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Bardoxolone methyl prevents the development and progression of cardiac and renal pathophysiologies in mice fed a high-fat diet

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Abstract

Obesity caused by the consumption of a high-fat (HF) diet is a major risk factor for the development of associated complications, such as heart and kidney failure. A semi-synthetic triterpenoid, bardoxolone methyl (BM) was administrated to mice fed a HF diet for 21 weeks to determine if it would prevent the development of obesity-associated cardiac and renal pathophysiologies. Twelve week old male C57BL/6J mice were fed a lab chow (LC), HF (40% fat), or a HF diet supplemented with 10 mg/kg/day BM in drinking water. After 21 weeks, the left ventricles of hearts and cortex of kidneys of mice were collected for analysis. Histological analysis revealed that BM prevented HF diet-induced development of structural changes in the heart and kidneys. BM prevented HF diet-induced decreases in myocyte number in cardiac tissue, although this treatment also elevated cardiac endothelin signalling molecules. In the kidneys, BM administration prevented HF diet-induced renal corpuscle hypertrophy and attenuated endothelin signalling. Furthermore, in both the hearts and kidneys of mice fed a HF diet, BM administration prevented HF diet-induced increases in fat accumulation, macrophage infiltration and *tumour necrosis factor alpha (TNFα)* gene expression. These findings suggest that BM prevents HF diet-induced developments of cardiac and renal pathophysiologies in mice fed a chronic HF diet by preventing inflammation. Moreover, these results suggest that BM has the potential as a therapeutic for preventing obesity-induced cardiac and renal pathophysiologies.

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Abstract

Obesity caused by consumption of a high-fat (HF) diet is a major risk factor for the development of associated complications, such as heart and kidney failure. A novel semi-synthetic triterpenoid, bardoxolone methyl (BM) was administrated to mice fed a high-fat (HF) diet for 21 weeks to determine if it would prevent the development of obesity-associated cardiac and renal pathophysiologies. Twelve week old male C57BL/6J mice were fed a lab chow (LC), HF (40% fat), or a HF diet supplemented with 10 mg/kg/day BM in drinking water. After 21 weeks, the left ventricles of hearts and cortex of kidneys of mice were collected for analysis. Inflammatory and endothelin signalling molecules were examined in heart and kidney tissue using immunohistochemistry and RT-PCR. Histological analysis revealed that BM prevented HF diet-induced development of structural changes in the heart and kidneys. BM prevented HF diet-induced decreases in myocyte number in cardiac tissue and renal corpuscle hypertrophy in the kidney. Furthermore, in both the hearts and kidneys of mice fed a HF diet, BM administration prevented HF diet-induced increases in fat accumulation, macrophage infiltration and *TNFα* gene expression. These findings suggest that BM prevents HF diet-induced developments of cardiac and renal pathophysiologies in mice fed a chronic HF diet by preventing inflammation. Moreover, these results suggest that BM has the potential as a novel therapeutic for preventing obesity-induced cardiac and renal pathophysiologies

1. Introduction

Obesity caused by the consumption of a high-fat (HF) diet increases the risk of cardiorenal diseases. Cardiovascular disease is the leading cause of death worldwide, with the incidence expected to rise from 17.3 million per year in 2008 to over 23.6 million per year by 2030 (Mozaffarian, Benjamin et al. 2015). There is increasing evidence that obese individuals have an increased risk of developing cardiovascular disease (Kenchaiah, Evans et al. 2002). In addition, there is direct evidence that obesity from a HF diet can cause kidney injury, which also increases the associated cardiovascular disease risk (Prasad 2014). Therefore, there is an urgent need to find suitable therapeutics that can prevent HF diet-induced obesity-associated complications to the heart and kidney, in order to reduce the incidence of global mortality from cardiorenal disease.

The endothelin system has been suggested to play an important role in the development of cardiovascular pathophysiologies. In the heart, endothelin 1 (ET-1) acts through two receptors, endothelin receptor type a (ET_A) and endothelin receptor type b (ET_B). The key endothelin system molecules ET-1, ET_A and ET_B play a role in vasoconstriction, with ET_B also having an additional role in vasodilation (Kedzierski and Yanagisawa 2001). In the cardiac muscle, ET-1 activates ET_A which results in the promotion of cardiac hypertrophy leading to subsequent heart failure (Nasser and El-Mas 2014). Previous studies have demonstrated that there is therapeutic potential in targeting the endothelin system with ET_A or combined ET_A/ET_B antagonists in patients with congestive heart failure (Krum, Viskoper et al. 1998, Nakov, Pfarr et al. 2002). However, it is important to note that in the kidneys the endothelin pathway plays several important roles including the regulation of sodium and water homeostasis and renal blood flow (Kohan 2006). Therefore, over-suppression of the endothelin pathway by antagonistic drugs may lead to other complications in the kidneys such as fluid retention, which if not addressed can also lead to heart

failure (Kohan 2006). Therefore, the development of therapeutics that appropriately targets the endothelin pathway in the heart and kidneys is warranted, in order to prevent obesity-associated cardiovascular disease and renal failure.

Obesity from HF diet is known to result in the development of fat accumulation in peripheral organs, such as the heart and kidneys (Montani, Carroll et al. 2004). Furthermore, peripheral fat accumulation is associated with macrophage infiltration into adipose tissue, which promotes the release of proinflammatory cytokines including tumour necrosis factor alpha (TNFα) (Wellen and Hotamisligil 2005). In a recent study, significantly higher levels of inflammatory markers, including TNFα, were found in the cardiac tissue of Tibetan mini pigs as a result of being fed a HF diet for 24 weeks (Yongming, Zhaowei et al. 2015). Furthermore, rats fed a HF diet for 10 weeks demonstrated increased TNFα levels in the cortex of their kidneys (Elmarakby and Imig 2010). Therefore, novel pharmaceuticals that attenuate TNFα levels may provide a potential therapy for preventing obesity-induced inflammation and tissue damage such as to the heart and kidneys.

In recent years, BM has been extensively studied in both preclinical rodent studies and human clinical trials, and shows promise for the treatment of renal diseases such as chronic kidney disease, and colitis-induced colon cancer due to its anti-inflammatory effects (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011, de Zeeuw, Akizawa et al. 2013, Camer, Yu et al. 2014, Choi, Kim et al. 2014). Specifically, studies have demonstrated that BM can reduce inflammation induced by modulating TNFα levels in rodents fed a HF diet (Saha, Reddy et al. 2010, Dinh, Szabo et al. 2015). In addition, our previous studies have highlighted BM as a potential novel therapeutic for preventing HF diet-induced obesity, visceral fat accumulation, and associated development of insulin resistance, hepatic steatosis and cognitive deficits (Camer, Yu et al. 2015, Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). However these positive findings were overshadowed by the recently terminated phase III human clinical trial where there were adverse

cardiovascular events seen in patients with advanced chronic kidney disease treated with BM (de Zeeuw, Akizawa et al. 2013). The mechanisms contributing to these adverse events in the clinical trial were speculated to be via the modulation of the endothelin pathway (Chin, Reisman et al. 2014). However, this pathway was not investigated in the heart tissue (Camer and Huang 2014) and in the kidney following chronic BM treatment, suggesting that further investigation into this drug was vital. In addition, the therapeutic effects of BM treatment on the hearts and kidneys of mice fed a chronic HF diet have not been examined previously.

Here, we provide the first evidence that oral administration of BM prevents HF diet-induced cardiac hypertrophy in mice fed a chronic HF diet. In addition, the development of HF diet-induced kidney pathophysiologies was prevented by BM administration. Specifically, BM administration prevented HF diet-induced macrophage infiltration and elevation of *TNFα* gene expression in the heart and kidneys of mice fed a HF diet. Furthermore, BM treatment suppressed endothelin signalling molecules in the kidney, but elevated expression of endothelin signalling molecules in the heart. These findings indicate the potential of BM as a future therapeutic for the prevention of obesity-related complications, such as cardiac hypertrophy and chronic kidney disease.

2. Materials and Methods

2.1 Animals and HF diet-induced obesity model

Twelve week old C57BL/6J male mice were purchased from the Animal Resource Centre (Perth, Western Australia) and kept in the animal research facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.* All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions at a constant temperature of 22 °C with a 12 hour light/dark

cycle. Following 1 week of acclimatisation, mice were randomly divided into 3 groups (n=7 per group). For the next 21 weeks one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). Mice in the treatment group were fed a HF diet for 21 weeks, along with a daily oral dose of BM (10 mg/kg) in their drinking water. The dose was chosen according to a previous study (Wu, Liu et al. 2014). Body weights of mice were measured weekly for the duration of the experiment (Final average body weight after 21 weeks: LC, 27.15g; HF, 40.84g; HF+BM, 28.13g).

2.2 Tissue collection

Mice were euthanised (n=7 per group) at week 21 of the experiment. The kidneys and heart were dissected from each mouse. The full hearts were weighed before the apex was cut and place in 10% formalin. The right kidneys of each mouse were cut in half before the inferior portion was place into 10% formalin. The remaining heart and kidney tissue were snap frozen in liquid nitrogen, and stored at -80 °C until use.

2.3 Microdissection

Frozen heart and kidney tissue were cut into 10 µm sections with a cryostat at -18 °C before being mounted on Polylysine™ microscope slides for histological staining. Specifically, the apex of the hearts and the superior portion of the cortex of the kidney were sectioned. The left ventricle of each mouse heart and inferior portion of the kidney cortex were micro-dissected from 500 µm thick frozen sections, and collected for RT-PCR. Kidney and heart tissue were both stored at a temperature of -80 °C until use.

2.4 Oil Red O staining

Oil Red O staining was used to examine lipid accumulation in the heart and kidneys as described previously (Kudo, Tamagawa et al. 2007, Camer, Yu et al. 2015). Briefly, frozen heart and kidney

sections (10 μm) were stained with 0.5% Oil Red O (Sigma-Aldrich) for 15 minutes and then washed. Three fields from three sections collected from each mouse were viewed under a Leica microscope, and digital photographs were captured. Image J software (http://imagej.nih.gov/ij/download.html) was used to quantify staining, which corresponded to the percentage of stained lipid droplets on an area of each slide (Mehlem, Hagberg et al. 2013).

2.5 Haematoxylin and Eosin (H&E) staining

Briefly, frozen kidney and heart sections (10 μm) were stained with Haematoxylin and Eosin (POCD Scientific, Artamon, Australia) for 30 seconds each. Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs captured. The histological parameters of glomerular and Bowman's capsule hypertrophy in the kidneys were calculated according to the methods described by previous studies (Al-Douahji, Brugarolas et al. 1999, Henegar, Bigler et al. 2001). In the heart tissue, myocytes were measured quantitatively using the software, Image J according to our previous study (Camer, Yu et al. 2015, Dinh, Szabo et al. 2015).

2.6 Immunohistochemistry

Immunohistochemistry was performed as described previously (Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). Briefly, heart and kidney sections fixed in 10% formalin were embedded in paraffin before being sectioned (5 μm) onto Polylysine™ slides. Slides were incubated overnight at 4 °C with anti-rabbit F4/80, anti-goat ET-1, anti-goat ET_B, or anti-rabbit ET_A primary antibody (1:150 Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer. Samples were then incubated consecutively at room temperature for 30 minutes with their respective secondary antibody (1:150 Santa Cruz Biotechnology, TX) and then streptavidin-HRP polymer conjugate (1:1000 2438, Sigma-Aldrich Pty Ltd, Sydney, Australia). A DAB peroxidise substrate kit (4100, Vector Laboratories Inc, Burlingame, CA) was used for the development of the stained sections

before counterstaining with H&E (POCD Scientific, Artarmon, Australia). Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs captured. Image J software was used to quantify the area of F4/80, ET-1, ET_A, or ET_B staining in heart and kidney tissue on each slide.

2.7 RNA isolation and RT-PCR

Total RNA was extracted from dissected mouse heart and kidneys using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) before being reversed transcribed to complimentary first strand DNA with a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, California, USA) according to the manufacturer's directions. Quantitative real-time PCR (RT-PCR) was performed using a Light cycler 480 real time PCR system (F.Hoffmann-La Roche Ltd, Switzerland). A 20µl final reaction volume containing cDNA sample and SYBR green I master mix was used for PCR Briefly, amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The expression of mRNA was normalised to an internal control, GADPH. The degree of mRNA expression was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt =ΔCt sample - ΔCt reference) as described previously (Camer, Yu et al. 2015, Cheng, Yu et al. 2015). The primers used are listed in Table 1.3.

2.8 Statistics

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality before differences between mice fed a LC diet, HF diet, or HF diet administered with BM diet were determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as mean ± SEM.

3. Results

3.1 Bardoxolone Methyl prevented the development of cardiac hypertrophy, fat accumulation, and inflammation in mice fed a high-fat diet

To assess whether BM treatment can prevent diet-induced cardiac hypertrophy, we analysed heart tissue in mice fed a HF diet for 21 weeks. Following 21 weeks on a HF diet, the hearts of mice had significantly higher weights than mice fed a LC diet (Final heart weight: -20.66%, $p<0.001$, Figure 1A). This increase in heart weight was prevented by oral administration of BM (Final heart weight: -27.04%, $p<0.001$, Figure 1A). However, there were no significant differences in heart to body weight ratios between any of the groups (Figure 1B). We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of BM on myocyte number and lipid content in the heart (Figure 1C). Histological examination of mouse hearts revealed that there was a significant decrease in myocyte number and significant increase in cytoplasmic lipid droplets in mice fed a HF diet for 21 weeks compared to LC fed mice (Myocyte count: -37.43%; lipid stained area: +80.12%, $p<0.001$, Figures 1D and 1E). This change in cardiac morphology was significantly attenuated by BM treatment compared to untreated mice fed a HF diet (Myocyte count: +28.11%; lipid stained area: -50.61%, $p<0.001$, Figures 1D and 1E). However, BM treatment failed to revert HF diet-induced alterations in myocyte number and lipid content in cardiac tissue to the levels present in control LC mice (Myocyte count: -12.97 %; lipid stained area: +59.75%, $p<0.001$, Figures 1D and 1E). These results suggest that cardiac hypertrophy and cellular lipid droplet accumulation induced by a HF diet is attenuated with BM treatment.

To investigate the effect of BM on macrophage accumulation in the left ventricle of the heart in HF diet fed mice, we performed immunohistochemistry with an anti-F4/80 antibody. We found that macrophage numbers increased in the left ventricle of HF diet fed mice as indicated by accumulation of F4/80 positive cells (HF vs. LC difference: -53.17%, $p<0.05$, Figure 1F). BM

administration significantly prevented an increase in the numbers of F4/80 positive cells in the left ventricle of the heart in HF diet fed mice (HF vs. BM difference: -67.40%, p<0.05, Figure 1F and 1G). Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IKKβ* mRNA expression in the left ventricle of the heart in mice fed a HF diet (HF vs. LC difference: *TNFα*, -38.70%; *IKKβ*, -26.41%; p<0.05, Figure 1H). The alterations in *TNFα* mRNA levels were significantly prevented by BM administration (HF vs. BM difference: -63.12%, p<0.05, Figure 1H). However, BM treatment was unable to prevent HF diet-induced elevations in *IKKβ* mRNA expression (LC vs. BM difference: -25.47%, p<0.05, Figure 1H). No significant differences were found in the mRNA expression of *IL-6*, and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet-induced cardiac macrophage infiltration by downregulating proinflammatory signalling molecules in the left ventricle of the heart.

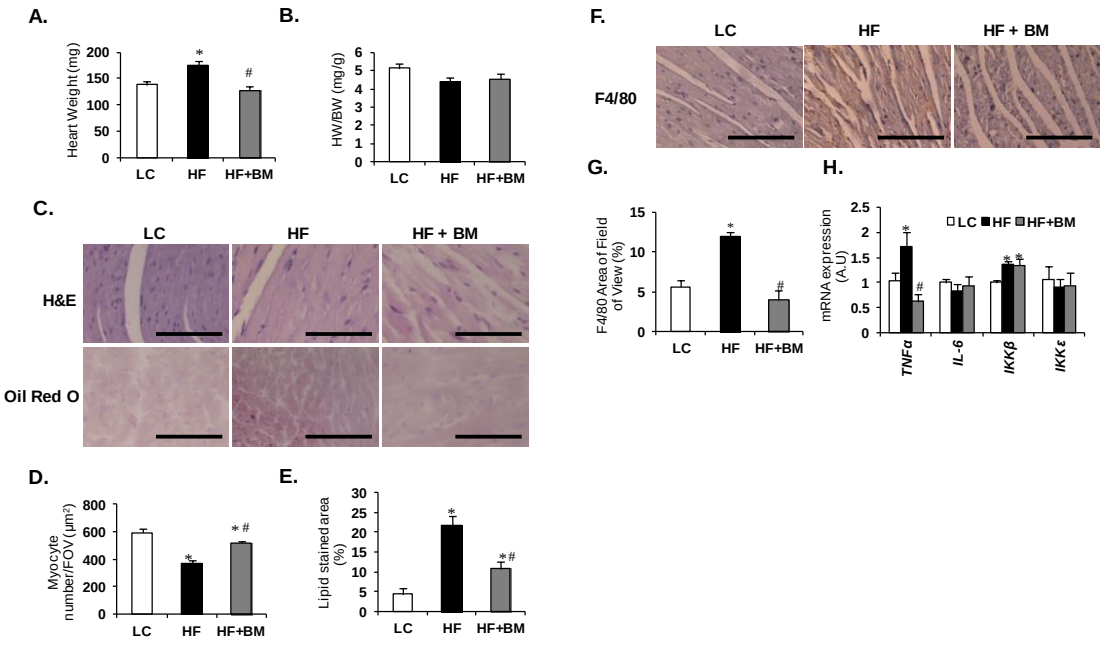

Figure 1. Bardoxolone methyl (BM) attenuated the development of cardiac hypertrophy, lipid accumulation, macrophage infiltration and inflammation in mice fed a chronic high-fat (HF) diet. (A) Heart weights showing significantly lower weights in BM treated mice compared to untreated mice fed a HF diet. (B) Heart to body weight ratio (HW/BW) (C) H&E and Oil Red O staining showing improved cardiac histomorphology and reduced lipid accumulation in mice treated with BM and fed a HF diet. Scale bar= 50μm. (D) Myocyte number per field of view. (E) Cardiac lipid accumulation (F and G) Histology and area of F4/80 immunoreactivity in the hearts of mice. Scale bar= 100μm. (H) RT-PCR analysis of inflammatory genes, *TNFα*, *IL-6*, *IKKβ* and *IKKε*. *, p = <0.05 vs. lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM. (n= 7 mice per group).

3.2 Bardoxolone methyl prevented the development of renal corpuscle hypertrophy, fat accumulation and inflammation in mice fed a high-fat diet

We evaluated whether BM treatment can prevent the development of diet-induced renal hypertrophy and fat accumulation in mice fed a HF diet for 21 weeks through analysis of kidney histomorphology. We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of BM on the cellular morphology of the renal corpuscle, and lipid content in the renal cortex (Figure 2A). Compared to LC mice, in mice fed a HF diet for 21 weeks there was a significant increase in thickening of the bowman's capsule and glomerular tuft area in the renal corpuscle that was coupled with a significant increase in cytoplasmic lipid droplets (Bowman's capsule thickening: +91.01%; Glomerular tuft area: +41.96%; lipid stained area: +54.89%, p<0.001, Figures 2B-2D). This change in kidney cellular morphology was significantly attenuated by BM treatment compared to untreated mice fed a HF diet (Bowman's capsule thickening: -53.34%; Glomerular tuft area: -36.73%; lipid stained area: -56.85%, p<0.001, Figures 2B-2D). However, BM treatment failed to restore the thickness of the Bowman's capsule to normal levels found in LC fed mice (Bowman's capsule thickening: +80.74%, p<0.001, Figure 2B). These results suggest that renal corpuscle hypertrophy and cellular lipid droplet accumulation caused by a HF diet are attenuated with BM treatment.

To investigate the effect of BM on macrophage accumulation in the renal cortex of HF diet fed mice, we performed immunohistochemistry with an anti-F4/80 antibody. We found that macrophage numbers increased in the renal cortex of HF diet fed mice as indicated by the accumulation of F4/80 positive cells (HF vs. LC difference: -52.79%, p<0.05, Figure 2E). BM administration prevented this increase in F4/80 positive cells (HF vs. BM difference: -46.01%, p<0.05, Figure 2E and 2F). Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IL-6* mRNA expression in the cortex of kidney tissue in mice fed a HF diet (HF vs. LC

difference: *TNFα*, -66.91%; *IL-6*, -67.79%; $p<0.05$, Figure 2G). The alterations in *TNFα* mRNA levels were prevented by BM administration (HF vs. BM difference: -62.38%, $p<0.05$, Figure 2G). However, there were no significant differences in *IL-6* mRNA levels between BM and the other groups ($p>0.05$, Figure 2G). No significant differences were found in the mRNA expression of *IKKβ* and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet-induced renal macrophage infiltration by regulating proinflammatory signalling molecules in the cortex of the kidneys.

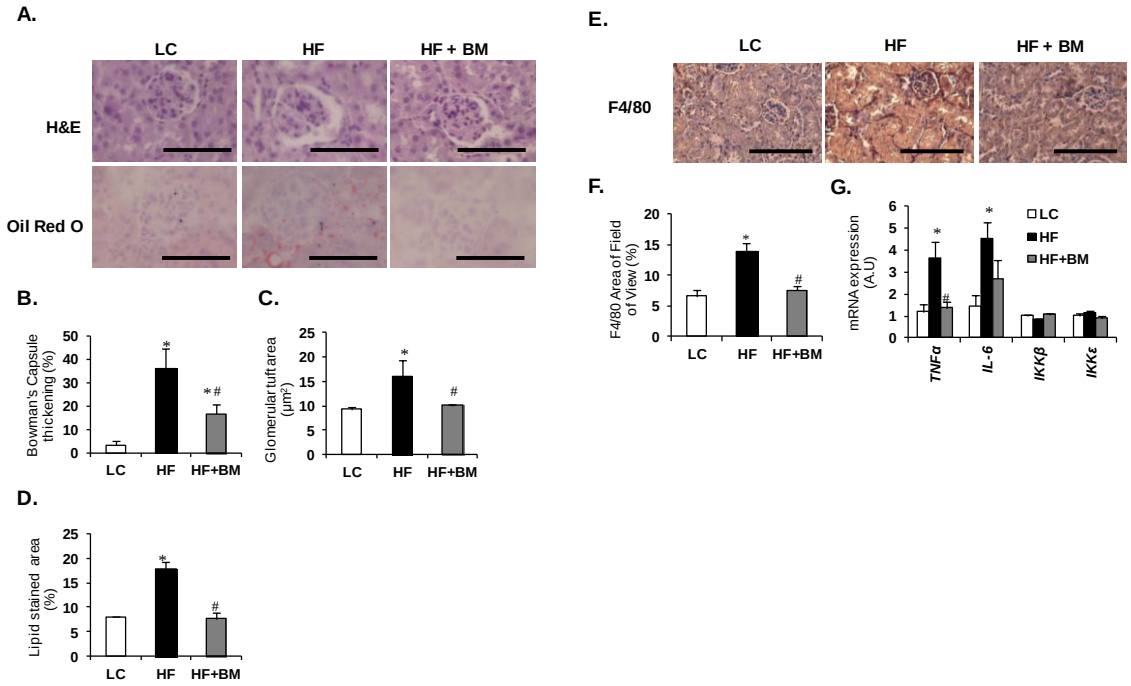

Figure 2. Bardoxolone methyl (BM) attenuated the development of renal corpuscle hypertrophy, lipid accumulation, macrophage infiltration and inflammation in mice fed a chronic high-fat (HF) diet. (A) H&E and Oil Red O staining showing improved renal histomorphology and reduced lipid accumulation in mice treated with BM and fed a HF diet. Scale bar= 50μm. (B and C) Percentage of Bowman's Capsule thickening and Glomerular Tuft Area demonstrating that BM administration prevented renal corpuscle hypertrophy in mice fed a HF diet (D) Renal lipid accumulation (E and F) Histology and area of F4/80 immunoreactivity in the kidneys of mice. Scale bar= 100μm (G) RT-PCR analysis of inflammatory genes, *TNFα*, *IL-6*, *IKKβ* and *IKKε*. *, p = <0.05 vs. lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM. (n= 7 mice per group).

3.3 Bardoxolone methyl treatment failed to restore ET_A protein expression to normal levels and elevated cardiac endothelin signalling genes in mice fed a high-fat diet

Endothelin signalling proteins in the left ventricles of mouse hearts were assessed using immunohistochemistry in order to examine whether BM could prevent HF diet-induced increases

in endothelin signalling. Protein levels of ET_A were significantly elevated in mice fed a HF diet compared to LC diet fed mice (HF vs. LC difference: -93.35%, $p<0.05$, Figure 3A and Table 1). BM treatment failed to restore HF diet-induced elevations in ET_A protein to normal levels present in LC fed mice (BM vs. LC difference: -92.62%, $p<0.05$, Figure 3A and Table 1). There were no differences in ET-1 or ET_B protein levels found between any of the groups ($p>0.05$, Figure 3A and Table 1).

Endothelin signalling gene transcription in the left ventricle of mouse hearts were examined using RT-PCR. Mice fed a HF diet were found to have increased expression of the ET_A gene (HF vs. LC difference: -45.07%, $p<0.05$, Figure 3B), and decreased expression of ET_B gene compared to LC diet fed mice (HF vs. LC difference: -63.04%, $p<0.05$, Figure 3B). BM treatment prevented the HF diet-induced decrease in ET_B gene expression (HF vs. BM difference: -60.62%, $p<0.05$, Figure 3B). However, BM treatment also resulted in a significant increase in *ET-1* gene expression compared to untreated HF diet fed mice (HF vs. BM difference: -56.34%, $p<0.05$, Figure 3B). There were no significant differences in *ET-1* gene expression between LC fed mice and mice fed a HF diet treated with BM ($p>0.05$, Figure 3B). Furthermore, BM administration elevated ET_A gene expression to levels higher than both the LC fed mice and the untreated HF diet fed mice (LC vs. BM difference: -60.72%, $p<0.05$; HF vs. BM difference: -28.49%, $p<0.05$, Figure 3B). These results suggest that BM fails to restore HF diet-induced elevations of ET_A receptor protein levels, and elevates the expression of the endothelin signalling genes, *ET-1*, ET_A, and ET_B in the left ventricles of mice fed a HF diet.

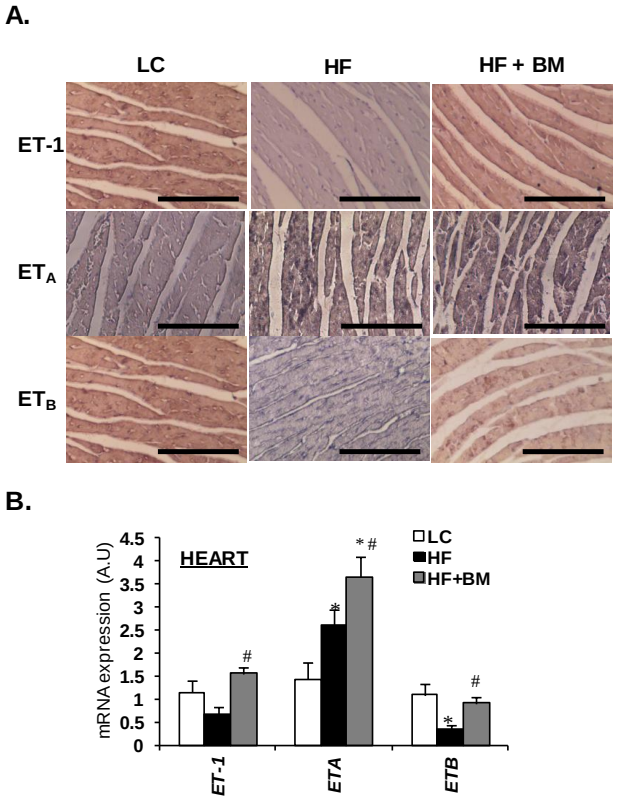

Figure 3. Bardoxolone methyl (BM) elevated cardiac endothelin signalling in mice fed a chronic high-fat (HF) diet. (A) Cardiac ET-1, ET_A and ET_B proteins detected by immunohistochemistry. (B) RT-PCR analysis of cardiac *ET-1*, *ET_A* and *ET_B* genes. *, p = <0.05 vs. lab chow (LC), #, p= <0.05 vs. HF group, values are means ±SEM. Scale bar= 50µm. (n= 7 per group).

Table 1 Endothelin protein levels in mouse heart and kidneys following 21 weeks of LC, HF or HF + BM diet

	Protein	LC	HF	HF+BM	F value	P value
Heart	ET-1	68.0±2.8	54.4±9.7	70.4±3.9	2.101	0.193
	ET_A	0.95±0.5[b]	14.4±3.8[a]	12.9±3.1[a]	7.857	0.013
	ET_B	68.7±3.8	47.9±8.4	59.0±3.4	2.813	0.119
Kidney	ET-1	43.1±7.3	50.8±6.7	25.1±1.9 [b]	5.153	0.032
	ET_A	59.6±4.6	59.4±3.3	41.9±8.8	2.824	0.112
	ET_B	53.5±6.0	43.7±6.3	52.8±6.7	0.728	0.512

Values are means ±SEM. LC, lab chow diet, HF, high-fat diet, HF+BM, high-fat diet and bardoxolone methyl treatment. [a]p<0.05 vs LC, [b]p<0.05 vs HF.

3.4 Bardoxolone methyl treatment reduced renal endothelin signalling in mice fed a high-fat diet

Endothelin signalling proteins in the cortex of mouse kidneys were examined using immunohistochemistry in order to assess if BM could prevent HF diet-induced renal dysfunction. There were no significant differences in the protein levels of ET-1 in HF diet fed mice compared to mice fed a LC diet (p>0.05, Figure 4A and Table 1). However, ET-1 protein levels were

significantly reduced in mice treated with BM compared to untreated mice fed a HF diet (HF vs. BM difference: -51.63%, p<0.05, Figure 4A and Table 1). There were no differences in ET_A or ET_B protein levels found between any of the groups (p>0.05, Figure 4A and Table 1).

Furthermore, endothelin signalling genes in the cortex of mouse kidneys were measured using RT-PCR. Mice fed a HF diet were found to have increased expression of the ET_A gene compared to LC diet fed mice (HF vs. LC difference: -49.31%, p<0.05, Figure 4B). This HF diet-induced increase in ET_A gene expression was prevented by BM administration (HF vs. BM difference: -46.70%, p<0.05, Figure 4B). There were no difference in *ET-1* or ET_B gene expression found between any of the groups (p>0.05, Figure 4B). These results suggest that BM prevents HF diet-induced elevations in ET_A gene expression, and significantly reduces ET-1 protein levels in the cortex of the kidneys of mice fed a HF diet.

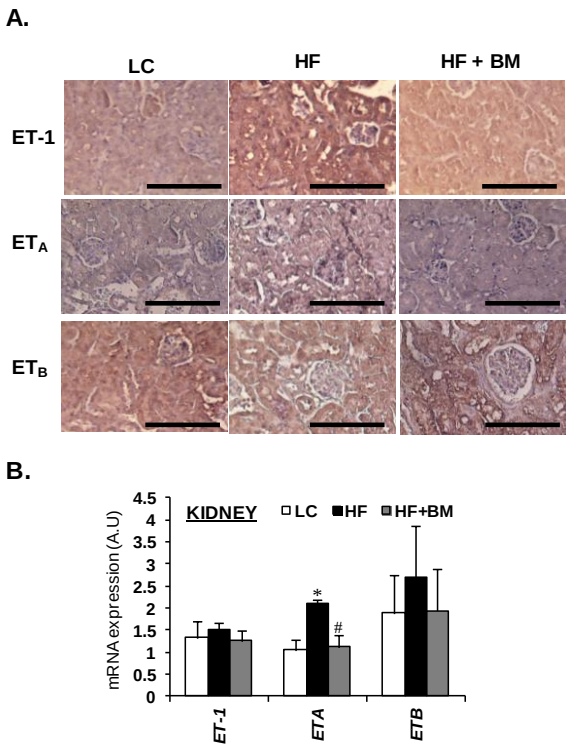

Figure 4. Bardoxolone methyl (BM) reduced renal endothelin signalling in mice fed a chronic high-fat (HF) diet. (A) Renal ET-1, ET_A and ET_B proteins detected by immunohistochemistry (B) RT-PCR analysis of renal *ET-1*, ET_A and ET_B genes. *, p = <0.05 vs. lab chow (LC), #, p= <0.05 vs. HF group, values are means ±SEM. Scale bar= 50µm. (n= 7 per group).

4. Discussion

It is well established that a HF diet can lead to the development of complications in the heart and kidneys, such as cardiac hypertrophy and chronic kidney disease (van Bilsen and Planavila 2014, Hariharan, Vellanki et al. 2015). Rodents fed a chronic HF diet have increased body weight gain, along with structural and functional changes in the kidneys and heart (Deji, Kume et al. 2009, Dhahri, Drolet et al. 2014). Previously, BM has shown promise in treating chronic kidney disease in phases I and II of human clinical trials via anti-inflammatory mechanisms (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011). Furthermore, the therapeutic benefits of BM have been demonstrated in HF diet-induced obese animal models such as preventing HF diet-induced visceral fat accumulation, insulin resistance, hepatic steatosis and recognition memory decline in mice (Camer, Yu et al. 2015, Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). The effects of chronic BM administration on the prevention of renal and cardiac pathophysiologies in mice fed a chronic HF diet have not been examined previously. In this present study, we found that feeding a chronic HF diet to mice induces fat accumulation, structural changes and inflammation in the heart and kidneys, which was attenuated by BM administration. These results suggest that BM has the potential to prevent the development of renal and cardiac complications of HF diet-induced obesity.

There is compelling evidence that overweight or obese individuals have an increased risk of heart failure due to left ventricular cardiac hypertrophy (Levy, Garrison et al. 1990, Russo, Jin et al. 2011, Barton, Baretella et al. 2012). Cardiac hypertrophy is characterised by an increase in myocyte size (Chien, Knowlton et al. 1991) and the activation of ET-1 (Huang, Zhang et al. 2011). Along with an increase in heart weight, Zucker fatty rats have an increase in ET-1, ET_A and ET_B gene expression in the left ventricle of the heart (Huang, Yang et al. 2005). In addition, mice fed a HF diet for 10 weeks have significantly elevated cardiac mRNA expression of ET-1, ET_A and ET_B

genes (Catar, Muller et al. 2014). However, in dogs with congestive heart failure, inhibition of ET_B by an antagonist resulted in increased cardiac pressure and decreased cardiac output, suggesting that the vasodilative actions of ET_B are functionally more important than their vasoconstrictive actions (Wada, Tsutamoto et al. 1997). In our study, BM administration prevented HF diet-induced increases in myocyte size in mice, which was indicated by an increase in myocyte number. However, BM did not prevent HF diet-induced increases in ET_A protein expression and worsened HF diet-induced increases in ET_A and ET-1 gene expression. Despite this, BM prevented HF diet-induced decreases in the expression of the ET_B gene. These results suggest that the therapeutic effects of BM on preventing HF diet-induced cardiac hypertrophy may be as a result of targeting the vasodilative functions of ET_B, or a mechanism other than the endothelin pathway in the heart, such as inflammation.

Previous studies have demonstrated that obesity can lead to the development of significant structural and functional changes to the kidneys that can progress to renal or even heart failure (Weisinger, Kempson et al. 1974, Hall, Brands et al. 1993). Obese dogs fed a HF diet were found to have an expansion in Bowman's capsule area and glomerular tuft area in their kidneys compared to lean dogs (Henegar, Bigler et al. 2001). Our study demonstrated that chronic BM administration can prevent the expansion in Bowman's capsule area and glomerular tuft area induced by HF diet in obese mice, suggesting BM has potential to prevent obesity associated kidney damage. Along with alterations in the structure and function of the kidneys, obesity induced by a chronic HF diet is associated with activation of the renal endothelin pathway (Barton 2014). For example, mice fed a chronic HF diet develop obesity, which is coupled with an increase in mRNA expression of ET_A and increased protein expression of ET-1 in the kidneys (Zhang, d'Uscio et al. 2001). BM has been found to suppress the renal endothelin pathway in the kidneys of rodents induced with chronic kidney disease by reducing the protein expression of ET_A

(Chin, Reisman et al. 2014). In this study, we also found that chronic BM administration prevented HF diet-induced increases in mRNA expression of ET_A. In addition, our study demonstrated that BM treatment significantly decreased the protein expression of ET-1. Our results support findings from previous research that demonstrate that BM suppresses renal endothelin signalling molecules. Furthermore, our results add additional knowledge that this drug can also prevent HF diet induced increases in molecules involved in modulating the endothelin signalling pathway.

There is extensive scientific evidence that BM can improve kidney function by inhibiting inflammation in a number of rodent studies and human clinical trials (Pergola, Krauth et al. 2011, Wu, Wang et al. 2011, Ruiz, Pergola et al. 2013). However, no study has investigated the effects of BM on the heart, and thus we investigated the potential preventative effects of BM on inflammation in the hearts and kidneys of mice fed a chronic HF diet. Along with increased fat accumulation, we found that there was elevated macrophage infiltration that was coupled with an increase in the proinflammatory *TNFα* gene in both the left ventricle of the heart and the cortex of the kidneys of mice fed a chronic HF diet. Furthermore, our results demonstrated that chronic BM treatment prevented HF diet-induced fat accumulation, macrophage infiltration and elevated *TNFα* gene expression in the left ventricular area of the heart and cortex of the kidneys of mice. A possible mechanism for these anti-inflammatory effects of BM in these regions of the heart and kidneys includes preventing *TNFα* gene expression and macrophage infiltration, resulting in the attenuation of the proinflammatory response and organ fat accumulation.

In conclusion, our findings suggest that chronic supplementation with BM can prevent HF diet-induced development of cardiac and renal pathophysiologies in mice. Since obesity-induced peripheral fat accumulation and inflammation has been implicated in the progression of heart failure, BM may have beneficial effects in preventing the progression of HF diet-induced cardiac

and renal hypertrophy. With further research and human clinical trials, the possibility of using BM for the prevention of obesity-induced development of renal and cardiac pathophysiologies appears promising.

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