Effect of quercetin on hypoxic injury in freshly isolated rat proximal tubules

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The bioflavonoid quercetin, which has antioxidant properties, protects renal tubular epithelial cells from oxidant-induced injury by inhibiting lipid peroxidation. We examined the effect of quercetin on hypoxia-induced injury in freshly isolated rat renal proximal tubules. Hypoxia induced rapid loss of cellular ATP, followed by functional and structural alterations measured as a decrease in tubular potassium content and sequentially by an increase in lactate dehydrogenase release. Furthermore, hypoxia increased lipid peroxidation, measured as thiobarbituric acid–reactive substances. Quercetin significantly inhibited hypoxia-induced functional and structural tubular injury in addition to lipid peroxidation but did not alter hypoxia-induced ATP depletion. These results demonstrate the potency of the bioflavonoid quercetin in protecting proximal tubules from hypoxic injury, which is independent of tubular energy metabolism and may be related to the inhibition of lipid peroxidation. (J Lab Clin Med 2003;142:106-12)

Abbreviations: ARF = acute renal failure; BSA = bovine serum albumin; DMSO = dimethylsulfoxide; EDTA = ethylenediaminetetraacetate; HEPES = *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; LDH = lactate dehydrogenase; MDA = malondialdehyde; NBT = nitroblue tetrazolium; O_2^- = superoxide anion radical; ROS = reactive oxygen species; SDS = sodium dodecyl sulfate; SEM = standard error of the mean; TBARS = thiobarbituric acid–reactive substances

Acute renal failure affects almost 5% of all hospitalized patients and is associated with a mortality rate greater than 50%.[1–4] Impairment of proximal tubules by ischemia is the most frequent cause of ARF.[5] Because of their highly aerobic metabolism, the proximal tubules are most susceptible to ischemic injury.[1–3] They play a pivotal physiological role in the excretion and reabsorption of organic solutes, xenobiotics, and electrolytes. Tubular dysfunction is reflected by the clinical features of ARF, including uremic toxin and drug accumulation, fluid overload, and electrolyte and acid-base imbalances.[1–3]

An established model used to study ARF is the clamping of the renal artery after contralateral nephrectomy. In this in vivo setting of renal ischemia-reperfusion injury, as well as in vitro studies mimicking this condition, generation of ROS has been suggested to contribute to renal injury.[6–8] ROS have numerous deleterious effects on cells, including lipid peroxidation.[9] Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes and, consecutively, to cell death. Inhibition of lipid peroxidation is accompanied by prevention of cell death.[10]

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Bioflavonoids are a group of polyphenolic compounds of plant origin that are viewed as biological radical scavengers.[11] Structure-activity studies have identified quercetin as one of the bioflavonoids with the highest antioxidative capacity.[12,13] This antioxidative function may be a result of quercetin's ability to inhibit lipid peroxidation.[14] In addition, quercetin possesses antiproliferative and antiinflammatory properties, making it an interesting substance for a variety of clinical applications, including anticancer activity, immunosuppression, and cardiovascular protection. In the kidney, quercetin ameliorates ischemia-reperfusion injury after renal-artery clamping.[15] In cultured rat proximal tubular cells, quercetin reduces nuclear factor-kappaB (NFκB)-induced cytokine transcription,[16] protects against cisplatin toxicity,[13] and inhibits apoptosis in H_2O_2-induced oxidant injury.[17,18]

Because we have observed that quercetin also inhibits oxidant-induced lipid peroxidation in cultured renal tubular (LLC-PK1) cells,[13] it is tempting to speculate that the ultimate cause of all these effects of quercetin is its antioxidant function, especially the inhibition of lipid peroxidation. Proximal tubules and LLC-PK1 cells differ with regard to their energy metabolism. Proximal tubules are highly oxygen-dependent because they are not able to perform anaerobic glycolysis. We therefore investigated in freshly isolated rat renal proximal tubules whether quercetin has a protective effect on hypoxic injury, whether hypoxia induces oxidative damage measured as lipid peroxidation, and whether quercetin inhibits this lipid peroxidation.

METHODS

Preparation of freshly isolated proximal tubules. Rat proximal tubules were freshly isolated as recently described, with slight modification.[19,20] Male Sprague-Dawley rats (220-300 g) were anesthetized with intraperitoneal ketamine hydrochloride (100 mg/kg body wt i.p.) and xylazine hydrochloride (5 mg/kg body wt), after which the kidneys were flushed with 40 mL of an ice-cold (4°C) oxygenated and heparinized (1% of a 5000 IE/mL heparin solution) buffer (solution A) containing (in millimoles per liter): 112 NaCl, 20 $NaHCO_3$, 5 KCl, 1.6 $CaCl_2$, 2 NaH_2PO_4, 1.2 $MgSO_4$, 5 glucose, 10 HEPES, 10 mannitol, 1 glutamine, 1 sodium butyrate, and 1 sodium lactate, pH 7.3, at 37°C. Perfusion was continued with 30 mL of ice-cold solution A containing 5 mg of collagenase and 12.5 mg of hyaluronidase (both from Boehringer Mannheim Biochemical, Mannheim, Germany). After perfusion, the kidneys were removed and transferred into ice-cold solution A. Renal cortexes were dissected and minced on an ice-cold Petri dish. Tissue fragments were washed twice with 50 mL of oxygenated solution A (164*g* at 4°C for 1 minute) followed by incubation for 30 minutes with 30 mL of oxygenated solution A containing 10 mg of collagenase and 7.5 mg of hyaluronidase in a shaking water bath at 37°C. Separation was stopped by means of incubation for 20 minutes in 15 mL of ice-cold solution A containing 0.5 g of fatty acid–free BSA (Serva, Heidelberg, Germany). After the tissue was filtered through a tea strainer and washed to remove the albumin, proximal tubules were separated by means of Percoll-gradient centrifugation (10 minutes at 11,000 *g*) with 45% Percoll (Pharmacia).[20] Proximal tubules were recovered from the lowest band, which was mainly composed of proximal tubules (>95%) without containing any glomeruli. After 3 washes in solution A, tubules were suspended in oxygenated solution B, containing (in millimoles per liter): 106 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 2 NaH_2PO_4, 1 $MgSO_4$, 5 glucose, 10 HEPES, 2 glutamine, 10 sodium butyrate and 4 sodium lactate, adjusted to pH 7.05 at 4°C in a siliconized Erlenmeyer flask (200 mL). The tubule suspension, containing 0.5 to 1.0 mg/mL of protein, as determined with the Lowry method,[21] was gassed on ice for 5 minutes with 95% O_2/5% CO_2. After equilibration to room temperature with the Erlenmeyer flask capped, tubules were placed in a shaking water bath at 37°C for 10 minutes, whereafter pH had increased to 7.35. Aliquots (usually 4–6 mL) were transferred to siliconized Erlenmeyer flasks (25 mL) in a shaking water bath at 37°C, after which 0.5-mL samples for the assessment of baseline LDH release (see below) were removed.

Hypoxia studies. We achieved hypoxia by gassing the tubule suspension for 5 minutes with 95% N_2/5% CO_2 and gassing the control normoxic tubules for 5 minutes with 95% O_2/5% CO_2[20] both at a rate of 1 L/min. After gassing, flasks were kept in a shaking water bath at 37°C for an additional 5 minutes, after which samples were removed (t = 10 minutes). This procedure was repeated at 20 and 30 minutes. Induction of hypoxia resulted in a decrease of PO_2 in the tubule suspension to 10 to 15 mm Hg after 10 minutes and to 0 to 5 mm Hg after 30 minutes. Experiments were performed with no additions or in the presence of 100 μmol/L quercetin dissolved in 0.1% DMSO.

Cellular ATP content. To measure cellular ATP content, we added 0.5 mL of tubule suspension to a microcentrifuge tube filled with 50 μL of 10 mol/L ice-cold perchloric acid, shock-frozen in liquid nitrogen and stored at −70°C until measurements could be taken. After careful thawing in an ice bath, tubes were centrifuged for 10 minutes at maximum speed. Thereafter, the amount of ATP was measured with a luciferase assay (ATP-Bioluminescence Assay Kit; Boehringer Mannheim) with the method suggested by the manufacturer. Samples were diluted 1:100 in buffer containing 100 mmol/L Tris and 4 mmol/L EDTA, pH 7.75, and mixed immediately with equal amounts of luciferase reagent. Light emitted by the luciferase was measured in a luminometer (Berthold Detection Systems, Pforzheim, Germany). The pellet was resuspended with 1.2% SDS/0.4% NaOH, and samples were taken for protein measurement.[21] Cellular ATP content is expressed as nanomoles of ATP per milligram of protein.

Cellular potassium content. To measure cellular potassium content, we rapidly separated tubules from the medium by layering 0.5 mL of tubule suspension in a microcentrifuge

tube containing 0.75 mL of bromododecane (Sigma-Aldrich Chemicals, Steinheim, Germany) on top of 0.2 mL of 275 mmol/L sucrose and 4% Ficoll (Amersham Biosciences, Uppsala, Sweden). The tube was centrifuged in an Eppendorf microcentrifuge at maximal speed at room temperature for 1 minute to pellet the tubules into the bottom sucrose layer. The top layer was removed, replaced with deionized water, and centrifuged again in an Eppendorf microcentrifuge at maximal speed for 1 minute to remove as much of the remaining potassium in the medium as possible. Subsequently, the water and oil layers were removed. The remaining sucrose/Ficoll layer was diluted with 0.5 mL of deionized water. After vortexing, two 25-μL samples were taken for the measurement of protein.[21] Potassium in this bottom layer was measured by means of atomic-absorption spectroscopy and expressed as nanomoles per milligram of protein. We corrected the results for the contribution of medium potassium to the pellet using carbon 14–labeled inulin as described.[20,22]

LDH release. We assessed structural cellular damage by measuring the release of LDH from the tubules into the medium. Aliquots (0.5 mL) from the tubule suspension were centrifuged at 3000*g* for 1 minute, after which LDH was assayed spectrophotometrically both in the supernatant and in the pellet, which was subjected to lysis with 1% Triton X-100 (Sigma).[20,22] We expressed hypoxia-induced LDH release as a percentage of total content by dividing supernatant LDH by total LDH (supernatant LDH plus pellet LDH).

Lipid peroxidation. To assess lipid peroxidation, we measured the formation of MDA, an end product of nonenzymatic lipid peroxidation, as TBARS, using the thiobarbituric acid reaction. We added 200 μL of supernatant to 2 μL of butylated hydroxytoluene (200 mmol/L) and stored it at −20°C. TBARS formation was assessed in accordance with the method of Aust[23] as described recently[13] and expressed as nanomoles per milligram of protein. Protein content was determined with the use of Bradford's method.[24]

O_2^- formation as assessed with the NBT-reduction assay. The NBT-reduction assay was used to determine the amount of formazan formation as a measure of tubular O_2^- generation under hypoxic conditions.[25] NBT was added to the tubule suspension in solution B in a final concentration of 0.1 mg/mL. Tubules were incubated under normoxic (95% O_2/5% CO_2) and hypoxic (95% N_2/5% CO_2) conditions. After 30 minutes of incubation, 1 mL aliquots were taken and centrifuged at 3000*g* for 1 minute, after which the pellet was lysed with 5% SDS in phosphate buffer (100 mmol/L, pH 7.8) containing 0.45% gelatin. Samples were centrifuged for 5 minutes at 10,000*g*. Absorbance at 540 nm (formazan) and 450 nm was determined against a lysis-buffer blank. Formazan concentration was calculated from E_{540} corrected for unspecific adsorbance/turbidity ($E_{450} = 0.51 \times E_{540}$ + unspecific absorbance, as determined spectrophotometrically with an NBT solution treated with solid potassium superoxide and lysing the precipitated formazan in the lysis buffer; $E_{540corr} = (E_{540} - E_{450})/0.49$) using (540 = 7.2 $cm^2/\mu mol$).[25] Data are expressed as nanomoles of formazan per milligram of protein.

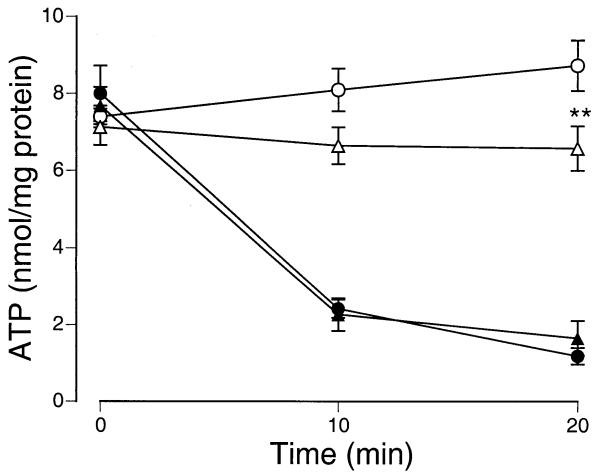

Fig 1. Cellular ATP content in rat renal proximal tubules under normoxic (*open circles* and *triangles*) and hypoxic (*solid circles* and *triangles*) conditions. Cellular ATP content was measured in the absence (*circles*) or presence (*triangles*) of 100 μmol/L quercetin in the medium. Data expressed as mean ± SEM; $n = 6$. *$P < .05$ vs normoxia alone.

Statistical analysis. Data are expressed as mean ± SEM from a minimum of 6 separate experiments performed at least in duplicate. We used analysis of variance, with the Bonferroni-Dunn post hoc test for multiple comparisons. Differences were regarded as significant when P was less than .05. Calculations were performed with the computer program Stat View (version 5.0 for Macintosh; Abacus Concepts, Inc, Berkeley, Calif).

RESULTS

Effect of quercetin on tubular ATP content. Under normoxic conditions, ATP content in freshly isolated proximal tubules showed a small increase over time, with slightly lower values in the presence of 100 μmol/L quercetin (Fig 1). Within 10 minutes of the induction of hypoxia, ATP content markedly decreased, from 8.0 ± 0.7 to 2.4 ± 0.2 nmol/mg protein ($P < .05$, $n = 6$; Fig 1). Quercetin did not alter this effect (Fig 1).

Effect of quercetin on tubular potassium content. Under normoxic conditions, cellular potassium content remained stable throughout the experiments (Fig 2). In the presence of quercetin, a transient increase in cellular potassium was observed at 10 minutes of normoxia; it was no longer detectable at 20 minutes. Hypoxia led to substantial time-dependent reduction in cellular potassium content, which was already significant at 10 minutes (269 ± 16 vs 379 ± 16 nmol/mg protein in normoxic tubules; $P < .001$, $n = 16$; Fig 2). This reflects functional tubular alteration. In the presence of quercetin, tubular potassium depletion was considerably delayed. At 10 minutes, the potassium content of quercetin-treated hypoxic tubules was similar to that of

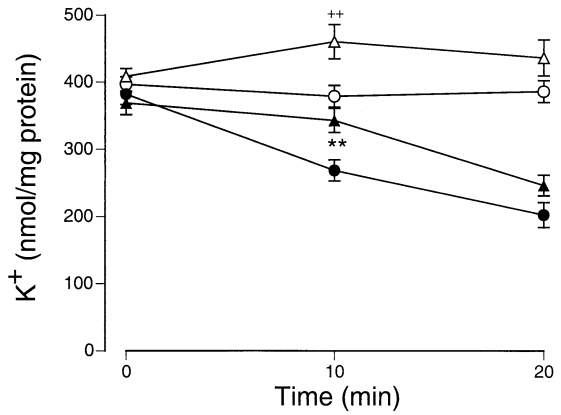

Fig 2. Cellular K^+ content in rat renal proximal tubules under normoxic (*open circles* and *triangles*) and hypoxic (*solid circles* and *triangles*) conditions. Cellular K^+ content was measured in the absence (*circles*) or presence (*triangles*) of 100 μmol/L quercetin in the medium. Data expressed as mean ± SEM; $n = 8–16$. $++P < .01$ vs normoxia alone, $**P < .01$ vs hypoxia alone.

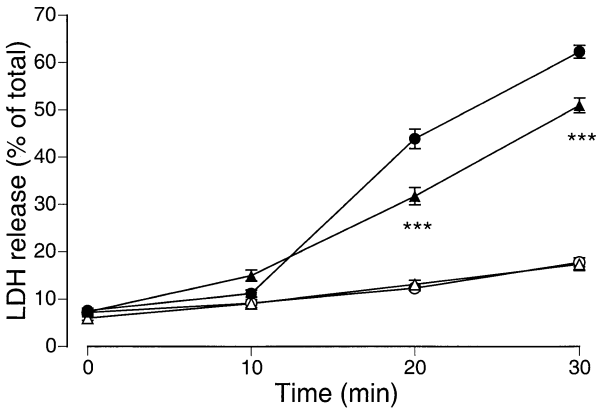

Fig 3. Structural damage in freshly isolated rat renal proximal tubules during 30 minutes of hypoxia. LDH release was determined under normoxic (*open circles* and *triangles*) and hypoxic (*solid circles* and *triangles*) conditions, without (*circles*) or with (*triangles*) 100 μmol/L quercetin added to the medium. Data expressed as mean ± SEM; $n = 34$. $***P < .001$ vs hypoxia.

quercetin-free normoxic control tubules (343 ± 18 vs 379 ± 16 nmol/mg protein; P = NS, $n = 15$; Fig 2).

Effect of quercetin on LDH release. Under normoxic conditions, basal LDH release by oxygenated control tubules slightly increased (Fig 3). The presence of quercetin in the medium had no effect on LDH release in oxygenated control tubules (Fig 3). Hypoxia resulted in considerable structural tubular injury, as reflected by significant increases in LDH release at 20 and 30 minutes (Fig 3). Quercetin significantly inhibited hypoxia-induced LDH release, both at 20 (31.7% ± 1.8% vs 43.9% ± 2.1%; $P < .001$, $n = 34$; Fig 3) and 30 minutes (51.0% ± 1.6% vs 62.2% ± 1.4%; $P < .001$, $n = 34$; Fig 3), resembling a relative reduction of cell damage by 38.6% ± 5.2% at 20 minutes of hypoxia.

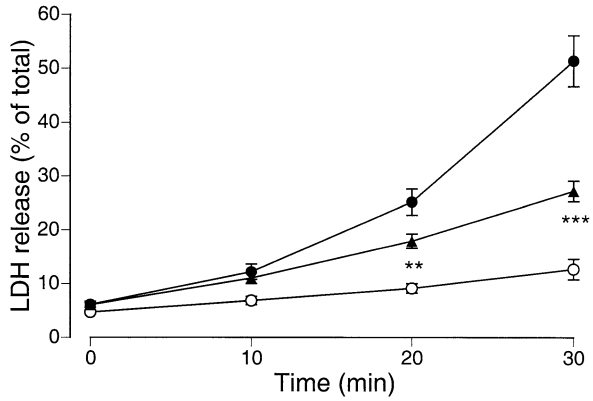

Fig 4. Structural damage in normoxic rat renal proximal tubules during H_2O_2-induced injury. LDH release was determined under normoxic control conditions (*open circles*), in the presence of 10 mmol/L H_2O_2 (*solid circles*), and in the presence of H_2O_2 and 100 μmol/L quercetin (*solid triangles*) in the medium. Data expressed as mean ± SEM; $n = 5–7$. $**P < .01$. $***P < .001$ vs tubules subjected to H_2O_2 alone.

Addition of 10 mmol/L H_2O_2 to continuously oxygenated tubules resulted in significant structural tubular injury, reflected by an increase in LDH release (Fig 4). Quercetin significantly reduced this H_2O_2-induced structural tubular damage (Fig 5). H_2O_2-induced LDH release averaged 17.9% ± 1.3% in the presence of quercetin, compared with 25.1% ± 2.4% without it ($P < .01$, $n = 7$) at 20 minutes and 27.2% ± 1.9% vs 51.4% ± 4.7% ($P < .001$, $n = 7$) at 30 minutes (Fig 6).

Effect of quercetin on lipid peroxidation. Under normoxic control conditions, we noted some baseline lipid peroxidation, as assessed on the basis of TBARS formation ($n = 22$; Fig 5), that was not significantly affected by quercetin (P = NS, $n = 5$). Hypoxia significantly stimulated lipid peroxidation, from 0.25 ± 0.03 to 0.41 ± 0.04 nmol/mg protein at 20 minutes and from 0.30 ± 0.03 to 0.55 ± 0.04 nmol/mg protein at 30 minutes ($P < .001$ for each comparison, $n = 22$). In the presence of quercetin, hypoxia-induced lipid peroxidation was completely abolished (Fig 6).

In normoxic tubules exposed to 10 mmol/L H_2O_2, considerable TBARS formation was observed (0.21 ± 0.04 nmol/mg protein at baseline vs 2.25 ± 0.26 nmol/mg protein at 30 minutes; $P < .001$, $n = 6$; Fig 5). H_2O_2-induced lipid peroxidation was completely inhibited in the presence of quercetin (0.43 ± 0.06 nmol/mg protein in normoxic controls vs 0.57 ± 0.7 nmol/mg protein at 30 minutes; P = NS, $n = 6$; Fig 5).

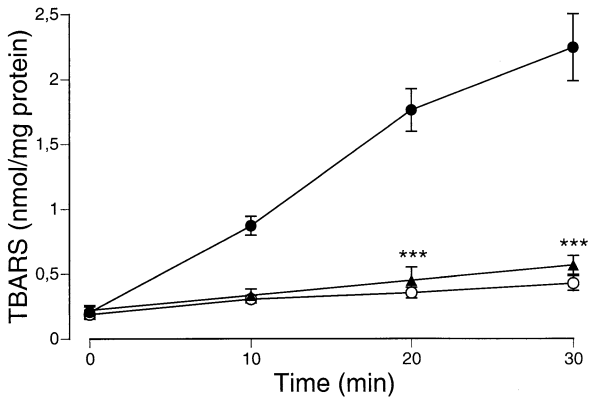

Fig 5. H_2O_2-induced lipid peroxidation in rat renal proximal tubules. TBARS formation was measured under normoxic control conditions (*open circles*), in the presence of 10 mmol/L H_2O_2 (*solid circles*), and in the presence of 10 mmol/L H_2O_2 and 100 μmol/L quercetin (*solid triangles*) in the medium. Data expressed as mean ± SEM; $n = 6$. ***$P < .001$ vs H_2O_2 alone.

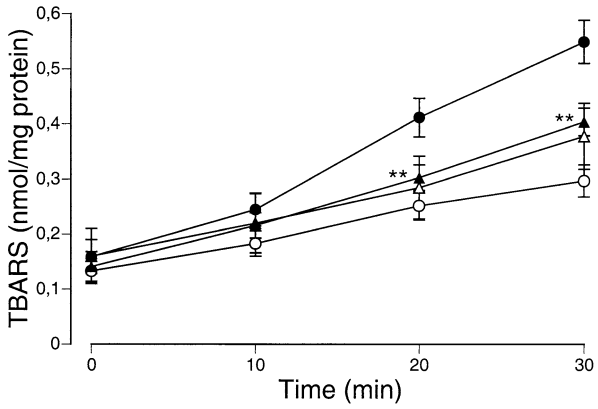

Fig. 6. Hypoxia-induced lipid peroxidation in rat renal proximal tubules. TBARS formation was measured under normoxic (*open circles* and *triangles*) and hypoxic (*solid circles* and *triangles*) conditions, without (*circles*) or with (*triangles*) 100 μmol/L quercetin in the medium. Data expressed as mean ± SEM; $n = 22$. **$P < .01$ vs hypoxia.

O_2^- formation as assessed with the NBT-reduction assay. We observed threefold greater formazan formation of the proximal tubules under hypoxic conditions compared with that under normoxic conditions (118 ± 18 vs 42 ± 5.5 nmol/mg protein; $P < .01$, $n = 6$). These results indicate that under hypoxia with oxygen tensions decreasing below 5 mm Hg, significantly greater tubular generation of O_2^- occurred compared with that under normoxic conditions.

DISCUSSION

In this study, we investigated the effect of the bioflavonoid quercetin on hypoxic tubular injury. Adopting the model of freshly isolated renal proximal tubules allowed us to examine the effects of quercetin on a well-defined intact renal structure subjected to oxygen deprivation. Quercetin significantly inhibited hypoxia-induced functional and structural cell injury in freshly isolated rat proximal tubules. However, quercetin had no effect on the hypoxia-induced alteration in tubular energy metabolism with a drastic loss of cellular ATP. Therefore the protective effect of quercetin on functional and structural cell injury cannot be ascribed to conserved tubular energy metabolism.

Oxygen deprivation increases the permeability of the plasma membrane in a time- and size-dependent manner from smaller to larger molecules.[26] In this process of progressive tubular injury, the cytoplasmic protein LDH, with a molecular size of 140 kD, is released, reflecting structural cell damage with the loss of membrane integrity.[26] In a previous study, LDH release was not observed before 15 minutes of hypoxia had elapsed.[22] This finding is in accordance with the observations of our investigation, in which an increase in LDH was first observed after 20 minutes of hypoxia. This structural cell injury was inhibited by quercetin.

To investigate early alterations of membrane dysfunction, which may reflect changes in cellular function rather than structural injury, we measured the loss of cellular potassium.[20,22] Hypoxia induced a decrease in tubular potassium content within 10 minutes. This may have been a result of direct interactions of ROS with ion-transport mechanisms[27] or may reflect redistribution of Na^+/K^+-ATPase resulting from cytoskeletal changes under ATP depletion[28]; inhibition of the Na^+/K^+-ATPase by exposure to free radicals, including H_2O_2 with a concomitant increase in lipid peroxidation has been observed.[29] Quercetin significantly inhibited potassium depletion before the occurrence of structural cell injury. Therefore quercetin may have preserved cellular potassium content by reducing the oxidant-induced inhibition of Na^+/K^+-ATPase activity. Under normoxia, inhibition rather than stimulation of Na^+/K^+-ATPase by flavonoids, including quercetin, has been reported.[30,31] However, these investigators did not investigate the effect of quercetin or other polyphenols in the context of hypoxic conditions or exposure to ROS.

The induction of ischemia-reperfusion injury to the kidney leads to substantial generation of ROS, with the consequence of oxidative damage to the kidney. Production of the 3 major ROS—superoxide anion radical, hydrogen peroxide, and hydroxyl radical—has been demonstrated in hypoxic primary cultures of rat renal epithelial cells.[32] Direct measurements of rates of ROS production by mitochondria harvested from the kidney subjected to ischemia-reperfusion injury demonstrate

that such a production rate is increased 1.5-fold during the ischemic and fourfold during the postischemic phase.[33] Because in our experiments oxygen tension was 10 to 15 mm Hg after 10 minutes of hypoxia, it can be assumed that under these conditions, important mechanisms of ROS formation, such as conversion of xanthine dehydrogenase to xanthine oxidase, are still active.[34,35] That ROS formation indeed took place under these conditions could be demonstrated by the increased formation of O_2^- we observed. Protective effects of flavonoids on oxidant-induced injury in cultured renal tubular cells (LLC-PK1) have recently been demonstrated.[13] Among a variety of structurally related compounds, quercetin was identified as the one flavonoid most effectively preventing oxidative injury. Structure-activity experiments indicated that the cytoprotective effects of quercetin were related to membrane affinity and the presence of lipid peroxidation.[13] Lipid peroxidation has also been detected in situ in the rat kidney during renal ischemia, localized to the proximal tubules.[36] In parallel, we have observed a significant increase in lipid peroxidation under hypoxia in freshly isolated rat proximal tubules. Induction of oxidant-induced injury by means of exposure to H_2O_2 also leads to structural tubular damage and to a significant increase in lipid peroxidation. Under both conditions, LDH release, as well as lipid peroxidation, was significantly decreased by quercetin. The pronounced effect of quercetin on lipid peroxidation supports previous findings suggesting that quercetin directly interferes with nonenzymatic lipid peroxidation.[13,37] On the basis of these data, we hypothesize that the protective effect of quercetin in renal proximal tubules during hypoxia is related to inhibition of lipid peroxidation. The incomplete effect on structural tubular injury indicates that in addition to ROS formation, other factors that are not affected by quercetin contribute to tubular injury during hypoxia.

The congruous results of a protective effect of the bioflavonoid quercetin in a "vertical" series of experiments (eg, a cell-culture model with LLC-PK1 cells,[13] freshly isolated rat proximal tubules (this study), and ischemia-reperfusion injury in rat kidney[15]) indicate that quercetin may be clinically relevant in the treatment of ARF.[38]

Taken together, the results of this study demonstrate protective effects of quercetin in a model of hypoxia-induced tubular injury. In addition to functional and structural damage, lipid peroxidation, which was completely inhibited by quercetin, was observed under hypoxia. Therefore the protective effects of quercetin on functional injury and structural damage may be related to inhibition of oxidant-induced nonenzymatic lipid peroxidation. The given probability of having a clinical relevance would open the opportunity for therapeutic advances in the setting of human ARF.

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