Bardoxolone Methyl Decreases Megalin and Activates Nrf2 in the Kidney

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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, heightened renin-angiotensin activity, and albuminuria, activate oxidative stress–mediated inflammation.1–5 Indeed, oxidative stress and impaired antioxidant capacity intensify with progression of CKD,6,7 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.\textsuperscript{9–11} In animals with CKD, oxidative stress and inflammation are associated with impaired Nrf2 activity.\textsuperscript{12–14} Pharmacologic or genetic activation of Nrf2 results in a phenotypic shift toward heightened 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.\textsuperscript{15} Nrf2 also regulates the endogenous antioxidant glutathione by regulating its synthesis (glutamate-cysteine ligase, catalytic subunit [GCLC]) and recovery (glutathione reductase [GSR]).\textsuperscript{16,17} Other Nrf2 targets, such as sulfiredoxin 1 (SRXN1) and thioredoxin reductase 1 (TXNRD1), are protective against reactive oxygen species and promote protein repair.\textsuperscript{18,19} During the past 15 years, substantial evidence has accumulated demonstrating that activation of the Nrf2 pathway can protect against oxidative and electrophilic insult.\textsuperscript{20–23} In contrast, lack of Nrf2 leads to markedly increased susceptibility to many oxidative stress–related abnormalities,\textsuperscript{23} including lupus-like nephropathy in aged animals.\textsuperscript{24} In addition, Nrf2 is suppressed in cardiac tissue from diabetic rodents, as well as patients with type 2 diabetes.\textsuperscript{25}

The synthetic triterpenoid bardoxolone methyl and its analogues (e.g., CDDO-Im) are the most potent known activators of the Nrf2 pathway.\textsuperscript{26–28} They mimic the cyclopentenone prostaglandins (such as 15-deoxy–Δ12, 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.\textsuperscript{29} Correspondingly, mice deficient in 15-deoxy–Δ12, 14 prostaglandin J2 develop glomerular hypertrophy and increased basement membrane thickening, TGFβ protein expression, lipid deposition, and renal fibrosis.\textsuperscript{30} Moreover, bardoxolone methyl and its analogues have many beneficial effects and are protective in rodent models of kidney disease. For example, CDDO-Im protects mice from iron nitrolotriacetate–induced AKI.\textsuperscript{31} 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.\textsuperscript{32} 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.\textsuperscript{33} However, in concert with improved kidney function, albuminuria was increased. The degree of

![Figure 1. Effect of bardoxolone methyl on megalin and cubilin protein expression in monkey kidney. After 28 days of daily oral administration of vehicle (n=6) or bardoxolone methyl (n=6, 30 mg/kg), kidneys were collected, formalin-fixed, and paraffin-embedded for immunohistochemistry. Immunohistochemistry is presented for megalin (A) and cubilin (C) in monkey kidney with representative photomicrographs (original magnification, ×5 and ×20). Staining was primarily localized to the tubules. Densitometry of staining intensity of megalin (B) and cubilin (D) protein in monkey kidney was determined by a board-certified pathologist and represented as percentage change from vehicle control. Densitometry data are presented as mean ± SEM. Asterisks indicate a statistically significant difference from vehicle control group (**P<0.01).](image-url)
RESULTS

**Bardoxolone Methyl Decreases Renal Expression of Megalin but Not Cubilin**

Immunohistochemical examination of kidney sections from cynomolgus monkeys demonstrated staining patterns of megalin and cubilin in the proximal tubules, consistent with previous reports. 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 3B). 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.

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**Figure 3.** Effect of bardoxolone methyl administration on body weight. (A) Bardoxolone methyl (30 mg/kg) or vehicle (sesame oil) was administered to female cynomolgus monkeys by oral gavage once daily for 28 days with body weights taken before dose and weekly after dose. (B) Bardoxolone methyl (5, 30, or 300 mg/kg) or vehicle (sesame oil) was administered to male and female cynomolgus monkeys by oral gavage once daily for 350 days with body weights taken before dose and weekly after dose. No sex differences were observed, so male and female data were combined. For simplicity, only body weights every 5 weeks are depicted. Weight data were analyzed by calculating the area under the weight-versus-time curve for each animal using the linear trapezoid method with Phoenix WinNonLin software, version 6.3 (Pharsight, Cary, NC). Data are presented as mean ± SEM. Asterisks indicate a statically significant difference in the calculated areas under the curve from vehicle-treated monkeys (*P<0.05).
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,
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

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**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>
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<tbody>
<tr>
<td>Sodium (mg/dl)</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>146.8±3.5</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
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<td>Potassium (mEq/L)</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
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<td>Chloride (mEq/L)</td>
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<td>Vehicle</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
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<td>Calcium (mg/dl)</td>
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<td>10.08±0.53</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
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<td>Phosphorous (mg/dl)</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
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<td>Magnesium (mg/dl)</td>
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<td>Bardoxolone methyl (30 mg/kg)</td>
<td>1.80±0.09</td>
<td>1.65±0.15</td>
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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 ($P>0.05$).

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**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>
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<tbody>
<tr>
<td>Sodium (mg/dl)</td>
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<tr>
<td>Vehicle</td>
<td>145.7±2.7</td>
<td>146.4±1.8</td>
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<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>146.8±3.5</td>
<td>146.9±2.1</td>
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<tr>
<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>146.8±2.7</td>
<td>147.8±2.2</td>
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<tr>
<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>146.7±2.6</td>
<td>147.3±2.3</td>
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<tr>
<td>Potassium (mEq/L)</td>
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<tr>
<td>Vehicle</td>
<td>4.83±0.45</td>
<td>5.17±0.30</td>
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<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>4.84±0.49</td>
<td>4.94±0.61</td>
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<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>5.05±0.59</td>
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<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>5.04±0.59</td>
<td>4.79±0.50</td>
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<tr>
<td>Chloride (mEq/L)</td>
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<tr>
<td>Vehicle</td>
<td>105.0±2.0</td>
<td>109.3±2.2</td>
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<tr>
<td>Bardoxolone methyl (5 mg/kg per day)</td>
<td>105.9±1.7</td>
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<td>Bardoxolone methyl (30 mg/kg per day)</td>
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<td>106.3±2.6</td>
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<td>Bardoxolone methyl (300 mg/kg per day)</td>
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<tr>
<td>Calcium (mg/dl)</td>
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<td>Bardoxolone methyl (5 mg/kg per day)</td>
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<td>9.90±0.29</td>
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<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>9.99±0.34</td>
<td>10.01±0.46</td>
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<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>9.99±0.40</td>
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<tr>
<td>Phosphorous (mg/dl)</td>
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<tr>
<td>Vehicle</td>
<td>6.04±0.64</td>
<td>5.57±0.64</td>
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<td>Bardoxolone methyl (5 mg/kg per day)</td>
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<td>Bardoxolone methyl (30 mg/kg per day)</td>
<td>6.12±0.86</td>
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<td>Bardoxolone methyl (300 mg/kg per day)</td>
<td>6.06±0.62</td>
<td>5.10±0.78</td>
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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.

$aP<0.01$ versus vehicle.

$bP<0.001$ versus vehicle.

$cP<0.05$ versus vehicle.
important role in tubular uptake of filtered proteins, including albumin. In addition, interaction of protein-bound lipids with megalin-cubilin complexes facilitates NFκB activation and release of profibrotic and proinflammatory mediators, such as TNFα and monocyte chemotactic protein-1, which can contribute to interstitial inflammation and fibrosis. In contrast, bardoxolone methyl and its analogues inhibit NFκB activation and production of proinflammatory mediators.

Animals with CKD and proteinuria exhibit lipid accumulation in proximal tubular epithelial cells, accompanied by marked upregulation of megalin. 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. Oxidized lipids and foam cells are even prominent in kidney biopsy specimens from humans with kidney disease. 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. 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. Such changes are consistent with alterations in energy and lipid metabolism caused by the pharmacological activity...
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).

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BASIC RESEARCH


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SUPPLEMENTAL-DETAILED METHODS

**Animal Studies.** Two separate in-life studies were conducted in cynomolgus monkeys. In one study, cynomolgus monkeys (n=9/gender/dose group) were administered amorphous bardoxolone methyl by oral gavage, using sesame oil as the vehicle, at 5, 30, and 300 mg/kg once daily for 12 months in a GLP environment. Observations for morbidity, mortality, injury, and the availability of food and water were conducted twice daily for all animals. Clinical observations and body weights were conducted and recorded weekly. Weight data were analyzed by calculating the area under the weight versus time curve using the linear trapezoidal method with Phoenix® WinNonLin® v.6.3 (Pharsight, Cary, NC). Blood samples for clinical chemistry evaluations were collected from all animals pretest and from all animals prior to interim (6-month) and terminal (12-month) necropsies. An additional group of monkeys for each dose group were allowed to recover for 4 weeks.

A follow-up study was conducted to assess the pharmacodynamic effects of bardoxolone methyl after a shorter dosing period. For this, female cynomolgus monkeys (n=6 for vehicle and n=12 for treated) were administered bardoxolone methyl (30 mg/kg/day) by oral gavage, using sesame oil as the vehicle, once daily for 28 days. Clinical observations and body weight were handled as above. Blood samples for clinical chemistry evaluations were collected from all animals pretest and on Day 28. Urine samples (24 hours) were collected on the same days as blood. Animal welfare for both studies was compliant with the U.S. Department of Agriculture’s (USDA) Animal Welfare Act (9 CFR Parts 1, 2 and 3). The Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources was followed. The protocols were both reviewed and approved by an Institutional Animal Care and Use Committee (IACUC).
**Clinical and Urine Chemistries.** Serum BUN and serum creatinine were determined on a commercially available clinical chemistry analyzer (Alternative Biomedical Solutions, Dallas, TX). Urine chemistries in the 28-day study were determined using a Clintek 500 urine analyzer (Siemans, Tarrytown, NY). Urine creatinine (Cayman Chemical, Ann Arbor, MI, Catalog # 500701) and albumin (Active Motif, Carlsbad, CA, Catalog # 15002) were determined using commercially available kits. Monkeys with poor urine collections or with creatinine clearance values outside of two times the standard deviation were excluded from the analysis.

**Histology.** Kidneys from the 12-month GLP study were fixed in neutral buffered formalin and processed for H&E staining according to standard histological techniques. Slides were evaluated by a board-certified pathologist. Slides from all animals were captured digitally using an Aperio ScanScope (Aperio, Vista, CA). Representative photomicrographs are presented at 5X and 20X.

**Messenger RNA Quantification.** Kidneys were collected from the 28-day study, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Kidney tissue was prepared and analyzed as previously described using the Quantigene™ Plex 2.0 assay from Affymetrix (Santa Clara, CA) (1). A modified panel (Catalog # 312050) with targets designed against the human genome was used. A description of the panel with accession numbers can be found at http://www.panomics.com. Specifically, NQO1, SRXN1, TXNRD1, GSR, GCLC, megalin, and cubilin mRNA were quantified. All data were standardized to the internal control polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and presented as fold vehicle control.

**Glutathione (GSH) Quantification.** Total GSH content in kidneys was determined by using a commercially available kit (Catalog #703002) from Cayman Chemical (Ann Arbor, MI). Total GSH content was normalized to protein determined using the DC Protein Assay (Catalog#500-0112) from Biorad (Hercules, CA). Data are presented as mean nmol GSH/mg protein.
**Enzyme Activity Assays.** Kidney tissue from the 28-day monkey study was snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Kidney tissue was homogenized at 250 mg tissue/mL ice cold PBS (pH 7.2), containing 2 mM ethylenediaminetetraacetic acid (EDTA). Homogenates were then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatants were collected and stored at -80°C until analysis. Protein concentrations of tissue homogenates were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit from Pierce Biotechnology (Rockford, IL, Catalog #23225). NQO1 enzyme activity was determined by quantifying the rate of reduction of 2,6-dichlorophenol-indophenol (DCPIP), as previously described (2-4). Gsr enzyme activity was determined using a commercially available kit (Cayman Chemical, Ann Arbor, MI, Catalog# 703202). All enzyme activities were normalized to protein and presented as fold vehicle control.

**Immunohistochemistry.** Kidney tissue from the 28-day female monkey study (n=6/group) was fixed in neutral-buffered formalin and processed according to standard histological techniques. Formalin-fixed paraffin-embedded tissue antigens were retrieved using high- or low-pH heat-mediated retrieval. After enzyme and protein blocking steps, tissues were incubated with primary antibody (megalin, Abcam, ab76969; cubilin, Santa Cruz, sc-20607; or NQO1, Abcam, ab28947) at room temperature. A horseradish peroxidase (HRP)-conjugated polymer goat anti-Rabbit IgG (Dako Carpinteria, CA , K4003) was applied to all slides either as a secondary or tertiary reagent after anti-mouse or anti-goat IgG secondary linker. Slides were then developed using 3,3-diaminobenzidine+ (DAB+) solution (Dako, Catalog# K3468), counterstained with Mayer’s hematoxylin, dehydrated, and permanently cover-slipped. Representative photomicrographs are presented at 5X and 20X. For densitometry, the whole slide sections of kidney were 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. Areas of artifactually altered staining, such as tissue edge fading or stain particle aggregation were omitted from the region of analysis in each kidney section. Manually annotated regions of cortex were then analyzed using a deconvolution algorithm with threshold stain levels set to encompass all levels of stain and also to include all negative and positive staining in all background tissue area such that appropriate area percent calculations could be made. The lowest level of stain intensity (1+) was adjusted to its lowest level and utilized as background area staining, and medium positive (2+) and strong positive (3+) were used to denote actual intensity levels of positive DAB+ staining. Data are presented as percent change from vehicle-treated monkeys.

**Statistics.** Data presented graphically are represented as mean ± standard error of the mean (S.E.M.). Data presented in tabular form are represented as mean ± standard deviation (SD). Data were analyzed by t-test or one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range *post-hoc* test with p values <0.05 considered statistically significant. The analyses were conducted using Sigmaplot 12.0 (Systat, Inc, San Jose, CA).
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