Early detection of ketoprofen-induced acute kidney injury in sheep as determined by evaluation of urinary enzyme activities

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Objective—To evaluate early indicators of renal tissue destruction and changes in urinary enzyme activities in sheep during the first hours after acute kidney injury induced by administration of an overdose of an NSAID.

Animals—12 adult female sheep.

Procedures—Acute kidney injury was induced in 6 sheep by administration of ketoprofen (30 mg/kg, IV) and detected by evaluation of urinary protein concentration, iohexol clearance, and results of histologic examination. Six sheep served as control animals. Blood and urine samples were collected for up to 24 hours after administration of ketoprofen. Plasma concentrations of urea, creatinine, albumin, and total protein; plasma activities of alkaline phosphatase, acid phosphatase, γ-glutamyl transpeptidase (GGT), matrix metalloproteinase (MMP)-2, and MMP-9; and urinary creatinine and protein concentrations, specific gravity, and activities of alkaline phosphatase, acid phosphatase, GGT, lactate dehydrogenase, N-acetyl-β-D-glucosaminidase (NAG), MMP-2, and MMP-9 were measured. Urinary protein concentration and enzyme activities were normalized on the basis of urinary creatinine concentrations and reported as ratios.

Results—Many urinary enzyme-to-creatinine ratios increased before the plasma creatinine concentration exceeded the reference value. Urine NAG, lactate dehydrogenase, and acid phosphatase activities were increased beginning at 2 hours after ketoprofen administration, and alkaline phosphatase, GGT, and MMP-2 activities were increased beginning at 4 hours after ketoprofen administration. Most peak urinary enzyme-to-creatinine ratios were detected earlier than were the highest plasma creatinine and urea concentrations.

Conclusions and Clinical Relevance—Urinary enzyme activities were sensitive early indicators of acute kidney injury induced by an overdose of an NSAID in sheep. (*Am J Vet Res* 2010;71:1246–1252)

Accidental overdosing of NSAIDs is relatively common in animals, especially dogs, and it can induce ARF. In humans, ARF is generally defined as an abrupt and sustained decrease in renal function that leads to an accumulation of nitrogenous waste products and uremic toxins.[1] However, indicators (such as serum creatinine and urea concentrations) routinely used to evaluate renal function will increase above reference values only after severe renal damage, and there is a delay in this increase. These indicators can also be affected by factors other than renal insufficiency. Therefore, early biomarkers of AKI are needed to enable clinicians to detect patients with injury or at risk for ARF because of an overdose of an NSAID, which will allow for early

ABBREVIATIONS

ACP	Acid phosphatase
AKI	Acute kidney injury
ALP	Alkaline phosphatase
ARF	Acute renal failure
GGT	γ-Glutamyl transpeptidase
HPLC	High-performance liquid chromatography
LDH	Lactate dehydrogenase
MMP	Matrix metalloproteinase
NAG	N-acetyl-β-D-glucosaminidase
TBS	Tris-buffered saline

therapeutic intervention. An ideal indicator can be measured rapidly from a single urine sample without the need to collect urine for 24 hours.

Activities of a variety of enzymes, such as GGT,[2–4] ALP,[2,3] LDH,[2] and NAG,[2–7] have been evaluated in urine as biomarkers of AKI in humans, and they have been used to evaluate nephrotoxicosis in dogs.[8] Increases of urinary GGT and NAG activities have been suggested as indicators of early renal damage in dogs with diro-

filariasis (ie, heartworm disease)[9] and leishmaniasis.[10] In 1 study,[11] ALP and GGT were sensitive early indicators of nephrotoxic damage experimentally induced by administration of mercuric chloride in sheep. The ACP activity in renal tissue has been investigated after experimentally induced tubular injury.[12] The gelatinases, MMP-2 and -9, have been localized to the renal tubules, interstitial cells, and tubulointerstitial space.[13] Urinary total gelatinolytic activity, comprising both MMP-2 and -9, was increased in humans with diabetic nephropathy, compared with activity in healthy control subjects.[14] The gelatinases also may play a role in AKI because MMP-9 has been associated with AKI[6] and MMP-2 and -9 activities are enhanced in postischemic kidney tissue in rats.[13] Only limited information exists describing the detection of enzyme activities in urine during the early phases of clinical or experimentally induced AKI.[6,7,11]

The primary objective of the study reported here was to evaluate noninvasive indicators of renal tissue destruction that could be used to predict the risk of AKI as early as possible after an overdose of an NSAID before increases in plasma concentrations of creatinine and urea would suggest renal insufficiency. A second objective was to determine changes in urinary enzyme activities during the first hours after induction of AKI by administration of an overdose of an NSAID (ie, ketoprofen). Acute renal failure was detected by evaluation of traditional markers, such as urinary protein concentration that indicated increased glomerular permeability, glomerular filtration rate that reflected decreased renal function, and results of histologic examination that revealed morphological changes. We hypothesized that increases in enzyme activities in urine would indicate tissue destruction before the first signs of renal failure were detected.

Materials and Methods

Animals—Twelve female Finnish Landrace sheep were purchased from a commercial breeder for use in the study. All sheep were > 18 months old and were not pregnant. Body weight ranged from 48.5 to 59.0 kg (mean, 52.5 kg). The sheep had previously been used in a pharmacodynamic study, but they had not received clinical doses of medications for 4 months before the onset of the study reported here, and we presumed that the medications administered in that previous study did not cause irreversible lesions. The sheep were provided ad libitum access to good-quality hay and water before and during the experiment. At the start of the experiment, the sheep were healthy and were not receiving any treatments. No changes indicative of renal disease were detected in plasma or urine. The study protocol was approved by the Ethics Committee of the University of Helsinki.

Study design and procedures—Each sheep was allocated to a treatment or control group (n = 6 sheep/group) by use of a simple randomization procedure (ie, by drawing lots). The 6 sheep in the treatment group were each administered an overdose of ketoprofen[a] (30 mg/kg, IV). The ketoprofen was injected into a jugular vein, and the contralateral jugular vein was used for collection of blood samples. The 6 sheep in the control group did not receive any injection.

Blood samples (9 mL) were collected into tubes containing lithium heparin before treatment (time 0 [baseline]) and at 1, 2, 4, 6, 8, and 24 hours. Urine samples (10 mL) were collected before treatment (baseline) and at 2, 4, 6, and 8 hours via a urinary catheter[b] inserted into the bladder. After collection of the urine sample at 8 hours, the catheter was removed. The next day, after collection of the blood sample at 24 hours and performing the iohexol test, each sheep was anesthetized by IV administration of xylazine hydrochloride[c] (0.2 mg/kg) and ketamine hydrochloride[d] (5 mg/kg). Sheep then were euthanatized by IV administration of a solution of embutramide and mebezoniumiodide.[e] A final urine sample (ie, sample at 24 hours) was collected via cystocentesis immediately after the sheep were euthanatized.

Laboratory assays—Blood and urine samples were kept in ice water until centrifuged (1,300 × g for 10 minutes at 4°C) to remove cellular components and debris. Plasma and urine supernatant were harvested. Plasma concentrations of urea, creatinine, albumin, and total protein and activities of aspartate aminotransferase, ALP, ACP, and GGT and urine concentrations of creatinine and protein and activities of GGT, ALP, and LDH were measured by use of a clinical chemistry analyzer.[f] Urine specific gravity was determined via refractometry. The remainder of the plasma and urine samples was frozen at –80°C until subsequent use of plasma and urine in MMP-2, MMP-9, and ACP assays and use of urine in an NAG assay. Activities of ACP were analyzed with a commercial kit,[g] and urinary NAG activity was measured with a commercial kit[h] by use of a colorimetric method.

Activities of MMP-2 and -9 were measured via semiquantitative gelatin zymography, SDS-PAGE electrophoresis, and western blotting. Purification of urinary gelatinases was performed as described elsewhere,[15] with slight modifications. Briefly, 20-mg creatinine-equivalent urine samples were mixed with 200 μL of gelatin-sepharose beads[i] equilibrated with binding buffer (50mM Tris [pH, 7.5], 0.5M NaCl, 10mM CaCl$_2$, 0.01% Tween 20, and 5mM EDTA) and incubated overnight at 4°C with gentle rotation. The beads then were extensively washed, and the bound material was eluted with electrophoresis loading buffer. Plasma samples were diluted with distilled deionized water (1 part plasma:5 parts distilled deionized water) to minimize the effect of plasma proteins on detection of bands with gelatin zymography. Plasma and urinary MMP activities were measured by use of gelatin zymography.[16] The samples were loaded into the wells of a 10% gelatin gel.[j] For each gel, 3 concentrations of purified human MMP-2 and -9 mixture[k] as well as prestained protein standard[l] were assayed in parallel with the samples. After electrophoresis, gels were incubated with renaturing buffer (2.5% Triton X-100) for 30 minutes at 20°C with gentle rotation. Thereafter, the gels were incubated with developing buffer (50mM Tris-HCl [pH, 7.5], 0.02M NaCl, 5mM CaCl$_2$, and 0.02% polyoxyethylene lauryl ether) overnight at 37°C. After incubation, gels were stained with Coomassie Brilliant Blue R-250, which revealed clear bands against a blue background. Gels were photographed, and density of the bands was measured with a spot-density tool of an imaging system.[m] A standard curve was created on the basis of the values for the standard mixtures, and samples obtained from the sheep were quantified by

comparing their mean pixel value (after background correlation) with values for the standard curve. Results were reported as intensities of the gelatinolytic bands.

Native PAGE electrophoresis and SDS-PAGE electrophoresis were performed on ice with 10% gels and Tris-glycine buffer (pH, 8.3) at 125 V for 90 minutes, as described elsewhere.[17] Proteins were transferred onto a polyvinylidene fluoride membrane[n] by use of a transfer apparatus.[o] Nonspecific binding was reduced by incubating blots in blocking buffer (5% nonfat dry milk, 0.1% Tween 20, and 1X TBS) for 1 hour at 20°C. Immunodetection was performed by incubating blots with a mouse anti-human MMP-2[18,p] or goat anti-human MMP-9[p] primary antibody at appropriate dilution in a buffer of 1X TBS plus 0.1% Tween 20. Blots then were washed 3 times with 1X TBS plus 0.1% Tween 20

and incubated with horseradish peroxidase–conjugated goat anti-mouse[k] or donkey anti-goat[k] primary antibody in 1X TBS plus 0.1% Tween 20 (dilution, 1:5,000) for 3 hours at 20°C. After the third wash, detection of the signal was achieved by use of an enhanced chemiluminescent substrate,[q] and the signal was evaluated with a luminescent image analyzer.[r]

To correct for variations in urine flow, urinary protein concentration and enzyme activities were normalized on the basis of urinary creatinine concentrations. Results were reported as ratios.

An iohexol test was performed twice (2 to 4 weeks before and 24 hours after treatment). Iohexol[s] was administered (3.0 g/sheep, IV) into a jugular vein, and blood samples were collected from the contralateral jugular vein at 2, 3, and 4 hours after injection. For iohexol analysis,

Table 1—Median (range) values for urinary concentration of creatinine, protein-to-creatinine ratio, enzyme activity indices (normalized on the basis of urinary creatinine concentrations), and specific gravity in samples collected from 6 sheep after administration of ketoprofen (30 mg/kg, IV) and from 6 control sheep that did not receive any treatment.

Variable	Group	Baseline	Time (h)				
			2	4	6	8	24
Creatinine (mmol/L)	Ketoprofen	17.8 (8.0–21.8)	10.9 (3.7–16.6)	7.2 (4.5–10.1)*†	3.9 (2.2–7.9)*†	2.7 (1.4–6.5)*†	2.7 (2.2–3.5)†
	Control	12.4 (8.9–14.9)	13.4 (10.9–25.4)	14.9 (6.3–27.1)	15.3 (12.7–25.6)	12.8 (6.6–24.8)	9.1 (2.1–20.0)
Protein-to-creatinine ratio (g/g)	Ketoprofen	0.11 (0.09–0.18)	0.96 (0.56–1.50)†	7.72 (4.09–19.6)*†	18.0 (6.69–63.1)*†	27.2 (3.05–112.9)*†	3.76 (0.97–16.5)†
	Control	0.14 (0.08–0.38)	0.49 (0.18–1.38)†	0.78 (0.34–1.77)†	1.41 (0.38–2.67)†	1.36 (0.69–3.58)†	0.77 (0.23–3.60)†
GGT (U/mmol)	Ketoprofen	0.86 (0.50–1.18)	0.86 (0.79–1.38)	1.66 (0.49–4.20)	3.02 (1.27–8.17)*†	6.17 (2.62–17.53)*†	2.62 (0.89–16.13)
	Control	0.94 (0.56–1.14)	0.81 (0.39–1.98)	1.01 (0.37–2.95)	1.07 (0.56–3.69)	1.34 (0.40–3.35)	1.33 (0.78–2.93)
ALP (U/mmol)	Ketoprofen	3.2 (1.9–5.0)	4.3 (1.9–9.7)	13.9 (9.4–42.4)*†	42.3 (27.7–135.8)*†	98.2 (16.2–420.8)*†	27.4 (8.7–86.3)†
	Control	3.7 (2.6–7.4)	4.3 (1.6–10.6)	5.2 (1.4–13.2)	5.8 (2.5–18.6)	9.6 (3.0–20.1)	8.5 (4.4–9.9)
ACP (U/mmol)	Ketoprofen	8.3 (7.3–9.1)*	13.9 (9.9–22.2)*†	26.8 (11.9–39.2)*†	34.4 (11.6–53.8)*†	30.9 (9.3–56.5)*†	9.5 (8.7–20.5)*†
	Control	7.1 (6.8–8.1)	7.2 (5.7–7.7)	5.9 (4.6–6.4)†	6.3 (5.3–6.8)	6.1 (5.8–8.1)	7.5 (7.1–9.6)
LDH (U/mmol)	Ketoprofen	0.95 (0.12–2.77)	5.98 (2.67–22.64)*†	51.05 (0–150.46)†	87.7 (40.2–228.2)*†	185.2 (28.2–535.4)*†	105.0 (39.9–305.7)*†
	Control	0.89 (0–4.36)	1.67 (0–2.19)	2.05 (0.97–2.69)	2.44 (0.68–6.88)	1.71 (1.09–17.60)	8.09 (0–59.33)
Proenzyme MMP-2 (intensity)	Ketoprofen	0 (0–0.40)	1.07 (0.88–2.60)†	6.27 (0.89–11.43)*†	13.3 (5.87–28.9)*†	21.8 (5.63–40.1)*†	8.62 (0.83–40.6)*†
	Control	0 (0–0.00)	0.56 (0–1.29)	0.63 (0–2.00)	1.03 (0–2.40)†	0.89 (0–1.96)	0 (0–0.62)
Activated MMP-2 (intensity)	Ketoprofen	0 (0–0)	0.10 (0–1.36)	0.56 (0–1.92)	2.64 (0.12–5.89)*†	2.70 (0.41–9.46)*†	1.47 (0–4.28)*†
	Control	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
NAG (U/mmol)	Ketoprofen	0.03 (0.02–0.04)	0.12 (0.04–0.26)*†	NM	0.73 (0.17–1.40)*†	NM	0.26 (0.05–0.60)*†
	Control	0.03 (0.02–0.04)	0.02 (0.02–0.03)	NM	0.02 (0.02–0.04)	NM	0.04 (0.02–0.15)
Specific gravity	Ketoprofen	1.050 (1.028–1.055)	1.041 (1.021–1.055)	1.031 (1.027–1.036)	1.020 (1.016–1.030)*†	1.016 (1.014–1.019)*†	1.013 (1.012–1.016)†
	Control	1.030 (1.023–1.044)	1.041 (1.032–1.052)†	1.036 (1.020–1.052)	1.041 (1.028–1.044)	1.035 (1.027–1.049)	1.037 (1.006–1.046)

Urine samples were collected at 0 (baseline), 2, 4, 6, and 8 hours via a urinary catheter inserted into the bladder; urine samples at 24 hours were collected via cystocentesis immediately after the sheep were euthanatized.
*Within a time point within a variable, value differs significantly ($P < 0.05$) from the value for the control sheep. †Within a row, value differs significantly ($P < 0.05$) from the value for baseline.
NM = Not measured.

Table 2—Median (range) values for plasma concentrations of urea, creatinine, total protein, and albumin and activities of ALP, ACP, and GGT in samples obtained from 6 sheep after administration of ketoprofen (30 mg/kg, IV) and from 6 control sheep that did not receive any treatment.

| Variable | Group | Baseline | Time (h) | | | | | |
			1	2	4	6	8	24
Urea (mmol/L)	Ketoprofen	5.3 (3.9–6.9)	5.2 (4.1–7.0)	5.8 (4.5–7.6)†	7.2 (5.6–8.8)*†	8.8 (6.6–9.6)*†	9.8 (7.1–10.4)*†	20.6 (14.0–22.3)*†
	Control	4.7 (3.5–5.2)	5.3 (3.8–6.3)	4.9 (3.6–6.1)	4.6 (3.7–5.7)	4.1 (3.4–5.7)	3.4 (2.8–5.0)	4.1 (2.6–7.2)
Creatinine (μmol/L)	Ketoprofen	86 (80–106)	88 (79–105)	89 (82–109)	120 (100–140)*†	151 (131–191)*†	184 (150–208)*†	390 (131–414)*†
	Control	92 (81–113)	96 (80–110)	89 (79–106)	91 (74–101)	90 (71–102)	88 (74–105)	90 (71–101)
Albumin (g/L)	Ketoprofen	31.6 (30.8–32.6)	31.5 (31.0–32.8)	30.1 (28.0–31.1)*†	29.6 (28.6–30.4)*†	29.4 (28.7–31.5)*†	28.6 (27.6–31.2)*†	26.4 (23.5–31.9)*†
	Control	31.8 (30.2–33.9)	31.9 (30.3–33.1)	31.6 (30.7–32.4)	31.6 (30.9–32.8)	32.6 (30.1–34.4)	31.8 (30.9–32.8)	32.0 (30.8–33.6)
Total protein (g/L)	Ketoprofen	64.8 (61.0–68.7)	64.9 (61.0–66.5)†	62.1 (59.3–63.1)*†	60.1 (56.4–60.1)*†	60.5 (54.7–66.0)*†	57.6 (52.0–65.2)*†	51.8 (42.9–68.1)*†
	Control	67.0 (63.0–69.5)	66.2 (63.4–68.7)	66.4 (64.8–68.2)	66.5 (63.7–67.9)	67.1 (63.5–72.3)	67.0 (64.8–68.9)	66.3 (60.9–68.3)
ALP (U/L)	Ketoprofen	229 (175–296)	196 (159–296)	202 (164–296)	198 (131–288)†	205 (136–302)	186 (128–312)	121 (72–312)*†
	Control	202 (184–337)	212 (199–259)	201 (189–252)	186 (167–276)	203 (187–266)	201 (169–300)	200 (171–300)
ACP (U/L)	Ketoprofen	3.8 (3.4–4.1)	NM	NM	NM	NM	9.3 (5.0–11.2)*†	7.5 (4.3–10.2)*†
	Control	3.7 (3.5–4.0)	NM	NM	NM	NM	3.8 (3.6–4.1)	3.8 (3.4–3.9)
GGT (U/L)	Ketoprofen	46 (37–69)	44 (36–67)	42 (35–65)†	43 (35–62)†	43 (36–64)	40 (34–59)†	36 (30–48)*†
	Control	46 (41–52)	45 (40–49)	47 (41–50)	47 (41–51)	48 (42–50)	46 (42–50)	46 (41–49)

See Table 1 for key.

plasma samples were precipitated with 10% trifluoroacetic acid and analyzed with an HPLC.[19,20,t] The internal standard was 4-aminobenzoic acid. Iohexol clearance was calculated as described elsewhere.[21]

Plasma concentrations of ketoprofen were measured with an HPLC,[22] with slight modifications. Determinations were made on 4 samples in parallel. The HPLC system was equipped with a piston pump,[u] autosampler,[v] tunable absorbance UV detector,[w] and workstation.[x] Sample separation was conducted on a 4.6 × 150-mm column packed with 5 μm of reversed-phase silica.[y] Flow rate of the isocratic mobile phase (which consisted of acetonitrile and 0.03% phosphoric acid [ratio, 1:1]) was 1.5 mL/min. Samples were analyzed at a wavelength of 254 nm. Accuracy and precision of the method were evaluated (as recommended in another study[23]) by analyzing 6 parallel plasma samples spiked with ketoprofen. The standard curve was found to be linear ($R^2 > 0.998$) over the concentration range used (0.36 to 50 mg/L). Mean values at the extremes of the concentration range were 0.39 mg/L (coefficient of variation for accuracy and precision was 4.1% and 8.3%, respectively) and 50.8 mg/L (4.9% and 1.7%, respectively). No interfering peaks were detected for the plasma blanks. The elimination half-life was calculated[z]

by dividing the natural logarithm of 2 by β, where β is the elimination rate constant.

Histologic examination—A necropsy was performed immediately after each sheep was euthanatized. Tissue samples for histologic examination were collected from the kidneys and placed in neutral-buffered 10% formalin. The fixed samples were embedded in paraffin, sectioned in 4-μm-thick slices, and stained with H&E, van Gieson, periodic acid–Schiff, and von Kossa stains for histologic examination via light microscopy.

Statistical analysis—Differences in iohexol clearance between groups were analyzed via a Student *t* test. Because the plasma enzyme activities and urinary enzyme indices were not normally distributed, all differences between groups at various time points were analyzed via a Mann-Whitney *U* test and the values were compared with values at time 0 via a Wilcoxon rank test. Significance was set at values of $P < 0.05$.

Results

Urinary enzyme-to-creatinine ratios for GGT, ALP, ACP, LDH, proenzyme MMP-2, activated MMP-2, and NAG increased after administration of ketoprofen
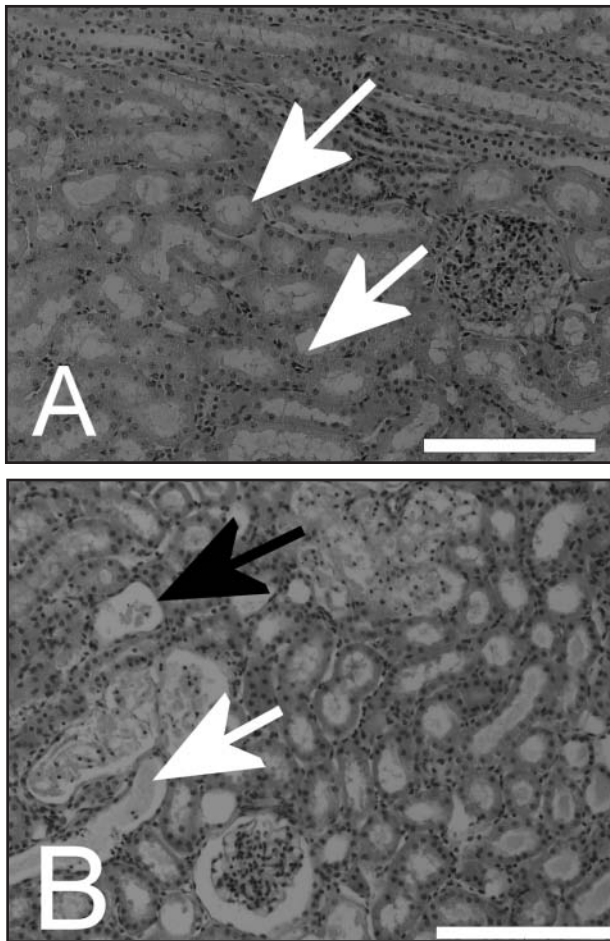
Figure 1—Photomicrographs of sections of renal tissues obtained from a representative control sheep that did not receive any treatment (A) and a representative sheep 24 hours after administration of ketoprofen (30 mg/kg, IV; B). In panel A, notice the histologically normal tubular epithelium (white arrows). In panel B, notice the thinning of the tubular epithelium (black arrow) and the cellular debris in the tubular lumina (white arrow). H&E stain; bar = 200 µm.

(Table 1). Activity of MMP-9 was not detected in any urine samples obtained at time 0 or in urine samples obtained from the control sheep; however, MMP-9 activity was detected in urine samples obtained from 3 of 6 sheep after ketoprofen injection. Some urine samples had a reddish tint after administration of ketoprofen, which suggested hematuria or hemoglobinuria. Proteinuria was detected and urine specific gravity decreased after administration of an overdose of ketoprofen.

Plasma ALP and GGT activities decreased and ACP activity increased after administration of ketoprofen (Table 2). Plasma MMP-2 and -9 activities did not change over time; in addition, they did not differ between groups (data not shown).

Plasma concentrations of urea and creatinine increased after administration of ketoprofen, and they remained higher than in the control sheep beginning at 4 hours after treatment until the end of the study (Table 2). Plasma concentrations of creatinine were > 1.5 times as high as the value at time 0 in all ketoprofen-treated sheep from 4 to 8 hours (median, 6 hours) after injection until the end of the study, at which time

they were > 3 times as high as the value at time 0 in 5 of 6 ketoprofen-treated sheep. Plasma concentrations of creatinine exceeded the reference range[24] for sheep (168 µmol/L) in 5 of 6 sheep at 6 to 8 hours (median, 6 hours) after ketoprofen treatment. Plasma concentrations of urea were higher than the reference range[24] for sheep (7.1 mmol/L) in all 6 sheep at 24 hours after ketoprofen treatment. Elimination of iohexol was significantly reduced the day after administration of ketoprofen; at that time, mean ± SD iohexol clearance was 3.7 ± 4.5 mL/h/kg and 23.2 ± 1.8 mL/h/kg in treated and control sheep, respectively.

Mean ± SD plasma concentration of ketoprofen was 87.5 ± 33.4 µg/mL and 22.1 ± 7.9 µg/mL at 1 and 2 hours after administration, respectively. Mean elimination half-life of ketoprofen was 2.1 ± 0.6 hours.

Histologic examination of renal tissues collected from the carprofen-treated sheep revealed acute tubular injury. This was not detected in tissues collected from the control sheep. Lesions concerning the cortical region (especially the proximal tubules) included degeneration and necrosis of individual tubular epithelial cells, swelling of the tubular epithelium, detachment of the tubular epithelium from the underlying tubular basement membrane, loss of the periodic acid–Schiff–positive brush border of proximal tubular epithelial cells, thinning of the tubular epithelium, dilatation of the tubular lumina, interstial edema, sloughed epithelial cells in the tubular lumens, cellular debris, and casts in the distal tubules (Figure 1). Other histologic results were consistent for both groups, and only mild incidental unspecific findings, such as interstitial medullary calcification, were detected.

Discussion

The increase in plasma concentrations of urea and creatinine and the decrease in iohexol clearance in ketoprofen-treated sheep indicated a reduced glomerular filtration rate. Proteinuria suggested increased glomerular permeability attributable to an overdose of ketoprofen, and it was probably associated with the decrease in the plasma concentration of albumin. Histologic examination revealed ketoprofen-induced AKI. Thus, the dose of ketoprofen administered was sufficient to induce AKI in sheep. The slight increases detected in urinary protein concentration and proenzyme MMP-2 activity in some sheep in the control group suggested that the urethral catheter, which remained in situ for 8 hours, may have caused local irritation in the bladder, but catheterization did not markedly affect the other variables measured in the study.

The elimination half-life of ketoprofen in plasma was higher after the overdose of ketoprofen in the sheep of the present study than in sheep in other studies[25,26] after administration of a frequently used dose of racemic ketoprofen (3 mg/kg) or its enantiomers. Approximately 2 hours after administration of ketoprofen at a dose of 30 mg/kg, the plasma concentration of ketoprofen in our study was at the same value as the maximum concentration reported in sheep after administration at the frequently used dose of 3 mg/kg.[25,26] Thus, exposure to the toxic plasma concentration of ketoprofen

was probably relatively short, and most of the injuries in the tubular cells can be expected to have taken place during the early phase of our follow-up period.

Although the plasma concentration of creatinine was significantly higher than the concentration at time 0 and the concentration in the control sheep by 4 hours after administration of ketoprofen, it did not exceed criteria for AKI in humans (ie, a 2- to 3-fold increase from the baseline value)[2,27] and it was not above the reference range for sheep[24] in any of the sheep in our study until several hours later. Therefore, the plasma concentration of creatinine was not a sensitive indicator of AKI during the first few hours after exposure to ketoprofen.

In general, urinary enzyme-to-creatinine ratios seemed to be sensitive early indicators of the risk of AKI because many of them increased sooner than did the plasma concentration of creatinine. Brush border enzymes are typically released into the urine during less severe injuries, compared with release of lysosomal or cytosolic enzymes.[3] Thus, the increases in urinary activities of the lysosomal enzymes NAG and ACP and the cytosolic LDH suggested relatively severe injuries of tubular cells within 2 hours after administration of ketoprofen. In addition, the sporadic hematuria observed in some sheep may have contributed to the increase in LDH activity, which is an enzyme known to be found in RBCs. According to a recent systematic review,[4] evaluation of GGT activity performs well for early diagnosis of AKI in humans, and in another study[11] in sheep, evaluation of GGT activity appeared to be more sensitive than did evaluation of LDH activity after injection with mercuric chloride. However, in the study reported here, NAG, LDH, and ACP activities were increased in urine earlier than were activities of the brush border enzymes ALP and GGT, which indicated that the former may reveal cell injuries earlier than do the latter. On the other hand, the decrease in plasma ALP and GGT activities detected after administration of ketoprofen suggested that at least a part of these urinary enzyme activities originated from plasma. In humans, urinary NAG activity may be useful for assessing initial malfunction or damage of the proximal tubular epithelial cells in the early stages of potentially progressive diseases.[5] In addition, urinary NAG activity has predictive value in functional outcome and response to treatment,[5] and it can be used for prediction of the risk of death after AKI.[4] The use of LDH, ACP, and ALP activities should be evaluated in clinical settings.

In the study reported here, increased activities of proenzyme and activated MMP-2 were found in urine, whereas MMP-9 activity was detected only in a few samples. In another study[6] in which MMP-2 activity was not measured, MMP-9 activity could not be used to differentiate human patients with AKI from those with urinary tract infection, despite the fact that MMP-9 activity was associated with AKI. On the other hand, in human patients with diabetic nephropathy, MMP-9 was more extensively converted to the active form than was MMP-2 and the proportion of active MMP-9 in urine was significantly higher than that in healthy control subjects.[14] These findings suggest that MMP-2 may be released into the urine and activated, especially during the acute phase of injuries to tubular cells.

Most of the urinary enzyme-to-creatinine ratios measured in our study were already decreasing when the highest plasma concentrations of creatinine and urea were detected at 24 hours (ie, the day after induction of AKI). This indicated a delay between cellular damage and the accumulation of creatinine and urea in plasma. Our results also suggested that peak urinary enzyme activities may not have been detected in a clinical study[5] in humans because the first urine sample in that study was collected 24 hours or more after exposure.

In the study reported here, urinary enzyme-to-creatinine ratios were found to have great potential as early biomarkers of AKI induced by an overdose of an NSAID. Activities of NAG, LDH, and ACP were increased in the urine within 2 hours after ketoprofen administration, and ALP and GGT activities increased significantly at 4 hours after ketoprofen administration. Activity of MMP-2 may be a better indicator of AKI than is activity of MMP-9. However, we did not evaluate cell injuries via histologic examination until the end of the study; thus, we could not compare the stage of the injury and its progression to the urinary enzyme indices at the various time points. Therefore, we could not estimate the specificities of these enzymes as biomarkers of AKI and their possible cutoff points, and they should be investigated in clinical studies.

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