Pathophysiological studies on canine renal papillary necrosis induced by nefiracetam

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Abbreviations

ACP, acid phosphatase

COX, cyclooxygenase

CRE, creatinine

DMSO, dimethyl sulfoxide

γ-GTP, γ-glutamyl transpeptidase

H&E, hematoxylin and eosin

HPLC, high performance liquid chromatography

LC/ESI-MS/MS, liquid chromatography electrospray ionization-tandem mass spectrometry

LDH, lactate dehydrogenase

NAG, *N*-acetyl-*β*-D-glucosaminidase

NSAID, nonsteroidal anti-inflammatory drugs

PG, prostaglandin

RPN, renal papillary necrosis

RT-PCR, reverse transcription-polymerase chain reaction

SDS-PAGE, sodium dodecyl sulfate-polyarylamide gel electrophoresis

UN, urea nitrogen

Introduction

Nefiracetam [*N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide], a novel pyrrolidone derivative, possesses a pharmacologically unique nootropic action, and facilitates cognitive functions in various animal models (1, 2, 17, 26, 42, 44, 51, 63). Regarding the mechanisms underlying its pharmacological action, nefiracetam has been reported to increase the release of neurotransmitters by activation of the long-lasting N/L-type Ca^{2+} channel (63), interact with γ-amininobutyric acid (GABA)-ergic and cholinergic neuronal systems, and enhance protein synthesis in the brain (42). Recently, the involvement of an unknown G_i-protein pathway relevant to activation of cAMP-dependent protein kinase and G-protein-independent protein kinase C pathways has been demonstrated (44). Prevention of the accumulation of intracellular calcium through N-methyl-D-aspartate receptor channels may also contribute in part to the action of nefiracetam (2).

In both clinical and non-clinical pharmacokinetic studies, nefiracetam was found to be extensively metabolized; more than twenty metabolites were found in serum, urine, and tissues (55). The major metabolic pathways of nefiracetam are shown in Fig. 1. In serum, nefiracetam and the metabolite-3 (M-3) are identified as the primary components in all species tested, and make up more than 80% of the total concentration of nefiracetam and its metabolites. In urine, nefiracetam itself accounted for below 4.4%, 1.9%, and 2.8% of the dosages administered to rats, dogs and monkeys, respectively; while the distribution (concentration) of each metabolite following a single oral administration of nefiracetam at 30 mg/kg was M-3 (35.9%, 0.86 mM), M-4 (6.7%, 0.16 mM), M-11 (4.0%, 0.09 mM) and others for rats, M-10 (21.2%, 0.37 mM), M-3 (8.7%, 0.20 mM), M-11 (4.3%, 0.10 mM) and others for dogs, and M-3 (15.3%, 0.37 mM), M-11 (10.0%, 0.23 mM), M-4 (9.9%, 0.24 mM) and others for monkeys. The metabolic pattern seen in human urine is essentially similar to that in monkeys although M-20 was also identified in serum and urine. In human urine, M-20 occupied approximately 7 % next to M-3 and M-11. These metabolic data raise the possibility that 3-hydroxylation (M-18) and subsequent sulfation (M-10) may be one of the key metabolic pathways for dogs.

According to previous toxicological investigations, changes in the urinary bladder and renal papillary necrosis (RPN) were observed only in dogs given a large amount of nefiracetam (over 180 mg/kg/day) with a delayed onset (28, 32, 56). In the urinary bladder, although degeneration and desquamation of epithelial cells and edema and hemorrhage of

the lamina propria were observed in week 1, these changes tended to recover and had almost disappeared by week 10, even with continuous treatment. In the kidney, degeneration and hyperplasia of epithelial cells in the papilla and collecting ducts with interstitial congestion and hemorrhage were seen at necropsy performed in week 11. In addition, extensive hemorrhage and papillary necrosis were seen in a few dogs that died during repeated treatment periods (week 10 or 11). Hence, epithelial cells in the urinary bladder, renal papilla and collecting ducts were considered to be an early target, and metabolite(s) in urine were inferred to play a central role in the development of RPN (32).

RPN is known to be caused by different kinds of compounds such as analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs, 3, 11, 35, 62), 2-bromoethylamine hydrobromide (7, 49), or D-ormaplatin (36). Regarding NSAIDs-induced RPN, direct cytotoxic action (47, 61) or ischemic injury to renal medullary cells through inhibition of the vasodilator effects of renal prostaglandins as a result of cyclooxygenase inhibition (3, 50) has been indicated as an important contributor. However, the process of renal damage is generally insidious and renal functions are often severely compromised before the condition becomes obvious (6, 14, 25, 58).

Therefore, the present study was performed to elucidate pathophysiological characteristics of nefiracetam-induced RPN and its underlying mechanism.

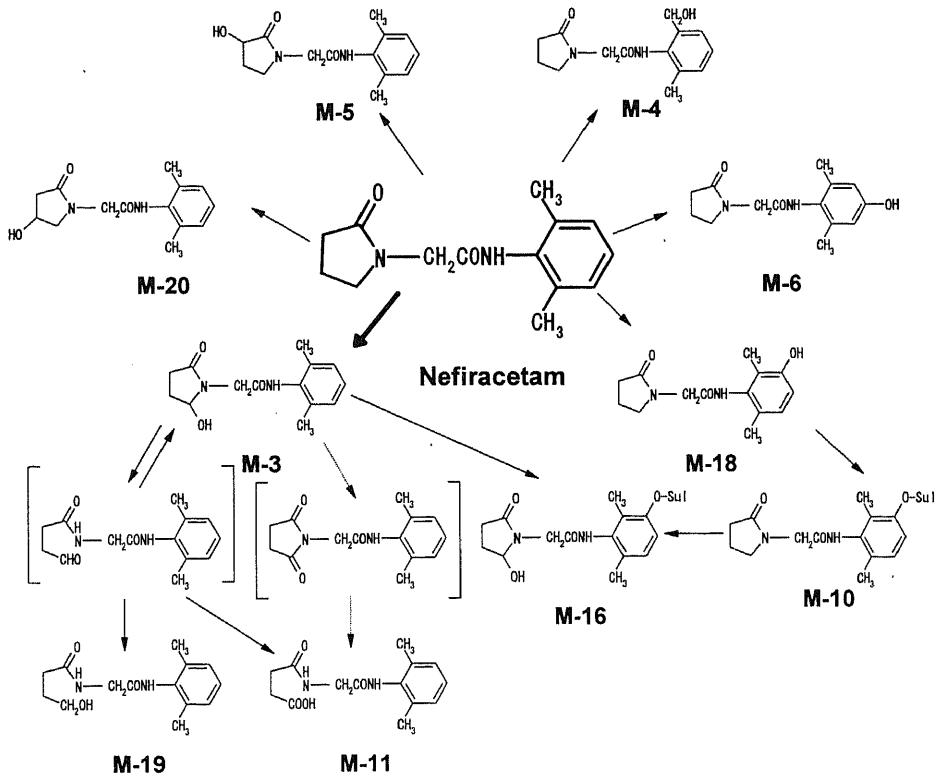


Fig. 1 Major metabolic pathways of nefiracetam. M: metabolite.

Chapter 1. Comprehensive evaluation of canine renal papillary necrosis induced by nefiracetam, a neurotransmission enhancer

Summary

Effects of nefiracetam, a neurotransmission enhancer, on renal biochemistry and morphology with toxicokinetic disposition were investigated in both in vivo and in vitro systems. In the in vivo studies with rats, dogs, and monkeys, only the dog exhibited RPN. Briefly, when beagle dogs were orally administered 300 mg/kg/day of nefiracetam over 11 weeks, decreased urinary osmotic pressure was noted from week 5, followed by increases in urine volume and urinary lactate dehydrogenase (LDH) from week 8. The first morphological change was necrosis of ductal epithelial cells in the papilla in week 8. In toxicokinetics after 3-week repeated oral administration to dogs, nefiracetam showed somewhat high concentrations in serum and the renal papilla as compared with rats and monkeys. As for metabolites, although metabolite-18 (M-18) concentration in the renal papilla of dogs was between that in rats and monkeys, the concentration ratios of M-18 in the papilla to cortex and papilla to medulla were remarkably high. In the in vitro studies, while nefiracetam itself showed no effects on the synthesis of prostaglandin (PG) E_2 and 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, in canine renal papillary slices, only M-18 among the metabolites clearly decreased both PG synthesis. The basal PG synthesis in canine renal papillary slices was extremely low relative to those in rats and monkeys. Taken together, certain factors such as basal PG synthesis, M-18 penetration into the renal papilla leading to an intrarenal gradient, and inhibitory potential of M-18 on PG synthesis were considered to be crucial for the occurrence of RPN in dogs.

Objective

The present study was carried out to elucidate the clinicopathological characteristics in the development of nefiracetam-induced RPN seen only in dogs, and to shed light on the proposed mechanisms contributing to the species differences in both in vivo and in vitro systems.

Materials and methods

Chemicals

Nefiracetam and its 4 metabolites, M-3, M-11, M-18, and M-20 were synthesized in

the Akita Factory or Research Technology Center of Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan). M-3 was the main metabolite in serum of all species tested including human and in urine of rats and monkeys, and was also a precursor of M-11. In dog urine, the main metabolite, M-10, was an extremely unstable sulfate of M-18 and conjugation usually led to detoxication. M-20 was identified in human serum and urine, and its metabolite pattern in human was essentially similar to that in monkeys. Therefore, M-3, M-11, M-18 and M-20 were selected for the present study as main metabolites of nefiracetam. Ibuprofen, an NSAID, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were the highest grade available from commercial sources.

Animals

Male Crj:CD(SD)IGS rats (211-255 g) aged 7 weeks, male LRE strain beagles (6.9-13.0 kg) aged approximately 7 months to 5 years, and female cynomolgus monkeys (2.9-3.4 kg) aged approximately 6 years were used. Since there was no sex difference in the onset of RPN in dogs (27, 31, 55), either sex was used in the present investigation. The animals were housed under controlled conditions at a temperature of 20-26°C, a relative humidity of 35-80%, a lighting cycle of 12 h (from 8:00 to 20:00 each day) and 15 or more change filtered air/h. Commercial diets were given *ad libitum* and the animals were allowed free access to water except on the sampling day for urine or serum. In this study, the first dosing week was designated as week 1. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co, Ltd.

Time course changes in renal toxicity in dogs after repeated oral administration of nefiracetam.

Nefiracetam was administered orally at 300 mg/kg/day in a gelatin capsule to 3 groups of 3-4 dogs each: group 1, treated for 8 weeks; group 2, treated for 11 weeks; group 3, treated for 8 weeks, and untreated for 32 weeks. In an additional group, the animals receiving a gelatin capsule alone, corresponding to group 3, were used as controls. The dose of 300 mg/kg/day was chosen because RPN in dogs given this dose was morphologically observed over an 11-week treatment (32). At the end of each treatment period, the dogs were killed for clinicopathological examination. Periodic urinalyses and blood biochemical analyses were performed before the start of treatment, once a week from weeks 1 to 12,

every two weeks from weeks 13 to 24, and every four weeks from weeks 25 to 40. Urine was accumulated in a collecting tray cooled on ice for approximately 17 h. During the collection, animals were deprived of food and water. After centrifugation (4°C, 1500 rpm, 10 min) of collected urine, approximately 5 mL of the supernatant was filtered through a membrane filter (0.8 μm, DISMIC-25cs, ADVANTEC, Tokyo, Japan). A 2.5-mL sample of the filtered supernatant was applied on the disposable column PD-10 (Sephadex® G-25 M, Amersham Biosciences UK Ltd., Buckinghamshire, UK) and 3.5 mL of 0.0625 M Tris-HCl (pH 6.8) was used for elution. In fresh urine, osmotic pressure was measured with an osmometer (model 3C2, Advanced Instruments, Inc., Norwood, MA, USA). Protein, creatinine (CRE), γ-glutamyl transpeptidase (γ-GTP), and *N*-acetyl-β-D-glucosaminidase (NAG) contents in the filtered urine supernatant; LDH and acid phosphatase (ACP) levels in the eluted urine were measured with commercially available kits purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Shionogi & Co., Ltd. (Osaka, Japan), or KAINOS Laboratories, Inc. (Tokyo, Japan) according to the manufacturer's instructions. Approximately 3.5 mL of blood was withdrawn from the cephalic vein into a tube containing polymer gel as a serum separating agent. Serum urea nitrogen (UN) and CRE were measured with a Hitachi 7350 auto-analyzer (Hitachi Ltd., Tokyo, Japan). Pathological examination was performed after the termination of each treatment period. The left kidney of dogs was fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd.), embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E), and examined light-microscopically.

Toxicokinetics in dogs after repeated oral administration of nefiracetam in comparison with those in rats and monkeys.

Serum and renal concentrations of nefiracetam, M-3, M-11, M-18, and M-20 were measured after repeated oral administration of nefiracetam at 180 mg/kg/day to 10 rats, 4 dogs, and 4 monkeys for 3 weeks. The dose of 180 mg/kg/day was selected as a steady dose that caused RPN in dogs after a 13-week treatment (56) without severe clinical changes seen at 300 mg/kg/day, and the treatment period was set at 3 weeks to obtain biodistribution of nefiracetam before the occurrence of severe tissue damage such as RPN. At termination, blood and the kidneys from all species were taken 2 h after the final dose. The kidneys were divided into three portions: cortex (cortex), outer medulla (medulla), and inner medulla including papilla (papilla). Each portion was minced and homogenized with a 2-fold

volume (v/w) of 0.0167 M KH_2PO_4 containing 7 vol% methanol. Only in rats, serum samples were obtained from 5 animals and kidney homogenates were prepared from the pooled tiny samples from 10 animals. Serum or kidney homogenate samples (100 μL), calibration standard samples (200 μL), or quality control samples (200 μL) was put into a tube to which 50 μL of an internal standard solution (20 vol% methanol for the blank), 100 μL of 50 vol% methanol, and 750 μL of 0.0167 M KH_2PO_4 were added. The mixture was vortexed for approximately 30 s. The kidney homogenate sample was then centrifuged at 2000 rpm for 3 min. The mixture (serum) or supernatant (kidney homogenate) was then applied to an activated Bond Elut C18 100 mg/1 mL cartridge (Varian Sample Preparation Products, Harbor City, CA, USA), and washed with 1 mL of 0.0167 M KH_2PO_4. A sample was eluted with 1 mL of 100% methanol and evaporated to dryness under conditions providing N_2 gas at approximately 40°C. The residue was dissolved in 1 or 2 mL of 15 vol% methanol and 20 μL of this solution was then injected into a high-performance liquid chromatography (HPLC) system comprising a 600S controller, 616 pump, 717 plus auto-sampler, CHM-D column heater, and symmetry C_{18} column (4.6 mm x 75 mm, 3.5 μm or 2.1 mm x 150 mm, 3.5 μm, Waters Corporation, Milford, MA, USA). The mobile phase was 10 mM ammonium formate buffer (pH 4.7)/methanol (1:1, v/v) or 0.2 vol% formate/methanol (3:1 or 5:1, v/v). The flow rate was 0.2 to 0.3 mL/min by gradient flow. Electrospray ionization liquid chromatography-mass spectrometry (ESI/LC/MS) was performed with a TSQ7000 mass spectrometer (Finnigan MAT, San Jose, CA, USA).

Analysis of PG synthesis in canine renal papillary slices

To investigate PG synthesis in renal papillary slices, PG concentrations in the conditioned culture medium were measured as follows. Non-treated dogs were killed by exsanguination under pentobarbital anesthesia (25 mg/kg, iv, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). The right kidney was removed and was divided vertically into 2 parts. Renal papillary slices were prepared according to the method described previously with some modifications (37, 38). Briefly, pieces of 5-mm diameter were cut with a coring tool (VITRON Inc., Tuscon, AZ, USA), and slices of approximately 300-μm thickness were prepared with a Brendal/Vitron tissue slicer (VITRON Inc., Tuscon, AZ, USA) in ice-cooled slicing buffer (pH 7.4). The slicing buffer was composed of 13 mM HEPES, 132 mM NaCl, 10 mM CH_3COONa, 4.8 mM KCl, 1.3 mM KH_2PO_4, 2.5 mM $CaCl_2 \cdot 2H_2O$, and 1.2 mM $MgSO_4 \cdot 7H_2O$. Renal papillary slices were placed in a non-tissue culture-treated 12-well

plate (Becton Dickinson and Company, Franklin Lakes, NJ, USA) with 1 mL of the conditioned medium comprising serum-free and phenol red-free Dulbecco's modified Eagle medium/F-12 Ham's medium (1:1) containing 50 U/mL penicillin, 50 μg/mL streptomycin, and 125 ng/mL amphotericin B (LIFE TECHNOLOGIES ORIENTAL, INC., Tokyo, Japan). Then the slices were pre-incubated at 37°C for 2 h under a 95% O_2/5% CO_2 atmosphere. After the pre-incubation period, the medium was replaced by 1 mL of the conditioned medium containing nefiracetam, M-3, M-11, M-18, and M-20, 0.2, 1, and 5 mM, dissolved in 0.5% dimethyl sulfoxide (DMSO) solution. Ibuprofen as a positive control was used at 0.1 mM. Slices were cultured for further 24 h, and the culture medium was collected and stored at -80°C until assay. PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations in the culture medium were determined with a commercial enzyme immunoassay system (Amersham Biosciences UK, Ltd.). Slices were weighed and homogenized in 0.5 mL of ice-cooled distilled water with an ULTRASONIC PROCESSOR (VC-130, IEDA TRADING CORPORATION, Tokyo, Japan). To 0.5 mL of ice-cooled distilled water, 0.1 mL of slice homogenate was added and passed through a membrane filter (0.8 μm, DISMIC-25CS, Advance Toyo Kaisha, Ltd., Tokyo, Japan). The solution was stored at -80°C until assayed for aspartate aminotransferase (AST) and LDH. A solution of 70% perchloric acid (Wako Pure Chemical Industries, Ltd.) was added (20 μL) to the remaining homogenate and mixed well. After centrifugation (4°C, 14,000 rpm) for 6 min, the resultant supernatant was used for the measurement of potassium. AST and LDH in the medium and the supernatant of slices were measured with a Hitachi 7350 automatic analyzer (Hitachi Ltd.) and potassium was measured with an automatic electrolyte analyzer Model 710 (Hitachi Ltd.). Additionally, to elucidate the basal PG synthesis in naive rats and monkeys, renal papillary slices were prepared and incubated with the conditioned medium containing 0.5% DMSO as in the dog study. PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations in the culture medium were determined.

Statistical analyses

All quantitative data are shown as the means ± standard deviation (S.D.) at each sampling point. In the two-group comparison, the homogeneity of the variance between groups was analyzed by F-test at each sampling point. Student's *t*-test or the Aspin-Welch's test was subsequently used, depending on homogeneity. In the multiple comparison, Bartlett's test was used for the analysis of variance, followed by Dunnett's test. For the qualitative urine data, Wilcoxon's rank sum test was used. A two-tailed p value less than 5%

was considered to be statistically significant. The in-house GLP computer system (FUJITSU LIMITED, Tokyo, Japan) or EXSAS ver. 5.10 (Arm Corporation, Osaka, Japan), a software package, was used for these analyses. For toxicokinetic data, no statistical analyses were performed because of the small number of animals (n = 1-5).

Results

Renal changes in dogs due to nefiracetam

Table 1 gives the summarized renal clinicopathological findings for rats, dogs, and monkeys. In the 13-week repeated oral treatment studies, neither rats nor monkeys showed any renal changes (30, 59). Conversely, according to previous data, dogs administered 180 mg/kg/day of nefiracetam had increases in urine volume and urinary protein and RPN with hemorrhage and cell infiltration of the medulla at the scheduled killing time after a 13-week treatment (56).

In the present study, dogs administered nefiracetam at 300 mg/kg/day over 8 weeks had occult blood in urine from weeks 5 to 32 (corresponding to recovery week 24). The severity of this change became less from week 16. Urinary osmotic pressure tended to decrease from week 5, and was significantly and progressively decreased from week 7. After reaching the nadir in week 12 (recovery week 4), it increased gradually, but did not return to the control level (Fig. 2). Urine volume was significantly increased in the treated dogs from week 8 with a peak in week 11, and thereafter decreased very slowly. However, this change had not returned to the control level by week 40 (recovery week 32, Fig. 2). The urinary LDH level rose in week 8, reached its highest value in week 11, began to decrease thereafter, and returned to the control level in week 36 (recovery week 28, Fig. 2). Urinary protein in the treated dogs increased from weeks 5 to 12 with individual fluctuation and statistically significant changes were seen after week 11. After week 12 (recovery week 4), although statistical significance was observed, the values of the urinary protein decreased.

Small but significant increases in serum UN and CRE were observed from weeks 12 to 40 (recovery week 32). Urinary CRE, ACP, γ-GTP, and NAG fluctuated significantly during the course of the experiment, but these changes were minor and of doubtful toxicological importance.

Table 1 Overview of renal clinicopathological findings in rats, dogs, and monkeys following repeated oral administration of nefiracetam

Animal species	Dose (mg/kg/day)	Treatment period (week)	Urinalysis	Serum creatinine	Renal pathology
Rat	480	13	No change	No change	No channge
Dog	60	13	No change	No change	No change
	180	13	Pro ↑, Vol ↑	No change	Renal papillrary necrosis, hemorrhage in the medulla, cell infiltration in the medulla
	300	5	OP (↓), Pro (↑)	No change	Not examined
		8	OP ↓, Vol ↑ Pro (↑), LDH ↑	(↑)	Necrosis of ductal epithelia in the papilla
		11	OP ↓, Vol ↑, Pro ↑, LDH ↑	(↑)	Renal papillary necrosis, hemorrhage in the papilla, cell infiltration in the medulla
Monkey	180	13	No change	No change	No change

Five-week-old SD rats of both sexes (10 rats of each sex/group) were orally received nefiracetam, 30, 120, or 480 mg/kg/day in a suspension with 0.5% carboxymethyl cellulose. Seven- to eleven-month-old beagles of both sexes (3-4 dogs of each sex/group) were orally received nefiracetam, 20, 60, 180, or 300 mg/kg/day in gelatin capsules. Approximately five-year-old male cynomolgus monkeys (3 monkeys/group) were orally received nefiracetam, 5, 30 or 180 mg/kg/day in a suspension with 0.5% carboxymethyl cellulose. Pro: urinary protein; Vol: urine volume; OP: urinary osmotic pressure; LDH: lactate dehydrogenase. ↑: statistically significant increase; ↓: statistically significant decrease; (↑): tendency to increase; (↓): tendency to decrease. [Tsuchiya et al., 2003 (59)].

At necropsy, dark red foci in the renal papilla were seen in 2 of 3 dogs killed in week 11. After a 32-week recovery period, the kidneys had a rough surface and irregular shape with dark red foci of the cortex, medulla, and/or papilla in all dogs. Light-microscopically, necrosis of ductal epithelial cells in the papilla was observed in the dogs killed in week 8 (Fig. 3B). In week 11, hemorrhage and necrosis of ductal epithelial cells in the papilla were observed in 2 of 3 and 3 of 3 dogs, respectively. One of these dogs had severe RPN accompanied by medullary tubular dilatation, epithelial hyperplasia of the collecting ducts and focal fibrosis (Fig. 3C). After a 32-week recovery period, all dogs showed slight to moderate RPN with tubular dilatation of the medulla, epithelial hyperplasia of the collecting ducts, necrosis of ductal epithelia in the papilla, and desquamation at the tip of the papilla (Fig. 3D).

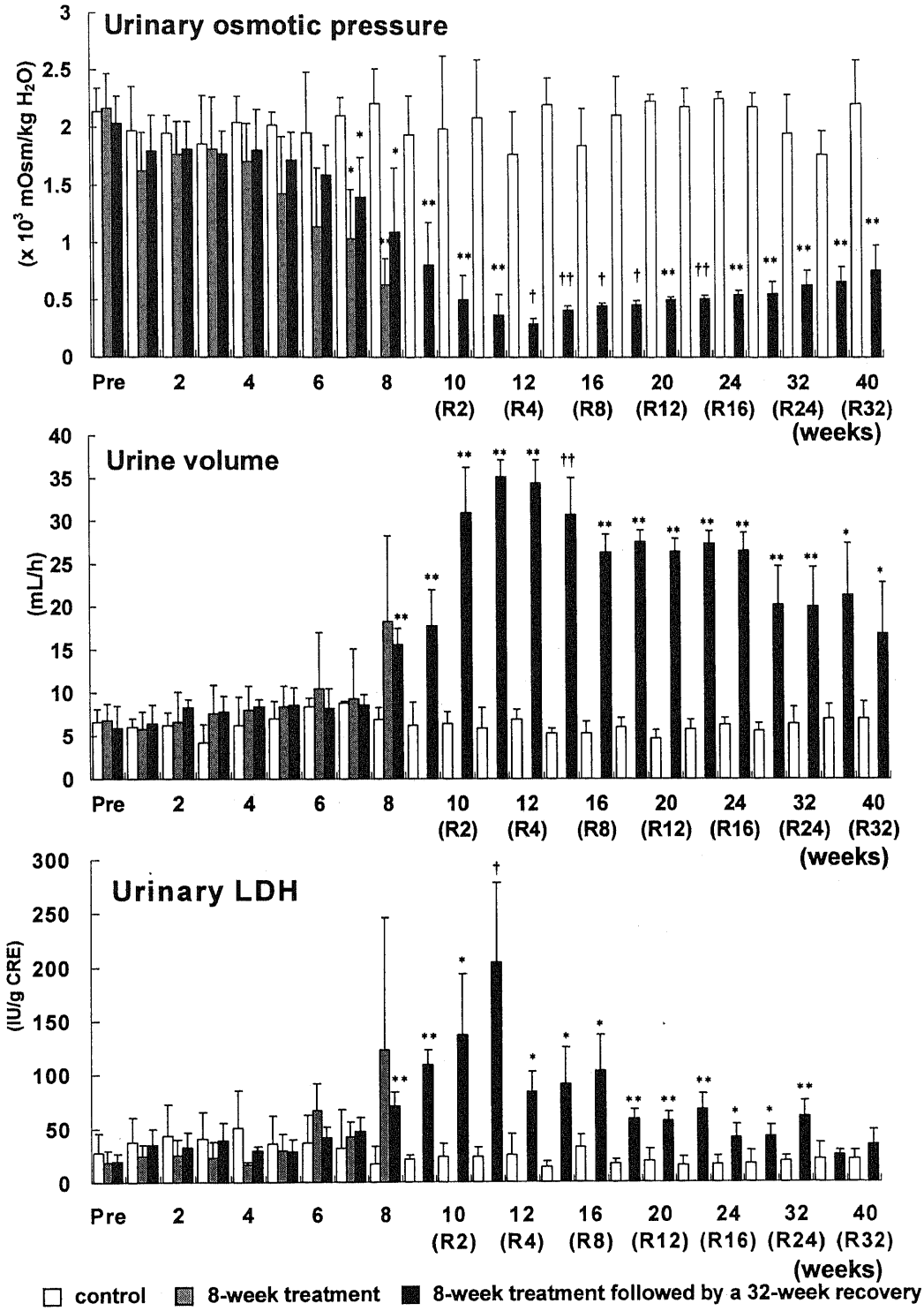


Fig. 2 Urinary osmotic pressure, urine volume, and urinary lactate dehydrogenase (LDH) values in male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day for 8 weeks, followed by a 32-week recovery period.

CRE: creatinine; Pre: pre-administration; R: recovery. Values are shown as the means ± S.D. (n = 3-4). *P<0.05, **P<0.01, significantly different from the control (Student's *t*-test). †P<0.05, ††P<0.01, significantly different from the control (Aspin-Welch's test).

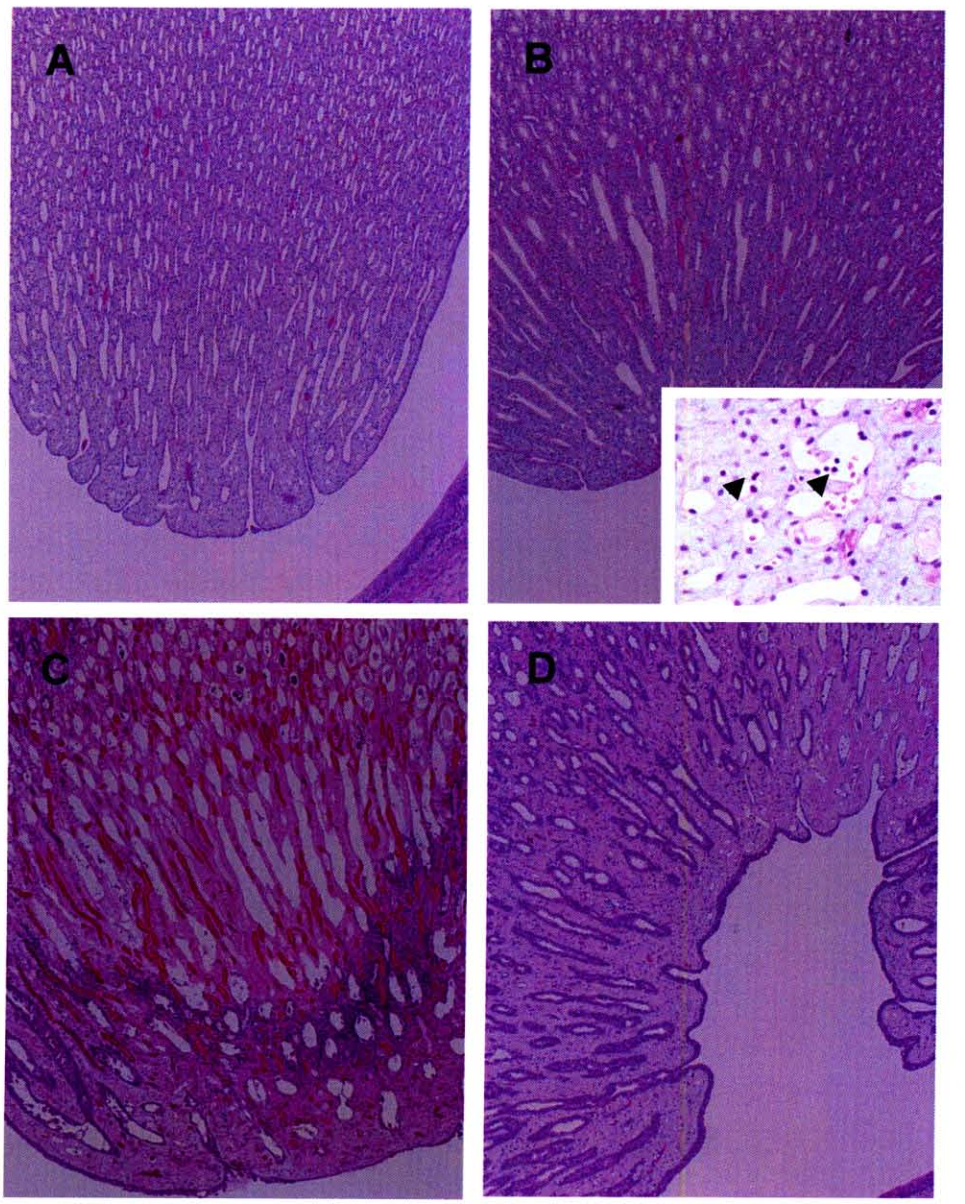


Fig. 3 Light micrograph of the renal papilla from male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day.
H&E staining. (A) Control in week 40, x 4. (B) Nefiracetam in week 8, x 4. Inset: necrosis of ductal epithelia in the papilla (arrow head), higher magnification, x 40. (C) Nefiracetam in week 11, x 4. Severe renal papillary necrosis with hemorrhage was seen. (D) Nefiracetam in week 40 (8-week treatment, followed by a 32-week recovery period), x 4. Only slight to moderate renal papillary necrosis with desquamation at the tip of the papilla was seen.

Toxicokinetics in dogs in comparison with those in rats and monkeys

Nefiracetam concentrations in sera and in each renal portion were somewhat higher in dogs than in rats and monkeys (Fig. 4). Of the 4 main metabolites, M-3 showed higher concentrations in all portions without species differences. M-11 and M-20 concentrations in each renal portion from rats and dogs were equal to or less than those from monkeys. M-18 showed higher concentrations in each renal portion only in monkeys; 0.78 μg/g for rat papilla, 3.30 ± 1.38 μg/g for dog papilla, and 13.41 ± 10.35 μg/g for monkey papilla (Fig. 4). However, both the papilla to cortex ratio and papilla to medulla ratio of M-18 were remarkably high in dogs (17.63 and 14.68, respectively) relative to those in rats (3.71 and 3.25) and monkeys (1.18 and 0.98). Ratios of the medulla to cortex, papilla to cortex, or papilla to medulla for nefiracetam and other metabolites were almost identical (0.85 to 2.75 for rats, 0.94 to 4.02 for dogs, and 1.02 to 1.40 for monkeys).

PG synthesis in canine renal papillary slices

The metabolite M-18 at 5 mM significantly decreased both PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis to 17.2% and 38.5%, respectively, as compared with the vehicle controls (Fig. 5). On the contrary, nefiracetam and its 3 other metabolites had no significant effects on either PG synthesis, even at 5 mM. Ibuprofen used as a positive control showed potent inhibitory effects on both PGE_2 and 6-keto-$PGF_{1\alpha}$ syntheses up to 3.9% and 6.4%, respectively. No changes were seen in AST and LDH leakage to the medium or in potassium content of all treated slices.

There was a great interspecies difference in basal PG synthesis in renal papillary slices. The rank order of PGE_2 (pg/mL/mg slice) and 6-keto-$PGF_{1\alpha}$ (pg/mL/mg slice) concentrations in the medium (from highest to lowest) was: monkeys (10394.7 ± 1265.0 and 12858.2 ± 1992.8, respectively) > rats (7668.2 ± 3516.0 and 4153.9 ± 1966.0) >> dogs (563.2 ± 223.2 and 751.3 ± 195.2). Of the species used, dogs showed the lowest basal PG synthesis.

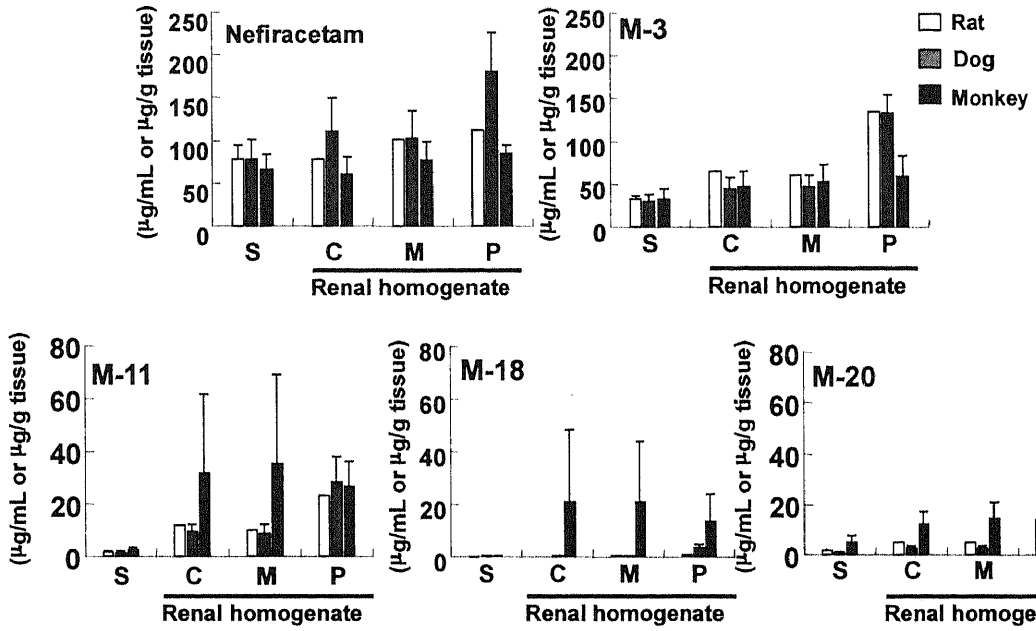


Fig. 4 Concentrations of nefiracetam and metabolites (M-3, M-11, M-18, and M-20) in sera (S) and renal cortex (C), medulla (M), and papilla (P) homogenates in rats, dogs, and monkeys.

Nefiracetam was given orally to 10 male rats, 4 male dogs, and 4 female monkeys at 180 mg/kg/day for 3 weeks. Two hours after the final administration (day 21), the kidneys were divided into 3 portions (cortex, medulla, and papilla), minced and homogenized with a 2-fold volume (v/w) of 1/60M KH_2PO_4 containing 7 vol% methanol. Concentrations were measured with an LC/MS/MS system. Values are shown as the means ± S.D. (n = 4-5) except for rat data (n = 1) from 10 animals, pooled.

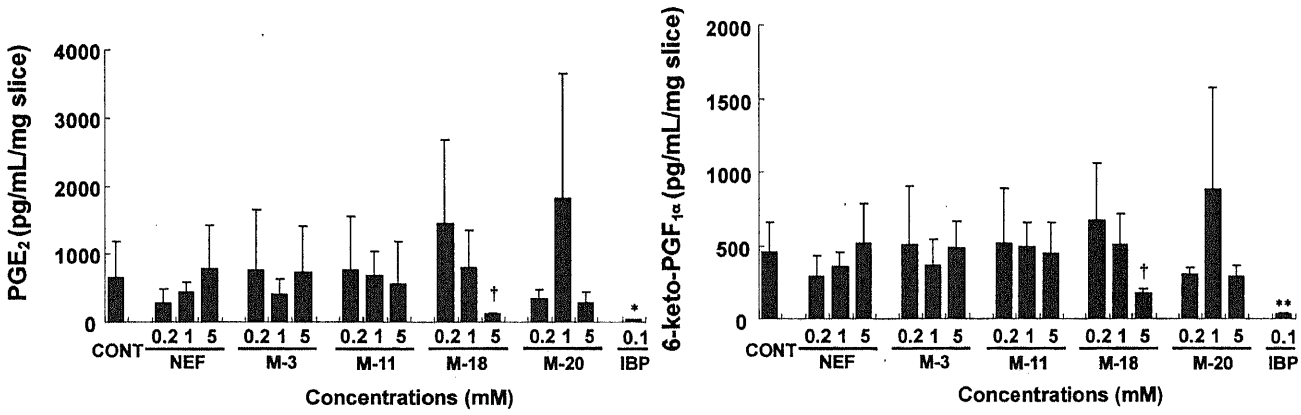


Fig. 5 Effects of nefiracetam (NEF) and metabolites (M-3, M-11, M-18, and M-20) on prostaglandin (PG) E_2 and 6-keto-prostaglandin (PG) $F_{1\alpha}$ synthesis in canine renal papillary slices.

NEF, M-3, M-11, M-18, and M-20 (0.2 to 5 mM), and ibuprofen (IBP, 0.1 mM) as a positive control were dissolved in 0.5% DMSO solution, and the slices were cultured with the test solutions for 24 h. Control (CONT) slices were treated only with the conditioned medium containing 0.5% DMSO solution. PGE_2 and 6-keto-$PGF_{1\alpha}$ in the medium were measured with enzyme immunoassay kits. Values are shown as the means ± S.D. (n = 4-8). *P<0.05, **P<0.01, significantly different from the control (Aspin-Welch's test). †P<0.05, significantly different from the control (Dunnett's test).

Discussion

Repeated oral administration of nefiracetam at a high dose caused RPN only in dogs. Briefly, decreased urine osmotic pressure was first noted in week 5; subsequently, urine volume and urinary LDH increased from week 8. These changes became most severe in week 11 or 12. Histopathologically, necrosis of ductal epithelial cells in the renal papilla was an initial change observed in week 8 when increased urinary LDH occurred. Although compounds such as analgesics and NSAIDs have been shown to evoke RPN, there are no specific and sensitive diagnostic biomarkers available by which changes in the papilla can be detected before irreversible lesions emerge. LDH, a cytosolic enzyme, has been reported to be relatively specific to the distal tubules (18, 48). In contrast, urinary CRE, ACP, or γ-GTP, a brush border enzyme in the proximal tubules, and NAG, a lysosomal enzyme in the proximal tubules and papilla, did not show any changes that would indicate progress in renal injury. Likewise, small but significant increases in serum UN and CRE were observed from week 12. Therefore, urinary LDH was thought to be a useful biomarker for detecting severe RPN induced by nefiracetam. Furthermore, based on the facts that urinary osmotic pressure, a marker for concentrating abilities in the distal tubules, started to decrease from week 5 prior to the observed changes in urinary LDH and that there were no evident morphological changes in the renal papilla at this time (32), we regard the decreased urinary osmotic pressure as an initial functional event in RPN. Though the recovery of decreased urinary osmotic pressure and increased urine volume was very slow, there was a tendency to recuperation by week 40 (recovery week 32).

In a previous study, of the dogs that died during repeated treatment periods (week 10 or 11) some showed severe RPN with hemorrhage (32). In the present study, severe RPN with hemorrhage was also observed in week 11, and slight to moderate RPN remained by week 40 (recovery week 32), suggesting that it takes a long period of time to repair the incurred morphological change. Meanwhile, the aforementioned change in urinary osmotic pressure was seen from week 5, and no macroscopic or light-microscopic changes were seen by week 4 (32). In view of these observations, it is considered that if the treatment were stopped when urinary osmotic pressure is decreased, nefiracetam-induced RPN could be preventable.

In our in vitro work, ibuprofen markedly decreased both PGE_2 and 6-keto-$PGF_{1\alpha}$ levels in canine renal papillary slices, in which either of these PGs induces vasodilation (39,

60). While nefiracetam itself showed inhibitory effects on neither PGE_2 nor 6-keto-$PGF_{1\alpha}$ synthesis in papillary slices, of the metabolites only M-18 decreased the synthesis of both PGs. Evidence thus suggests that M-18 may be a determinant for the induction of RPN in dogs.

RPN provoked experimentally by analgesics and NSAIDs has been noted mainly in rats, occasionally in rabbits and dogs, and rarely in monkeys (3, 5, 40). Alden and Frith (3) have also explained the high susceptibility of rats to RPN on the basis of renal anatomical and physiological characteristics (e.g., well-developed inner medulla, unipapillary kidney as in the dog, unlike monkeys and humans with multipapillary kidney, and high urine concentrating capacity). In addition, Khan et al. (34) have suggested that the difference in cyclooxygenase (COX) distribution within the kidney can also contribute to interspecies differences in the susceptibility to RPN. In the present study, the species difference in basal levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the renal papillary slices was also confirmed, and dogs had the lowest basal PG synthesis.

Nefiracetam-induced RPN was not seen in rats and monkeys (27, 30, 31, 59). It is considered that rats did not develop RPN because of the low concentration (0.78 μg/g) of M-18 in the renal papilla, in spite of the high sensitivity to RPN as mentioned above. In monkeys, higher PG contents may protect from the onset of RPN, even though M-18, which provokes a decrease in renal PGs, resulted in the highest concentration (13.4 μg/g) in the papilla. In dogs with the lowest basal PG synthesis, M-18 (3.30 μg/g) in the papilla was higher than that (0.78 μg/g) in rats. In addition, the papilla to cortex or papilla to medulla concentration ratio of M-18 was remarkably high only in dogs. From these results, extensive penetration and retention of M-18 into the renal papilla were considered to be crucial for the onset of nefiracetam-induced RPN. In fact, RPN in dogs was observed only after repeated administration of nefiracetam for a long period, but not after the single large dose at 500 mg/kg (57). These phenomena implied that a close relation among basal PG synthesis, M-18 penetration into the renal papilla with an intrarenal gradient, and inhibitory potential of M-18 on PG synthesis may explain the species difference. In human clinical trials, there has so far been no report dealing with the occurrence of RPN. This is supported by the assumption that metabolic patterns in human urine were essentially similar to those in monkeys.

There was a considerable gap between the M-18 concentration (3.30 μg/g) in the canine renal papilla in vivo and the concentration (5 mM: *ca* 1311.5 μg/mL) that inhibited

PG synthesis in vitro. This can be, at least partly, explained by the finding that an extremely high concentration under severe in vitro conditions (exposure time to M-18: 24 h) may be required since the long-term constant exposure of the canine renal papilla to M-18 was needed in the in vivo study. As only one time point of around C_{max} (2 h after administration) was selected as a toxicokinetic sampling time in the present study, further investigation will be needed to resolve these hypotheses.

No changes in AST or LDH leakage or in potassium contents were seen in the canine renal papillary slices exposed to nefiracetam and its 4 metabolites, suggesting the absence of direct cytotoxic effects.

As a proposed mechanism, the occurrence of RPN seen only in dogs may involve decreased levels of PGs, preceded by penetration of M-18 into the papilla.

Chapter 2. Analyses of urinary proteins and renal mRNA expression in canine renal papillary necrosis induced by nefiracetam

Summary

The occurrence of RPN seen only in dogs after repeated oral administration of nefiracetam at a relatively high dose is due to inhibition of renal PG synthesis by the nefiracetam metabolite M-18. In the present study, analyses of urinary proteins and renal mRNA expression were carried out to investigate the possible existence of a specific protein expressing characteristics of RPN evoked by nefiracetam. In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of urinary proteins from male dogs given nefiracetam at 300 mg/kg/day over weeks 5 to 11, a protein of around 40 kD, which was not seen in the control urine, and protein of around 30 kD emerged in distinct bands. Consequently, clusterin precursor was identified in the former band, and tissue kallikrein precursor was recognized in the latter band by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). By quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis with renal morphological aspects, individual findings showed that renal clusterin mRNA was increased in certain dogs showing severe renal injuries, and renal tissue kallikrein also increased presumably related to hemodynamics. These results demonstrate that changes in renal clusterin mRNA may reflect the progression or severity of RPN, whereas upregulation of tissue kallikrein mRNA may play a subsequently compensating role in the prevention of RPN.

Objective

In Chapter 1, dogs receiving nefiracetam at 300 mg/kg/day showed decreased urinary osmotic pressure, increased urine volume and urinary LDH from week 5, 8 and 8, respectively. These urinary parameters were considered to be useful biomarkers for the onset of RPN. The present study was performed to find out specific proteins expressing characteristics of RPN and to seek for the pathophysiological mechanism from a viewpoint of these analyses.

Materials and methods

Chemicals

Nefiracetam was synthesized in the Akita Factory of Daiichi Pharmaceutical Co., Ltd.

All other chemicals and reagents used were the highest grade available from commercial sources.

Animal treatment

A total of 16 male LRE strain beagles (6.9-13.0 kg) aged approximately 7 months to 5 years were used. Dogs were housed individually under controlled conditions at a temperature of 21-25°C, a relative humidity of 40-80%, a lighting cycle of 12 h (from 8:00 to 20:00 each day), and 15 or more changes filtered air/h. The animals were given *ca.* 300 g daily of commercial diets and were allowed free access to water except for the time of urine collection. Nefiracetam was administered orally at 300 mg/kg/day in a gelatin capsule to 3 groups of 4 animals each: group 2, treated for 8 weeks (animal Nos. 5, 6, 7 and 8); group 3, treated for 11 weeks (Nos. 9, 10, 11 and 12); group 4, treated for 8 weeks, untreated for 32 weeks (Nos. 13, 14, 15 and 16). In an additional group (group 1), animals receiving gelatin capsules alone, corresponding to group 4, served as a control (Nos. 1, 2, 3 and 4). In this study, the first dosing week was designated as week 1. At the end of the respective study periods, the dogs were killed by exsanguination from the axillary artery under pentobarbital anesthesia (25 mg/kg, iv), and the left kidney was removed. The dog No. 9 had only the unilateral kidney (left) at necropsy. The excised kidney was divided into two parts for renal mRNA analysis and histopathological examinations. For histopathological examinations, the kidney was fixed with 10% neutral buffered formalin, trimmed, embedded in paraffin wax, cut into 5-μm thick slices and stained with H&E. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co, Ltd.

SDS-PAGE of urinary proteins

Urine samples were collected before the start of treatment and once a week from weeks 1 to 11 from dogs in both the control (group 1) and 11-week treatment (group 3) groups. Urine was accumulated in a collecting tray cooled on ice for approximately 17 h. During the collection, the animals were deprived of food and water. After centrifugation (4°C, 1500 rpm, 10 min), approximately 5 mL of the urine supernatant was filtered through a membrane filter (0.8 μm, DISMIC-25cs, ADVANTEC), and a 2.5-mL sample was applied onto a PD-10 disposable column (Sephadex® G-25 M, Amersham Biosciences UK Ltd.) to remove interfering materials. The sample was eluted with 3.5 mL of 0.0625 M Tris-HCl (pH

6.8) and concentrated by ultrafiltration using an Ultrafree-4 centrifugal filter unit (Nihon Millipore Ltd.). The concentrated urine sample was diluted with 0.0625M Tris-HCl buffer (pH 6.8) to achieve a concentration of 3 mg/mL and was mixed with 2-fold Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) after adding 2-mercaptoethanol (19:1). The sample was then heated at 100°C for 15 min, and 10 μg of protein samples were loaded to PAGEL (10-20% gradient gel, ATTO CORPORATION, Tokyo, Japan) or 15% and 20% isocratic gels. The gels were stained by Coomassie G-250 (urine from control dogs, GelCode Blue stain reagent, PIERCE, Rockford, IL, USA) or silver (urine from treated dogs). The analysis was applied to the urine samples prior to treatment and in weeks 5, 8 and 11. As described in Chapter 1, dogs given nefiracetam revealed decreased urinary osmotic pressure in week 5, increased urinary LDH in week 8, and RPN in week 11; therefore, these time points (4 sampling points) were selected for the SDS-PAGE analysis. The urine from control dogs taken at 11 sampling points (pre-dose to week 40) was also analyzed as a basal reference.

Analysis of urinary proteins by an LC/ESI-MS/MS method

The several distinct bands showing much higher intensity in post-dose than in pre-dose or appearing only after treatment in SDS-PAGE analysis were chosen, cut off with a razor blade, and digested with 25 ng/μL trypsin. Afterward, peptides obtained were analyzed by an LC/ESI-MS/MS method using Magic 2002 HPLC systems (Michrom Bioresources Inc. CA, USA), HTC PAL auto-sampler (CTC Analytics, Zwingen, Switzerland) and LCQ Deca XP (Thermo Electron K.K., Yokohama, Japan) equipped with an electrospray ion source. The ESI parameters were as follows: Ion spray voltage, 2.0 kV; electrospray tip, fused silica (PicoTip FS360-50-15-CE, New Objective Inc., MA, USA); capillary temperature, 200°C; mass range, 350-2000 m/z. The MS spectra were interpreted and matched by using a MASCOT search engine from Matrix Science Ltd. (46).

Quantitative real-time RT-PCR

Renal expression of clusterin or tissue kallikrein mRNA, which was identified in the distinct bands of urinary proteins by the LC/ESI-MS/MS method and considered to be candidates for specific proteins representing characteristics of nefiracetam-induced RPN, was measured by a quantitative real-time RT-PCR method. Total RNA was extracted from a portion of the renal papilla with an Absolutely RNA^{TM} RT-PCR Miniprep Kit

(STRATAGENE, La Jolla, CA, USA), which included DNase treatment to remove genomic DNA. Extracted total RNA was converted to cDNA with cocktails comprising 1x PCR buffer (Applied Biosystems, Foster City, CA, USA), 5 mM $MgCl_2$, 1 mM dNTP mixture (TOYOBO CO., LTD., Osaka, Japan), 1 U/μL RNase inhibitor (TOYOBO CO., LTD.), 2.5 U/μL Random Primer (TAKARA BIO INC. Shiga, Japan), and 0.125 U/μL AMV Reverse Transcriptase XL (TAKARA BIO INC.). The primers and TaqMan probes for each target were designed by using of a Primer Express (Applied Biosystems) for the detection of target gene sequences. Real-time PCR data and analysis were collected in Applied Biosystems 7700 Sequence Detection System instruments (Applied Biosystems). The amount of each gene target is normalized to an endogenous control (18S rRNA, 20 x Pre-Developed TaqMan Assay Reagents, Applied Biosystems), and then expressed as the ratio to the mean control value (each value in a treated dog / the mean value of 3 control dogs).

The following primers and probes were used for PCR.

Clusterin:

forward primer, 5'-AAGTGACCAGGCCTTGCCT-3'; reverse primer, 5'-GCGGAGTCC AGAGGAAGGA-3'; TaqMan probe, 5'-AGGCGCTCCTGTCTCCTAACCCAG C-3'.

Tissue kallikrein:

forward primer, 5'-GCTGGGTCGCTACAACCTGT-3'; reverse primer, 5'-GGGAAGCTC TCTCTGACCTGG-3'; TaqMan probe, 5'-CGAGCATGAAGACACGGCCCAGTT T-3'.

Statistical Analyses

The quantitive data from renal gene expression analysis are represented as the group mean ± S.D. at each sampling point. The homogeneity of the variance between groups was analyzed by F-test at each sampling point. Student's *t*-test or Aspin-Welch's test was subsequently used, depending on homogeneity. A two-tailed p value less than 5% was considered statistically significant. EXSAS ver. 6.10 (Arm Corporation) was used for this analysis.

Results

Urinary protein analysis by SDS-PAGE and LC/ESI-MS/MS methods

In urine from a representative control dog (No. 3), the following two patterns were recognized by a combination procedure of SDS-PAGE and LC/ESI-MS/MS. The profile of pattern 1 (weeks 2, 4, 5 and 6 corresponding to 2 W, 4 W, 5 W and 6 W, upper panel in Fig. 6) comprised 2 distinct bands at around 15 kD (bands a and b), and prostatic tissue kallikrein precursor was identified in both bands (Table 2). Pattern 2 (Pre, 8 W, 11 W, 16 W, 24 W, 32 W and 40 W) consisted of 6 major distinct bands (c to h) except for the 2 bands in pattern 1. Numerous proteins such as albumin, serum albumin precursor and tissue kallikrein precursor were identified in those bands (Table 2).

Eighteen distinct bands in urine from 3 representative treated dogs (bands i to z, middle and lower panels in Fig. 6) showing different severities of renal injuries after 11-week treatment with nefiracetam, were also analyzed by LC/ESI-MS/MS. Briefly, these individual animals incurred only slight necrosis of ductal epithelia in the papilla (No. 10), slight necrosis of ductal epithelia in the papilla, hemorrhage in the papilla, and tubular dilatation in the medulla (No. 11), or severe RPN (No. 12). Albumin, clusterin precursor, tissue kallikrein precursor, prostatic tissue kallikrein precursor, hemoglobins, haptoglobins, apolipoproteins, and some other proteins were recognized in these distinct bands (Table 2).

Among these proteins, clusterin precursor was seen in two bands (k and o) at around 40 kD over weeks 5 to 11, and tissue kallikrein precursor was noted in two bands (j and n) at around 30 kD over weeks 8 to 11 (middle panel in Fig. 6). Thus, clusterin precursor, which was not observed in the control urine, and tissue kallikrein precursor, which has been known as a vasodilative protein were chosen for the following gene expression analysis.

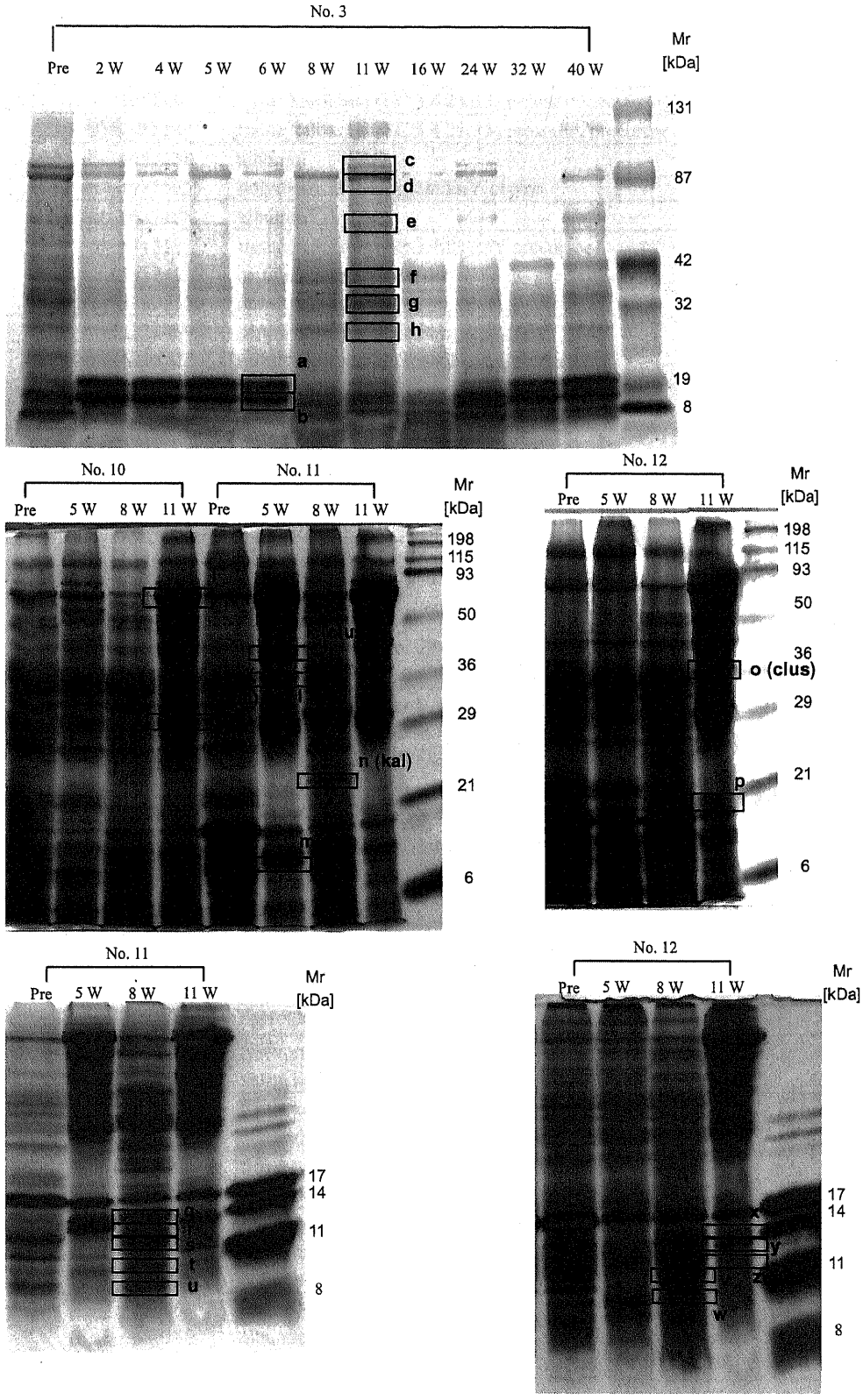


Fig. 6 SDS-PAGE patterns of urinary protein (10 μg) in male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day over 11 weeks. In the upper panel (control dog No. 3), a 0-20% gradient gel was used and stained by Coomassie G-250. In the middle (treated dog Nos. 10, 11, and 12) and lower (treated dog Nos. 11 and 12) panels, 15% and 20% isocratic gels were utilized for bands i to p and q to w, respectively, and stained with silver. The right lane in each panel showed a molecular marker. Pre: pre-administration; W: weeks; clus: clusterin precursor; kal: tissue kallikrein precursor.

Table 2 Canine urinary proteins identified by LC/ESI-MS/MS method for selected bands (a-z seen in Fig. 6)

Band No.	GI No.	Protein	Molecular weight
a	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
b	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
d	6687188	albumin	71046
	19715659	polymeric immunoglobulin receptor	40584
e	6687188	albumin	71046
f	631512	tissue kallikrein (EC 3.4.21.35), precursor	29675
	13124699	serum albumin precursor	71048
	37695552	annexin 2	38985
g	13124699	serum albumin precursor	71048
	631512	tissue kallikrein (EC 3.4.21.35), precursor	29675
	22347541	neutrophil elastase	30470
	123774	Ig heavy chain V region MOO	12837
h	13124699	serum albumin precursor	71048
	631512	tissue kallikrein (EC 3.4.21.35), precursor	29675
i	6687188	albumin	71046
j	6687188	albumin	71046
	631512	tissue kallikrein (EC 3.4.21.35) precursor	29675
k	6687188	albumin	71046
	116531	clusterin precursor (glycoprotein 80)	52467
l	123511	haptoglobin alpha and beta chains	37002
	6687188	albumin	71046
m	122368	hemoglobin alpha chain	15189
	122579	hemoglobin beta chain	16128
	70191	hemoglobin alpha-II chain	15380
	3915607	apolipoprotein A-I precursor	30178
	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
n	631512	tissue kallikrein (EC 3.4.21.35) precursor	29675
	3121745	major allergen Can fl precursor	19449
o	123511	haptoglobin alpha and beta chains	37002
	6687188	albumin	71046
	116531	clusterin precursor (glycoprotein 80)	52467
p	6687188	albumin	71046
	123511	haptoglobin alpha and beta chains	37002
q	8928189	lysozome C, spleen isoenzyme (1, 4-beta-N-acetylmuramidase C)	15137
	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
r	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
u	5441519	ubiquitin-ribosomal protein L40 fusion protein	15057
v	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
	125781	Ig kappa chain V region GOM	12140
w	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603
	6687188	albumin	71046
	3121745	major allergen Can fl precursor	19449
x	122579	hemoglobin beta chain	16128
	6687188	albumin	71046
	1304047	fibrinogen A-alpha-chain	46175
y	70191	hemoglobin alpha-II chain	15380
	122579	hemoglobin beta chain	16128
	122368	hemoglobin alpha chain	15189
	258499	haptoglobin heavy chain, HpH chain	26965
	123511	haptoglobin alpha and beta chains	37002
	6687188	albumin	71046
z	6687188	albumin	71046
	3915607	apolipoprotein A-I precursor	30178
	108118	tissue kallikrein (EC 3.4.21.35) prostatic, precursor	29603

No canine proteins were identified in bands c, s and t.

Gene expression analysis of clusterin and tissue kallikrein in canine renal papilla

Clusterin mRNA was increased in weeks 8, 11, and 40 (8-week treatment followed by a 32-week recovery) as shown in Table 3. The strongest increase in expression was seen in week 11 when RPN in dogs given nefiracetam initially became apparent (32), although statistical significance was noted only in week 40. Tissue kallikrein mRNA in the papilla also increased in weeks 8, 11 and 40. However, the level was relatively low as compared with that of clusterin mRNA in week 11. There were large individual variations in both papillary clusterin and tissue kallikrein mRNA expression.

Individual mRNA expression and renal histopathological findings are given in Table 4. In week 11, renal clusterin mRNA showed strong increases in dogs with severe morphological changes, such as ductal epithelial necrosis with hemorrhage in the papilla, and RPN (Nos. 9 and 12), while renal tissue kallikrein mRNA was extremely low. In animal No. 9, the renal injuries induced by nefiracetam may be deteriorated by the unilateral kidney. For two other dogs (Nos. 10 and 11) with slight to moderate necrosis of ductal epithelia and/or hemorrhage in the papilla without RPN, the expression patterns of clusterin and tissue kallikrein differed from those of the aforementioned dogs (Nos. 9 and 12). One dog (No. 10) exhibited no apparent increase in either clusterin or tissue kallikrein, consistent with only slight morphological changes; whereas the other dog (No. 11) displayed relatively high clusterin mRNA expression with an equivalent increase in tissue kallikrein mRNA (clusterin: 61.20 vs. tissue kallikrein: 42.75). Of dogs (Nos. 5, 6, 7 and 8) receiving 8-week treatment, one dog (No. 6) showed the strongest increase in clusterin mRNA and displayed slight ductal epithelial necrosis in the papilla with hemorrhage. Three remaining dogs with exceedingly mild renal injuries demonstrated different patterns of clusterin and tissue kallikrein mRNA expression; namely, the dog No. 5 had decreased clusterin and markedly increased tissue kallikrein. Two others (Nos. 7 and 8) showed somewhat increased clusterin, and further the dog No. 8 had slightly increased tissue kallikrein. From these results, the meaning of increases in renal clusterin and tissue kallikrein mRNA was equivocal in dogs with no apparent renal injuries at the early stage of nefiracetam treatment.

After the 32-week recovery period, the dogs showed similarly mild increases in both clusterin and tissue kallikrein, except for one dog (No. 16) showing extremely high tissue kallikrein expression. These dogs exhibited desquamation of the papillary tip and slight to moderate RPN, which suggests that there was very slow progression of RPN or none after

the 32-week recovery.

Table 3 Group mean renal papillary clusterin and tissue kallikrein mRNA expression of male dogs given repeated oral administration of nefiracetam at 300 mg/kg/day over 11 weeks

Dose (Treatment period)	Normalized by 18S rRNA	
	Clusterin	Tissue kallikrein
Control (8 W + 32 RW)	0.265 ± 0.127	0.0969 ± 0.110
Nefiracetam 300 mg/kg/day (8 W)	11.9 ± 12.6	10.2 ± 19.7
Nefiracetam 300 mg/kg/day (11 W)	14.7 ± 13.4	1.07 ± 2.04
Nefiracetam 300 mg/kg/day (8 W + 32 RW)	2.30 ± 1.06*	22.2 ± 43.6

Values are shown as the mean ± S.D. (n = 4).

N/A: not applicable.

W: weeks; RW: recovery weeks.

Statistical significance: *P<0.01 by Aspin-Welch's test.

Table 4 Individual renal papillary clusterin and tissue kallikrein mRNA expression and renal histological findings of male dogs given repeated oral administration of nefiracetam at 300 mg/kg/day over 11 weeks

Dose	Control				Nefiracetam 300 mg/kg/day				Nefiracetam 300 mg/kg/day				Nefiracetam 300 mg/kg/day			
(Treatment period)	(8 W + 32 RW)				(8 W)				(11 W)				(8 W + 32 RW)			
Animal No.	1	2	3	4	5	6	7	8	9[a]	10	11	12	13	14	15	16
Renal papillary mRNA expressions (normalized by the mean control value)																
Clusterin	N/A	N/A	N/A	N/A	0.891	101.00	10.2	68.0	37.3	1.28	61.2	122	6.37	14.6	6.23	7.46
mean ± S.D.	N/A				45.0 ± 47.7				55.4 ± 50.6				8.67 ± 3.99*			
Tissue kallikrein	N/A	N/A	N/A	N/A	410	3.03	1.76	4.27	0.144	1.23	42.8	0.254	3.62	2.70	6.90	904
mean ± S.D.	N/A				105 ± 204				11.1 ± 21.1				229 ± 450*			
Renal histopathological findings																
papillary necrosis	-	-	-	-	-	-	-	-	+++	-	-	+++	+	++	+	+
tubular dilatation, cortex	-	-	-	-	-	+	-	-	+++	-	-	-	-	-	-	-
tubular dilatation, medulla	-	-	-	-	-	+	-	-	+	-	+	-	+	+	+	+
hemorrhage, papilla	-	-	-	-	-	+	-	-	+++	-	+	+	+	+	+	-
ductal epithelial necrosis, papilla	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+
mineralization, papilla	-	-	-	-	+	+	+	+	-	+	-	-	-	+	+	-
focal fibrosis	-	-	-	-	-	-	-	-	-	+	-	-	+	+	++	+

-: no change; +: slight; ++: moderate; +++: marked.

W: weeks; RW: recovery weeks. N/A: not applicable.

[a]: The animal No. 9 had the unilateral kidney.

Statistical significance: *P<0.01 by Aspin-Welch's test.

Discussion

Urinary proteins obtained from dogs given nefiracetam at 300 mg/kg/day over 11 weeks were analyzed by the combination of SDS-PAGE and LC/ESI-MS/MS methods. Clusterin and tissue kallikrein precursors were identified in the distinct bands of urinary proteins by SDS-PAGE from weeks 5 to 11 and from weeks 8 to 11, respectively. In gene expression analysis by a quantitative real-time RT-PCR with renal morphological aspects, papillary clusterin mRNA was markedly increased in dogs showing severe morphological changes, such as ductal epithelial necrosis in the papilla with hemorrhage and RPN. In contrast, tissue kallikrein mRNA was only slightly increased in the papilla of those animals or markedly increased in the papilla of animals showing mild changes.

Clusterin is an 80 kD heterodimeric glycoprotein existing in many tissues, which has been known by numerous names including sulfated glycoprotein (SPG-2), glycoprotein 80 (GP-80), human serum protein (SP 40, 40), testosterone-repressed prostate message-2 (TRPM-2) or protein-2 (TRPP-2) and ApoJ (4, 9). This protein has been reported to show bands at 35-45 kDa, corresponding to the reduced individual subunits (22). In the present study, 2 bands appearing at around 40 kD were recognized to contain clusterin precursor. Although the precise functions of clusterin have not been completely understood, it has been proposed to function in the regulation of complement activity, the protection of cells from stress, the transport of lipids, and sperm maturation. Furthermore, its production is also increased in the early stages of normal organ development and after tissue injuries (24). Sensiber et al. (53) and Conner et al. (16) have suggested that clusterin acts as a marker for cell death, and that its expression is upregulated in various normal and malignant tissues undergoing apoptosis. Aulitzky et al. (4) have shown that levels of urinary and renal clusterin proteins increase prior to changes in serum creatinine in rats treated with gentamicin. In the present work, strong increases in renal papillary clusterin mRNA consistent with the severe renal injuries were seen in week 11, suggesting that renal necrotic changes under the rapid progression of RPN are reflected in increases in clusterin mRNA expression.

Kallikrein is one of the serine proteases, which release vasodilative kinins from kininogens, and is generally classified into 2 groups, i.e., plasma kallikrein (EC 3.4.21.34) and tissue kallikrein (EC 3.4.21.35). Plasma kallikrein is a basic glycoprotein with a molecular weight of about 100kD, which is present in blood and releases bradykinin, while

tissue kallikrein is an acidic glycoprotein with a molecular weight of about 40kD, which is mainly located in the submaxillary glands, kidney, and pancreas, and releases kallidin (45). Recently, in addition to the known classical tissue kallikrein, kallikrein-related enzymes have also been recognized in other tissues, especially in the prostate of mammalian species. This enzyme is clearly a member of the kallikrein gene family, but has unknown physiological substrates (13). Murthy et al. (41) has demonstrated that mammalian urinary kallikreins are tissue kallikreins of renal origin and a kallikrein of 38.5 kD is found in canine urine. In our investigation, a tissue kallikrein precursor was identified in distinct bands at around 30 kD over weeks 8 and 11 of treatment with nefiracetam, while prostatic tissue kallikrein precursor was detected mainly in the control urine as two distinct bands of approximately 15 kD, indicating that tissue kallikrein precursor originating from the prostate was generally predominant in normal dogs.

Renal hemodynamics as well as sodium and water homeostasis were well regulated by the renin-angiotensin system (RAS), renal PGs, kallikrein-kinin system (KKS), and renal dopamines (10). The RAS stimulation reduces the renal blood flow, glomerular filtration rate, and urinary sodium excretion, whereas KKS promotes an increase in renal hemodynamic and excretory functions (54). The kinins are degraded by several enzymes, among which the most important one is kininase II, identical to angiotensin converting enzyme (ACE). Furthermore, the kinins stimulate PG synthesis in the kidney, and PG may enhance the effect of the kinins and modulate the effects of the vasoconstrictor, angiotensin II (10). In our previous study (Chapter 1), PG synthesis in canine renal papillary slices was decreased by M-18 among the metabolites of nefiracetam, and this was thought to cause ischemic injury, followed by RPN. During the treatment period (until week 11), dogs showing mild renal injury without RPN revealed an upregulation of renal papillary tissue kallikrein mRNA. These results suggested that after the metabolite M-18 inhibited PG synthesis in the renal papilla, compensatory synthesis of kallikrein for vasodilative kinin production would occur and consequently the progression of RPN may be prevented. Renal kallikrein has been known to be predominant in the cortex, whereas the medulla and papilla have a very little kallikrein (12). In our results, tissue kallikrein mRNA expression was generally higher in the cortex than in the papilla, and changes in the intra-group expression were milder in the cortex than in the papilla. The initial renal morphological change seen in dogs given nefiracetam was necrosis of ductal epithelial cells in the papilla (Chapter 1). Since the constitutional level of tissue kallikrein in the papilla is extremely low and further

PG synthesis in this area was inhibited by nefiracetam metabolite M-18, RPN in dogs showing decreased tissue kallikrein expression in the papilla may reflect an attenuation of hemodynamics. In other words, these findings demonstrate that increased tissue kallikrein plays a crucial role in the improvement of hemodynamics in the renal papilla.

In conclusion, clusterin and tissue kallikrein precursors were identified in the distinct bands of urinary proteins from dogs given nefiracetam. The changes in renal clusterin mRNA may reflect the progression or severity of RPN, whereas upregulation of tissue kallikrein mRNA may play a subsequently compensating role in the prevention of RPN.

Chapter 3. Early pathophysiological features in canine renal papillary necrosis induced by nefiracetam

Summary

To ascertain the early pathophysiological features in RPN, nefiracetam was administered orally to male beagle dogs at 300 mg/kg/day for 4 to 7 weeks. Additionally, to compare the process in its onset, ibuprofen, an NSAID that was well-known to induce RPN, was administered orally to another dogs at 50 mg/kg/day for 5 weeks. During the dosing period, the animals were subjected to laboratory tests, light-microscopic, immunohistochemical, and electron-microscopic examinations and/or COX-2 mRNA analysis. Of the laboratory tests, urinary osmotic pressure, volume, and LDH were confirmed to be the most susceptible biomarkers to RPN. Light-microscopically, nefiracetam induced epithelial swelling and degeneration in the papillary ducts in week 7, while ibuprofen displayed degeneration and necrosis in the papillary interstitium in week 5. In immunohistochemical stainings with COX-2 antibody, nefiracetam elicited a positive reaction within interstitial cells around the affected epithelial cells in the papillary ducts (upper papilla) in week 7, and ibuprofen exhibited a positive reaction within interstitial cells adjacent to the degenerative/necrotic lesions in week 5. Ultrastructurally, nefiracetam displayed reductions of intracellular interdigitation and infoldings of epithelial cells in the papillary ducts, whereas ibuprofen showed no changes in the identical portions. Thus, the early morphological change in the papilla brought about by nefiracetam was quite different from that elicited by ibuprofen. In the renal papillary COX-2 mRNA expression analysis, nefiracetam markedly decreased its expression in week 4, but increased in week 7, suggesting an induction of COX-2 mRNA by renal papillary lesions. These results demonstrate that the epithelial cells in the papillary ducts is the primary target site for the onset of RPN evoked by nefiracetam.

Objective

The present study was performed to clarify the early pathophysiological features in RPN seen in dogs orally given nefiracetam at 300 mg/kg/day for 4 to 7 weeks, in comparison with those in ibuprofen, an NSAID, at 50 mg/kg/day for 5 weeks.

Materials and methods

Chemicals

Nefiracetam was synthesized in the Akita Factory of Daiichi Pharmaceutical Co., Ltd. Ibuprofen was purchased from Sigma Chemical Co. All other chemicals and reagents were the highest grade available from commercial sources.

Animal Treatment

A total of 22 male LRE strain or Nosan beagles (8.9-12.6 kg) aged approximately 17 to 19 months were used. Dogs were housed individually under controlled conditions at a temperature of 21-25°C, a relative humidity of 40-80%, a lightning cycle of 12 h (from 8:00 to 20:00 each day) and 15 or more changes filtered air/h. The animals were given ca. 300 g daily of commercial diets and were allowed free access to water except for urine or serum collection. They were divided into 6 groups as follows. Group 1, intact control group (Nos. 1, 2 and 3); Group 2, nefiracetam treatment group at 300 mg/kg/day for 4 weeks (Nos. 4, 5, 6, and 7); Group 3, nefiracetam treatment group at 300 mg/kg/day for 5 weeks (Nos. 8, 9, 10 and 11); Group 4, nefiracetam treatment group at 300 mg/kg/day for 6 weeks (Nos. 12, 13, 14 and 15); Group 5, nefiracetam group at 300 mg/kg/day for 7 weeks (Nos. 16, 17, 18 and 19) and Group 6, ibuprofen group at 50 mg/kg/day for 5 weeks (Nos. 20, 21 and 22). In this study, the first dosing week was designated as week 1. Periodic laboratory tests were performed in dogs from groups 1 (non-treated control), 5 (nefiracetam for 7 weeks), and 6 (ibuprofen for 5 weeks) before the commencement of treatment and weekly thereafter. At the end of the respective treatment periods, the dogs were killed by exsanguination from the axillary artery under pentobarbital anesthesia (25 mg/kg, iv, Dainippon Pharmaceutical Co., Ltd.), and the bilateral kidneys were removed. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co, Ltd.

Laboratory tests

The laboratory tests were conducted to monitor the early alterations in nefiracetam-induced RPN which was characterized by decreased urinary osmotic pressure, increased urine volume and urinary LDH with trivial increases in serum CRE and UN as mentioned in Chapter 1. Urine and blood were collected, and urinary osmotic pressure,

volume, and LDH and serum CRE and UN were examined by the same procedure as described in Chapter 1.

Light microscopy

The left kidney was removed, fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd.), embedded in paraffin wax, sectioned at 5-μm thickness, stained with H&E and alcian blue, and examined microscopically.

Immunohistochemical staining of COX-1 or COX-2 in the renal papilla

A section from the left kidney was deparaffinized, and an antigen was retrieved with Dako Proteinase K solution (Dako Cytomation Co., Ltd., Kyoto, Japan). After incubation with 3% hydrogen peroxide for 5 min, the sections were preblocked with a Non-Specific Staining Blocking Reagent containing 0.25% casein and carrier protein (Dako Cytomation Co., Ltd.), and then were incubated with a primary antibody overnight at 4°C. As COX-1 and COX-2 primary antibodies, ovine COX-1 polyclonal rabbit antiserum (No. 160108, Cayman Chemical Co., Ann Arbor, MI, USA) and murine COX-2 polyclonal rabbit antibody (No. 160106, Cayman Chemical Co.), respectively, were used. These antibodies were diluted 1:400 with 0.1% Tween-Tris buffer. Immunoreactive complexes were detected via an LSAB 2 Systems-HRP (Dako Cytomation Co., Ltd.) and visualized with diaminobenzidine (DAB, Dako Cytomation Co., Ltd.), which reacts with peroxidase to give a brown reaction product. The sections were counterstained briefly with hematoxylin. All control sections were treated with no primary antibody.

Electron microscopy

Since apparent morphological alterations were light-microscopically noted in week 7 for nefiracetam and in week 5 for ibuprofen, electron-microscopic examination was performed only for these points. A tiny portion from the left kidney was removed, fixed in 3% glutaraldehyde and 2% osmic acid, dehydrated with alcohol, and embedded in epoxy resin. Thick sections were made and stained with 1% toluidine blue for light microscopic survey. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a transmission electron microscope (H-500, Hitachi Ltd.).

Quantitative real-time RT-PCR analysis of papillary COX-2 gene expression

Inasmuch as the early morphological features in RPN brought about by nefiracetam were quite different from those by ibuprofen, serial changes in the renal papillary COX-2 mRNA expression were assessed only in nefiracetam-treated groups. Total RNA was extracted from an approximately 40 mg portion of the papilla from the right kidney and converted to cDNA by the same method described in Chapter 2 with following primers and TaqMan probes designed by using a Primer Express (Applied Biosystems). Forward primer, 5'-CTG TGG GCC AGG AGG TCT T-3'; Reverse primer, 5'-CAC TCT GTT ATG CTC CCG CAG-3'; TaqMan probe, 5'-TGC CTG GTC TGA TGA TGT ATG CCA CC-3'. The real-time PCR data and analyses were collected in Applied Biosystems 7700 Sequence Detection System instruments. The amount of each gene target is normalized to an endogenous control (18S rRNA, 20 x Pre-Developed TaqMan Assay Reagents, Applied Biosystems).

Statistical analyses

The quantitative data from laboratory tests and COX-2 mRNA expression analysis are represented as the group mean ± S.D. at each sampling point. For the data of laboratory tests, the following 2 analyses were performed, and only the second method was applied for the renal gene expression data: 1) paired *t*-test compared with pre-dose values were applied; 2) the homogeneity of the variance between groups was analyzed by F-test at each sampling point. Student's *t*-test or Aspin-Welch's test was subsequently used, depending on the homogeneity. A two-tailed p value less than 5% was considered to be statistically significant. EXSAS ver. 6.10 (Arm Corporation), a software package, was used for these analyses.

Results

Laboratory tests

In dogs receiving nefiracetam, decreased urinary osmotic pressure and a tendency of increasing urine volume were observed in weeks 6 to 7. Urinary LDH tended to be higher from week 5. Serum UN showed somewhat higher values than that in the pre-dose or control group throughout the study period without any remarkable increase in serum CRE. In dogs given ibuprofen, a decrease in urinary osmotic pressure, increases in urine volume and urinary LDH and a slightly increase in serum UN were sporadically noted in weeks 1 to

5 (Fig. 7). All alterations seen in ibuprofen were earlier and severer than those in nefiracetam.

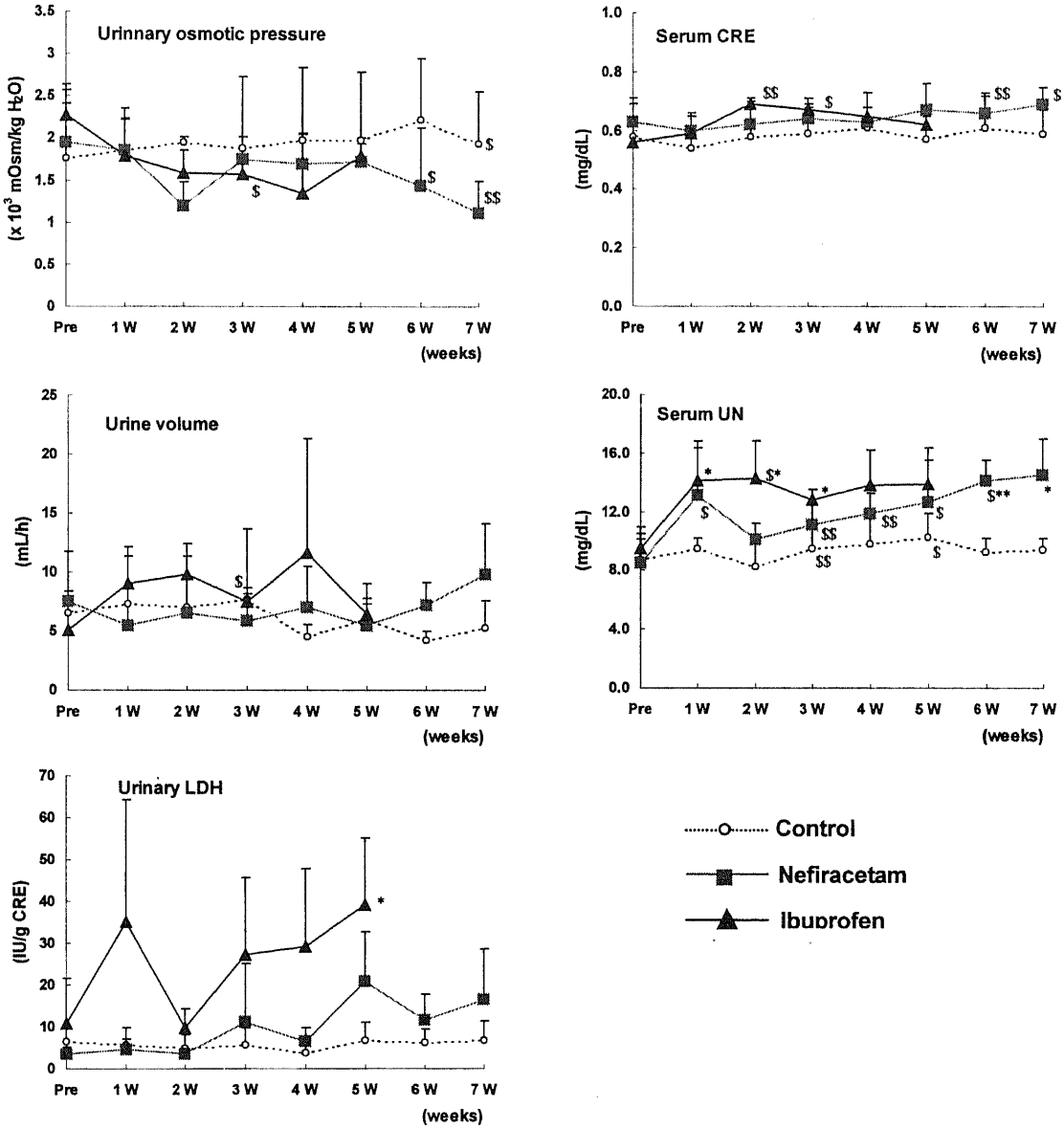


Fig. 7 Urinary osmotic pressure, urine volume, urinary lactate dehydrogenase (LDH), serum creatinine (CRE) and serum urea nitrogen (UN) values in male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day for 7 weeks or ibuprofen at 50 mg/kg/day for 5 weeks.

Pre: pre-administration. Values are shown as the mean ± S.D. (n = 3-4). *P<0.05, significantly different from the control (Student's *t*-test). $P<0.05, $$P<0.01, significantly different from the pre-value (Paired *t*-test).

Light microscopy

In dogs given nefiracetam, no treatment-related changes were observed by week 6. In week 7, however, epithelial swelling of the papillary ducts (upper papilla) was seen in 3 of 4 dogs with increased eosinophilic-staining intensity in the cytoplasm of epithelial cells, and nuclei located in the basal side (Table 5 and Fig. 8C). Degeneration and desquamation of epithelial cells was sometimes noted (Fig. 8C, inset). The alcian-blue staining intensity was comparable to the control throughout the dosing periods (Fig. 8D). Mineralization of the papilla without detectable change in interstitial cells was noted in 2 of 4 dogs (Table 5). In dogs receiving ibuprofen, degeneration and necrosis of the interstitium in the papillary tip were noted in 2 of 3 dogs (Table 5 and Fig. 8E); however, no remarkable changes were noted in the remaining papillary ducts. The alcian-blue staining intensity was markedly decreased in the degenerative/necrotic areas of the papillary interstitium (Fig. 8F). Mineralization of the papilla was seen in 1 of 3 dogs (Table 5).

Immunohistochemical staining of COX-1 or COX-2 in the renal papilla

In the COX-1 staining, there were no significant differences among the control, nefiracetam, and ibuprofen groups (Table 5).

In the COX-2 staining, the immunoreaction in control dogs was seen in the cytoplasm of interstitial cells in the papillary tip (Table 5 and Fig. 9A). In dogs receiving nefiracetam, though the immunoreaction was within the cytoplasm of interstitial cells in the papillary tip by week 6, similarly as in the control, interstitial cells around affected epithelial cells (upper papilla) were positively reacted in week 7 (Table 5 and Fig. 9B). In dogs given ibuprofen, a marked positive immunoreaction was seen within interstitial cells adjacent to the degenerative/necrotic areas of interstitium in the papilla (Table 5 and Fig. 9C).

Table 5 Light-microscopy and immunohistochemistry of the kidney in male dogs receiving repeated oral administration of nefiracetam or ibuprofen for 4 to 7 weeks

Dose	Control			Nefiracetam 300 mg/kg/day																Ibuprofen 50 mg/kg/day		
Treatment period	7 weeks			4 weeks				5 weeks				6 weeks				7 weeks				5 weeks		
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Light microscopy																						
Papillary duct																						
swelling, epithelia	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
degeneration, epithelia	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
Papillary tip																						
degeneration/necrosis, interstitium	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+
decrease, alcian-blue staining intensity, interstitium	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+
Papilla																						
mineralization	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
Immunohistochemistry in the papilla																						
COX-1																						
collecting ducts, epithelia, cytoplasm	+	N	+	N	+	+	N	+	+	N	N	+	+	N	N	N	N	+	+	N	+	+
ducts, epithelia, cytoplasm, tip of papilla	-	N	-	N	-	-	N	+	+	N	N	+	-	N	N	N	N	-	+	N	+	+
transitional epithelium, cytoplasm, pelvis	+	N	+	N	+	+	N	+	+	N	N	+	+	N	N	N	N	+	+	N	+	+
COX-2																						
interstitial cells, tip of papilla	+	N	+	N	+	+	N	+	+	N	N	+	+	N	N	N	N	-	-	N	-	-
interstitial cells, upper papilla	-	N	-	N	-	-	N	-	-	N	N	-	-	N	N	N	N	+	+	N	-	-
interstitial cells, around degenerative/necrotic lesions	-	N	-	N	-	-	N	-	-	N	N	-	-	N	N	N	N	-	-	N	++	++

-: no change; +: slight; ++: moderate.

NP: not examined because the papilla was not present. N: not examined.

Electron microscopy

In control dogs, epithelial cells of the papillary ducts showed a cuboidal appearance with the nucleus located in the center of the cell, and short microvilli were seen on the luminal surface. The lateral plasma membrane was interdigitated, and the basal membrane displayed infoldings. Small numbers of mitochondria and vacuoles were noted in the cytoplasm (Fig. 10A). In dogs receiving nefiracetam, epithelial cells of the papillary ducts showed swelling and the microvilli were decreased or absent. Membranous blebs were noted on the luminal surface. Intracellular interdigitation and infoldings in both lateral and basal membranes were inconspicuous. These epithelial cells revealed sparse cytoplasm containing large vacuoles (Fig. 10B). In dogs given ibuprofen, no remarkable changes in the epithelial cells of the papillary ducts were noted.

COX-2 mRNA expression in the renal papilla

Nefiracetam exceedingly decreased COX-2 mRNA expression in week 4, but it recovered to the basal control level in weeks 5 to 6. Afterward, in week 7, COX-2 mRNA markedly increased (Fig. 11).

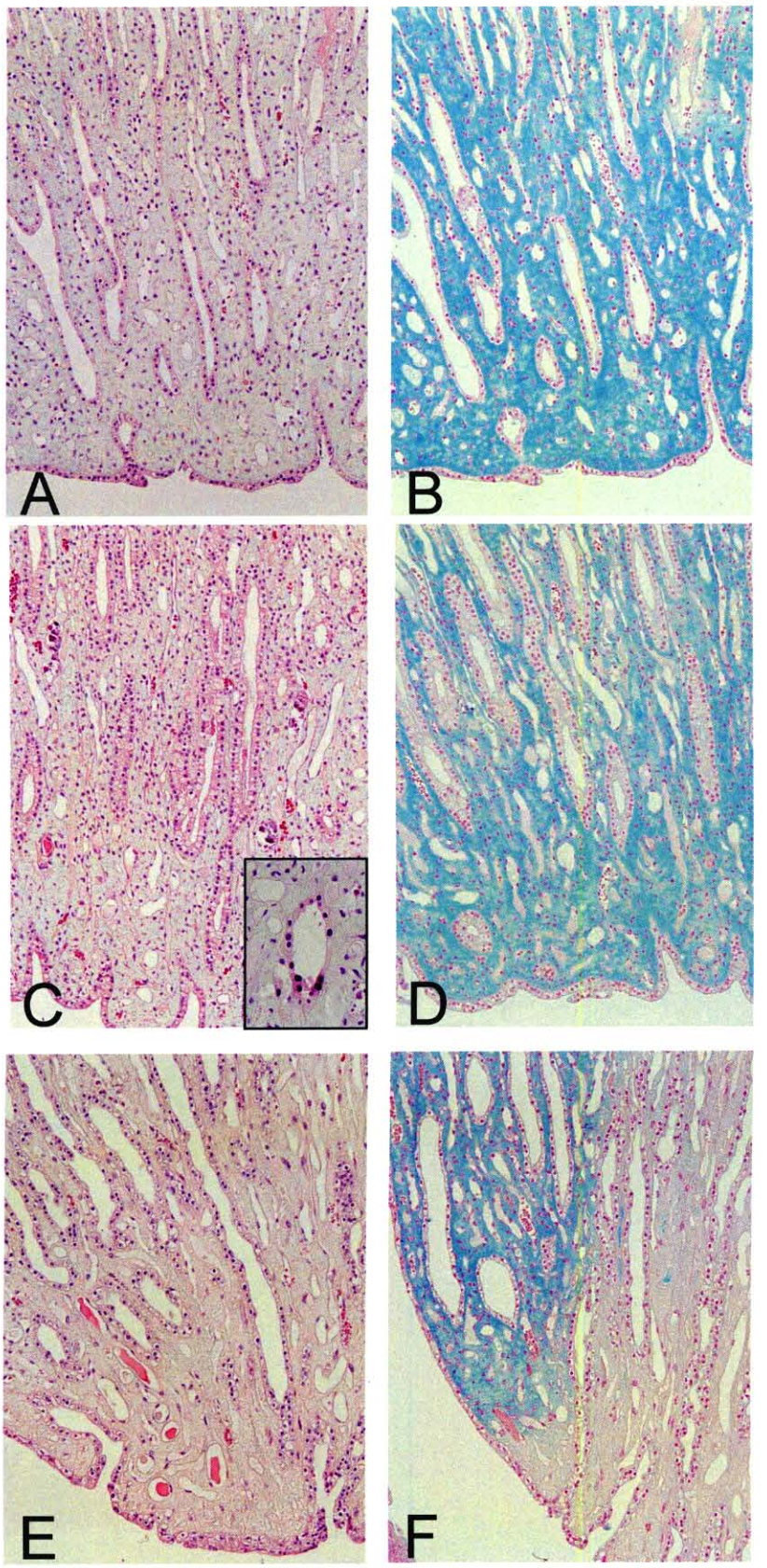
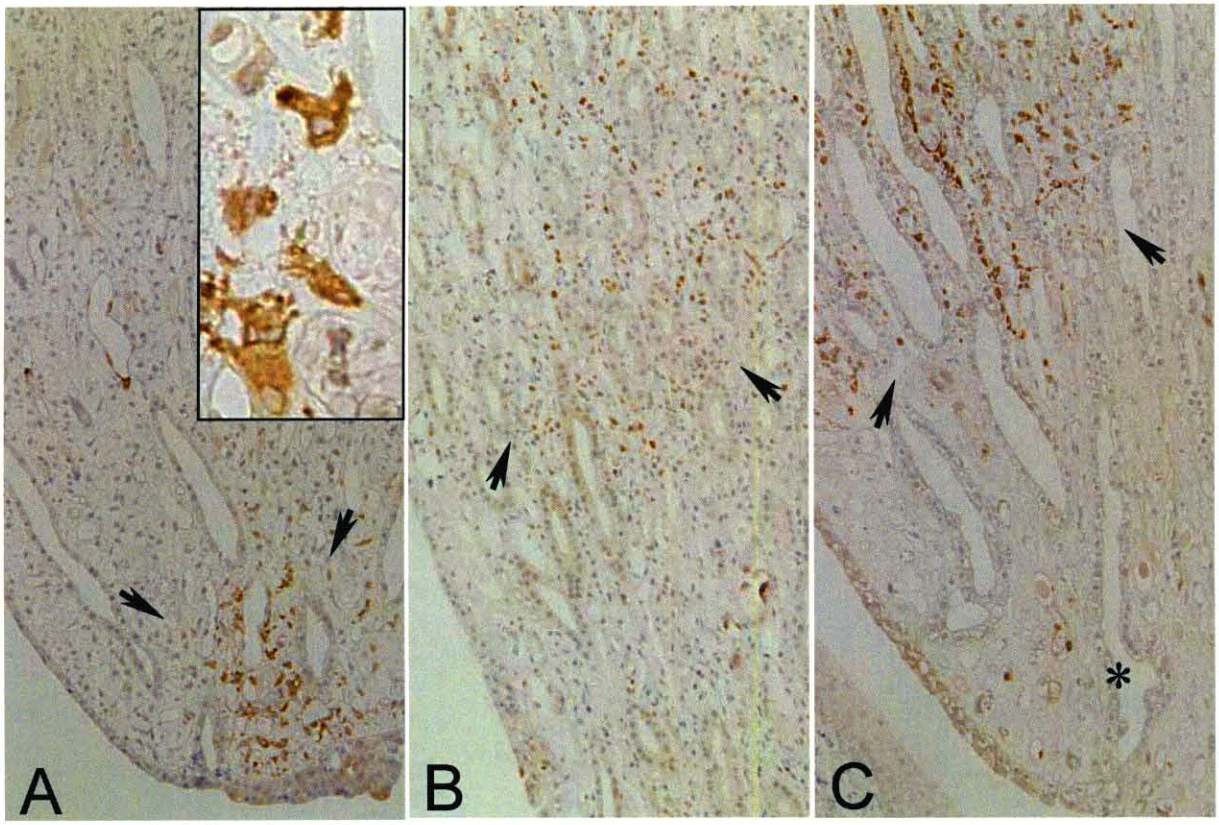


Fig. 8 Light micrograph of the renal papilla from male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day or ibuprofen at 50 mg/kg/day.
(A, C, and E) H&E staining. (B, D, and F) Alcian-blue staining. (A-B) Intact control in week 7, x 95. (C-D) Nefiracetam in week 7, x 95. Epithelial swelling of the papillary ducts were seen. Inset: degeneration of epithelial cells (higher magnification), x 170. (E-F) Ibuprofen in week 5, x 95. Degeneration of interstitium in the papilla was noted.



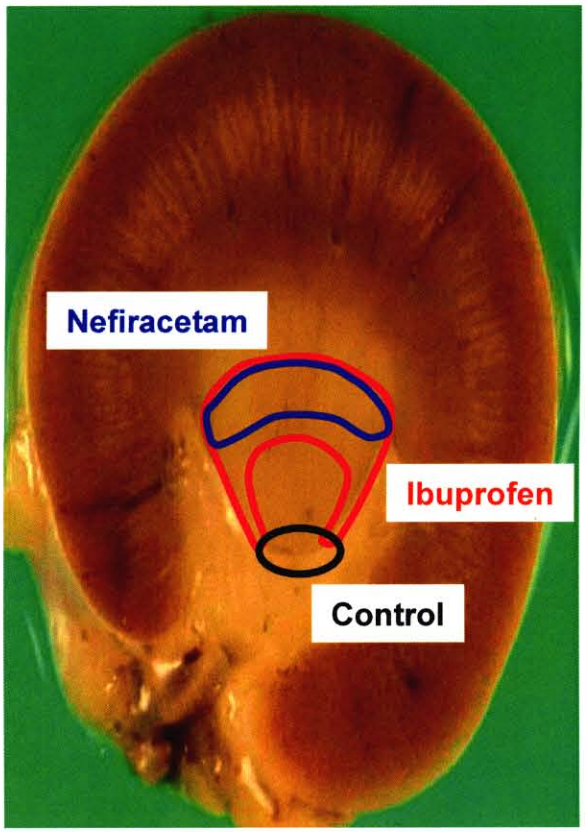


Fig. 9 Immunohistochemical staining of COX-2 in the renal papilla from male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day or ibuprofen at 50 mg/kg/day. Anti-COX-2, LSAB method. (A) Intact control in week 7, x 140. Positive reaction was noted in the cytoplasm of interstitial cells in the papillary tip. Inset: higher magnification, x 860. (B) Nefiracetam in week 7, x 140. Interstitial cells around affected epithelial cells in the papillary ducts showed positive reaction. (C) Ibuprofen in week 5, x 140. Strongly positive reaction was seen in interstitial cells adjacent to degenerative/necrotic interstitium in the papilla (asterisk). (D) Schematic area showing positive immunoreactions. The area surrounded by a black line (control), blue line (nefiracetam), or red line (ibuprofen).

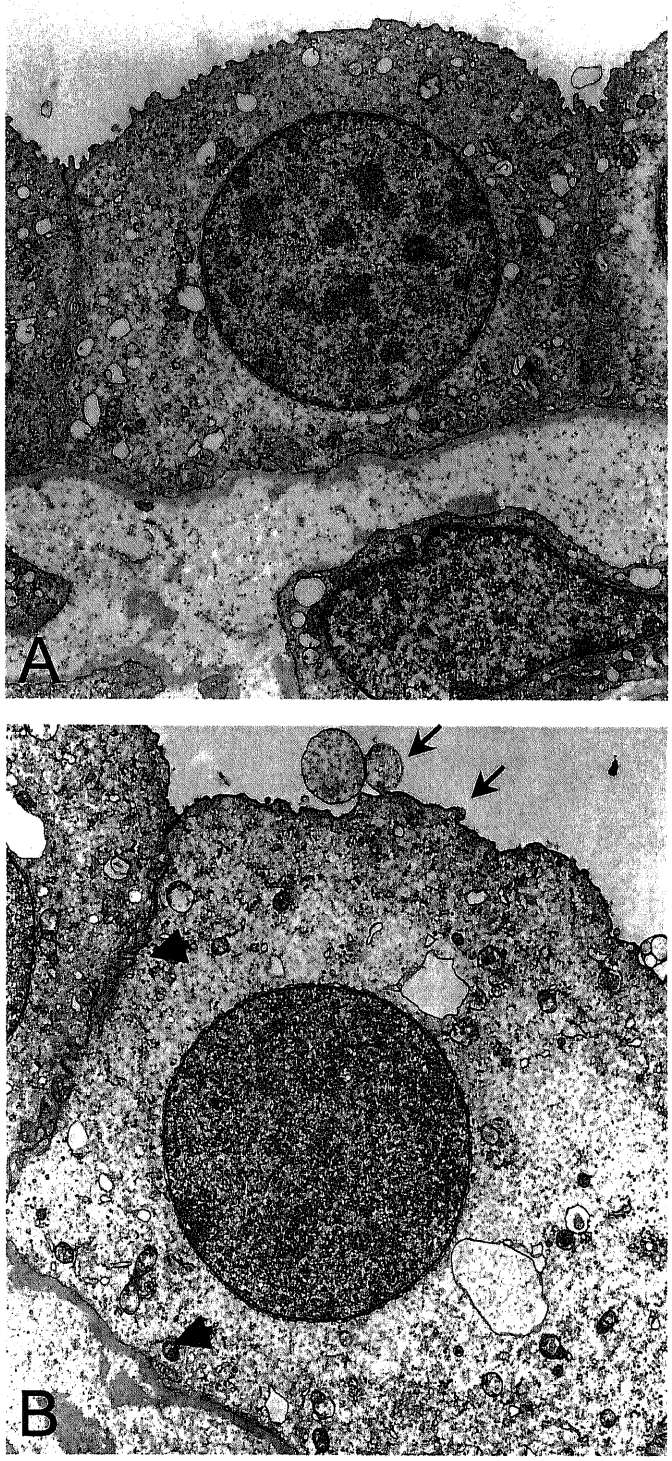


Fig. 10 Electron micrograph of the papillary duct from male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day. Glutaraldehyde-osmium fixation. (A) Intact control in week 7, x 6500. Normal structures of epithelial cells were seen. (B) Nefiracetam in week 7, x 6500. Epithelial cells of the papillary ducts showed swelling and decreased microvilli. Membranous blebs were noted on the luminal surface (arrow). Interdigitation and infoldings in both lateral and basal membranes were inconspicuous (arrow head).

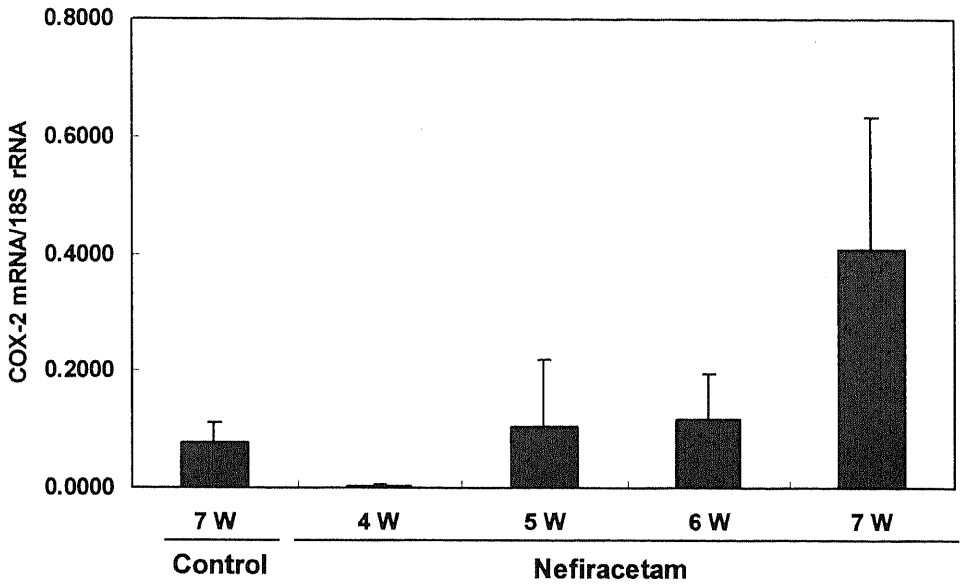


Fig. 11 COX-2 mRNA expression of the renal papilla in male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day.
Values are shown as the mean ± S.D. (n = 3-4).

Discussion

As described in Chapter 1, nefiracetam caused RPN only in dogs administered at 300 mg/kg/day over 11 weeks, namely, decreases in urinary osmotic pressure were observed from week 5, followed by increases in urine volume and urinary LDH from week 8. The initial renal morphological change was necrosis of ductal epithelial cells in the papilla in week 8. In the present study, to clarify the earlier pathophysiological features in RPN, male beagle dogs were given orally 300 mg/kg/day of nefiracetam for 4 to 7 weeks, in comparison with 50 mg/kg/day of ibuprofen, a NSAID, for 5 weeks, and the laboratory tests, light-microscopic, immunohistochemical and electron-microscopic examinations, and COX-2 mRNA analysis were periodically performed.

In laboratory tests, decreased urinary osmotic pressure and increased urine volume in weeks 6 to 7 of nefiracetam treatment were essentially consistent with those obtained in week 8 of the previous study in Chapter 1, but an increased urinary LDH level was observed earlier (week 5). Alternatively, these parameters were reconfirmed to be useful early biomarkers to detect the onset of RPN evoked by nefiracetam. Ibuprofen also elicited decreased urinary osmotic pressure, increased urine volume and increased urinary LDH, although there was a great interindividual variance. The onset and severity of these changes by ibuprofen were earlier and stronger than those by nefiracetam, which would be closely linked with necrosis of the interstitium in the papillary tip observed only in the ibuprofen group as mentioned below.

Light-microscopically, in dogs receiving nefiracetam, no changes were seen in the kidney by week 6. In week 7, however, epithelial swelling and degeneration of the papillary ducts appeared accompanied with a positive immunoreaction to COX-2 within interstitial cells around the affected epithelial cells in the upper papilla. Ultrastructurally, epithelial swelling, decreased or disappeared microvilli, and blebbing on the luminal surface were noted with reductions of intracellular interdigitation and infoldings in both lateral and basal membranes. In contrast, treatment with ibuprofen resulted in degeneration and necrosis of the interstitium in the papillary tip in week 5; however, no remarkable changes were observed in the remaining papillary ducts. In the COX-2 staining, unlike the case with nefiracetam, ibuprofen showed a positive reaction within interstitial cells adjacent to degenerative/necrotic areas in the papillary tip. Ultrastructurally, no changes in epithelial cells of the papillary ducts were noted. Thus, these data implied that the early

morphological alteration in the papilla brought about by nefiracetam was quite different from that elicited by ibuprofen. Both nefiracetam and ibuprofen showed mineralization in the renal papilla suggesting the pre-existence of damaged regions.

As for the COX-1 staining, there were no significant differences among the control, nefiracetam and ibuprofen groups. Generally, COX-1 has been reported as a constitutive isoform having housekeeping functions, whereas COX-2 is an inducible isoform induced by inflammatory stimuli or growth factors (21, 23, 62). More recent studies have suggested that COX-2 is also expressed in the renal medulla and is the major isoform contributing to medullary PG generation in vivo, especially in dehydrated subjects (33, 43). Sellers et al. have further shown that COX-1 is the main isoform involved in the synthesis of renal PGs in non-human primates and humans while both COX-1 and COX-2 contribute in the synthesis of renal PGs under basal conditions in rats and dogs (52). Hence, COX-2 was confirmed to play more important roles than COX-1 in the progression of RPN in dogs given nefiracetam.

In the renal papillary COX-2 expression, nefiracetam markedly decreased its expression in week 4, but returned to the basal control level in weeks 5 to 6. In week 7, COX-2 mRNA extremely increased as compared with the control. Therefore, it was considered that nefiracetam exceedingly depressed COX-2 mRNA expression at the early phase, followed by a compensating increase, and then marked upregulation was seen in response to the progression of renal injuries with continuous treatment. This assumption was partly supported by the fact that the positive immunoreaction to COX-2 was increased in the interstitial cells around the affected epithelial cells of the upper papilla in week 7.

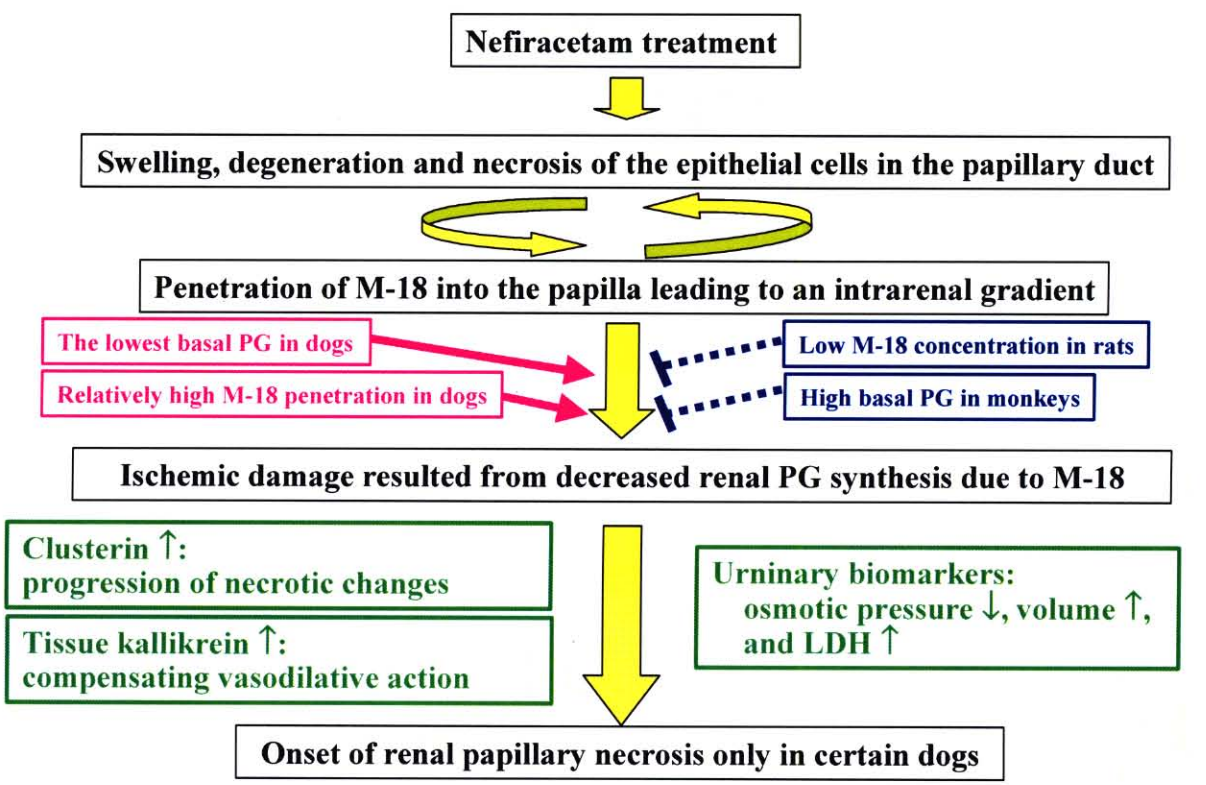
It has been reported that the epithelia promote organ homeostasis by restricting the flow of ions and solutes between cells across the epithelial cells, and the intercellular junctional complexes such as the tight junction, adherens junction, and desmosomes are important to maintain the cell-cell contact (15, 19, 29). Bachmann et al. have demonstrated that the epithelia (especially when salt secretion is stimulated) exhibit an elaborate "intracellular membranous labyrinth" created by cellular interdigitation and infoldings of the basolateral membrane (8). Goto et al. have previously shown that M-18, a metabolite of nefiracetam, reduces transepithelial electric resistance (TER) in the primary cultured uroepithelial cells of canine urinary bladder, and M-10 and M-18 displayed a deformation of uroepithelial cells and a slight reduction of the ZO-1 band, which is an essential protein associating with the tight junction (20). Likewise, we have reported that the concentration

ratio of M-18 in the papilla to medulla or cortex is remarkably higher in dogs than in rats and monkeys, and that M-18 possessed inhibitory effects on PG synthesis in canine renal papillary slices (Chapter 1). By taking into account aforementioned findings, we propose the following mechanism underlying RPN evoked by nefiracetam: the reductions of intracellular interdigitation and infoldings at both lateral and basal membranes in epithelial cells of the papillary ducts may initially inactivate the paracellular transport. This is followed by decreased cellular integrity, and then M-18 penetration and retention into the renal papilla occur. M-18 inhibits renal papillary PG synthesis, and certain animals finally incur ischemic injury due to decreased PG synthesis.

In conclusion, the earliest event in RPN evoked by nefiracetam is quite different from that by ibuprofen. The epithelial cells in the papillary ducts are the primary target site for the onset of RPN evoked by the nefiracetam.

Conclusion

The results obtained in the present investigation lead to following conclusions (see a scheme described below). In nefiracetam-induced RPN, urinary osmotic pressure, volume and LDH were useful biomarkers in the early phase of the onset. From the analyses of the urinary and renal constituents, changes in renal papillary clusterin mRNA reflected the progression or severity of RPN, whereas upregulation of tissue kallikrein mRNA played a subsequently compensating role in the prevention of RPN. As the mechanism underlying the onset of RPN, nefiracetam first altered cellular integrity of ductal epithelia in the renal papilla and consequently M-18, a metabolite of nefiracetam, penetrated and retained into the renal papilla leading to an intrarenal gradient only in dogs. Finally, M-18 inhibited renal papillary PG synthesis resulting in induction of ischemic injuries in individuals having the lowest basal PG synthesis and eventually RPN.



Possible mechanism underlying the onset of canine RPN induced by nefiracetam

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Article Lists

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Referees

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