Maleate nephrotoxicity: mechanisms of injury and correlates with ischemic/hypoxic tubular cell death

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Submitted 15 September 2007; accepted in final form 11 October 2007

Zager RA, Johnson AC, Naito M, Bomsztyk K. Maleate nephrotoxicity: mechanisms of injury, and correlates with ischemic/hypoxic tubular cell death. Am J Physiol Renal Physiol 294: F187–F197, 2008. First published October 17, 2007; doi:10.1152/ajprenal.00434.2007.—Maleate injection causes dose-dependent injury in proximal tubular cells. This study sought to better define underlying pathogenic mechanisms and to test whether maleate toxicity recapitulates critical components of the hypoxic/ischemic renal injury cascade. CD-1 mice were injected with maleate or used as a source for proximal tubule segments (PTS) for in vitro studies. Maleate induced dose-dependent PTS injury [lactate dehydrogenase (LDH) release, ATP reductions, non-esterified fatty acid (NEFA) accumulation]. These changes were partially dependent on maleate metabolism (protection conferred by metabolic inhibitors: succinate, acetocetate). Maleate toxicity reproduced critical characteristics of the hypoxia/ATP depletion-induced injury cascade: 1) glutathione (GSH) conferred protection, but due to its glycine, not cysteine (antioxidant), content; 2) ATP reductions reflected decreased production, not Na-K-ATPase-driven increased consumption; 3) cell death was completely blocked by extracellular acidosis (pH 6.6); 4) intracellular Ca2+ chelation (BAPTA) mitigated cell death; 5) maleate and hypoxia each caused plasma membrane cholesterol shedding and in both instances, this was completely glycine suppressible; 6) maleate + hypoxia caused neither additive NEFA accumulation nor LDH release, implying shared pathogenic pathways; and 7) maleate, like ischemia, induced renal cortical cholesterol loading; increased HMG CoA reductase (HMGCR) activity (statin inhibitable), increased HMGCR mRNA levels, and increased RNA polymerase II recruitment to the HMGCR locus (chromatin immunoprecipitation, ChIP, assay) were involved. These results further define critical determinants of maleate nephrotoxicity and suggest that it can serve as a useful adjunct for studies of ischemia/ATP depletion-induced, proximal tubule-specific, cell death.

hypoxic/ischemic renal injury cascade; proximal tubule; chIP assay; HMG CoA reductase

DESPITE MAJOR ADVANCES in our understanding of pathogenic events in ischemic and toxic acute renal failure (ARF), clinical outcomes in this disease, and our prophylactic/therapeutic approaches to it, have not substantively changed over the past 30 years. This is perhaps most graphically illustrated by the fact that saline-induced intravascular volume expansion, renal vasodilators, and diuretics remain the most widely employed agents for preventing/treating this disease. There are a number of potential explanations for a failure to translate basic, laboratory-derived, mechanistic insights into clinical practice. These have been summarized in a recent series of editorials, assembled by Lieberthal and Nigam (13). Perhaps the dominant opinion emerging from this compendium is that currently employed animal models of ARF are “imperfect,” i.e., they do not accurately simulate clinical disease (13). Thus it was suggested that new models, new insights into existing models, and corollaries between models are needed before firm mechanistic conclusions are drawn.

When injected into rodents, maleic acid undergoes relatively selective proximal tubule (PT) cell uptake via organic anion transporters (OATs) (9, 10, 30). Once intracellular accumulation occurs, maleate is a preferred substrate for succinyl-CoA: 3-oxoadic CoA transferase (SCOTase) (24–26, 32). This results in the formation of maleyl-coenzyme A. With subsequent conversion of maleyl CoA into a stable thioether, severe coenzyme A (CoA) depletion results (25, 29). Ample levels of CoA are essential for fatty acid “activation,” allowing for their subsequent metabolism through the Krebs cycle, yielding ATP. In the absence of this process, PT ATP depletion and cell injury result (10, 23). Additionally, maleate conjugates the sulphydryl group of glutathione (GSH) (19, 23), culminating in GSH depletion and potential oxidant tubular stress (20, 27, 33). Depending on the severity of these processes, either functional transport defects (Fanconi syndrome) or proximal tubular necrosis/filtration failure result (3, 10, 13).

The maleate model of ARF, and its subcellular determinants, has received comparatively scant attention in the ARF literature. This is likely because no direct clinical equivalent of maleate toxicity exists (in sharp contrast to widely employed ARF models such as glycerol-induced rhahdomyolysis, amnoglycoside nephrotoxicity, or cisplatin-induced ARF). Despite the lack of a direct clinical correlate, the maleate model has theoretical appeal because it can induce dose-dependent, PT-specific, ATP and GSH depletion (8, 10). Thus we hypothesized that it might represent a useful and underutilized ARF model that can simulate selected components of the ischemic tubular injury cascade.

Given this possibility, the present study was conducted with two goals in mind: first, to gain new insights into mechanisms and determinants of maleate-induced proximal tubular injury; and second, to ascertain whether maleate-induced tubular injury does, in fact, recapitulate some widely accepted determinants of ischemic/ATP depletion-induced renal damage. If so, then maleate nephrotoxicity could represent a useful adjunct to the widely used renal artery occlusion (RAO) model of ischemic ARF for which multiple experimental and theoretical limitations exist (13, 54).
METHODS

General

All in vivo and isolated tubule experiments were conducted using 25- to 35-g male CD-1 mice (Charles River Laboratories, Wilmington, MA). Protocols were approved by the Fred Hutchinson Cancer Research Center IACUC. The mice were maintained under routine vivarium conditions with free food and water access. For the purpose of kidney harvesting, the mice were deeply anesthetized with pentobarbital sodium (50 mg/kg ip), followed by bilateral renal resection performed through a midline abdominal incision. In selected experiments, a heparinized blood sample was obtained from the abdominal vena cava just before renal resection [for blood urea nitrogen (BUN) analysis].

Isolated Tubule Preparations

Isolated tubules were prepared from renal cortex as previously described (49, 51). In brief, following bilateral renal resection, the kidneys were iced and both cortices were resected. The tissues were minced with a razor blade, digested with collagenase, passed through a stainless steel sieve, and pelleted by centrifugation (4°C). Viable PTs were recovered by centrifugation through 32% Percoll (Pharmacia, Piscataway, NJ). After multiple washings in iced buffer, the PTs were suspended (~2–3 mg protein/ml) in experimentation buffer (in mmol/l: 100 NaCl, 2.1 KCl, 25 NaHCO3, 2.4 KH2PO4, 1.2 MgSO4, 1.2 CaCl2, 5 glucose, 1 alanine, 4 Na lactate, 10 Na butyrate, 0.6% 36-kDa dextran, gassed with 95% O2-5% CO2, final pH 7.4). They were rewarmed from 4°C to 37°C in a heated shaking water bath over 15 min and then divided into four to six equal aliquots for experimentation. Each preparation consisted of tubules isolated from a single mouse.

Maleate Toxicity in PTs: Dose-Response Relationship

Five sets of PTs were divided into six equal aliquots and incubated under control oxygenated conditions or with 1, 2.5, 5, or 10 mM Na maleate (final pH 7.4). After 30-min incubations, the extent of lethal cell injury was determined by calculating percent cellular Na maleate (final pH 7.4). After 30-min incubations, the extent of cell injury was determined by calculating percent cellular

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GSH Effects on Maleate Toxicity

The following experiments were performed to test GSH’s impact on maleate toxicity.

GSH addition. Six sets of PTs were each divided into four aliquots: 1) control incubation; 2) incubation with 5 mM GSH; 3) 2.5 mg/ml maleate; or 4) maleate + GSH. At the completion of 30-min incubations, percent LDH release was determined.

N-acetyl cysteine addition. The above experiment was repeated in four sets of tubules, substituting 5 mM N-acetyl cysteine (NAC) for GSH. This was done to ascertain whether GSH’s cysteine component (glutamate-cysteine-glycine) was responsible for the latter’s cytoprotective effect.

Glycine addition. The above experiment was repeated in six additional sets of tubules, substituting 5 mM glycine for GSH (to assess the impact of GSH’s glycine content).

Maleate Effects on Tubule Adenine Nucleotide Profiles

Adenine nucleotides. Four sets of PTs were incubated under control conditions or with 2.5 mg/ml maleate. After 30 min, percent LDH release was determined, tubule adenine nucleotides were extracted in trichloroacetic acid, and analyzed for ATP, ADP, AMP, and degradation products [adenosine (ADO) and inosine (INO)] by HPLC (52).

Glycine-mediated protection. To explore whether the maleate-induced suppression in ATP levels (see RESULTS) was due to the expression of lethal cell injury, four additional sets of tubules were incubated ×30 min under control conditions ± 5 mM glycine or with 2.5 mg/ml maleate ± glycine (to prevent lethal cell damage) (38). Percent LDH release and ATP concentrations were then assessed.

Ouabain-mediated Na-K-ATPase inhibition. To ascertain whether increased Na-K-ATPase-driven ATP consumption was responsible for the ATP depressions (45, 46), four additional sets of tubules were incubated ×30 min under control conditions ± 0.5 mM ouabain, or with 2.5 mg/ml maleate ± ouabain. Percent LDH release and ATP levels were then assessed.

Maleate Effects on Nonesterified Fatty Acid Levels

Four sets of PTs were each divided into four equal aliquots and incubated under: 1) control conditions, 2) with 2.5 mg/ml maleate, 3) with 5 mg/ml maleate, or 4) with 5 mg/ml maleate ± 5 mM glycine. After 30 min, percent LDH release was determined. Tubule aliquots underwent lipid extraction in 2:1 chloroform:methanol and were assayed for total nonesterified fatty acid (NEFA) content (Wako Chemicals, Osaka, Japan).

To gain an insight into relative degrees of maleate-induced NEFA accumulation, the above results were contrasted with those produced by hypoxia. Four sets of PTs were each divided into four aliquots: 1) control incubation, 2) hypoxia ×30 min (gassing with 95% N2-5% CO2), 3) 2.5 mg/ml maleate, and 4) combined hypoxia + maleate. Percent LDH release and NEFA levels were then assessed.

Unsaturated vs. Saturated Free Fatty Acid Effects on Maleate Toxicity

It was previously demonstrated that unsaturated, but not saturated, fatty acids confer protection against hypoxia-mediated PT cell death (1, 47, 50). This experiment tested whether this same cytoprotective profile is expressed against maleate cytotoxicity. Twelve sets of PTs were prepared and divided into three sets for testing each of the following three fatty acids: arachidonic acid (C20:4); linoleic acid (C18:2); or stearic acid (C18:0), as follows: 1) control incubation; 2) incubation with a test fatty acid; 3) incubation with 2.5 mg/ml maleate; or 4) incubation with the same test fatty acid + maleate. Each fatty acid was added to a final concentration of 100 μM in 0.2% DMSO vehicle. (DMSO was also added to the nonfatty acid-treated control aliquots.) After 30-min incubations, percent LDH release was assessed.

Extra-Intracellular Ca2+ As Potential Determinant(s) of Maleate Cytotoxicity

The following experiment was undertaken to ascertain whether extracellular—intracellular Ca2+ leak or intracellular Ca2+ levels are potential determinants of maleate cytotoxicity. Eight sets of PTs were each divided into two sets of experiments: 1) control incubation, 2) incubation with either 100 μM BAPTA AM (an intracellular Ca2+ chelator) or 2.5 mM EGTA (extracellular Ca chelator), 3) 2.5 mg/ml maleate, or 4) maleate + either BAPTA or EGTA. Thirty minutes later, percent LDH release was assessed.

Competitive Inhibition of SCOTase

LDH release. The following experiment assessed whether SCO-Tase activity is critical to maleate-induced PT damage. Four sets of tubules were divided into four equal aliquots: 1) control incubation, 2) incubation with 1 mg/ml (7.2 mM) maleate, 3) incubation with 7.2 mM Na succinate, or 4) combined maleate/succinate incubation (succinate used as a competitive SCOTase inhibitor) (24–26). After 45 min, percent LDH release was assessed.

An additional eight sets of tubules were treated exactly as above, but either equimolar acetooacetate (a second SCOTase inhibitor) (32) or non-SCOTase Krebs cycle substrates (7.2 mM Na citrate or malate) were employed.

ATP levels. To ascertain whether succinate-mediated SCOTase inhibition mitigates maleate-induced ATP depletion, the above exper-
iment was repeated in four additional sets of tubules with two exceptions: 1) 5 mM glycine was added to all aliquots to prevent lethal cell injury (see RESULTS) and 2) the samples were extracted for ATP after completing a 20-min (not 45-min) incubation.

**Acidosis Effects on Expression of Maleate Toxicity**

It has previously been demonstrated that acidosis protects against hypoxic tubular injury, presumably by stabilizing the plasma membrane (28, 49). The following experiment tested whether similar protection is observed against maleate cytotoxicity. Four sets of tubules were each divided into four aliquots and incubated as follows: 1) control incubation, 2) incubation with 1, 2.5, or 5 mg/ml of maleate (added as maleic acid, allowing pH to fall to ~7.2, ~6.6, and ~6.0, respectively). After 45-min incubations, percent LDH release and ATP concentrations were assessed.

To determine whether the acidosis-mediated protection extended to oxidant injury (i.e., is acidosis-mediated protection nonspecific), two additional sets of tubules were divided into four aliquots as follows: 1) control incubation, 2) incubation with a known oxidant challenge (50 μM ferrous ammonium sulfate; complexed with the Fe siderophore, hydroxyquinoline at pH 7.4), 3) incubation with 2.5 mg/ml maleic acid (pH 6.6), and 4) Fe incubation with pH lowered to 6.6 by 2.5 mg/ml maleic acid addition. After 45 min, lethl cell injury was assessed by percent LDH release.

**In Vivo Renal Cortical Cholesterol Assessments**

Previous studies demonstrated that renal cholesterol accumulation follows heterogeneous forms of renal injury (42–44, 48). The following experiments tested whether maleate-induced PT injury also causes cholesterol accumulation, and if so, whether increased cholesterol synthesis is involved. Mice were injected intraperitoneally with Na maleate, 600 mg/kg (in ~0.7 ml saline, pH adjusted to 7.4) or with the same volume of saline vehicle. Either 6 or 24 h later, the following groups were created: 1) 6-h control mice (n = 12), 2) 6-h maleate mice (n = 8), 3) 24-h control mice (n = 6), 4) 24-h maleate mice (n = 6), 5) 6-h maleate mice (n = 6) treated for the preceding 3 days with mouse chow to which was added atorvastatin (Lipitor, Pfizer, New York, NY; 1.5 mg/g standard mouse chow) (see Ref. 48), or 6) 6-h maleate mice, treated with the same mouse chow without atorvastatin addition (44). At these designated time points, the animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip), the kidneys were resected through a midline abdominal incision, the cortices underwent lipid extraction in chloroform:methanol, and the extracts were assayed for both free cholesterol (FC) and cholesteryl esters (CEs) by gas chromatography (48). Terminal BUN concentrations were determined to gauge the severity of renal damage. Three control mice and four 24-h maleate-treated mice also had renal sections fixed in 10% formalin. Four-micrometer sections were stained with hematoxylin/eosin and examined by light microscopy.

**Renal Cortical HMG CoA Reductase mRNA Expression**

Mice were injected intraperitoneally with maleate or vehicle (n = 20 each). At 6 or 24 h postinjection, half of the controls and half of the maleate-injected mice were anesthetized and subjected to left nephrectomy. Renal cortical samples were extracted for total RNA and assayed for HMG CoA reductase (HMGCR) mRNA by RT-PCR (43). The results were factored by simultaneously obtained GAPDH product, used as a housekeeping gene. Terminal BUNs were determined in the 24-h postmaleate mice and the results were correlated with the prevailing HMGCR mRNA levels.

**Renal Cortical Chromatin Immunoprecipitation Assay**

The following experiments were undertaken to ascertain whether: 1) cortical chromatin immunoprecipitation (CHIP) assay can be used to localize RNA polymerase II (Pol II) at genomic DNA sites in nuclear extracts from in vivo cortex and 2) if so, to define whether maleate-induced increases in HMGCR mRNA (see RESULTS) reflected increased HMGCR gene transcription, as assessed by Pol II recruitment along the HMGCR gene. The method used for these studies is an adaptation of the Fast ChIP assay developed by Nelson et al. (22).

**Tissue preparation.** Fifteen mice were injected with maleate. Five normal mice served as controls. Either 1, 2, or 4 h postmaleate injection (n = 5 mice at each time point), renal cortices were harvested, as noted above. Tissues (25–30 mg) were placed in 1 ml PBS/1% formaldehyde and minced with forceps. Following 20-min incubations (22°C), tissue suspensions were centrifuged (1 min at 4,000 rpm). After the supernatant was discarded, cross-linked tissue was suspended in 1 ml of 125 mM glycine ×5 min. After being vortexed, the slurry was centrifuged (1 min at 4,000 rpm) and the supernatant was discarded. Tissues were washed ×2 with 1 ml of PBS and after centrifugation (1 min at 4,000 rpm) and they were stored at ~70°C.

**Shearing chromatin.** One milliliter of immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol; with the following inhibitors: 10 μg/ml leupeptin, 0.5 mM PMSF, 30 μM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na3VO4, 0.1 mM Na2MoO4, 10 mM β-glycerophosphate) was added to Eppendorf tubes on ice containing 50 μl of tissue sample. Tissues were sheared with a sonicator probe for 6 rounds of 15-s pulses at output level 5 (Misonix 3000). Sheared chromatin was cleared by centrifugation (10 min at 10,000 rpm 4°C) and immediately stored at ~70°C.

**Chromatin immunoprecipitations.** Sheared chromatin aliquots (100 μl) were diluted with IP buffer containing all the inhibitors to a final volume of 250 μl. After addition of anti-Pol II antibody (Santa Cruz Biotechnology, SC899) or mock immune IgG (Vector Laboratories, #1-1000), the sheared chromatin fraction was incubated in an ultrasonic water bath (15 min, 4°C; Bronson 3510). Tubes were centrifuged (10 min at 17,000 g) and the supernatant was transferred to fresh tubes containing 20 μl of washed protein A beads (Pharmacia). The slurry was rotated for 45 min (4°C) and then the beads were washed once with 10 μl of IP buffer (without inhibitors) in 16-ml tubes. Washed beads were transferred to fresh 1.5-ml Eppendorf tubes and then 100 μl of 10% Chelex in H2O containing 100 μg proteinase K buffer were added to each tube. After incubation for 30 min at 55°C and boiling for 15 min, the tubes were centrifuged, the supernatants were transferred to fresh tubes, diluted with water (to 200 ml), and stored at ~20°C.

**Real-time PCR.** The reaction mixture contained 2.7 μl 2 × SYBR Green PCR master mix (SensiMix, Quantace), 2.3 μl DNA template, and 0.3 μM primers (5 μl final volume) in 384-well Optical Reaction Plates (Applied Biosystems). Amplification (3 step; 40 cycles), data acquisition, and analyses were done using the 7900HT real-time PCR system and SDS Enterprise Database (Applied Biosystems). Pol II localizations at the following genes/exons were sought: 1) heme oxygenase-1 (HO-1; sought as a presumptive positive control); 2, 3) HMGCR gene (exons 1 and 19: i.e., the start and end exons); and 4) 18S ribosomal genes (as a negative control). The primers used are listed in Table 1. Pol II density at genomic sites was expressed as a signal-to-noise ratio, R, using the following formula: R = exp2(CTmock − CTspecific), where CTmock and CTspecific are mean thresholds of PCR done in triplicates on DNA samples from specific and mock immunoprecipitations.

**Cholesterol Shedding**

The following experiment tested: 1) whether plasma membrane cholesterol, a cytoprotectant (42), is acutely shed from PTs in response to maleate, 2) whether a similar phenomenon occurs with hypoxic cell injury, and 3) whether potential cholesterol shedding is a glycerine-suppressible event. Eight sets of tubules were each divided into four aliquots and incubated ×90 min as follows: 1, 2) control, oxygenated, incubation ± 5 mM glycine; 3, 4) with either 2.5 mg/ml
maleate ± glycine; 5, 6) 30 min of hypoxia (95% N2-5% CO2 gassing) followed by 60-min reoxygenation (95% O2-5% CO2) ± glycine. Percent LDH release was determined, the aliquots were centrifuged, the supernatants were extracted in chloroform:methanol, and then the extracts were assayed for total cholesterol. Results were expressed per milligram of tubule protein content.

Calculations and Statistics

All values are presented as means ± 1 SE. Statistical comparisons were performed by paired (isolated tubule data) or unpaired (renal cortical data) Student’s t-test. If multiple comparisons were made, the Bonferroni correction was applied. Significance was judged at a P value of <0.05.

RESULTS

Maleate Toxicity in Isolated Tubules

Maleate induced significant dose-dependent cytotoxicity over 30-min incubations (Fig. 1). It did not independently alter the LDH assay, artifactually altering this endpoint.

Influence of GSH, NAC, and Glycine on Maleate Cytotoxicity

As shown in Fig. 2, left, GSH conferred ~50% protection against maleate cytotoxicity without independently altering LDH release (i.e., in the absence of maleate). This protection could not be ascribed to GSH’s sulfhydryl (–SH) content because NAC tended to exacerbate (P < 0.07), rather than protect against, maleate cytotoxicity (Fig. 2, middle). Conversely, glycine (Gly) conferred total protection against maleate-induced LDH release (Fig. 2, right). Thus these studies indicate that GSH-mediated protection stems from its glycine, not sulfhydryl (cysteine) content.

Maleate Effects on Adenine Nucleotides

Maleate reduced tubule ATP by ~70%. This was accompanied by reciprocal increases in AMP, ADO, and INO (Fig. 3, left). Although glycine totally prevented maleate-induced lethal cell injury (as shown above), this protection occurred...
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without any preservation of ATP (Fig. 3, right). Ouabain also failed to conserve ATP (Fig. 3, right).

Maleate Effects on NEFA Accumulation

As shown in Fig. 4A, maleate cytotoxicity was associated with an approximate fourfold increase in NEFA concentrations. Although glycine (Gly) prevented lethal cell injury (Fig. 4B), it did not significantly decrease NEFA levels (Fig. 4A).

Hypoxia (N2) also raised NEFA concentrations by about fourfold (Fig. 4C). However, the combination of hypoxia + maleate did not cause greater NEFA accumulation than either one alone (Fig. 4C). Furthermore, hypoxia + maleate did not cause more LDH release than did hypoxia alone (Fig. 4D). The absence of additive NEFA accumulation/LDH release suggests that hypoxia and maleate share common injury pathway(s).

Exogenous Fatty Acid Effects on Maleate Toxicity

None of the fatty acids (C20:4, C18:2, C18:0) exerted an independent effect on percent LDH release in the absence of maleate (Fig. 5, left). C20:4 mitigated maleate cytotoxicity by ~50% (Fig. 5, right). A second unsaturated fatty acid (C20:2) conferred somewhat lesser protection (~33%), whereas a saturated fatty acid (C18:0) failed to mitigate maleate-induced cell death (Fig. 5, right). Thus the degree of fatty acid protection was exactly, and inversely, correlated with the degree of fatty acid desaturation (# unsaturated bonds vs. % LDH release, r = −0.99).

Ca2+ Chelator Effects on Maleate Toxicity

As shown in Fig. 6, left, neither BAPTA nor EGTA altered tubule viability in the absence of maleate. BAPTA decreased maleate cytotoxicity by ~50%, whereas EGTA had no protective effect (Fig. 6, right). Of note, in data not shown, BAPTA induced similar protection against hypoxic, but not Fe-mediated, oxidant death indicating a relatively specific action against maleate/hypoxic injury pathways.
Succinate Effects on Maleate Cytotoxicity

As shown in Fig. 7, left, succinate caused an approximate 50% reduction in maleate cytotoxicity (LDH release). This is consistent with the expected 50% reduction in maleate/SCO-Tase metabolism (given that equimolar amounts of succinate and maleate were added). This cytoprotection was largely dissociated from changes in mitochondrial performance, given that succinate caused only a trivial increase in tubule ATP (Fig. 7, right). In the absence of maleate, succinate did not decrease LDH release or increase ATP.

As shown in Fig. 8, acetoacetate, another SCOTase competitive inhibitor, recapitulated succinate’s cytoprotective effect. Conversely, neither citrate nor malate (additional Krebs cycle substrates/non-SCOTase inhibitors) failed to reproduce the protective effects seen with succinate or acetoacetate.

pH Effects on Maleate Cytotoxicity

Addition of maleic acid (rather than Na maleate as used above) caused dose-dependent reductions in tubule suspension pH (7.2, 6.6, 6.0 at maleic acid concentrations of 1, 2.5, and 5.0 mg/ml). At pH 7.2 (1 mg/ml maleic acid), 72% LDH release was apparent. However, 2.5- and 5-mg/ml maleic acid doses (pH 6.6 and 6.0) failed to induce any LDH release (see Fig. 9, left). This was despite the fact that increasing maleic acid concentrations caused progressive declines, rather than preservation of, tubule ATP (Fig. 9, right). This was despite the fact that increasing maleic acid concentrations caused progressive declines, rather than preservation of, tubule ATP (Fig. 9, right).

Acidosis-mediated protection against maleate toxicity was relatively specific: when the ferrous ammonium sulfate challenge was imposed under acidotic (6.6 pH) conditions, a worsening of, rather than protection against, Fe injury was observed: (21 ± 2 % vs. 65 ± 10% LDH release at pH 7.4 vs. 6.6, respectively; P < 0.02; Fig. 9, left).

Renal Cortical Cholesterol Concentrations

6 and 24 h Postmaleate: renal cortical cholesterol content. As shown in Fig. 10, left, maleate induced a progressive rise in renal cortical FC content over time, reaching ~125% of control values by 24 h. A corresponding four- to fivefold increase in CE (the cholesterol “storage” form) was observed (Fig. 10, right). A wide variation in 24-h BUN levels was observed in the maleate-treated mice (mean = 97 ± 17 mg/dl; range = 20–138 mg/dl). Statistically significant correlations were observed between the 24-h BUNs and corresponding FC (r = 0.70; P < 0.05) and CE (r = 0.90; P < 0.02) levels.
Statin effects on renal cortical cholesterol content. Statin therapy did not alter renal FC or CE levels in normal mice (Fig. 11, open bars). However, it prevented the maleate-induced FC/CE increases, as assessed at the 6-h time point (confirming that increased synthesis plays a dominant role in maleate-induced cholesterol accumulation). Statin treatment did not significantly alter the extent of renal injury, as assessed 6 h postmaleate injection (statin 56 ± 8, no statin 41 ± 7 mg/dl; \( P = 0.2 \); control mice = 25 ± 2 mg/dl).

Renal Cortical HMGCR mRNA Levels

Maleate injection caused an approximate doubling of HMGCR mRNA levels at both the 6- and 24-h postinjection time points (Fig. 12, left). The 24-h mRNA levels significantly correlated with the severity of renal injury (24-h BUNs vs. mRNA values, \( r = 0.85; P < 0.001 \); Fig. 12, right).

RNA Pol II Recruitment Along the HMGCR Locus

A bell-shaped curve in Pol II recruitment to Exon 1 of HO-1 was observed, peaking at 2 h postmaleate injection (i.e., positive control; Fig. 13). Quantitatively comparable, but earlier (1 h peak), Pol II recruitment along the HMGCR gene (exons 1 and 19, last exon) was observed. No Pol II recruitment to ribosomal genes was found, serving as the negative control.
**Cholesterol Shedding in Response to Maleate or Hypoxic Reoxygenation Injury**

Both maleate and hypoxia-reoxygenation injury caused significant, and comparable, lethal cell injury (65 and 58% LDH release, respectively) and cholesterol shedding into the media (Fig. 14). In both instances, cholesterol shedding was completely glycine suppressible. Glycine did not independently alter cholesterol content.

**Renal Histology**

Unlike renal artery occlusion, maleate caused no obvious renal medullary vascular congestion (Fig. 15). Maleate induced obvious tubular necrosis, but this was largely confined to the S3 (outer medullary stripe) proximal tubular segment (Fig. 15). Only rare foci of tubular necrosis were seen in S1/S2 (renal cortical) segments. No other renal histological injury was observed.

**DISCUSSION**

When trying to induce renal tubular ATP depletion injury in vivo, investigators are confronted with a number of experimental limitations that may confound the results that are obtained. The first is the lack of a method to evoke ATP reductions specifically within proximal tubular cells. For example, during RAO, all nephronal segments and the vasculature are involved. Even deep medullary structures develop marked ATP losses despite their inherent glycolytic capacity (53). A second limitation of the RAO model is that the ATP reductions are “all-or-none” in nature. Within seconds of cross clamping the renal artery, ATP concentrations plummet to ~5% of baseline and remain at or below these levels until reperfusion occurs. Conversely, during systemic shock, far less severe ATP losses occur (~50% during hemorrhage, sepsis, cardiopulmonary failure), and minute-to-minute ATP fluctuations exist (52). Therefore, it is likely that RAO-induced ATP depletion does not accurately reproduce the changes evoked by more clinically relevant renal hypoperfusion states. A third limitation of the RAO model is the need for anesthesia and major surgery.

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**Fig. 13.** RNA polymerase II recruitment to specific gene loci, as determined by chromatin immunoprecipitation (CHIP) assay. **A:** “positive control”: because heme oxygenase-1 (HO-1) is induced by maleate injection (e.g., Ref. 26), Pol II recruitment to the HO-1 gene (exon 1) was sought. A bell-shaped curve of recruitment was observed, peaking at 2 h postmaleate injection. **B:** “negative control.” As expected, no Pol II recruitment to ribosomal 18S genes (rDNA) was observed. **C and D:** HMGCoA reductase (HMGCR): Pol II recruitment was observed in a time-dependent fashion at both exons 1 and 19 of the HMGCR gene, peaking at 1 h postmaleate injection. The relative increases over baseline were approximately as great as those seen in the HO-1 “positive control.” All values were normalized to control values of 1. Statistics were run by unpaired Student’s t-test using the absolute numerical values that were obtained.

**Fig. 14.** Cholesterol shedding into tubule buffer in response to either 90 min of maleate exposure or 30 min of hypoxia/60 min of reoxygenation (N₂/O₂) injury. In both forms of injury, there was an approximate doubling of media cholesterol content, indicative of membrane shedding. In both forms of injury, this cholesterol shedding was completely blocked by glycine.

**Fig. 15.** Left: gross appearance of kidneys sectioned at 24 h post-30 min of renal artery occlusion (RAO; top), a control kidney (middle), or 24 h postmaleate injection. A kidney section obtained from a mouse which was subjected to 30 min of RAO + 24-h reperfusion is shown for comparison to a postmaleate and control kidney. RAO induced marked medullary vascular congestion, which was not seen in maleate-treated kidneys. Right: renal histology observed 24 h postmaleate injection. The top half of the photomicrograph illustrates renal cortex, and virtually no necrosis/injury is apparent. The bottom half of the slide is outer medulla, where extensive S3 segment proximal tubular necrosis is seen (*). The remainder of the kidney appeared normal.
Anestheses are well-known to impact ischemic renal damage (e.g., Refs. 2, 14, 15, 46), and the abdominal or bilateral flank incisions through which RAO is induced can release injury-provoking Toll receptor ligands (11, 12, 16, 41). Because of these limitations, the present study attempted to address whether maleate toxicity might have potential utility as a complementary model for testing selected components of a proximal tubular “ischemic” injury cascade. 

As illustrated by the current histological assessments, a major advantage of the maleate model is that it induces relatively specific PT damage, particularly in the S3 segment. Especially noteworthy in this regard is the absence of microvascular injury and the attendant blood trapping (Fig. 15) that routinely occurs following RAO (5, 17, 18). At least three factors account for the predominance of PT segment involvement. First, because maleate is an organic acid, it is transported via OATs that are preferentially expressed along the PT basolateral membrane (9, 10, 30). This allows for toxic intracellular maleate concentrations to be attained. Second, maleate is freely filtered by the glomerulus, yielding high intraluminal concentrations. This may induce further PT damage, possibly via interactions with gp330/megalin (4). Third, maleate has been shown to be a preferred substrate for the SCOTase isofrom within PT cells (26, 32). Indeed, the present study confirms the critical role that this enzyme plays in maleate toxicity, given that succinate and acetoacetate, competitive SCOTase inhibitors, mitigated maleate-induced cell death. That this protection was due to SCOTase inhibition, rather than provision of a Krebs cycle substrate, is indicated by the fact that neither malate nor citrate recapitulated this cytoprotective effect.

In 1993, Kellerman (10) confirmed that maleate injection into rats causes dose-dependent ATP losses (up to 70%) within whole kidney tissue. The pathophysiological significance of these reductions was indicated by the fact that maintenance of renal ATP levels via intravenous ATP-MgCl2 infusion conferred functional (TNaR; glomerular filtration rate) and morphologic protection. The present observation that maleate acutely reduces ATP in isolated renal tubules, and to a comparable degree (~70%) to that observed in vivo (10), indicates that Kellerman’s whole kidney ATP results reflected, in large part, proximal tubular events. Renal tubular ATP declines can reflect either decreased production or increased consumption. The latter can be driven by accelerated Na-K-ATPase activity, as can result from increased cellular Na influx, followed by active efflux (45). However, that ouabain-mediated Na-K-ATPase inhibition failed to conserve ATP during maleate exposure indicates that suppressed mitochondrial ATP generation was the dominant, if not the sole, cause for the maleate-induced energy declines. 

A frequently advanced concept is that maleate toxicity stems, at least in part, from its ability to deplete GSH, culminating in oxidant stress (8, 19, 23). Nath’s observations (20, 34) that maleate induces HO-1, a redox-responsive stress protein, underscores this point. Given these considerations, we sought evidence for the concept that GSH depletion is a critical determinant of maleate cytotoxicity within PTs. As one might predict, based on the above considerations, a modicum of protection was produced by exogenous GSH addition. However, in pilot experiments, we found no evidence for maleate-induced tubule lipid peroxidation, as assessed by malondialdehyde content. Additionally, (n-acetyl) cysteine, the antioxidant component of GSH, slightly worsened, rather than protected against, maleate-induced cell death. In an elegant series of studies, Weinberg et al. (38) made the seminal observation that glycine, rather than cysteine, is responsible for GSH’s protective action against ATP depletion/hypoxic cell death. Furthermore, glycine is inactive against most forms of oxidant attack (Ref. 31; additional unpublished observations, R. A. Zager). Thus we tested whether glycine could recapitulate GSH’s protective action. As in vivo, where glycine mitigates maleate nephrotoxicity (23), glycine completely blocked LDH release from isolated tubules. This further supports the concept that maleate primarily induces an ATP depletion (glycine suppressible), rather than an oxidant form of attack. That Nath et al. (20) found that maleate decreases, not increases, renal cortical H2O2 content further supports this concept. Finally, it should be noted that a 90–95% ATP decline is considered by some to be the critical threshold for lethal tubule damage, as assessed in cultured LLC-PK1 cells (36). That maleate caused marked LDH release from isolated tubules with just 70% ATP reductions suggests that other pathogenic pathways (e.g., NEFA accumulation; maleate-induced thioester formation) might act synergistically with ATP depletion to simulate an “hypoxic” (“glycine suppressible”) form of cell death.

Intracellular NEFA accumulation is a well-defined consequence, and mediator, of mitochondrial respiratory blockade (6, 7, 47). A predictable result of maleate-induced CoA deple tion should be NEFA increases due to a failure of their metabolism. To seek similarities between hypoxic and maleate-induced tubule injury, we compared NEFA levels in both forms of tubule damage. Each caused striking, and highly equivalent (4- to 5-fold), NEFA elevations. However, when these two forms of injury were imposed simultaneously, no additive NEFA increases resulted. Furthermore, no additive lethal cell injury (LDH release) was observed. Thus these findings imply that hypoxia and maleate evoked NEFA accumulation and cell death via shared pathogenic pathways. It is notable that glycine addition did not abrogate either maleate-mediated NEFA increases or ATP declines despite completely blocking LDH release. That this is the same as with glycine-mediated protection against hypoxic injury (38) underscores the similarity between maleate-induced cytotoxicity and hypoxic renal tubular cell death.

To gain further support for the concept that maleate recapitulates critical pathogenic components of the ATP depletion/injury cascade, additional commonalities were sought. Under each situation tested, identical findings were obtained. First, maleate induced preferential injury to the S3 segment of the PT, exactly as does the RAO model of ischemic ARF (35). Second, acidosis completely blocked maleate-induced tubular cell death, just as it does against hypoxia (37, 49). Furthermore, that acidosis exacerbated Fe-mediated oxidant stress (Fig. 9) indicates that this blockade of maleate- and hypoxia-induced LDH release does not simply reflect a nonspecific cytoprotective effect. Third, unsaturated fatty acids, when added to isolated tubules, mitigate hypoxic tubule injury (1, 47, 50), whereas saturated fatty acids have no protective effect. That this same profile was observed with maleate toxicity further underscores the similarity of these two forms of renal damage. Fourth, cytosolic Ca2+ overload, evoked by a failure of mitochondrial sequestration, is a known determinant of hypoxic/ATP depletion-mediated tubular cell death (39, 40). That an
intracellular (BAPTA), but not extracellular (EGTA), Ca\textsuperscript{2+} chelator mitigated maleate toxicity again implies commonality between maleate and hypoxic injury pathways. And fifth, in the present experiments, we sought to further define the nature of the plasma membrane injury that develops during hypoxic and maleate-induced cell death. Specifically, we tested whether plasma membrane cholesterol, a previously defined membrane cytoprotectant (42–44), is shed from the membrane, and if so, might glycine potentially protect by inhibiting this pathway? As shown in Fig. 14, both situations were observed: hypoxia and maleate each caused PT cholesterol release, and in both instances, glycine completely prevented this event. Thus these findings not only underscore the commonality of maleate and hypoxic injury pathways, but they provide a potential new insight into mechanisms by which glycine confers its cytoprotective/plasma membrane “stabilizing” effects. The exact mechanism(s) by which glycine prevents lethal membrane damage still remains unknown.

Finally, this laboratory previously observed that proximal tubular cholesterol accumulation, mediated in part via increased cholesterol synthesis, is a critical adaptive response to hypoxic/ischemic forms of attack (42–44). For maleate to recapitulate ATP depletion-induced membrane injury, it would need to increase plasma membrane cholesterol content. However, maleate-induced CoA depletion could theoretically suppress cholesterol synthesis by decreasing CoA availability for acetyl CoA and acetoacetyl CoA formation, the condensation of which represents the first step in cholesterol production. To address this possibility, cholesterol/cholesterol ester levels were measured at different time points following maleate injection, and the following conclusions can be drawn: 1) maleate does indeed induce renal cholesterol loading, 2) the degree of cholesterol accumulation correlates well with the severity of renal damage (BUN vs. cholesterol levels, \( r = 0.85 \)), 3) increased cholesterol synthesis is operative (given that atorvastatin blocked maleate-induced cholesterol increases), and 4) increased gene expression is clearly involved (based on the findings of increased levels of HMGR mRNA). That these mRNA increases likely reflected, at least in part, increased transcription, and not just potential message stabilization, can be inferred by the finding of increased levels of Pol II along the HMGR gene, as assessed by ChIP assay. To our knowledge, this is the first documentation of increased DNA–Pol II recruitment at any specific gene locus in studies of in vivo tubular injury/ARF. Thus these findings not only support the concept of increased cholesterol synthesis in ARF, but more importantly, they underscore the potential utility of ChIP assay in future studies of in vivo renal damage.

In conclusion, the present studies indicate that maleate-induced PT injury recapitulates critical components of ischemic/hypoxic renal cascade: 1) it is glycine suppressible, and as with hypoxic injury this protection is independent of any reductions in NEFAs or preservation of ATP. 2) Like hypoxia, maleate toxicity is mitigated by unsaturated, but not saturated, fatty acids. 3) Hypoxia + maleate do not induce additive tubular injury (LDH release, NEFA accumulation), implying shared mechanistic pathways. 4) Acidosis completely blocks lethal maleate-induced injury, just as it does against hypoxic tubular cell death. Furthermore, as with hypoxia, acidosis-mediated protection is independent of changes in ATP. 5) As with hypoxia, intracellular Ca\textsuperscript{2+} overload helps mediate maleate cytoxicity. 6) Both hypoxia and maleate cause plasma membrane cholesterol shedding, and in both instances, this is glycine suppressible. 7) As with other forms of acute renal tubular injury, maleate causes renal cortical cholesterol loading, at least in part, by increased HMGR gene transcription/ increased cholesterol synthesis. Given these similarities between ischemic and maleate-induced tubular injury, we posit that the maleate model may be a useful complementary approach for studying specific components of the “ischemic” PT injury cascade. Finally, the current Pol II findings indicate that the ChIP assay represents a powerful new tool for studying in vivo renal injury and ARF.

ACKNOWLEDGMENTS

The authors thank N. Kim and S. Lund for expert technical assistance with this project.

GRANTS

This work was supported by National Institutes of Health Research Grants R37-DK-38432, DK-68520, and DK-68520 (to R. A. Zager), and DK-45978 and GM-45134 (to K. Bomsztyk).

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