Bardoxolone Methyl Decreases Megalin and Activates Nrf2 in the Kidney

Scott A. Reisman,* Glenn M. Chertow,† Sudarshan Hebbar,* Nosratola D. Vaziri,‡ Keith W. Ward,* and Colin J. Meyer*

*Reata Pharmaceuticals, Inc., Irving, Texas; †Division of Nephrology, Stanford University School of Medicine, Palo Alto, California; and ‡Division of Nephrology and Hypertension, Schools of Medicine and Biological Science, University of California-Irvine, Orange, California

ABSTRACT

Inflammation and oxidative stress are hallmarks and mediators of the progression of CKD. Bardoxolone methyl, a potent activator of the nuclear factor erythroid 2–related factor 2 (Nrf2)–mediated antioxidant and anti-inflammatory response, increases estimated GFR and decreases BUN, serum phosphorus, and uric acid concentrations in patients with moderate to severe CKD. However, it also increases albuminuria, which is associated with inflammation and disease progression. Therefore, we investigated whether this bardoxolone methyl–induced albuminuria may result from the downregulation of megalin, a protein involved in the tubular reabsorption of albumin and lipid-bound proteins. Administration of bardoxolone methyl to cynomolgus monkeys significantly decreased the protein expression of renal tubular megalin, which inversely correlated with the urine albumin-to-creatinine ratio. Moreover, daily oral administration of bardoxolone methyl to monkeys for 1 year did not lead to any adverse effects on renal histopathologic findings but did reduce serum creatinine and BUN, as observed in patients with CKD. Finally, the bardoxolone methyl–induced decrease in megalin corresponded with pharmacologic induction of renal Nrf2 targets, including NAD(P)H:quinone oxidoreductase 1 enzyme activity and glutathione content. This result indicates that Nrf2 may have a role in megalin regulation. In conclusion, these data suggest that the increase in albuminuria that accompanies bardoxolone methyl administration may result, at least in part, from reduced expression of megalin, which seems to occur without adverse effects and with strong induction of Nrf2 targets.


Pathogenic stimuli in patients with CKD and type 2 diabetes, including hypertension, obesity, height-ened renin-angiotensin activity, and albuminuria, activate oxidative stress–mediated inflammation.1–5 Indeed, oxidative stress and impaired antioxidant capacity intensify with progression of CKD,5,7 and production of reactive oxygen species and oxidative stress result in activation of the transcription factor nuclear factor κB (NFκB). NFκB regulates expression of proinflammatory cytokines and chemokines, and its pathologic activation is a hallmark of many inflammatory disorders, including CKD. Activated NFκB is present in the kidneys of patients with diabetic nephropathy but is undetectable in normal healthy kidneys.8 Thus, oxidative stress facilitates proinflammatory signaling, which frequently results in further oxidative stress, thereby creating a destructive feedback loop and, often, perturbation of normal physiologic processes and disease progression.

To respond to oxidative and electrophilic stimuli, organisms have developed elaborate cytoprotective pathways that are directly regulated by the
transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2). Its central role in the maintenance of redox balance and protection against oxidative stress is now well recognized. Unfortunately, long-term inflammatory signaling can result in decreased Nrf2 activity, decreased antioxidant defense capacity, chronic inflammation, and disease progression. In animals with CKD, oxidative stress and inflammation are associated with impaired Nrf2 activity. Pharmacologic or genetic activation of Nrf2 results in a phenotypic shift toward height-ened antioxidant defense, decreased inflammation, and improved survival. As such, Nrf2 regulates more than 250 genes, including many antioxidative and detoxifying enzymes. For example, NQO1 is a prototypical Nrf2 target gene important for the reduction of highly reactive quinones. Nrf2 also reg-ulates the endogenous antioxidant glutathione by regulating its synthesis (glutamate-cysteine ligase, catalytic subunit [GCLC]) and recovery (glutathione reductase [GSR]). Other Nrf2 targets, such as sulfiredoxin 1 (SRXN1) and thioredoxin reductase 1 (TXNRD1), are protective against reactive oxygen species and promote protein repair. During the past 15 years, substantial evidence has accumulated demonstrating that activation of the Nrf2 pathway can protect against oxidative and electrophilic insult. In contrast, lack of Nrf2 leads to markedly increased susceptibility to many oxidative stress–related abnormalities, including lupus-like nephropathy in aged animals. In addition, Nrf2 is suppressed in cardiac tissue from diabetic rodents, as well as patients with type 2 diabetes.

The synthetic triterpenoid bardoxolone methyl and its analogues (e.g., CDDO-Im) are the most potent known activators of the Nrf2 pathway. They mimic the cyclopen-tone prostaglandins (such as 15-deoxy-D12, 14 prostaglandin J2), which are produced during the resolution phase of inflammation and are the most potent endogenous activators of Nrf2 and inhibitors of NFκB. Correspondingly, mice deficient in 15-deoxy-D12, 14 prostaglandin J2 develop glomerular hypertrophy and increased basement membrane thickening, TGFβ protein expression, lipid deposition, and renal fibrosis. Moreover, bardoxolone methyl and its analogues have many benef-icial effects and are protective in rodent models of kidney disease. For example, CDDO-Im protects mice from iron nitrotoxic-reacte–induced AKI. In addition, short-term dosing of bardoxolone methyl (twice daily for 7 days) ameliorates ischemic AKI in mice, as assessed by both functional (i.e., BUN) and pathologic (i.e., renal histopathology) measures. However, it is now understood that rodent data with bardoxolone methyl itself may be confounded, especially with long-term administration, because bardoxolone methyl undergoes rodent-specific biotransformation to toxic metabolites not observed in humans or nonhuman primates (Reata Pharmaceuticals, Inc., data on file; manuscript in preparation). Therefore, a heavy nonclinical emphasis has focused on characterizing these differences and generating human-relevant pharmacodynamic and safety data in nonhuman primates.

In a recently completed phase 2, randomized, placebo-controlled, double-blinded clinical trial (NCT00811889), patients randomly assigned to bardoxolone methyl experienced significantly improved kidney function, with lower serum creatinine (corresponding to higher estimated GFR), along with lower concentrations of urea nitrogen, uric acid, and phosphorus. However, in concert with improved kidney function, albuminuria was increased. The degree of
albuminuria can indicate glomerular integrity and endothelial dysfunction and is often used to evaluate kidney function and risk for cardiovascular disease as CKD progresses.34,35 Thus, it is paradoxical that albuminuria would increase while other measures of kidney function improve. An increase in albuminuria with bardoxolone methyl may result from inhibition of tubular reabsorption of filtered protein caused by downregulation of megalin and/or cubilin, membrane receptors expressed on the apical membrane of proximal tubular epithelial cells.36 These proteins form complexes that participate in reabsorption of filtered albumin and other proteins.36 Furthermore, interaction of protein-bound lipids, such as those bound to albumin, with megalin-cubilin complexes, facilitates the release of profibrotic and pro-inflammatory mediators, such as NFκB, TNFα, and monocyte chemotactic protein-1, which can contribute to interstitial inflammation and fibrosis.37,38 Downregulation of megalin or cubilin may be cytoprotective and would be consistent with the pharmacology of bardoxolone methyl and the physiological function of Nrf2.

Two monkey studies were conducted to evaluate the effects of bardoxolone methyl on the kidney. The first was a 12-month toxicology study in cynomolgus monkeys, conducted under Good Laboratory Practice (GLP) conditions in accordance with international regulatory authorities, to evaluate the overall safety and tolerability of bardoxolone methyl in a relevant nonhuman primate species. However, because GLP toxicology studies can be somewhat restrictive in mechanistic exploration, an additional 28-day monkey study was performed to further evaluate the pharmacological effects of bardoxolone methyl. Thus, the 28-day study was conducted to explore the effects of bardoxolone methyl on renal protein handling (i.e., megalin and cubilin expression). To our knowledge, a detailed investigation of bardoxolone methyl on renal structure, function, and protein handling has not been performed to date. In addition, bardoxolone methyl–mediated activation of Nrf2 and induction of its targets genes in the kidney were evaluated to investigate whether megalin or cubilin expression was decreasing coordinately with Nrf2 engagement.

RESULTS

Bardoxolone Methyl Decreases Renal Expression of Megalin but Not Cubilin

Immunoistochemical examination of kidney sections from cynomolgus monkeys demonstrated staining patterns of megalin and cubilin in the proximal tubules, consistent with previous reports.39–41 Kidney sections from bardoxolone methyl–treated monkeys demonstrated decreased megalin protein expression (Figure 1A) despite similar mRNA expression across groups (data not shown). The visualized decrease in megalin protein expression was confirmed by densitometry analyses, which demonstrated that bardoxolone methyl administration significantly decreased megalin protein expression in the monkey kidney (Figure 1B). Bardoxolone methyl administration did not affect the protein expression of cubilin in the kidney (Figure 1, C and D) or the mRNA expression of cubilin in the kidney (data not shown).

Bardoxolone Methyl Increases Creatinine Clearance and Urinary Albumin-to-Creatinine Ratios

Twenty-four–hour urine collections demonstrated that bardoxolone methyl administration did not alter the fractional excretion of sodium or magnesium in urine or the total amount of excreted creatinine (data not shown). However, measured creatinine clearance in bardoxolone methyl–treated monkeys was significantly increased. The creatinine clearance in monkeys administered bardoxolone methyl significantly
differed from that at baseline and in vehicle-treated animals on day 28 (Figure 2A). After 28 days of bardoxolone methyl administration, urinary albumin-to-creatinine ratios (UACRs), determined from the 24-hour urine collections, were significantly increased compared with those in animals receiving vehicle (Figure 2B). Of note, UACRs decreased 53.3% in vehicle-treated animals and increased 27.9% in bardoxolone methyl–treated monkeys.

**Bardoxolone Methyl Did Not Produce Adverse Effects in Cynomolgus Monkeys**

Bardoxolone methyl demonstrated tolerability after once-daily oral administration to cynomolgus monkeys in both 28-day (30 mg/kg per day) and 12-month (5, 30, and 300 mg/kg per day) studies. In the 28-day study, monkeys experienced a minimal decrease in body weight, as assessed by area under the curve of the body weight–versus–time data (Figure 3A). In the 12-month study, all groups gained weight. The bardoxolone methyl–treated monkeys tended to gain slightly less weight over time relative to controls, although the differences were not significant or dose dependent (Figure 3B). Bardoxolone methyl significantly decreased serum creatinine in the 28-day study, a finding also observed with some dose dependency at both 6 and 12 months (and continued in the 4-week recovery group) in the 12-month study (Figure 4A). Furthermore, BUN tended to be lower after 28 days and was significantly lower after 6 and 12 months, with the trend continuing in the 4-week recovery group (Figure 4B). Of note, BUN in the vehicle-treated group tended to decrease but was not statistically different from baseline.

Serum electrolytes (sodium, potassium, chloride, calcium, phosphorus, and magnesium) in monkeys administered bardoxolone methyl for 28 days did not differ from those in controls (Table 1). In the 12-month study, serum sodium, potassium, and calcium were also unchanged from vehicle (Table 2). Serum magnesium was not measured in the 12-month study. Chloride values exhibited a statistically significant but minimal increase, which was unlikely to be biologically meaningful. Phosphorus tended to decline in a dose-dependent manner, with a significant difference between bardoxolone methyl–treated and placebo-treated monkeys at the 30-mg/kg dose.

Microscopic examination of kidney tissues obtained after 12 months of dosing showed normal renal histological features, with no evidence of injury, hypertrophy, or fibrosis in any group (Figure 5). Likewise, muscle (rectus femoris) histological findings were also within normal limits and did not differ from those in controls, with no evidence of injury (data not shown).

**Bardoxolone Methyl Induces Nrf2 Cytoprotective Targets in Monkey Kidneys**

Bardoxolone methyl administration significantly increased renal mRNA expression of NQO1, TXNRD1, GCLC, and GSR (Figure 6A). Bardoxolone methyl also increased SRXN1 approximately 43-fold, a finding that approached statistical
significance ($P=0.05$). Bardoxolone methyl administration also increased renal protein expression of NQO1, which was localized primarily to tubules, as determined by immunohistochemistry (Figure 6B). Densitometry evaluations demonstrated that bardoxolone methyl significantly increased NQO1 protein expression (Figure 6C). In addition, increased NQO1 mRNA and protein expression and GSR mRNA expression were accompanied by significant increases in corresponding enzyme activity (Figure 6D). Furthermore, bardoxolone methyl significantly increased total kidney glutathione content (Figure 6E).

**DISCUSSION**

Because the degree of albuminuria often indicates deficits in glomerular integrity and endothelial dysfunction, it is frequently used to evaluate kidney function and risk for cardiovascular disease as CKD progresses.$^{34,35}$ Of note, increased albuminuria was observed in patients with CKD treated with bardoxolone methyl, despite significantly improved kidney function, observed as lower serum creatinine (corresponding to higher estimated GFR), along with lower serum concentrations of BUN, uric acid, and phosphorus.$^{33}$ However, an increase in albuminuria does not necessarily indicate damage to the filtration barrier or increased intraglomerular pressure with resultant “hyperfiltration.” In addition to an increase in glomerular filtration of albumin, an increase in UACR can also be due to decreased renal tubular uptake of filtered albumin, which may actually protect against interstitial inflammation and fibrosis, often amplified by the reabsorption of filtered albumin. Because such marked improvements in kidney function probably would not have occurred if glomerular integrity and endothelial cell function were deteriorating,
Table 1. Effect of bardoxolone methyl on serum electrolytes in female cynomolgus monkeys after 28 days of oral administration

<table>
<thead>
<tr>
<th>Analyte per Treatment Group</th>
<th>Baseline</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>146.8±3.5</td>
<td>144.5±3.3</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>146.0±1.7</td>
<td>146.4±2.5</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.92±0.56</td>
<td>4.63±0.62</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>4.73±0.46</td>
<td>4.81±0.81</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>107.2±3.0</td>
<td>105.8±3.8</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>107.7±2.7</td>
<td>103.3±3.3</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>10.08±0.53</td>
<td>9.67±0.23</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>9.86±0.30</td>
<td>9.64±0.47</td>
</tr>
<tr>
<td>Phosphorous (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.03±0.25</td>
<td>5.20±0.56</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>4.91±0.45</td>
<td>3.91±1.88</td>
</tr>
<tr>
<td>Magnesium (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.83±0.20</td>
<td>1.62±0.08</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg)</td>
<td>1.80±0.09</td>
<td>1.65±0.15</td>
</tr>
</tbody>
</table>

Serum electrolyte levels for female cynomolgus monkeys dosed orally once daily for 28 days with vehicle (sesame oil) or bardoxolone methyl (30 mg/kg/day) are presented. Values were determined using a commercially available clinical chemistry analyzer (Alternative Biomedical Solutions, Dallas, TX). Data are presented as mean ± SD. with vehicle-treated animal data compared with day 28 data by t test using Sigmaplot 12.0 (Systat, Inc., San Jose, CA). There were no significant differences from the vehicle control group (P>0.05).

Further characterization was needed to determine whether there was a pharmacologic explanation for the increased albuminuria. Therefore, this study was designed to explore the effects of bardoxolone methyl on renal protein handling and to determine whether megalin or cubilin was downregulated. Because kidney biopsy specimens are often difficult to collect from patients with CKD, these studies were performed in nonhuman primates, which metabolize bardoxolone methyl similarly to humans and recapitulate the observed human pharmacology.

Bardoxolone methyl decreased megalin protein expression in monkey kidney, which occurred along with a modest but significant increase in UACR. These changes were not accompanied by any apparent adverse structural or functional consequences after 1 year of high-dose bardoxolone methyl administration. In fact, bardoxolone methyl administration significantly increased creatinine clearance and decreased BUN, suggesting improved kidney function and excluding kidney injury as a cause of increased albuminuria with bardoxolone methyl treatment. Bardoxolone methyl did not alter cubilin protein expression, suggesting a somewhat specific mechanism of megalin regulation. In addition, if bardoxolone methyl caused “hyperfiltration,” it is reasonable to hypothesize that some structural changes in the kidney should have been evident after 12 months of high daily dosing; however, no structural deficits were detected on kidney histology. These observations were also independent of creatinine production because 24-hour creatinine excretion did not differ among groups. Moreover, with the exception of decreased phosphorus, bardoxolone methyl administration did not alter electrolyte balance—electrolyte concentrations (including magnesium) and the fractional excretion of sodium and magnesium were unaltered.

The observed decrease in megalin protein expression may be related to bardoxolone methyl–mediated Nrf2 activation. Megalin is a multiligand, endocytic receptor that belongs to the LDL receptor family and is expressed in the apical membrane of the proximal tubular epithelial cells, where it plays an

Table 2. Effect of bardoxolone methyl on serum electrolytes in male and female cynomolgus monkeys after 12 months of oral administration

<table>
<thead>
<tr>
<th>Analyte per Treatment Group</th>
<th>Baseline</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>145.7±2.7</td>
<td>146.4±1.8</td>
</tr>
<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>146.8±3.5</td>
<td>146.9±2.1</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>146.8±2.7</td>
<td>147.8±2.2</td>
</tr>
<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>146.7±2.6</td>
<td>147.3±2.3</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.83±0.45</td>
<td>5.17±0.30</td>
</tr>
<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>4.84±0.49</td>
<td>4.94±0.61</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>5.05±0.59</td>
<td>4.83±0.63</td>
</tr>
<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>5.04±0.59</td>
<td>4.79±0.50</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>105.0±2.0</td>
<td>109.3±2.2</td>
</tr>
<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>105.9±1.7</td>
<td>108.3±2.0</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>105.9±1.9</td>
<td>106.3±2.6(^b)</td>
</tr>
<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>106.3±1.9</td>
<td>105.1±2.4(^b)</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>9.79±0.38</td>
<td>10.07±0.19</td>
</tr>
<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>9.98±0.39</td>
<td>9.90±0.29</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>9.99±0.34</td>
<td>10.01±0.46</td>
</tr>
<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>9.99±0.40</td>
<td>9.94±0.53</td>
</tr>
<tr>
<td>Phosphorous (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.04±0.64</td>
<td>5.57±0.64</td>
</tr>
<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>6.05±0.51</td>
<td>5.55±0.67</td>
</tr>
<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>6.12±0.86</td>
<td>4.67±1.06(^c)</td>
</tr>
<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>6.06±0.62</td>
<td>5.10±0.78</td>
</tr>
</tbody>
</table>

Serum electrolyte levels for male and female cynomolgus monkeys dosed orally once daily for 12 months under GLP conditions with vehicle (sesame oil) or bardoxolone methyl (5, 30, or 300 mg/kg/day) are presented. There were no sex differences, so male and female data were combined. Values were determined using a commercially available clinical chemistry analyzer (Alternative Biomedical Solutions, Dallas, TX). Data are presented as mean ± SD and were analyzed with Sigmaplot 12.0 (Systat, Inc., San Jose, CA) with an ANOVA, followed by the Duncan post hoc test. Asterisks indicate a statistically significant difference from vehicle.

\(^a\)P<0.01 versus vehicle.

\(^b\)P<0.001 versus vehicle.

\(^c\)P<0.05 versus vehicle.
important role in tubular uptake of filtered proteins, including albumin.\textsuperscript{36} In addition, interaction of protein-bound lipids with megalin-cubilin complexes facilitates NF\textsubscript{KB} activation and release of profibrotic and proinflammatory mediators, such as TNF\textalpha and monocyte chemotactic protein-1, which can contribute to interstitial inflammation and fibrosis.\textsuperscript{37,38} In contrast, bardoxolone methyl and its analogues inhibit NF\textsubscript{KB} activation and production of proinflammatory mediators.\textsuperscript{42–45} Animals with CKD and proteinuria exhibit lipid accumulation in proximal tubular epithelial cells, accompanied by marked upregulation of megalin.\textsuperscript{46,47} Moreover, accumulation of lipids in the tubular epithelium can lead to lipotoxicity and contribute to tubulo-interstitial injury and inflammation in CKD. Similar to the pathogenesis of atherosclerosis, oxidized lipids and lipoproteins can also be engulfed by macrophages in the kidney, leading to foam cell formation, glomerulosclerosis, and tubulo-interstitial injury.\textsuperscript{48} Oxidized lipids and foam cells are even prominent in kidney biopsy specimens from humans with kidney disease.\textsuperscript{49} Therefore, downregulation of megalin could contribute to enhanced urinary lipid excretion to alleviate oxidative stress and abnormalities associated with lipid accumulation. In addition, Nrf2 involvement in LDL receptor regulation has been suggested in a high-fat-diet model. Nrf2-null mice had larger increases in hepatic LDL receptor mRNA after being fed a high-fat diet than did wild-type mice; this finding suggests a role for Nrf2 in the negative regulation of LDL receptor, which could have subsequent effects on lipid uptake.\textsuperscript{50} Thus, the observed pharmacologic downregulation of megalin by bardoxolone methyl could be renoprotective because it would probably result in decreased lipid accumulation and increased lipid excretion.

In this study, bardoxolone methyl administration resulted in the profound upregulation of renal mRNA expression of critical Nrf2 target genes, NQO1 protein expression, and NQO1 and GSR enzyme activity, as well as total kidney glutathione content. Because the downregulation of megalin occurred in concert with induction of Nrf2 targets, Nrf2 may be involved, either directly or indirectly, with the regulation of megalin protein expression. However, these data are correlative and not causal in nature. Thus, this hypothesis requires further mechanistic evaluation.

Monkeys in the 28-day study had minimal but significant weight loss, whereas those in the 12-month study tended to gain less weight than controls. The latter finding was neither statistically significant nor dose dependent. This observation appears to recapitulate the observed changes in body weight experienced by humans administered bardoxolone methyl, which was directly dependent on body mass index and suggested adipose loss.\textsuperscript{33} Such changes are consistent with alterations in energy and lipid metabolism caused by the pharmacological activity of bardoxolone methyl.
Figure 6. Effect of 28 days of oral administration of bardoxolone methyl on Nrf2 targets in monkey kidney. After 28 days of daily oral administration of bardoxolone methyl (30 mg/kg), kidneys were collected and frozen in liquid nitrogen (for mRNA expression and enzyme activity) or formalin-fixed and paraffin-embedded (for immunohistochemistry). (A) Messenger RNA expression of NQO1, SRXN1, TXNRD1, GCLC, and GSR. Data were standardized to the internal control polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and presented as fold vehicle control. (B) Immunohistochemistry for NQO1 in monkey kidney. Representative photomicrographs are presented at original magnification ×5 and ×20. Staining was primarily localized to the tubules. (C) Densitometry of staining intensity of NQO1 protein in monkey kidney, as determined by a board-certified pathologist and represented as percentage change from vehicle. (D) NQO1 and GSR enzyme activity in monkey kidney presented as fold vehicle control. (E) Total glutathione content in monkey kidney normalized to protein concentration. All data are presented as mean ± SEM. Asterisks indicate a statistically significant difference from the vehicle control group (*P<0.05, **P<0.01, ***P<0.001). GSH, glutathione.
of synthetic triterpenoids. In mice fed a high-fat diet, the bardoxolone methyl analogue CDDO-Im prevented increases in body weight, adipose mass, and hepatic lipid accumulation and increased energy expenditure.51 These effects were not observed in Nrf2 knockout mice, suggesting that activation of Nrf2 has perceived positive effects on lipid and energy metabolism. Bardoxolone methyl–associated changes in body mass composition are being further evaluated in patients with type 2 diabetes and CKD.

Because megalin is also important in the renal reuptake of other substrates, such as vitamin D,36 there is also some theoretical concern of adverse effects related to the potential for increased vitamin D urinary excretion. Although vitamin D was not assessed clinically or in monkeys, bardoxolone methyl did not affect serum calcium. In addition, femur, rib, and sternum bone histologic characteristics in the 12-month study were normal in all bardoxolone methyl dose groups. Plasma vitamin D concentrations are also being monitored in ongoing clinical studies. Megalin is also expressed extrarenally and is important for the transcytosis of thyroglobulin, the thyroid hormone precursor; thus, megalin–deficient mice suffer from hypothyroidism.52 Although thyroid hormone levels have not been evaluated clinically or in monkeys, no obvious signs of hypothyroidism (e.g., goiter or weight gain) have been observed. As discussed earlier, an apparent controlled weight loss is often observed in humans33 and monkeys administered bardoxolone methyl (probably because of the pharmacological effects of bardoxolone methyl on lipid handling and energy homeostasis).

In conclusion, the observed reduction in renal megalin protein expression in cynomolgus monkeys may explain, at least in part, the seemingly paradoxical increase in albuminuria in patients with CKD and improving kidney function. Reduced megalin expression may be related to Nrf2 pharmacology, as the decrease in megalin is hypothesized to be renoprotective, and such a decrease occurred in the absence of any renal abnormality and in concert with induction of Nrf2 targets. Thus, the data suggest that the increase in UACR that develops in conjunction with a reduction in megalin protein expression is not damaging and may represent a beneficial response to bardoxolone methyl therapy.

**CONCISE METHODS**

**Animal Studies**

A detailed description of the study methods is provided in the Supplemental Material. Two separate studies were conducted in cynomolgus monkeys. In one, cynomolgus monkeys (n=9 per sex/dose group) were orally administered bardoxolone methyl at 5, 30, and 300 mg/kg once daily for 12 months, with an interim analysis at 6 months and a postdose recovery analysis 4 weeks after the final dose, in a GLP environment. In a second study, female cynomolgus monkeys (n=6 for vehicle and n=12 for treatment) were administered bardoxolone methyl (30 mg/kg per day), as above, once daily for 28 days.

**Clinical and Urine Chemistries**

BUN, serum creatinine, and urine chemistries were determined on commercially available clinical chemistry analyzers. Urine creatinine and albumin were determined using commercially available kits. Monkeys with poor urine collections or with creatinine clearance values outside of twice the SD were excluded from the analysis.

**Histology**

Kidneys from the 12-month study were fixed in neutral buffered formalin and processed for hematoxylin and eosin staining according to standard histologic techniques, with slides evaluated by a board-certified pathologist.

**Messenger RNA Quantification**

Kidney tissue was prepared and analyzed, as previously described, using the Quantigene Plex 2.0 assay from Affymetrix,53 with a modified panel of catalog #312050 with targets (NQO1, SRXN1, TXNRD1, GCLC, and GSR) designed against the human genome.

**Glutathione Quantification**

Total kidney glutathione content was determined by using a commercially available kit and normalized to protein concentrations.

**Enzyme Activity Assays**

NQO1 enzyme activity was determined by quantifying the rate of reduction of 2,6-dichlorophenol-indophenol, as previously described.54–56 Gsr enzyme activity was determined using a commercially available kit. All enzyme activities were normalized to protein concentrations and presented as fold vehicle control.

**Immunohistochemistry**

Kidney tissue from the 28-day monkey study (n=6 per group) was fixed in neutral-buffered formalin, processed according to standard histologic techniques, and incubated with primary antibodies against megalin, cubilin, or NQO1. Representative photomicrographs are presented at original magnification ×5 and ×20. For densitometry, the whole slide sections of kidney were blindly evaluated by a board-certified pathologist, and the largest possible representative region of cortex was demarcated by manual annotation for analytical determination of stain area and intensity.

**Statistical Analyses**

Data presented graphically are represented as mean ± SEM. Data presented in tabular form are represented as mean ± SD. Data were analyzed by t test or one-way ANOVA, followed by the Duncan multiple range post hoc test; P values < 0.05 were considered to represent statistically significant findings. The analyses were conducted using Sigmaplot 12.0 (Systat, Inc., San Jose, CA).

**ACKNOWLEDGMENTS**

We thank Dr. Justin L. Rains of Reata Pharmaceuticals, Inc., for his technical assistance. We would also like to thank Dr. Robert D. Toto and Dr. Jane Lee for their manuscript preparation assistance.
REFERENCES

36. Christensen EI, Birm H: Megalin and cubilin: synergistic endocytic recep-

37. Baines RJ, Brunskill NJ: The molecular interactions between
filtered proteins and proximal tubular cells in proteinuria. Nephron, Exp

38. Vaziri ND: Lipotoxicity and impaired high density lipoprotein-mediated
reverse cholesterol transport in chronic kidney disease. J Ren Nutr
20[Suppl]: S35–S43, 2010

39. Christensen EI, Devuyst O, Dom G, Nielsen R, Van der Smissen P,
Verroust P, Leruth M, Guggino WB, Courtoy PJ: Loss of chloride
canal ClC-5 impairs endocytosis by defective trafficking of megalin
and cubilin in kidney proximal tubules. Proc Natl Acad Sci U S A 100:
8472–8477, 2003

Jong M, Boerman OC: Renal uptake of different radiolabelled peptides
is mediated by megalin: SPECT and biodistribution studies in megalin-

41. Wilmer MJ, Christensen EI, van den Heuvel LP, Monnens LA,
Levchenko EN: Urinary protein excretion pattern and renal expression
of megalin and cubilin in nephropathic cystinosis. Am J Kidney Dis
51: 893–903, 2008

42. Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D: Triterpenoid CDDO-
Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on

triterpenoid, CDDO-Me, modulates the proinflammatory response to
in vivo lipopolysaccharide challenge. J Interferon Cytokine Res 30:
497–508, 2010

44. Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT,
Sporn MB, Yamamoto M, Klaassen CD: Increased Nrf2 activation in liters from Keap1-knockdown mice increases expres-
sion of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. Toxicol Sci
108: 35–47, 2009

45. Yore MM, Liby KT, Honda T, Gribble GW, Sporn MB: The synthetic
triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole
blocks nuclear factor-kappaB activation through direct inhibition of

46. Chen KH, Kim HJ, Kamanna VS, Vaziri ND: Niacin improves renal lipid
metabolism and slows progression in chronic kidney disease. Biochim
Biophys Acta 1800: 6–15, 2010

47. Kim HJ, Moradi H, Yuan J, Norris K, Vaziri ND: Renal mass reduction
results in accumulation of lipids and dysregulation of lipid regulatory
proteins in the remnant kidney. Am J Physiol Renal Physiol 296:
F1297–F1306, 2009

48. Abrass CK: Cellular lipid metabolism and the role of lipids in pro-


50. Tanaka Y, Aleksunes LM, Yeager RL, Gyamfi MA, Esterly N, Guo GL,
Klaassen CD: NF-E2-related factor 2 inhibits lipid accumulation and
oxidative stress in mice fed a high-fat diet. J Pharmacol Exp Ther

51. Shin S, Wakabayashi J, Yates MS, Wakabayashi N, Dolan PM, Aja S,
Liby KT, Sporn MB, Yamamoto M, Klaassen CD: Role of Nrf2 in pre-
vention of high-fat diet-induced obesity by synthetic triterpenoid

52. Lisi S, Segnani C, Mattii L, Botta R, Marocci C, Dolfi A, McCluskey RT,
Pinchera A, Bernardini N, Marinò M: Thyroid dysfunction in megalin
deficient mice. Mol Cell Endocrinol 236: 43–47, 2005

53. Reisman SA, Yeager RL, Yamamoto M, Klaassen CD: NF-E2-related factor 2 activation in liters from Keap1-knockdown mice increases expres-
sion of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. Toxicol Sci
108: 35–47, 2009

quinone oxidoreductase 1 during human liver injury. World J Gastro-
enterol 12: 1937–1940, 2006

55. Benson AM, Hunkeler MJ, Talalay P: Increase of NAD(P)H:quinone


See related editorial, “New Treatments for CKD—New Insights into Patho-
genesis,” on pages 1601–1603.

This article contains supplemental material online at http://jasn.asnjournals.