pathological role [18–21]. Oxidative stress and inflammatory responses are closely related in patients with ARF [22]. Studies have indicated the induction of redox-sensitive Nuclear Factor Kappa Beta (NF-κB) [23] following Mb-mediated oxidative stress induced by experimental RM.

Together these data support the idea that bolstering renal antioxidant capacity may protect the kidney from Mb-induced damage. Herein we have compared the renal protection of synthetic and naturally occurring phenolic antioxidants in an experimental model of RM that induces characteristics of ARF.

Materials and methods

Materials

Unless otherwise indicated, materials were from Sigma (Sydney, Australia). Chemicals used for transmission electron microscopy (TEM) were from ProSciTech (Queensland, Australia). Synthetic BP (99% purity) was from Polysciences Inc (Palo Alto, CA). Solutions were prepared with MilliQ® Water or analytical grade solvents. Male Sprague-Dawley rats (6–8 weeks old) were from the Animal Resources Centre (Perth, Australia). Studies were approved by the Local Ethics Committee.

Antioxidant supplementation

Animals were supplemented with BP or Vit E (*R*,*R*,*R*-α-TOH) at 0.12% w/w in the diet for 4 weeks. This level of BP supplementation yielded ~50–100 μM of the polyphenol in the blood [24,25]. Diets were prepared by dissolving 1.2 g of the antioxidant in ethanol and dispersing this solution over 1 kg of normal rodent chow then allowing the alcohol to thoroughly evaporate in a fume hood over 3 days. Chow preparations were routinely assessed for antioxidant content by extracting the adhered phenol from the surface of the chow. The fractional content of antioxidant per kg chow was then confirmed by liquid chromatography [24,25]. The level of *R*,*R*,*R*-α-TOH in normal chow was 32 mg/kg and this increased to 148 ± 8 mg/kg upon supplementation with *R*,*R*,*R*-α-TOH. Animals designated to the Control group were fed alcohol-treated chow. The Sham group received untreated normal chow. Feed and water was available *ad libitum*. To induce RM, animals were dehydrated for 18 h then anaesthetized with isofluorane (2% v/v $O_{2(g)}$, 1.5 L/min) and injected (hind leg) with 6 mL/kg of hypertonic glycerol (50% v/v). Animals assigned to the Sham group were administered an equal volume of sterile saline. All animals were then housed in metabolic cages and urine collected over 24 h.

Harvest of blood and organs

Animals were anaesthetized with isofluorane followed by *i.p.* injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Following a midline incision and thoracotomy, blood was collected into tubes containing 100 IU heparin via cardiac puncture. Plasma was isolated and stored at −80°C. Next, the animals were euthanized, the vasculature perfused with buffer A (50 mM phosphate buffer, pH 7.4) containing 1 mM EDTA, 10 μM butylated hydroxytoluene and one protease inhibitor tablet (Roche Pharmaceuticals, Sydney, Australia), then the kidneys were harvested.

The isolated left kidney was sectioned (~2 mm thickness) and immersed in a modified Karnovsky's fixative containing 4% w/v paraformaldehyde, 2% v/v glutaraldehyde and 1% w/v sucrose in neutral PBS for storage at 4°C. The right kidney was sectioned into two with the ventral half assigned to histology (fixed in 10% v/v formalin) and the dorsal portion sectioned into two. One sample was frozen in liquid nitrogen for mRNA analysis and the other immersed in buffer A and snap frozen for biochemical assays. Aortae were harvested, rinsed with saline (0.9% w/v) and immersed in buffer B (buffer A with phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine added (IBMX, 100 μM)) then frozen in liquid nitrogen.

Kidney morphological studies

An automatic tissue processor (Leica) was used to dehydrate specimens before paraffin embedding. Thin sections (5 μm) were cut and mounted onto Super-Frost slides for staining and immuno-histochemistry (IHC).

Images were captured using an Olympus inverted fluorescent microscope with a digital camera (Olympus Controller, v2.2.1.227). Images were converted to TIFF for manipulation with MS PowerPoint (2008, v7).

Histochemistry

Paraffin-embedded kidney sections were treated with periodic acid and stained in Schiff's reagent, counterstained with Mayer's hematoxylin, then dehydrated and mounted in *Di-n*-butyl-Phthalate in Xylene. Controls were stained in Schiff's reagent alone [26].

Immuno-histochemical visualization of HO-1 or Mb

Following antigen retrieval, sections were blocked with 3% v/v methanolic H_2O_2 and 10% v/v goat serum (in PBS) and then incubated with the primary antibody for 1 h (2 μg/mL of rabbit anti-human HO-1 or monoclonal mouse anti-human Mb; 1:500 v/v; Sigma). Controls were incubated with either PBS (primary negative control) or with rabbit or mouse IgG (isotype control, Santa Cruz). Sections were then washed and incubated in a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (0.5 μg/mL of goat anti-rabbit IgG, 30 min) to visualize HO-1 (excitation 450 nm, emission 510 nm). Sudan Black (0.6% v/v in PBS) was employed to decrease background staining. In the case of assessing immuno-active Mb, sections were washed and incubated in biotinylated secondary antibody (goat anti-mouse IgGB; Santa Cruz) for 1 h in a humidified chamber. Next, the signal was amplified using a commercial avidin-biotin complex kit (Vectastain ABC Kit; Vector Laboratories Inc, CA), before reaction with diaminobenzidine (DAB; DAKO Cytomation, CA). Sections were counterstained with Mayer's haematoxylin. Additonal control sections were treated with secondary alone before processing.

Transmission electron microscopy

Specimens were post-fixed in osmium tetroxide, dehydrated and embedded in Spurr's resin. Semithin (250 nm) and ultra-thin (90 nm) sections were dried onto glass slides and stained with toluidine blue then mounted on copper grids (200 mesh, ProSciTech) and stained with uranyl acetate and lead citrate to induce contrast. Images were obtained using the Philips CM12 TEM with iTEM imaging software (Electron Microscopy Unit, University of Sydney). Images were exported as tagged image files for further manipulation. Mitochondrial area was measured using equation (1):

$$\text{Mitochondrial area} = \pi.\frac{1}{2}(a \times b) \quad (1)$$

where a is length of longest diameter and b is length of shortest diameter.

Glomerular filtration rate

Plasma Na^+, K^+, Cl^-, urea, creatinine and protein were determined by the Diagnostic Pathology Unit (Concord Hospital). Serum and urinary creatinine were used to calculate the rate of creatinine clearance [27] to estimate glomerular filtration (equation 2) [28]:

$$C_{Cr} = (U_{Cr} \times U_{24V})/(P_{Cr} \times 1440) \quad (2)$$

where C_{Cr} = creatinine clearance in mL/min, U_{Cr} = urinary creatinine, mg/mL, U_{24V} = volume of urine passed in 24 h and P_{Cr} = plasma creatinine, mg/mL and 1440 min/24 h.

Plasma lipid and drug analysis

Analysis of vitamin E (as α-TOH), unesterified cholesterol (FC) and cholesteryl esters (CE) derived from arachidonic (C20:4) and linoleic acids (C18:2) and cholesteryl ester oxidation products (CE-O(O)H) in plasma and kidneys was carried out by high performance liquid chromatography (HPLC) as previously described [24,29]. Where required the plasma content of BP and DQ were measured by reversed phase HPLC [24,25].

Tissue studies

Kidney tissues were diced, frozen in $N_{(2)(liq)}$ pulverized with a mortar and pestle and then suspended in complete buffer A (2 mL), transferred to a glass tube and homogenized with a rotating piston (Wheaton, USA, 500 rpm) [24]. After 5 min, 50 μL was taken for protein analysis and the remainder was extracted into hexane/methanol (5:1 v/v) and the lipid-soluble fraction was isolated for HPLC analysis [29]. Renal lipids were normalized to total protein (BCA assay [29]) and expressed in units of nmol or ρmol/mg protein.

Enzyme assays

Where required, kidney homogenate was centrifuged (5000 × *g*, 5 min) and the supernatant taken and centrifuged a second time (5000 × *g*, 5 min) to remove any solid material. The clarified supernatant was collected for use in enzyme activity assays (see below).

Hemoxygenase activity

To complement gene and immuno-histochemical assessments of HO-1, total renal HO activity was

determined by measuring bilirubin production in renal homogenates [30]. Clarified homogenate was centrifuged (100 000 × *g*, 4°C, 1 h) with an Optima TLX Ultracentrifuge (Beckman-Coulter, Australia) and the microsomal fraction was suspended in Buffer C containing 250 mM sucrose and 20 mM Tris (pH 7.4). For total HO activity, 50–200 μg microsomal protein and 600 μg rat liver microsomes were mixed in 100 μL of ice-cold Buffer C containing 1 mM NADPH, 2 mM D-glucose-6-phosphate, 1U glucose-6-phosphate-dehydrogenase and 25 μM hemin in 25% v/v DMSO. Mixtures were incubated in the dark (1.5 h, 37°C) and reactions stopped with ethanol:DMSO (95:5, v/v), centrifuged (13 000 × *g*, 5 min) and bilirubin was determined with reversed-phase HPLC [30]. Total HO-1 activities were reported as the yield of bilirubin/mg protein/min.

Vascular cGMP

Activation of soluble guanylyl cyclase by •NO within vascular smooth muscle promotes the conversion of guanosine diphosphate to cGMP, which initiates vasorelaxation [31]. Where required, aortae were thawed then pulverized and homogenized in 1 mL of complete buffer A containing 100 μM IBMX. The resultant homogenate was split into two samples—50 μL was designated for protein assessment and the remainder for cGMP determination. Aortic cGMP was determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA). Absorbance (420 nm) was measured with an Ultramark reader (Bio·Rad, Sydney NSW, Australia) with Microplate Manager v5.1.

Tissue gene response

Total RNA was extracted from kidney homogenates with a commercial kit (GeneElute, Sigma, Sydney NSW, Australia). cDNA was constructed with BioScript Reverse Transcriptase (Bioline, Sydney NSW, Australia) and a PCR MasterCycler (Eppendorf, Sydney NSW, Australia). Mixtures containing 2 μL total RNA, 2 μL oligo (dT) (50 μm) and 8 μL of DEPC-treated Nanopure water were denatured (70°C, 5 min), chilled (4°C, 5 min), then treated with 0.25 μL RNase inhibitor (10U), 1 μL dNTP mix (10 mM, Proligo, Lismore, NSW, Australia), 4 μL of reaction buffer, 14.5 μL DEPC-treated Nanopure water and 0.25 μL Bioscript (Bioline, Sydney, Australia). Next, samples were heated (42°C, 60 min) and the reaction stopped by heating to 70°C (10 min).

Reverse transcriptase PCR

Gene-specific PCRs were performed with Biomix Red® (Bioline) and the primers shown in Table I. Cycling was initiated by denaturation (94°C, 5 min) followed by 25–33 cycles of denaturation (94°C, 30 s), annealing (0.5–1 min) and elongation (72°C, 1 min) then a final extension step (72°C, 10 min). Amplified cDNA was resolved on 1% w/v agarose containing ethidium bromide (2 μg/mL). Products were visualized and imaged under UV light and converted to TIFF for semi-quantitative densitometry using Image J v1.42 www.rsb.info.nih.gov/ij/ (NIH, USA).

Urinary myoglobin

Urine was diluted 10 × in PBS and urinary $[\text{globin}]_{\text{total}}$ was estimated by determining $A_{409\text{nm}}$ (Soret of hemoglobin and Mb) and using $\varepsilon = 188\ 000\ \text{mol}^{-1}\ \text{cm}^{-1}$.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (V5.0) using one-way ANOVA with Newman-Keuls post-hoc test; significance was accepted at $P < 0.05$.

Results

Injection of 6 mL/kg body weight of 50% v/v hypertonic glycerol into the rat hind leg induced RM. Animals in the Sham group received an equivalent volume of sterile saline. This model of RM yielded urinary $[\text{globin}]_{\text{total}} \sim 52 \pm 6$ μM (mean ± SD, $n = 16$, data not shown) that mimics urinary Mb levels in humans with severe electrical burn [32].

Table I. Primer sequences used for mRNA detection of antioxidant genes.*

Gene	Forward primer	Reverse primer	T_m (°C)
TNF	5′-AGGGTACCACAGAAAGATGC-3′	5′-GGAGATGAGACCCTTAGGTT-3′	56
NF-κB	5′-TTCCCCACACTGTAAACCAA-3′	5′-AGCAAGTGTAATCCAATAGC-3′	54
SOD-1	5′-CCACTGCAGGACCTCATTTT-3′	5′-TCTTCATTTCCACCTTTGCC-3′	59
SOD-2	5′-GGCCAAGGGAGATGTTACAA-3′	5′-GCTTGATAGCCTCCAGCAAC-3′	60
HO-1	5′-GAGATTGAGCGCAACAAGGA-3′	5′-AGCGGTAGAGCTGCTTGAACT-3′	59
β-Actin	5′-AGCCATGTACGTAGCCATCC-3′	5′-CTCTCAGCTGTGGTGGTGAA-3′	60

TNF, Tumour necrosis factor; NF-κB, nuclear factor kappa B; SOD-1/2, superoxide dismutase 1 and 2; HO-1, hemeoxygenase-1.
*Oligomers were synthesized by Sigma Aldrich (Sydney, Australia) and β-actin was employed as a housekeeping gene in all Reverse Transcriptase-PCR studies. The corresponding annealing temperatures employed in the RT-PCR reactions are as listed under T_m.

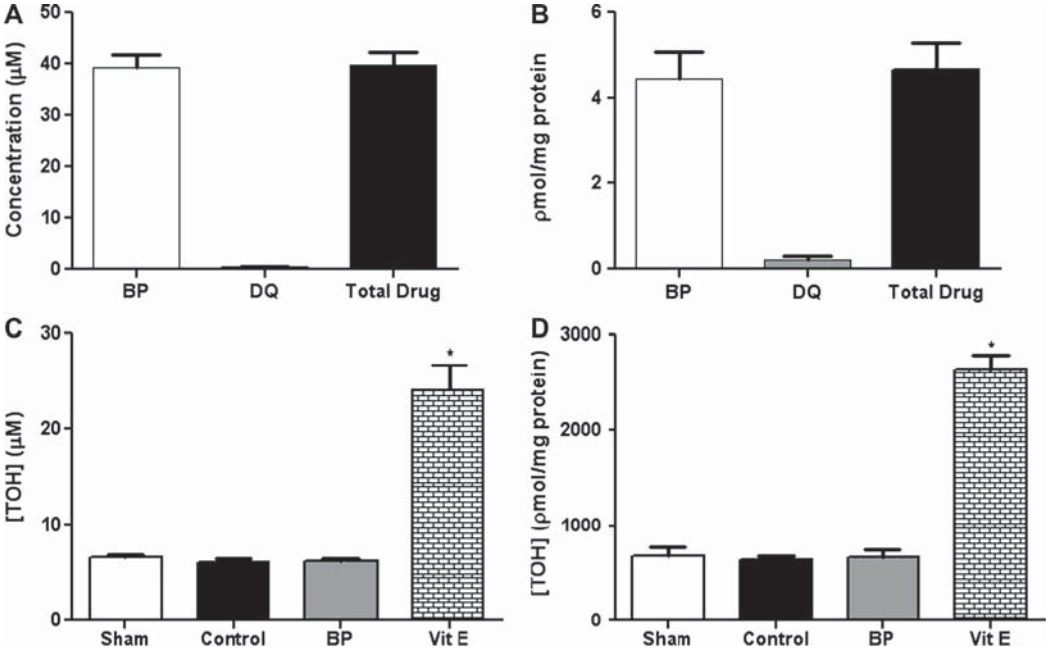

Figure 1. Supplementing rats with dietary BP or Vit E increases the content of the antioxidants in the circulating blood and renal tissues. After supplementation with normal, vehicle, BP or vitamin E-treated chow (antioxidants at 0.12% w/w) for 4 weeks, animals were euthanized and samples of blood and perfused kidneys were isolated. Figures represent BP and DQ levels within (A) plasma (ratio of active-to-inactive antioxidant BP/DQ~95%) and (B) kidney homogenates (BP/DQ~9%) and Vit E level (as α-TOH) measured in (C) plasma and (D) kidney homogenates. Data expressed as Mean ± SD for Sham ($n = 8$), Control ($n = 16$), BP ($n = 8$) and Vit E ($n = 8$). *Different to the Sham group, $p < 0.001$.

Antioxidant levels in supplemented animals

Consistent with the study design, BP was detected in plasma (reaching ~ 40 μM) and kidney (~ 4.3 ρmol/mg protein) with a significant proportion of the drug present as the active antioxidant rather than the oxidized diphenoquinone (DQ) (Figures 1A and B). The concentration of α-TOH was also elevated in the plasma and kidneys of the Vit E-treated animals compared to the other groups (Figures 1C and D and Tables II and III). Importantly, plasma and renal tissue levels of α-TOH in animals supplemented with BP were similar to that determined in the Sham and vehicle-Control groups. Thus, any affect attributed to BP is likely independent of Vit E.

Markers of oxidative stress

Plasma levels of FC and CE remained unchanged in the Sham and vehicle-Control groups after experimental RM (Tables II and III). However, CE-derived CE-O(O)H were elevated in the vehicle-Controls and this was irrespective of whether CE-O(O)H was

Table II. Plasma concentrations of native and oxidized lipids and Vitamin E.[a]

	Sham ($n = 8$)	Control ($n = 16$)	BP ($n = 8$)	Vit E ($n = 8$)
[FC] (mM)[b]	0.7 (0.3)	0.8 (0.3)	0.8 (0.13)	1.1 (0.2)*
[α-TOH] (μM)	6.7 (1.0)	6.2 (1.4)	6.3 (0.7)	24 (7.4)*
[CE-O(O)H] (μM)	0.04 (0.03)	3.9 (0.9)*†	0.1 (0.1)¶	2.0 (0.8)*†¶
[CE-O(O)H]/Total [CE] × 10^3	0.1 (0.04)	4.6 (4.4)*†	0.1 (0.1)¶	2.0 (0.7)*¶†
[C18:2] (mM)	0.12 (0.04)	0.13 (0.04)	0.1 (0.1)	0.1 (0.1)
[C20:4] (mM)	0.8 (0.3)	1.1 (0.4)	0.8 (0.3)	0.9 (0.2)

[a]Animals supplemented with normal chow (Sham), vehicle-treated chow (Control) or chow supplemented with Bisphenol (BP) or vitamin E (Vit E) (each antioxidant 0.12% w/w in the diet) for 4 weeks were subjected to experimental RM (except Sham) then euthanized and samples of blood were obtained and the parameters listed were measured by liquid chromatography as described in the Methods section. Data expressed as Mean ± (SD). FC = unesterified cholesterol; α-TOH = α-tocopherol (biologically active vitamin E); C18:2 = cholesteryl linoleate; C20:4 = cholesteryl arachidonate; CE = combined cholesteryl esters representing the sum of C18:2 and C20:4; CE-O(O)H = CE-derived lipid hydroperoxides and hydroxides. The proportion of CE-O(O)H/CE represents the proportion of oxidized lipid per parent lipid.

[b]Units of measurement and the numbers of samples tested (n) for all parameters are as indicated. *Increased compared to the Sham group; $p < 0.05$. ¶Decreased compared to the Control group; $p < 0.05$. †Increased compared to the BP-supplemented group; $p < 0.001$.

Table III. Kidney tissue concentrations of native and oxidized lipids and vitamin E.[a]

	Sham ($n=8$)	Control ($n=16$)	BP ($n=8$)	Vit E ($n=8$)
[FC] nmol/mg protein[b]	0.2 (0.04)	0.2 (0.1)	0.3 (0.05)	0.2 (0.1)
[α-TOH] ρmol/mg protein	683 (276)	643 (178)	666 (232)	2631 (406)*
[CE-O(O)H] ρmol/mg protein	0.3 (0.1)	7.8 (2.4)*††	0.7 (0.2)¶	3.1 (1.1)*¶†
[CE-O(O)H]/Total [CE] $\times 10^3$	0.01 (0.002)	0.2 (0.1)*	0.01 (0.002)¶	0.05 (0.01)¶†
[C18:2] ρmol/mg protein	3.0 (0.75)	3.6 (2.0)	4.2 (2.1)	7.0 (3.8)*
[C20:4] ρmol/mg protein	73.3 (31.3)	38.2 (19.3)*	85.7 (32.9)	64.1 (18.3)

[a]Animals supplemented with normal chow (Sham), vehicle-treated chow (Control) or chow supplemented with Bisphenol (BP) or vitamin E (Vit E) (0.12% w/w in the diet) for 4 weeks were subjected to experimental RM (except Sham) then euthanized. Kidneys were isolated and the biochemical parameters listed were measured by liquid chromatography as described in the Methods section. Data expressed as Mean ± (SD). FC = unesterified cholesterol; α-TOH = α-tocopherol (biologically active vitamin E); C18:2 = cholesteryl linoleate; C20:4 = cholesteryl arachidonate; CE = cholesteryl esters. It is the sum of C18:2 and C20:4; CE-O(O)H = CE-derived lipid hydroperoxides and hydroxides. CE-O(O)H/CE represents the proportion of oxidized lipid per parent lipid.
[b]Units of measurement and the numbers of samples tested (n) for all parameters are as indicated. *Increased compared to the Sham group; $p<0.05$. ¶Decreased compared to the Control group; $p<0.05$. †Increased compared to the BP-supplemented group; $p<0.001$.

normalized to protein or expressed as a proportion of the total CE. No alteration in renal FC or C18:2 was detected when comparing Sham and vehicle-Control groups. However, C20:4 decreased significantly upon RM-induction. Notably, renal CE-O(O)H increased markedly in the Control group, reaching ~ 40-fold greater than the Sham. Together, these data suggest that a majority of the oxidized CE in renal tissues comprised of oxidized C20:4 the most prevalent poly-unsaturated lipid in the kidney.

Compared to Sham, animals receiving dietary BP showed no change in lipid or α-TOH content in plasma or renal tissues after RM. As expected, the α-TOH content in the plasma and the kidneys increased significantly in animals receiving the Vit E-fortified diet, yet FC and C20:4 were unchanged. However, a selective increase in kidney C18:2 was observed. In the presence of the supplemented phenols, lipid oxidation in the plasma and renal tissues decreased significantly, irrespective of whether CE-O(O)H was normalized to protein or expressed as a fraction of total CE. Consistent with the idea that BP antioxidant activity was superior to Vit E, inhibition of lipid oxidation in the plasma and kidney was greater in animals supplemented with BP than those receiving Vit E.

Gene regulation in ARF

Of the genes studied, a ~ 2-fold increase in gene expression of HO-1 was determined for the Control group after RM-induction (Table IV). However, the low-molecular weight antioxidants BP and Vit E readily ameliorated HO-1 gene expression. In animals supplemented with BP, SOD-1 and SOD-2 decreased slightly relative to the Control and this reached significance for SOD-1. Consistent with previous reports [33,34], animals receiving a relatively low Vit E-supplemented diet showed increased expression of pro-inflammatory genes TNF and NF-κB.

Immuno-histochemistry

HO-1 protein increased in renal samples where enhanced HO-1 gene expression was detected (Figures 2C and D). A comparison of the bright field and fluorescent images for the same section indicated HO-1 was localized to the capillary network of the glomerular tufts, although some HO-1 was detected in the tubular epithelium (see arrows in Figure 2C). In contrast, only weak immuno-activity was detected in the BP- and Vit E-treated groups (Figures 2D, F and H). Consistent with previous studies of RM-induced ARF [35,36], IHC revealed that both crystalline casts, localized in the renal tubular network, and some hyaline casts contained precipitated Mb (Figure 3).

Table IV. Fold change in the expression of antioxidant response elements and pro-inflammatory genes in kidneys isolated from the different treatment groups.[a]

	Treatment group			
Gene	Sham	Control	BP	Vit E
HO-1	1 (0.56)¶	1.98 (1.15)	0.71 (0.29)¶	1.02 (0.28)¶
SOD-1	1 (0.35)	1.28 (0.65)	0.64 (0.20)¶	0.97 (0.64)
SOD-2	1 (0.45)	1.01 (0.41)	0.68 (0.36)	1.01 (0.45)
TNF	1 (0.19)	1.32 (0.57)	1.24 (0.40)	2.08 (0.95)*
NF-κB	1 (0.24)	1.04 (0.34)	0.86 (0.38)	1.50 (0.34)*

[a]Animals supplemented with normal chow (Sham), vehicle-treated chow (Control) or chow supplemented with BP or vitamin E (Vit E) (each antioxidant 0.12% w/w in the diet) for 4 weeks were subjected to experimental RM (or not, Sham) then euthanized. Kidneys were isolated and homogenized, then total mRNA was obtained and converted to cDNA for use in gene regulation studies as described in the Methods section. Data expressed as Mean ± (SD) for Sham ($n=6$), Control ($n=15$), BP ($n=8$) and Vit E ($n=8$) groups. Antioxidant stress elements (HO-1 and SOD-1/2) and inflammation (TNF and NF-κB) were measured and normalized against the corresponding β-actin housekeeping gene. Each treatment group was then expressed as a fold-change compared to the Sham (arbitrarily assigned unitary value). *Increased significantly compared to all other groups; $p<0.05$. ¶Different to the Control group; $p<0.05$.

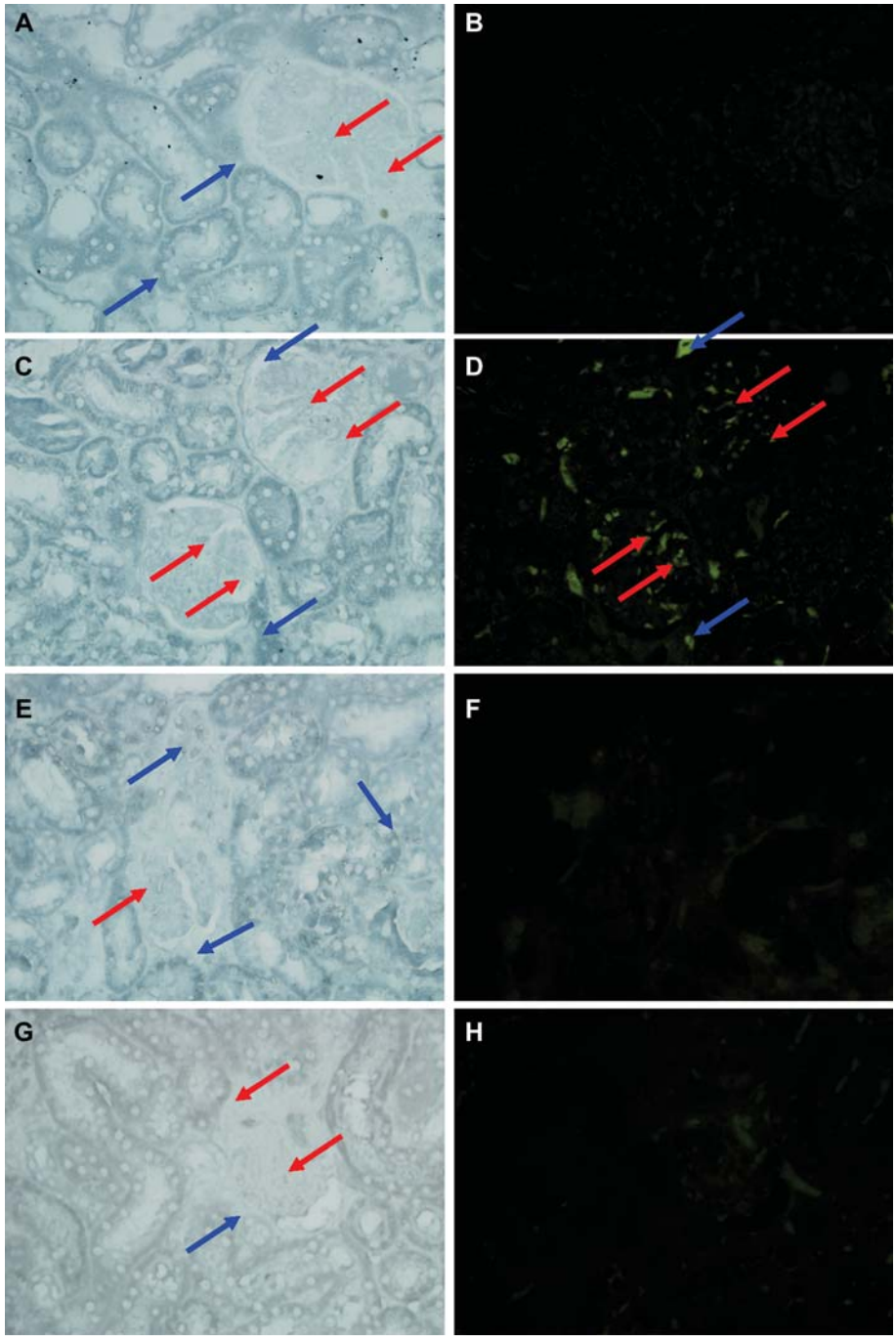

Figure 2. Detection of HO-1 in kidney tissues after experimental RM. Animals supplemented with vehicle, BP or vitamin E-treated chows (antioxidants 0.12% w/w in the diet) for 4 weeks were subjected to experimental RM or not (Sham). After 24 h, the animals were euthanized and samples of kidney were isolated, embedded, sectioned and stained for immuno-active HO-1 with a polyclonal antibody raised against human HO-1 and an anti-rabbit IgG-FITC secondary antibody (Excitation 450 nm, Emission 510 nm). Representative micrographs show: (A) bright field and (B) fluorescent views of HO-1 in a section of Sham kidney, (C) bright field and (D) fluorescent views of HO-1 in a section of vehicle-Control kidney, (E) bright field and (F) fluorescent views of HO-1 in a section of BP-treated kidney and (G) bright field and (H) fluorescent views of a section of vitamin E-treated kidney. Arrows highlight the capillary network of the glomerular tuft (red) and tubules (blue). Figures shown are representative of at least two independent samples from each treatment group. All images were captured under identical magnification and the scale of view is indicated in the figure.

Biochemistry analysis of renal function

Compared with the Sham, urinary Cl^- and K^+ ions decreased significantly in ARF groups while Na^+ remained unchanged. Plasma creatinine levels increased in the control after RM induction as did the urea content. As anticipated, proteinuria was evident in all glycerol-treated animals and GFR was significantly lower than that of Sham post RM-induction (Table V). Supplementation with synthetic or natural phenolic antioxidants had no effect on GFR or the extent of proteinuria while plasma levels of creatinine and urea decreased slightly albeit not significantly in

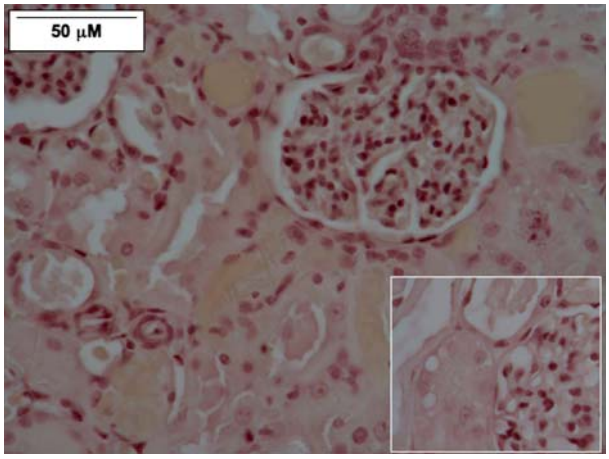

Figure 3. Accumulation of Mb in renal tubular casts after experimental RM induction. Animals supplemented with vehicle for 4 weeks were subjected to experimental RM or not (Sham). After 24 h, the animals were then euthanized and samples of kidney were isolated, embedded, sectioned and stained for immuno-active Mb shown as brown pigmentation present mainly in the tubular cast material. Inset shows sham tissue with no casts detected and no positive staining for immuno-active Mb.

the antioxidant supplemented groups. Urinary pH increased in animals supplemented with Vit E, whereas pH was unchanged for all other treatment groups (Table V).

Histochemistry

Renal sections obtained from Shams (Figure 4A) revealed structurally normal glomeruli and tubules exhibiting intact brush borders. However, tubular necrosis and both hyaline and crystalline casts were evident in the kidneys from the vehicle–Control and dietary antioxidants groups (Figures 4B–D). In addition, glomeruli showed evidence of swelling and/or deformation and discontinuous brush borders were accompanied with damaged epithelia (compare Figures 4A with B–D). These observations were consistent with the degree of ARF [37] established amongst these groups of animals (Table V).

Mitochondrial substructure in renal epithelial cells

TEM analysis of tubule epithelia in kidneys from the Sham group indicated normal mitochondria featuring compartmentalized inner membrane structures with clear cristae along with venules and basal lamina (Figure 5A). Kidneys from glycerol-treated animals showed mitochondrial damage with swollen matrixes and thickened cristae [26,37]—mitochondrial rupture was evident (Figure 5B). Consequently mitochondrial area increased (~1.8-fold) in glycerol-treated animals compared to Shams (Figure 5C) and regions of basal laminae were dissociated consistent with altered filtration capacity of the tubule epithelia. Mitochondrial swelling was unaffected by the presence of BP, suggesting that the polyphenol was unable to protect mitochondria from early dysfunction. The mitochondrial area of Vit E-treated samples was similar to that of the Sham group, despite the presence of some mitochondrial rupture.

Measurement of total HO activity

Induction of RM in the vehicle-Controls elicited a ~32-fold increase in bilirubin accumulation consistent with increased HO activity relative to the Sham (Figure 6A). Supplementation with BP or Vit E diminished HO activity to levels detected in the Sham with renal tissues from BP-supplemented animals recording a trend towards lower bilirubin accumulation than those receiving Vit E, but this did not reach significance.

Table V. Plasma and urinary biochemistry from animals exposed to experimental RM.[a]

Parameter	Sham (n = 8)	Control (*n* = 16)	BP (*n* = 8)	Vit E (*n* = 8)
Urine				
Na^+ (mmol/L)	34.1 (16.9)	40.1 (16.8)	36.1 (8.8)	53.3 (29.9)
K^+ (mmol/L)	218.1 (74.0)	136.0 (32.2)**	122.8 (22.4)**	115.8 (23.0)**
Cl^- (mmol/L)	154.0 (42.1)	94.0 (28.2)**	90.5 (27.7)**	107.4 (20.2)*
Protein (g/L)	2.2 (2.1)	9.9 (3.8)**	8.3 (1.9)**	8.7 (2.7)**
pH	7.4 (0.8)	7.6 (0.8)	6.9 (0.3)	9.0 (0.7)**
CCR (mL/min)	3.5 (1.4)	1.2 (0.9)*	1.4 (0.8)*	1.3 (0.9)*
Plasma				
Na^+ (mmol/L)	144.1 (4.2)	143.1 (2.0)	142.4 (2.9)	144.3 (1.3)
K^+ (mmol/L)	4.8 (1.4)	4.6 (0.9)	5.2 (0.7)	4.0 (0.6)
Cl^- (mmol/L)	102.3 (6.0)	103.7 (2.5)	103.1 (1.2)	106.6 (3.2)
Urea (mmol/L)	5.1 (1.1)	16.5 (10.6)*	13.9 (8.7)	15.9 (9.7)
Creatinine (μmol/L)	16.2 (4.0)	71.6 (56.6)*	50.0 (26.9)	65.4 (39.5)
Ca (mmol/L)	2.3 (0.3)	2.4 (0.2)	2.5 (0.2)	2.2 (0.1)

[a]Blood plasma and urine were collected 24 h after the induction of experimental RM and concentrations of different biochemical parameters were measured. Data expressed as Mean ± (SD). Units of measurement and the numbers of samples tested (*n* values for all parameters) are as indicated in the table. The creatinine clearance rate (CCR) was calculated as described in the Methods section. *Significantly different to the Sham group; $p < 0.01$. **Significantly different to the Sham group; $p < 0.001$.

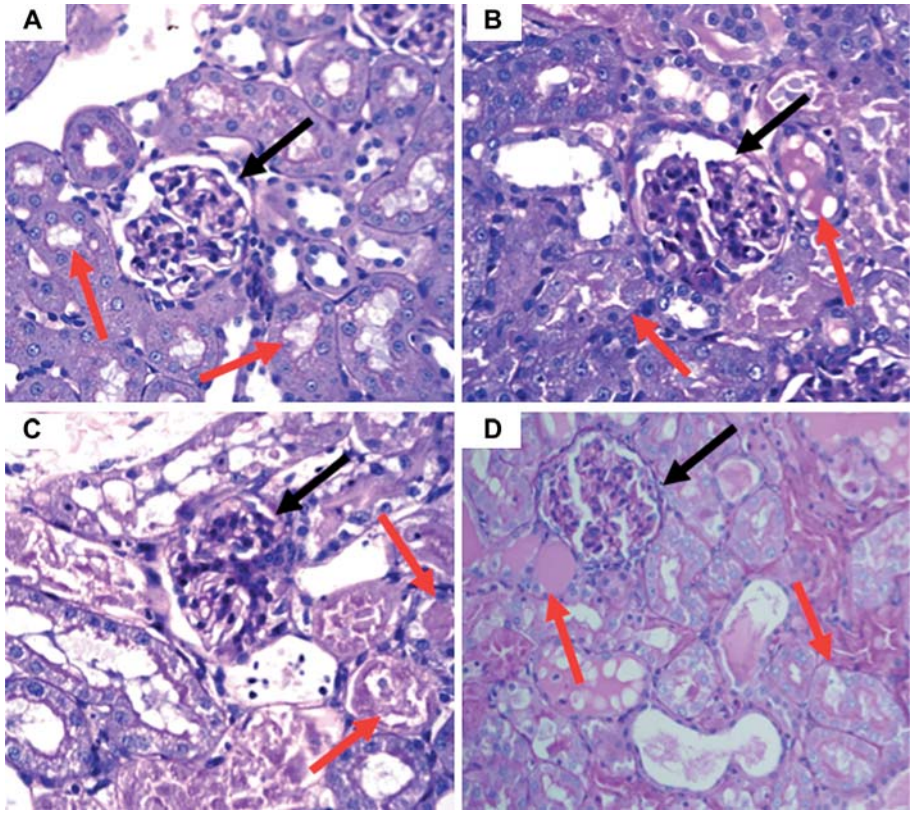

Figure 4. Experimental RM induces hallmarks of kidney damage associated with ARF. Animals supplemented with normal chow (Sham), vehicle-treated chow (Control) or chow fortified with BP or vitamin E (each 0.12% w/w in the diet) over 4 weeks were subjected to experimental RM (except Sham). After 24 h, the animals were euthanized and samples of kidney were isolated, embedded, sectioned and stained with PAS. Representative sections shown are obtained from (A) Sham, (B) vehicle-Control, (C) BP- and (D) vitamin E–treated animals. Arrows highlight the capillary network of the glomerular tuft (black) and tubules (red) contrasting the normal features in the Sham with condensed glomeruli and the presence of tubular atrophy and cast material in animals following RM induction. Figures are representative of at least three independent samples from each treatment group. Magnification was set to 40×. No evidence of infiltrating mononuclear cells was detected.

Aortic function

Relative to the Sham, vascular [cGMP] decreased significantly in all animals subjected to experimental RM (Figure 6B). In contrast, aortic cGMP was elevated in animals receiving dietary BP or Vit E compared to the vehicle-control, albeit that the restoration of cGMP did not reach corresponding levels in the Sham. Recovery of aortic cGMP in BP-treated animals was greater than that in Vit E-supplemented animals.

Discussion

Outcomes from this study confirm enhanced renal oxidative stress in this model of RM. Thus, lipid (per) oxidation increased in blood and renal tissues concomitant with release of extracellular Mb. This enhanced oxidative stress was characterized by a decrease in aortic cGMP, suggesting that circulating Mb impairs endothelial •NO bioavailability and that associated vascular dysfunction may be central to renal insufficiency in this model. Our studies show that Mb-insult induces the early antioxidant gene HO-1 linked to increased renal HO activity, whereas SOD enzymes remain unchanged. No inflammatory response was evident in the absence of antioxidant supplementation at least in the 24 h following RM. Renal tissues showed characteristic features of acute renal damage including the presence of tubular necrosis and tubular cast deposits rich in Mb, changes in glomerular structure, mitochondrial swelling and damage to the glomerular basement membrane, which manifested as increased proteinuria. These data demonstrate that RM leads to Mb-mediated damage in the kidneys and suggests that oxidative stress plays a role in decreasing renal blood flow and function in the absence of any marked inflammatory response as identified previously [38,39].

Effects of phenolic antioxidants in animal model of RM

The antioxidants BP and Vit E were selected for investigation here for several reasons. Both the phenols are bio-available and show biological activity associated with inhibiting lipid peroxidation [24,25, 40]. In addition, phenols are considered to be suitable substrates for peroxidase enzymes including the

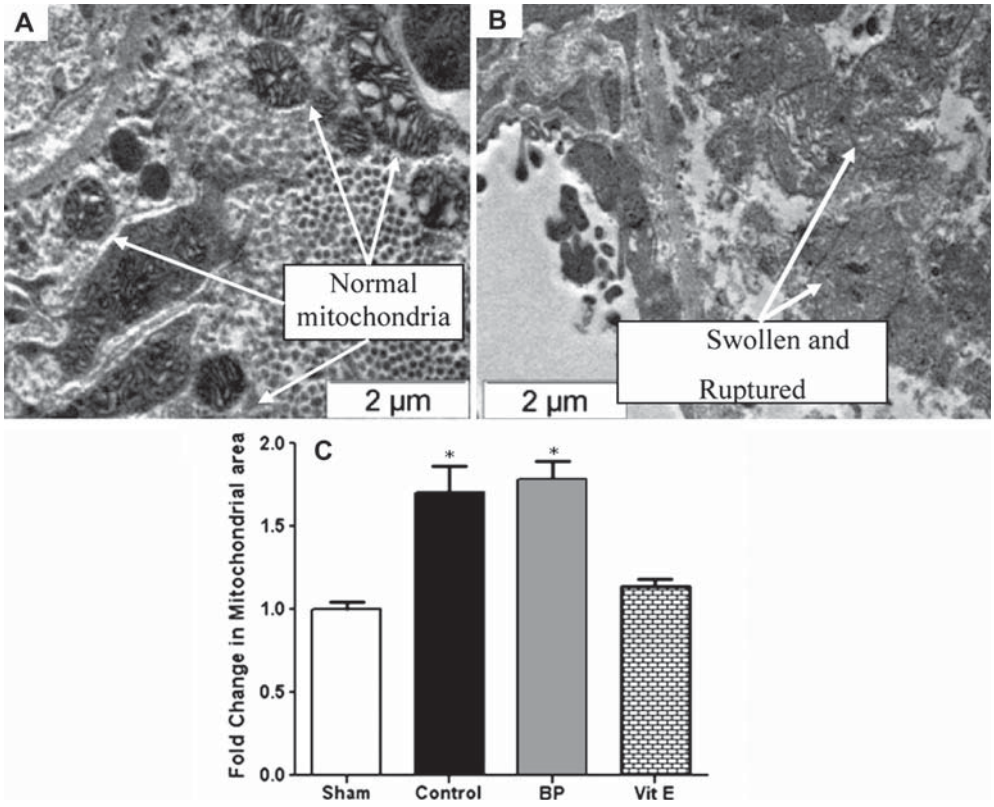

Figure 5. Transmission electron microscopy of kidney tubule epithelial cells. Animals supplemented with normal chow (*open bar*), vehicle-treated chow (*black bar*) or chow supplemented with BP (*grey bar*) (0.12% w/w in the diet) for 4 weeks were subjected to experimental RM (or not, Sham). After 24 h, the animals were euthanized and the kidneys isolated and prepared for TEM. Micrographs illustrate (A) mitochondria from a normal kidney, (B) swollen and/or ruptured mitochondria in an epithelial cell from a vehicle-Control kidney. (C) Mitochondrial swelling was determined by measuring area (assuming an oval shape), which was then expressed as fold-change relative to the Sham. The scale of view is indicated in the figure. For (A) and (B), data is representative of at least two different fields from each section with at least $n = 3$ samples from different animals. For (C), at least two different sections were viewed and every visible mitochondrion in the field was measured across its longest and shortest diameter to estimate its cross-sectional area (assuming an oval shape). Data expressed as Mean ± SD for Sham ($n = 219$), Control group ($n = 59$), BP ($n = 124$) and Vit E ($n = 512$). *Different to the Sham group, $p < 0.001$.

pseudo-peroxidase Mb [41]. Supplementing rats with BP or Vit E before the induction of RM prevented lipid oxidation in both the vasculature and kidneys. Antioxidant supplementation also reversed the decline in aortic cGMP and down-regulated HO-1 gene expression and activity in the kidneys. Supplementation with BP depressed SOD-1 gene expression in the kidney to below levels measured in the Sham.

As anticipated, BP showed greater antioxidant activity than Vit E, as judged by comparing most of the parameters tested. No change in pro-inflammatory gene regulation was detected up to 24 h post RM-induction, except for the animals supplemented with Vit E where TNF and NF-κB genes increased in response to supplementation. These latter observations effectively dissociate inflammation as a causal factor in the early stages of ARF.

HO-1 and renal disease

The promoter region of the HO-1 gene contains an antioxidant response element (ARE) that is responsible for induction during oxidative stress [42,43]—ARE are modulated by the redox-sensitive transcription factor NF-E2-related factor-2 (Nrf2). Under normal conditions cytosolic Nrf2 binds to the regulatory protein Keap1. Enhanced oxidative stress promotes the dissociation of Nrf2 from Keap1 and induces HO-1 transcription [44,45], so that if accumulating Mb caused renal oxidative stress, an increase in HO-1 gene and protein activity is anticipated, as previously noted [46]. Thus, our data documenting that BP and Vit E supplementation decreased HO-1 gene regulation in the kidney during experimental RM indicates that the supplemented phenols diminished oxidative stress.

Increasing evidence indicates that induction of HO-1 in response to nephrotoxicity is one mechanism that may protect renal tissues from damage. For example, macrophages over-expressing HO-1 protect renal tissues from ischemia-reperfusion injury [47], whereas renal dysfunction is enhanced in HO-1 deficient mice after renal ischemia [48]. Thus, acute kidney insult with extracellular Mb, which induces HO-1, can be considered a response to mitigate

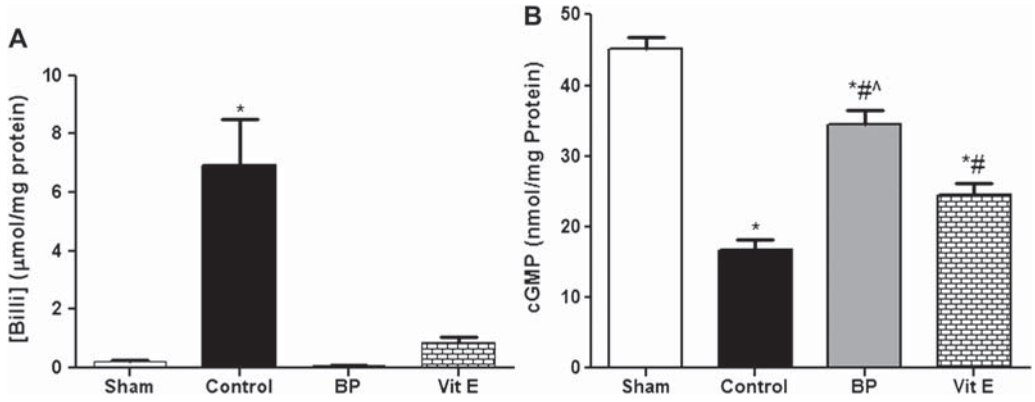

Figure 6. Quantitative assessment of renal HO activity and aortic [cGMP] from rats exposed to experimental RM. Animals supplemented with normal chow (Sham), vehicle-treated chow (Control) or chow supplemented with BP or vitamin E (Vit E) (each antioxidant 0.12% w/w in the diet) for 4 weeks were subjected to experimental RM (except Sham). After 24 h, the animals were euthanized and samples of kidney were homogenized and samples were tested for total HO activity or cGMP as described in the Experimental Procedures section. Data expressed as Mean ± SD, for HO activity: $n = 4$ for all groups or for cGMP: Sham ($n = 8$), Control ($n = 16$), BP ($n = 8$) and Vit E ($n = 8$). *Different to the Sham, $p < 0.001$. #Different to the Control, $p < 0.01$. ^Different to the Vit E-treated group, $p < 0.001$.

heme-toxicity associated with release of heme from Mb [49]. Therefore, antioxidants that down-regulate this innate response may promote nephrotoxicity and impaired renal function, albeit while inhibiting oxidative damage. On the other hand, ARF is established in this animal model despite the induction of HO-1 in response to RM suggesting that bolstering HO protein and activity in renal tissues in response to insult does not protect the kidney from renal dysfunction in this animal model. In addition, BP-supplementation down-regulated SOD-1 gene expression and marginally decreased SOD-2. Whether the supplemented synthetic phenol inhibited other endogenous antioxidant defense pathways (such as modulation of Fe stores) is not clear and further studies are warranted to determine if this phenol impacts on renal antioxidant systems in general.

Extracellular myoglobin and vascular dysfunction

Vascular dysfunction induced by myoglobinuria potentially limits renal blood supply, exacerbating damage to the renal tissues [50]. Our study demonstrated that extracellular Mb causes vascular dysfunction, and this is linked to enhanced oxidative stress. Thus, decreased aortic cGMP after RM-induction is indicative of diminished •NO bioavailability. In contrast, animals supplemented with BP and Vit E showed improved vascular reactivity through the inhibition of oxidative stress. Accepting this paradigm, it follows that a marked reduction in renal oxidative stress leads to improved renal function. However, renal dysfunction persisted even though BP or Vit E supplementation significantly inhibited oxidative damage in plasma and kidney tissues and diminished HO-1 gene and total HO activity. Therefore, we conclude that oxidative stress is not causally related to RM-induced ARF, consistent with other antioxidant supplementation studies in similar models of RM [51,52].

This conclusion is further supported by our assessments of biochemical markers of ARF including proteinuria and decreased GFR. Furthermore, histological assessment of renal tissues demonstrated structural disturbance of glomeruli and tubular epithelia along with tubular cast formation. TEM revealed increased frequency of lysosomal and other vesicular bodies (vacuoles) along with mitochondrial swelling and damage in the epithelial cells as documented previously [53,54]. With the exception of animals supplemented with Vit E, all groups of animals displayed the same extent of tissue damage, it is concluded that the antioxidants were unable to prevent renal impairment, despite the degree of protection against oxidative stress.

Although inflammation was not evident 24 h after RM-induction (judged by monitoring TNF and NF-κB gene expression), inflammation may play a role in renal damage at some later stage. Our data indicate that oxidative damage occurs early in the development of RM-induced ARF and for this reason the antioxidants were expected to exert a potential renal protective effect during the period where the importance of oxidative stress to disease progression would be most significant. Whether antioxidants can inhibit secondary inflammation and if so whether this improves the later stages of ARF induced by RM is not fully resolved in our study and remains to be evaluated.

The use of antioxidants to inhibit RM-induced ARF has been successful in some studies (refer to [55] and references therein) and this has been linked to the inhibition of Mb-induced oxidative stress [56]. However, while BP and Vit E inhibited lipid oxidation, improved aortic function and down-regulated the antioxidant response element HO-1 in this model of RM, both histochemical and physiological markers of ARF were unaffected. This outcome is in conflict with a recent publication that indicated acetamino-

phen (another substrate for Mb pseudo-peroxidase activity) inhibited Mb-mediated oxidative damage and renal dysfunction in a rat model of RM [57]. Therefore, our data add to the growing conflict in the literature that causally links Mb-derived oxidative stress and ARF. Interestingly, both BP and Vit E are likely to associate with the lipid compartment within renal tissues, whereas acetaminophen and other water-miscible agents, which inhibit both Mb peroxidase activity and ARF [58,59], are present largely in the extracellular compartment. Whether this difference in distribution of the supplemented antioxidant impacts on renal protection is not clear and systematic studies investigating the impact of increasing antioxidant lipophilicity on renal protective activity are required to address this issue.

Acknowledgements

This work was supported by a Discovery grant from the Australian Research Council (to PKW).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Kim GH, Oh KH, Yoon JW, Koo JW, Kim HJ, Chae DW, et al. Impact of burn size and initial serum albumin level on acute renal failure occurring in major burn. Am J Nephrol 2003;23:55–60.

[2] Mustonen KM, Vuola J. Acute renal failure in intensive care burn patients (ARF in burn patients). J Burn Care Res 2008;29:227–237.

[3] Holt S, Reeder B, Wilson M, Harvey S, Morrow JD, Roberts LJ, 2nd, Moore K. Increased lipid peroxidation in patients with rhabdomyolysis. Lancet 1999;353:1241–1242.

[4] Vertel RM, Knochel JP. Nonoliguric acute renal failure. J Am Med Assoc 1967;200:598–602.

[5] Davies DM, Pusey CD, Rainford DJ, Brown JM, Bennett JP. Acute renal failure in burns. Scand. J Plast Reconstr Surg 1979;13:189–192.

[6] Chrysopoulo MT, Jeschke MG, Dziewulski P, Barrow RE, Herndon DN. Acute renal dysfunction in severely burned adults. J Trauma 1999;46:141–144.

[7] D'Agnillo F, Alayash AI. A role for the myoglobin redox cycle in the induction of endothelial cell apoptosis. Free Radic Biol Med 2002;33:1153–1164.

[8] Zager RA. Rhabdomyolysis and myohemoglobinuric acute renal failure. Kidney Int 1996;49:314–326.

[9] Kavdia M, Tsoukias NM, Popel AS. Model of NO diffusion in an arteriole: impact of hemoglobin-based blood substitutes. AJP– Heart Circ Physiol 2002;282:H2245–H2253.

[10] Andriambeloson E, Witting PK. Chemical regulation of nitric oxide: a role for intracellular myoglobin? Redox Rep 2002;7:131–136.

[11] Vetterlein F, Hoffmann, F, Pedina J, Neckel M, Schmidt G. Disturbances in renal microcirculation induced by myoglobin and hemorrhagic hypotension in anesthetized rat. Am J Physiol 1995;268:F839–F846.

[12] Parry SN, Ellis N, Li Z, Maitz P, Witting PK. Myoglobin induces oxidative stress and decreases endocytosis and monolayer permissiveness in cultured kidney epithelial cells without affecting viability. Kidney Blood Press Res 2008; 31:16–28.

[13] Shanu A, Parry SN, Wood S, Rodas E, Witting PK. The synthetic polyphenol tert-butyl-bisphenol inhibits myoglobin-induced dysfunction in cultured kidney epithelial cells. Free Rad Res 2010;44:843–853.

[14] Witting PK, Westerlund C, Stocker, R. A rapid and convenient screening test for inhibitors of tocopherol-mediated peroxidation of LDL lipids. J Lipid Res 1996;37:853–867.

[15] Witting PK, Wu BJ, Raftery M, Southwell-Keely P, Stocker R. Probucol protects against hypochlorite-induced endothelial dysfunction: identification of a novel pathway of probucol oxidation to a biologically active intermediate. J Biol Chem 2005;280:15612–15618.

[16] Witting PK, Upston JM, Stocker R. The molecular action of alpha-tocopherol in lipoprotein lipid peroxidation. Pro- and antioxidant activity of vitamin E in complex heterogeneous lipid emulsions. Subcell Biochem 1998;30:345–390.

[17] Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med 2007;43:4–15.

[18] Chander V, Singh D, Chopra K. Catechin, a natural antioxidant protects against rhabdomyolysis-induced myoglobinuric acute renal failure. Pharmacol Res 2003;48:503–509.

[19] Seifi B, Kadkhodaee M, Karimian SM, Zahmatkesh M, Shams S, Bakhshi E. Reduction of kidney damage by supplementation of vitamins C and E in rats with deoxycorticosterone-salt-induced hypertension. Iran J Kidney Dis 2009; 3:197–202.

[20] Kobayashi N, Machida T, Takahashi T, Takatsu H, Shinkai T, Abe K, Urano S. Elevation by oxidative stress and aging of hypothalamic-pituitary-adrenal activity in rats and its prevention by vitamin E. J Clin Biochem Nutr 2009;45:207–213.

[21] Barbosa E, Faintuch J, Machado-Moreira EA, Goncalves da Silva VR, Lopes Pereima MJ, Martins Fagundes RL, Filho DW. Supplementation of vitamin E, vitamin C, and zinc attenuates oxidative stress in burned children: a randomized, double-blind, placebo-controlled pilot study. J Burn Care Res 2009;30:859–866.

[22] Himmelfarb J, Mcmonagle E, Freedman S, Klenzak J, McMenamin E, Le P, et al. Oxidative stress is increased in critically ill patients with acute renal failure. J Am Soc Nephrol 2004;15:2449–2456.

[23] Kim SH, Chang JW, Kim SB, Park SK, Park JS, Lee SK. Myoglobin induces vascular cell adhesion molecule-1 expression through c-src kinase-activator protein-1/nuclear factor-kappaB pathways. Nephron Exp Nephrol 2010;114:e48–e60.

[24] Witting P, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Wâgberg M, Stocker R. Dissociation of atherogenesis from aortic accumulation of lipid hydro(pero)xides in Watanabe heritable hyperlipidemic rabbits. J Clin Invest 1999;104: 213–220.

[25] Witting PK, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Eriksson AW, Stocker R. Inhibition by a coantioxidant of aortic lipoprotein lipid peroxidation and atherosclerosis in apolipoprotein E and low density lipoprotein receptor gene double knockout mice. FASEB J 1999;13:667–675.

[26] Bohle A, von Gise H, Schubert B, Wehrmann M, Mikeler E, Neuhaus G, Spath U. Transmission and scanning electron microscopy investigations on the structure of the ultrafiltrate of glomeruli in human acute renal failure. Am J Nephrol 1988;8:112–117.

[27] Rehberg PB. Studies on kidney function. I. The rate of filtration and reabsorption in the human kidney. Biochem J 1926;20:447–460.

[28] Miller BF, Leaf A, Mamby AR, Miller Z. Validity of the endogenous creatinine clearance as a measure of glomerular filtration rate in the diseased human kidney. J Clin Invest 1952;31:309–313.
[29] Sattler W, Mohr D, Stocker R. Rapid isolation of lipoproteins and assessment of their peroxidation by high performance liquid chromatography post-column luminescence. Methods Enzymol 1994;233:469–489.
[30] Rayner BS, Duong TT, Myers SJ, Witting PK. Protective effect of a synthetic anti-oxidant on neuronal cell apoptosis resulting from experimental hypoxia re-oxygenation injury. J Neurochem 2006;97:211–221.
[31] Thomas SR, Schulz E, Keaney JF, Jr. Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity—Role for iron-dependent oxidative stress. Free Radic Biol Med 2006;41:681–688.
[32] Zhu BL, Ishida K, Quan L, Taniguchi M, Oritani S, Kamikodai Y, et al. Post-mortem urinary myoglobin levels with reference to the causes of death. Forensic Sci Int 2001;115: 183–188.
[33] Berdnikovs S, Abdala-Valencia H, McCary C, Somand M, Cole R, Garcia A, et al. Isoforms of vitamin E have opposing immunoregulatory functions during inflammation by regulating leukocyte recruitment. J Immunol 2009;182:4395–4405.
[34] Bulger EM, Maier RV. An argument for Vitamin E supplementation in the management of systemic inflammatory response syndrome. [Review] Shock 2003;19:99–103.
[35] Ponraj D, Gopalakrishnakone P. Renal lesions in rhabdomyolysis caused by Pseudechis australis snake myotoxin. Kidney Int 1997;51:1956–1969.
[36] dos Reis MA, Costa RS, Coimbra TM, Teixeira VP. Acute renal failure in experimental envenomation with Africanized bee venom. Ren Fail 1998;20:39–51.
[37] Jones BD. Ultrastructure of human acute renal failure. Lab Invest 1982;46:254–264.
[38] Aydogdu N, Atmaca G, Yalcin O, Batcioglu K, Kaymak K. Effects of exogenous melatonin on myoglobinuric acute renal failure in the rats. Ren Fail 2004;26:479–486.
[39] Paller MS. Hemoglobin- and myoglobin-induced acute renal failure in rats: role of iron in nephrotoxicity. Am J Physiol – Renal Physiol 1988;255:F539–F544.
[40] Bräsen JH, Koenig K, Bach H, Kontush A, Heinle H, Witting PK, et al. Comparison of the effects of alpha-tocopherol, ubiquinone-10 and probucol at therapeutic doses on atherosclerosis in WHHL rabbits. Atherosclerosis 2002;163: 249–259.
[41] Witting PK, Willhite CA, Davies MJ, Stocker R. Lipid oxidation in human low-density lipoprotein induced by metmyoglobin/H2O2: involvement of alpha-tocopheroxyl and phosphatidylcholine alkoxyl radicals. Chem Res Toxicol 1999;12:1173–1181.
[42] Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24:449–455.
[43] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274: 26071–26078.
[44] Eggler AL, Liu G, Pezzuto JM, van Breemen RB, Mesecar AD. Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. Proc Natl Acad Sci USA 2005;102:10070–10075.
[45] Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, et al. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. Proc Natl Acad Sci USA 2004;101:2040–2045.
[46] Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME. Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. J Clin Invest 1992;90:267–270.
[47] Ferenbach DA, Ramdas V, Spencer N, Marson L, Anegon I, Hughes J, Kluth DC. Macrophages expressing heme oxygenase-1 improve renal function in ischemia/reperfusion injury. Mol Ther 2010;18:1706–1713.
[48] Tracz MJ, Juncos JP, Croatt AJ, Ackerman AW, Grande JP, Knutson KL, et al. Deficiency of heme oxygenase-1 impairs renal hemodynamics and exaggerates systemic inflammatory responses to renal ischemia. Kidney Int 2007;72:1073–1080.
[49] Tracz MJ, Alam J, Nath KA. Physiology and pathophysiology of heme: implications for kidney disease. J Am Soc Nephrol 2007;18:414–420.
[50] Wakabayashi Y, Kikawada R. Effect of L-arginine on myoglobin-induced acute renal failure in the rabbit. Am J Physiol 1996;270:F784–F789.
[51] Vlahović P, Cvetković T, Savić V, Stefanovic V. Dietary curcumin does not protect kidney in glycerol-induced acute renal failure. Food Chem Toxicol 2007;45:1777–1782.
[52] Chander V, Chopra K. Molsidomine, a nitric oxide donor and L-arginine protects against rhabdomyolysis-induced myoglobinuric acute renal failure. Biochim Biophys Acta 2005; 1723:208–214.
[53] Zager RA. Studies of mechanisms and protective maneuvers in myoglobinuric acute renal injury. Lab Invest 1989;60:619–629.
[54] dos Reis MA, Costa RS, Coimbra TM, Teixeira VP. Acute renal failure in experimental envenomation with Africanized bee venom. Ren Fail 1998;20:39–51.
[55] Koyner JL, Sher Ali R, Murray PT. Antioxidants. Do they have a place in the prevention or therapy of acute kidney injury? Nephron Exp Nephrol 2008;109:e109–117.
[56] Holt S, Moore K. Pathogenesis of renal failure in rhabdomyolysis: the role of myoglobin. Exp Nephrol 2000;8:72–76.
[57] Boutaud O, Moore KP, Reeder BJ, Harry D, Howie AJ, Wang S, et al. Acetaminophen inhibits hemoprotein-catalyzed lipid peroxidation and attenuates rhabdomyolysis-induced renal failure. Proc Natl Acad Sci USA 2000;107:2699–2704.
[58] Ustundag S, Yalcin O, Sen S, Cukur Z, Ciftci S, Demirkan B. Experimental myoglobinuric acute renal failure: the effect of vitamin C. Ren Fail 2008;30:727–735.
[59] Chander V, Chopra K. Protective effect of resveratrol, a polyphenolic phytoalexin on glycerol-induced acute renal failure in rat kidney. Ren Fail 2006;28:161–169.

This paper was first published online on Early Online on 27 June 2011.