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MODULATION OF APOPTOTIC AND INFLAMMATORY GENES BY BIOFLAVONOIDS AND ANGIOTENSIN II INHIBITION IN URETERAL OBSTRUCTION

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ABSTRACT

Objectives. Ureteral obstruction results in an injury response that can progress to irreversible renal fibrosis and tubular atrophy by apoptosis. The molecular events leading to apoptosis from obstruction are not well understood. We investigated the effect of bioflavonoids and angiotensin II inhibition on apoptotic and inflammatory gene expression in a model of unilateral ureteral obstruction (UUO).

Methods. Complete UUO was produced in rats by ureteral ligation. The rats were treated with dimethyl sulfoxide (control), enalapril, losartan, curcumin, or quercetin. The animals were killed on day 7 and both obstructed and contralateral unobstructed kidneys were harvested. Expression of the inflammatory chemokine monocyte chemotactic protein-1, apoptosis effector genes Fas and Fas ligand, and oxidative stress gene HO-1 was evaluated by reverse transcriptase-polymerase chain reaction.

Results. Ureteral obstruction was associated with a 6.3-fold increase in monocyte chemotactic protein-1 expression compared with sham-operated rats ($P = 0.01$). Monocyte chemotactic protein-1 expression was severely attenuated in all other treatment groups ($P < 0.05$). Similarly, Fas and Fas ligand expression were increased in control UUO kidneys compared with sham-operated ones ($P < 0.05$). Fas gene expression was significantly inhibited by quercetin but not enalapril, losartan, or curcumin compared with the control. The induction of Fas ligand was attenuated in all treatment groups ($P < 0.05$). HO-1 was expressed at low levels in both unobstructed and obstructed kidneys. Treatment with curcumin increased HO-1 expression fourfold ($P < 0.05$).

Conclusions. The expression of apoptotic and chemokine genes is significantly upregulated in UUO. Bioflavonoids and angiotensin inhibitors are able to attenuate the expression of these genes and thus may be beneficial in renal protection. UROLOGY **56:** 346–351, 2000. © 2000, Elsevier Science Inc.

Obstructive uropathy remains an important cause of renal injury in both adults and children and may progress to end-stage renal disease.[1] Despite timely surgical management of urinary tract obstruction, irreversible damage to the affected renal unit may occur. Currently, the molecular events leading to the progression of renal injury are poorly understood. To delineate the mechanism of renal injury and identify the agents that ameliorate the functional consequences of obstruction could therefore have significant clinical and financial impact.

Histologically, ureteral obstruction initiates an injury response characterized by leukocyte infiltration, progressive interstitial fibrosis, and tubular atrophy. This process is accompanied by a cascade of pro-inflammatory events that likely lead to intrarenal oxidative stress and, ultimately, tubular cell death through the energy-dependent process of apoptosis. Early activation of the renin-angiotensin system participates in the intrarenal hemodynamic changes during ureteral obstruction and has been shown to potentiate the expression of chemotactic and fibrogenic cytokines.[2] Furthermore, inhibition of the effects of angiotensin II by either angiotensin-converting enzyme (ACE) inhibitors or by angiotensin II receptor antagonists significantly ameliorates the histologic changes as-

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sociated with ureteral obstruction.[3] The effect of these agents on apoptosis is not known.

Bioflavonoids such as curcumin and quercetin with known antioxidant properties might ameliorate the renal injury in ureteral obstruction. Both curcumin and quercetin were shown to be effective in preventing histologic and functional deterioration and attenuating the expression of inflammatory chemokines in rat kidneys subjected to ischemia-reperfusion.[4] The goals of the present study were to investigate the effect of ACE inhibitors and bioflavonoid treatment on the induction of genes regulating inflammation and apoptosis in a rat model of ureteral obstruction.

MATERIAL AND METHODS

Experimental protocol: male Sprague-Dawley rats (weight 180 to 220 g) were used for all experiments. The animals were given free access to water and fed a standard rat chow diet. Under anesthesia with ketamine and xylazine, six groups of 5 rats each underwent midline laparotomy and either sham operation (group 1) or left proximal ureteral obstruction using 5-0 silk suture (groups 2 to 6). The midline incision was closed, and the animals were allowed to recover from the anesthesia. The animal care committee of our institutional review board approved this study.

Animals underwent the following procedures. Group 1 (sham) rats underwent laparotomy alone and received no treatment. Group 2 (control) rats were injected subcutaneously with 0.5 mL of dimethyl sulfoxide (DMSO, bioflavonoid vehicle) 1 day before surgery and 3 days postoperatively. Group 3 received the ACE inhibitor enalapril (200 mg/L) continuously in their drinking water beginning 24 hours before surgery. Group 4 received the angiotensin II type 1 receptor antagonist losartan (400 mg/L) continuously in their drinking water 24 hours before surgery. Groups 5 and 6 were injected with 0.5 mL of quercetin (in 30 mg/mL DMSO) or curcumin (in 30 mg/mL DMSO), respectively, 1 day before surgery and 3 days postoperatively.

All rats were killed on day 7 after surgery by craniocervical dislocation. Kidneys were perfused in situ by occluding the suprarenal aorta and perfusing normal saline through a cannula in the infrarenal aorta until the effluent was clear. Both the left and right kidneys were removed, dissected free of the surrounding fat and connective tissue, snap-frozen in liquid nitrogen, and stored at −70°C until used for the molecular studies.

RNA Isolation

Total RNA was extracted from renal tissue using the one-step guanidinium isothiocyanate method (TRIzol reagent, Life Technologies, Grand Island, NY) per the manufacturer's protocol.[5] The RNA pellet was reconstituted in RNase free water and quantified. RNA was separated into aliquots and stored at −70°C. All RNA preparations used in gene expression studies had a 260/280 ratio greater than 1.7.

Reverse Transcription

First strand complementary DNA (cDNA) synthesis was performed in a total volume of 25 μL. The reaction mixture was composed of 50 mM Tris-HCL, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.25 μg oligo(dt)-15 primer, 0.8 U/μL ribonuclease inhibitor, 1.2 mM dNTP, and 8 U/μL of Moloney murine leukemia virus (MMLV)-reverse transcriptase and 1 μg of total RNA. The mixture was incubated for 60 minutes at 42°C, and then the reaction was terminated by heating at 94°C for 5 minutes. All cDNA samples were stored at −20°C until use.

Polymerase Chain Reaction

The reactions were performed in a total volume of 50 μL containing polymerase chain reaction (PCR) buffer (50 mM Tris-HCL, 16 mM ammonium sulfate, 3.5 mM $MgCl_2$, and 150 μg/mL bovine serum albumin), 500 mM of each dNTP, 2 μL of cDNA, and 15 pmol oligonucleotide primers. All primers were obtained from Genosys. The primer sequences were as follows: Fas: 5′-GCA ATG CTT CTC TCT GTG ACC ACT G (sense) and 5′-GCT GTT GTG CTC GAT CTC ATC (antisense), Fas ligand (Fas-L): 5′-ATA GAG CTG TGG CTA CCG GTG (sense) and 5′-CTC CAG AGA TCA AAG CAG TTC C (antisense), glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-ATG TCA GAT CCA CAA CGG ATA CAT (sense) and ACT CCC TCA AGA TTG TCA GCA AT (antisense). Primer pairs for Fas, Fas-L, and GAPDH were designed using commercially available software (Amplify 1.2, Bill Engels, 1992). The accuracy of the primer pairs was verified by the size of the PCR products. The primer pairs for monocyte chemotactic protein-1 (MCP-1) and hemoxygenase-1 (HO-1) were adapted from published sequences.[6,7]

The optimized PCR conditions were as follows: 94°C (1 minute), 63°C (1 minute), and 72°C (45 seconds). PCR was performed for 22, 30, 32, 32, and 32 cycles for GAPDH, MCP-1, HO-1, Fas, and Fas-L, respectively. PCR products were visualized on 2% agarose gel with ethidium bromide staining. The molecular weights of the expected PCR products were estimated by the base pair size marker (run on the same gel). The band density of the target genes was determined by densitometry (Scan Analysis, Ferguson, Mo) and normalized by the band density of GAPDH. The results are presented as the mean ± SEM (n = 5/group) of the target gene/GAPDH ratio.

Statistical Analysis

The target gene/GAPDH ratio was compared between the control and treatment groups. Tests of significance were performed separately on the left (obstructed) kidneys and right (unobstructed) kidneys of each group. Each group contained the data from 5 treatment animals. The Mann-Whitney nonparametric test was used for comparison between the groups and the Kruskal-Wallis test for the analysis of variance. Significance was reported at $P < 0.05$.

RESULTS

At the time of death, all kidneys that underwent ureteral obstruction exhibited gross evidence of hydronephrosis. The unobstructed kidneys of all animals appeared grossly normal.

MCP-1 mRNA Expression

To monitor whether unilateral ureteral obstruction (UUO) would result in renal injury and inflammation, we measured the expression of MCP-1 by reverse transcriptase (RT)-PCR. A 6.3-fold and 4.7-fold increase in MCP-1 mRNA expression was evident in the obstructed kidneys ($P = 0.01$) and unobstructed kidneys ($P = 0.01$), respectively, of the control rats compared with the sham group (Fig. 1). However, in the enalapril, losartan, quercetin, and curcumin treatment groups, MCP-1

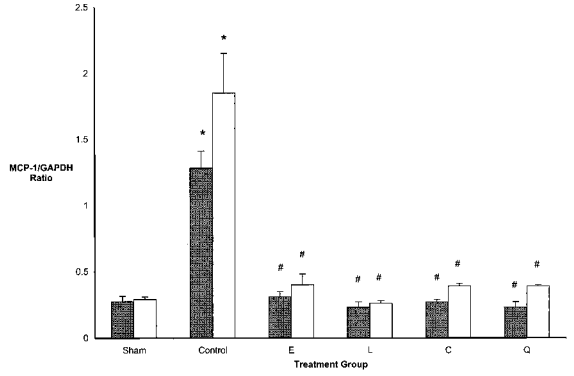

FIGURE 1. *Expression of the chemotactic cytokine MCP-1 in left (obstructed, open bars) and right (unobstructed, shaded bars) kidneys by RT-PCR corrected to GAPDH. Sham = sham operation, Control = vehicle control, E = enalapril, L = losartan, C = curcumin, Q = quercetin.* *P *<0.05 compared with sham.* #P *<0.05 compared with control.*

mRNA expression in the obstructed kidney was severely attenuated compared with the control rats ($P < 0.05$, Fig. 1). The level of expression was similar to levels seen in the sham-operated rats. These data suggest that UUO causes an upregulation of inflammatory chemokine, and the treatment with either bioflavonoids or angiotensin II inhibitors results in its downregulation.

Fas/Fas-L mRNA Expression

To investigate whether UUO causes cell damage or cell death, we measured the expression of Fas and Fas-L mRNA, because they are involved in T-cell-mediated cytotoxicity. The data demonstrated a 2.2-fold increase ($P < 0.05$) in Fas mRNA expression in the obstructed kidneys of the control rats compared with the sham rats (Fig. 2A). Treatment with enalapril, losartan, or curcumin had no significant effect on Fas mRNA expression in the obstructed kidneys compared with the control group. However, in the quercetin-treated group, Fas expression was three times lower ($P = 0.04$) than in the obstructed controls. The expression of Fas in the unobstructed kidneys of each of the treatment groups was not statistically different than that in the sham group (Fig. 2A).

The expression of Fas-L mRNA was increased 1.4-fold in obstructed kidneys of control animals compared with the sham rats ($P < 0.05$). Expression of Fas-L was lower in the enalapril, losartan, quercetin, and curcumin-treated groups compared with the control rats ($P < 0.05$). Although not statistically significant, Fas-L expression was lower in the unobstructed kidneys of all treatment groups compared with the sham group (Fig. 2B).

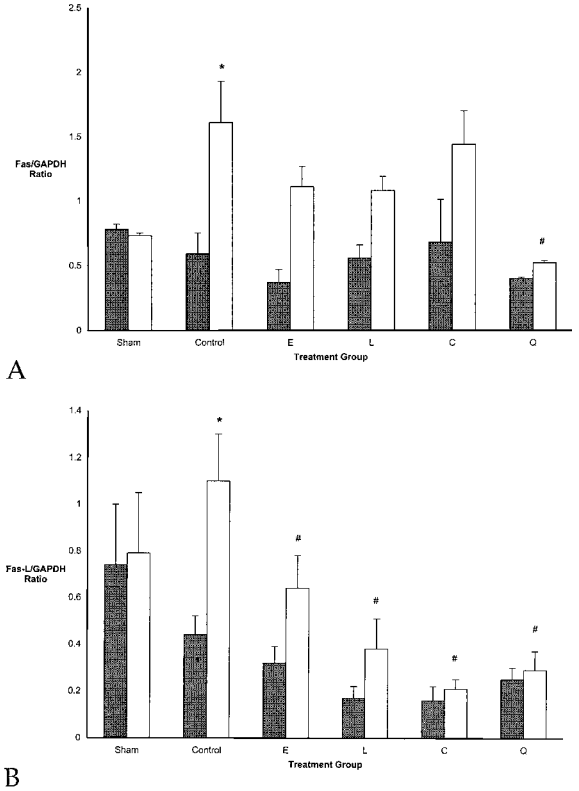

FIGURE 2. *Expression of Fas antigen and Fas-L in left (obstructed, open bars) and right (unobstructed, shaded bars) kidneys by RT-PCR corrected to GAPDH. Sham = sham operation, Control = vehicle control, E = enalapril, L = losartan, C = curcumin, Q = quercetin.* *P *<0.05 compared with sham.* #P *<0.05 compared with control. (A) Fas antigen expression by treatment group and (B) Fas-L expression by treatment group.*

HO-1 mRNA Expression

To investigate whether UUO causes an increase in oxidative stress, we measured the expression of HO-1, an inducible form of heme oxygenase. The expression of HO-1 mRNA was not increased in either the obstructed or unobstructed kidneys of the control animals compared with the sham rats (Fig. 3). Similarly, no change was observed in the enalapril, losartan, or quercetin-treated groups compared with control group. However, in the curcumin-treated rats, a threefold increase in HO-1 expression was evident in both the obstructed and unobstructed kidneys compared with the control rats ($P < 0.05$).

COMMENT

Obstructive nephropathy is a condition characterized by cell death and inflammatory repair. If obstruction is protracted, an injury response develops in the obstructed kidney characterized by increased cellularity of the tubulointerstitial compartment, followed by fibrosis and progressive

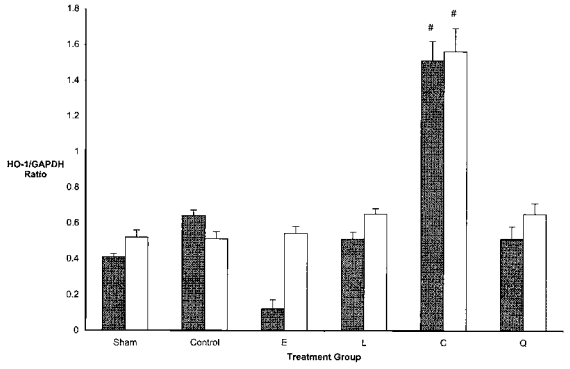

FIGURE 3. *Expression of the inducible stress protein HO-1 in left (obstructed, open bars) and right (unobstructed, shaded bars) kidneys by RT-PCR corrected to GAPDH. Sham = sham operation, Control = vehicle control, E = enalapril, L = losartan, C = curcumin, Q = quercetin.* #P *<0.05 compared with control.*

tubular cell loss through the energy-dependent process of apoptosis.[8] The eventual outcome of unrelieved obstruction is irreversible scarring and atrophy accompanied by impairment of renal function.[9]

The cascade of progressive renal injury is initiated by a monocytic infiltration of the glomerular and tubulointerstitial compartments. The recruitment of peripheral leukocytes represents a key feature in the developing injury response and is associated with the liberation of inflammatory chemokines and the potent chemotactic factor MCP-1.[10] The upregulation of renal MCP-1 expression seen in the present study has also been reported in other models of ureteral obstruction and can be inhibited by agents that interfere with the action of angiotensin II.[2,11] Curcumin has also been shown to suppress activation of nuclear factor-kB (NF-kB).[12]

The present study demonstrated attenuation of MCP-1 to the level seen in the sham group after treatment with enalapril (ACE inhibitor) or losartan (angiotensin II receptor antagonist). Interestingly, treatment with the polyphenolic bioflavonoids quercetin and curcumin, which have no known effect on the renin system, attenuated MCP-1 expression to a similar degree. Although such an effect has not been described in the context of ureteral obstruction, the finding is not surprising. These naturally occurring compounds have salutary effects on the histologic features and function after several types of tissue injury.[4,13] It has been suggested that the antioxidant and anti-inflammatory properties of quercetin and curcumin may be responsible for the observed effects. We have previously shown that quercetin and curcumin treatment inhibited the induction of several inflammatory cytokines, including the chemokines MCP-1 and RANTES after ischemia and reperfusion.[14]

Inflammatory cell infiltration with cell activation and cytokine release is a common feature of tissue injury. NF-kB is involved in the transcriptional regulation of a number of genes in the kidney, including MCP-1, and is activated during UUO. ACE inhibition has been shown to markedly decrease activation of NF-kB in the kidney with ureteral obstruction.[15] Curcumin has also been shown to suppress activation of NF-kB.[15] It is notable that these structurally and functionally distinct compounds are able to downregulate MCP-1 expression, suggesting that they may work at different levels on a common effector system.

Tubular cell death is the final common pathway of renal injury. Although the histologic and functional sequelae of apoptosis in ureteral obstruction are well described, the specific molecular events are not. Several potential mediators of cell death have been proposed, including mechanical injury, ischemia, cytokines, oxidative stress, and ligand-mediated cytotoxicity. These factors may act on a specialized family of cell membrane receptors that includes Fas antigen.[16] Fas antigen belongs to the tumor necrosis factor family of receptors and is activated when cross-linked by the membrane protein Fas-L, a major effector of CD8+ cytotoxic T lymphocytes and natural killer cells.[17]

In this study, we have, for the first time, demonstrated the increased expression of both Fas antigen and Fas-L in obstructed kidneys compared with sham-operated animals. Upregulation of Fas was significantly less in the quercetin-treated group, and Fas-L expression was reduced in all treatment groups. This suggests that these agents could protect the kidney from apoptosis-mediated injury. Fas-L expression was reduced in the treatment groups to levels similar to that in the normal control kidneys, suggesting that these agents may confer true renal protection against injury rather than simply interfering with the injury pathways after they are initiated.

The Fas/Fas-L pathway is known to mediate apoptosis in many tissues, including the kidney.[18–20] Recently, we have found that treatment with quercetin and curcumin decreases Fas and Fas-L expression (unpublished results), and when given together, they substantially inhibit apoptosis in the rat kidney after ischemia-reperfusion.[3] The mechanism of action of quercetin and curcumin on apoptosis is not known. However, both quercetin and curcumin are known to be potent antioxidants[21,22] and inhibitors of membrane-bound and cytosolic tyrosine kinases.[23] It is thought that the effect of quercetin and curcumin on the tyrosine kinases and their antioxidant properties may be involved.

Interestingly, only quercetin was associated with decreased levels of Fas gene expression. This finding suggests that distinct cellular pathways may mediate the cytoprotective features of quercetin and curcumin.

Immunologic reactions lead to an augmented generation of reactive oxygen species by leukocytes. Pro-oxidants such as superoxide anion, OH−, NO, and H_2O_2 may result in direct cellular damage by membrane peroxidation or may promote cell injury through their effect on the intracellular signaling pathways.[24] Recent evidence suggests that various cellular stresses induce a protective response that includes the induction of HO-1, a biomarker of oxidative stress. HO-1 is an inducible, microsomal, rate-limiting enzyme that catalyzes heme degradation into biliverdin, carbon monoxide, and iron.[25] Biliverdin is subsequently converted into bilirubin, a powerful antioxidant, by bilirubin reductase. HO-1 expression is upregulated by a variety of stimuli, including heme, heavy metals, endotoxins, hormones, cytokines, heat shock, oxidative stress, ultraviolet radiation, and hyperoxia. Upregulation of HO-1 expression is considered to be a protective response to cellular stress.[26] Overexpression of HO-1 has been shown to provide protection against lung injury[27] and to provide neuroprotection in a model of middle cerebral artery occlusion.[28] The present results show that treatment with curcumin induced the expression of HO-1 in both obstructed and contralateral unobstructed kidneys. It is speculated that the renal protective effects of curcumin may, in part, be mediated by HO-1 induction. It is noteworthy that HO-1 was not upregulated in other treatment groups of this model. In a recent report, induction of HO-1 mRNA and protein was demonstrated at 12 and 48 hours after ureteral obstruction in the mouse.[29] In the present study, however, rats were killed 7 days after obstruction. The induction of HO-1 mRNA may be an early event that does not normally persist until 7 days.

CONCLUSIONS

Progression of renal damage after ureteral obstruction results from a stereotypic injury response. Angiotensin II inhibition and treatment with polyphenolic bioflavonoids have beneficial effects on renal injury, as assessed by their effect on the expression of apoptotic and inflammatory genes. These agents share the ability to inhibit chemokine gene expression. The polyphenolic agents curcumin and quercetin have a superior ability to inhibit the expression of the apoptosis-related genes Fas and Fas-L. We have recently shown that quercetin treatment had a beneficial effect in patients with chronic pelvic pain syndrome and also reduced the level of 8-isoprostane F-2α (a marker of oxidative stress).[30] On the basis of these observations and the low toxicity of quercetin in humans, we believe that it may be of potential benefit to patients with partial or temporary complete ureteral obstruction and thus merits further studies.

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