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

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Abstract

The 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 renal papillary necrosis. Namely, when beagle dogs were orally administered with 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 from week 8. The first morphological change was necrosis of ductal epithelia in the papilla in week 8. In toxicokinetics after 3 weeks of 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 E_2 and 6-keto-prostaglandin $F_{1\alpha}$, a stable metabolite of prostaglandin I_2, in canine renal papillary slices, only M-18 among the metabolites clearly decreased both prostaglandin syntheses. The basal prostaglandin synthesis in canine renal papillary slices was extremely low relative to those in rats and monkeys. Taken together, certain factors such as basal prostaglandin synthesis, M-18 penetration into the renal papilla leading to an intrarenal gradient, and inhibitory potential of M-18 on prostaglandin synthesis were considered to be crucial for the occurrence of renal papillary necrosis in dogs.

Keywords: Nefiracetam; Metabolite; Renal papillary necrosis; Prostaglandin; Biomarker

1. Introduction

Nefiracetam [*N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide], a novel pyrroliodone derivative, possesses a pharmacologically unique nootropic action, and facilitates cognitive functions in various animal models. 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 (Yoshii and Watabe, 1994), interact with γ-amininobutyric acid (GABA)-ergic and cholinergic neuronal systems, and enhance protein synthesis in the brain (Nabeshima et al., 1991). 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 (Nishizaki et al., 1998). Prevention of the accumulation of intracellular calcium through *N*-methyl-D-aspartate receptor channels may also contribute in part to the action of nefiracetam (Aihara et al., 2001).

In both clinical and non-clinical pharmacokinetic studies, nefiracetam was found to be extensively metabolized; more than 20 metabolites were found in serum, urine, and tissues (Sudo et al., 1994). 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 concentration of each

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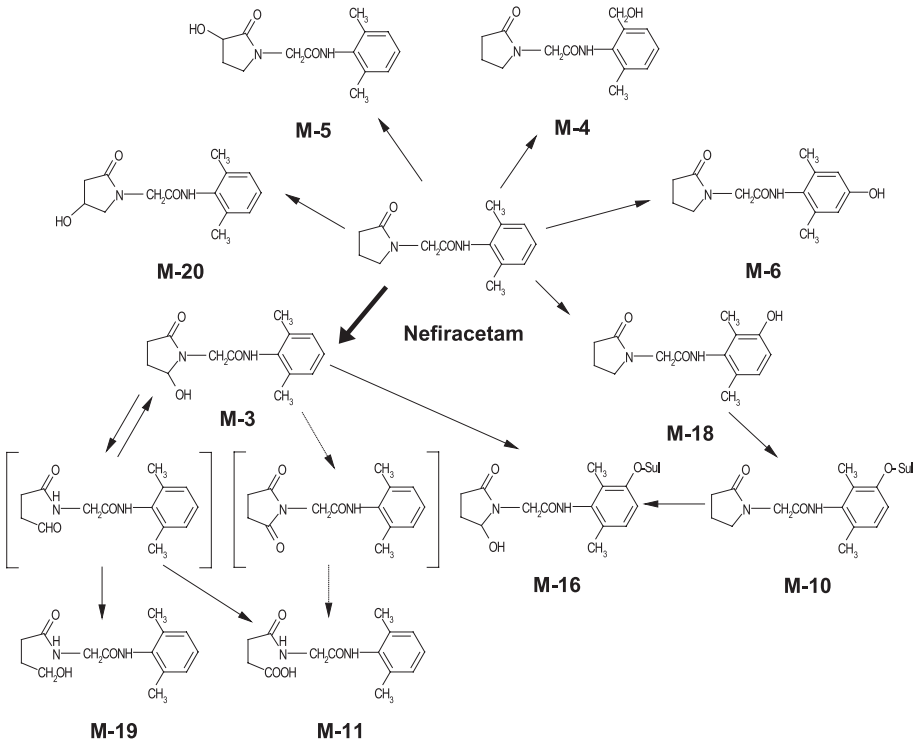

Fig. 1. The major metabolic pathways of nefiracetam. M: metabolite.

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 (unpublished data). 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 were observed only in dogs given a large amount of nefiracetam (over 180 mg/kg/day) with a delayed onset (Hooks et al., 1994; Sugawara et al., 1994a; Kashida et al., 1996). 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 on 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 renal papillary necrosis (Kashida et al., 1996).

Renal papillary necrosis is known to be caused by different kinds of compounds such as analgesic and non-steroidal anti-inflammatory drugs (NSAIDs, Alden and Frith, 1991; William et al., 1996; Bach and Thanh, 1998), 2-bromoethaniamine hydrobromide (Sabatini, 1984; Bach et al., 1991), or D-ormaplatin (Kolaja et al., 1994). Regarding NSAIDs-induced RPN, direct cytotoxic action (Whiting et al., 1999; Rocha et al., 2001) or ischemic injury to renal medullary cells through inhibition of the vasodilator effects of renal prostaglandins as a result of cyclooxygenase inhibition (Sabatini, 1988; Alden and Frith, 1991) 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 (Bach and Thanh, 1998; Clemo, 1998; Hildebland et al., 1999; Thanh et al., 2001).

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

2. Materials and methods

2.1. Chemicals

Nefiracetam and its four metabolites M-3, M-11, M-18, and M-20 were synthesized in the Akita Factory or Research Technology Center of Daiichi Pharmaceutical (Tokyo, Ja-

pan, Fig. 1). M-3 was the main metabolite in serum of all species tested 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. 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 (St. Louis, MO, USA). All other chemicals and reagents were the highest grade available from commercial sources.

2.2. 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 renal papillary necrosis in dogs (Hooks et al., 1994; Sugawara et al., 1994a; Kashida et al., 1996), either sex was used in the present investigation. The animals were housed under controlled conditions at a temperature of 20–26 °C, relative humidity of 35–80%, and lighting cycle of 12 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. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical.

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

Nefiracetam was orally administered at 300 mg/kg/day in a gelatin capsule to three 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 renal papillary necrosis in dogs given this dose was morphologically observed over an 11-week treatment (Kashida et al., 1996). 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 2 weeks from weeks 13 to 24, and every 4 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. For fresh urine, after volume was measured, urinary osmotic pressure was determined with an osmometer (model 3C2, Advanced Instruments, Norwood, MA, USA), and qualitative analyses were performed with N-Multistix SG-L (Bayer Medical, Tokyo, Japan) and Clinitek 200+ (Bayer Corporation, Pittsburgh, PA, USA). Approximately 5 ml of the supernatant of fresh urine after centrifugation (4 °C, 1500 rpm) for 10 min was filtered through a membrane filter (0.8 μm, DISMIC-25cs, ADVANTEC, Tokyo, Japan), and a 2.5-ml sample was applied to a PD-10 disposable column (Sephadex® G-25 M, Amersham Pharmacia Biotech, Buckinghamshire, UK) to remove interfering materials. The sample was eluted with 3.5 ml of Tris–HCl (0.0625 M, pH 6.8). In eluted urine, urinary protein, creatinine, lactate dehydrogenase, acid phosphatase, γ-glutamyl transpeptidase, and *N*-acetyl-β-D-glucosaminidase were measured with commercially available kits purchased from Wako Pure Chemical, (Osaka, Japan), Shionogi, (Osaka, Japan), or KAINOS Laboratories (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 and creatinine were measured with a Hitachi 7350 auto-analyzer (Hitachi, 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), embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin, and examined microscopically.

2.4. 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 renal papillary necrosis in dogs after a 13-week treatment (Sugawara et al., 1994a) 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 renal papillary necrosis. 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 five 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) were 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/1cc 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 × 75 mm, 3.5 μm or 2.1 × 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 0.2–0.3 ml/min by gradient flow. ESI/LS/MS was performed with a TSQ7000 mass spectrometer (Finnigan MAT, San Jose, CA, USA).

2.5. Analysis of prostaglandin synthesis in canine renal papillary slices

To investigate prostaglandin synthesis in renal papillary slices, prostaglandin 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, Osaka, Japan). The right kidney was removed and was divided vertically into two parts. Renal papillary slices were prepared according to the method described previously with some modifications (Leibbrandt and Wolfgang, 1995; Lupp et al., 2001). Briefly, pieces of 5-mm diameter were cut with a coring tool (VITRON, Tuscon, AZ, USA), and slices of approximately 300-μm thickness were prepared with a Brendal/Vitron tissue slicer (VITRON) 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, Franklin Lakes, NJ, USA) with 1 ml of the conditioned medium comprising serum-free and phenol red-free Dulbecco's modified Eagle's 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, 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 sulphoxide (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. Prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ concentrations in the culture medium were determined with a commercial enzyme immunoassay system (Amersham Pharmacia Biotech, Tokyo, Japan). Slices were weighed and homogenized in 0.5 ml of ice-cooled distilled water with an ULTRASONIC PROCESSOR (VC-130, IEDA Trading, 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, Tokyo, Japan). The solution was stored at −80 °C until assayed for aspartate aminotransferase and lactate dehydrogenase. A solution of 70% perchloric acid (Wako) was added (20 μl) to the remaining homogenate and mixed well. After centri-

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

Species	Dose (mg/kg/day)	Treatment period (weeks)	Urinalysis	Serum creatinine	Renal pathology
Rat[a]	480	13	No change	No change	No change
Dog	60[b]	13	No change	No change	No change
	180[b]	13	Pro ↑, Vol ↑	No change	Renal papillary 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[c]	180	13	No change	No change	No change

Five-week-old SD rats of both sexes (10 rats of each sex/group) 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) orally received nefiracetam, 20, 60, 180, or 300 mg/kg/day in gelatin capsules. Approximately five-year-old male cynomolgus monkeys (3 monkeys/group) 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.

[a] Jindo et al. (1994).

[b] Sugawara et al. (1994a).

[c] Unpublished data.

fugation (4 °C, 14,000 rpm) for 6 min, the resultant supernatant was used for the measurement of potassium. Aspartate aminotransferase and lactate dehydrogenase in the medium and the supernatant of slices were measured with a Hitachi 7350 automatic analyzer (Hitachi) and potassium was measured with an automatic electrolyte analyzer Model 710 (Hitachi). Additionally, to elucidate the basal prostaglandin 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. Prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ concentrations in the culture medium were determined.

2.6. Statistical analyses

All quantitative data are shown as the means ± S.D. 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 test was subsequently used, depending on homogeneity. In 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, Tokyo, Japan) or EXSAS ver.

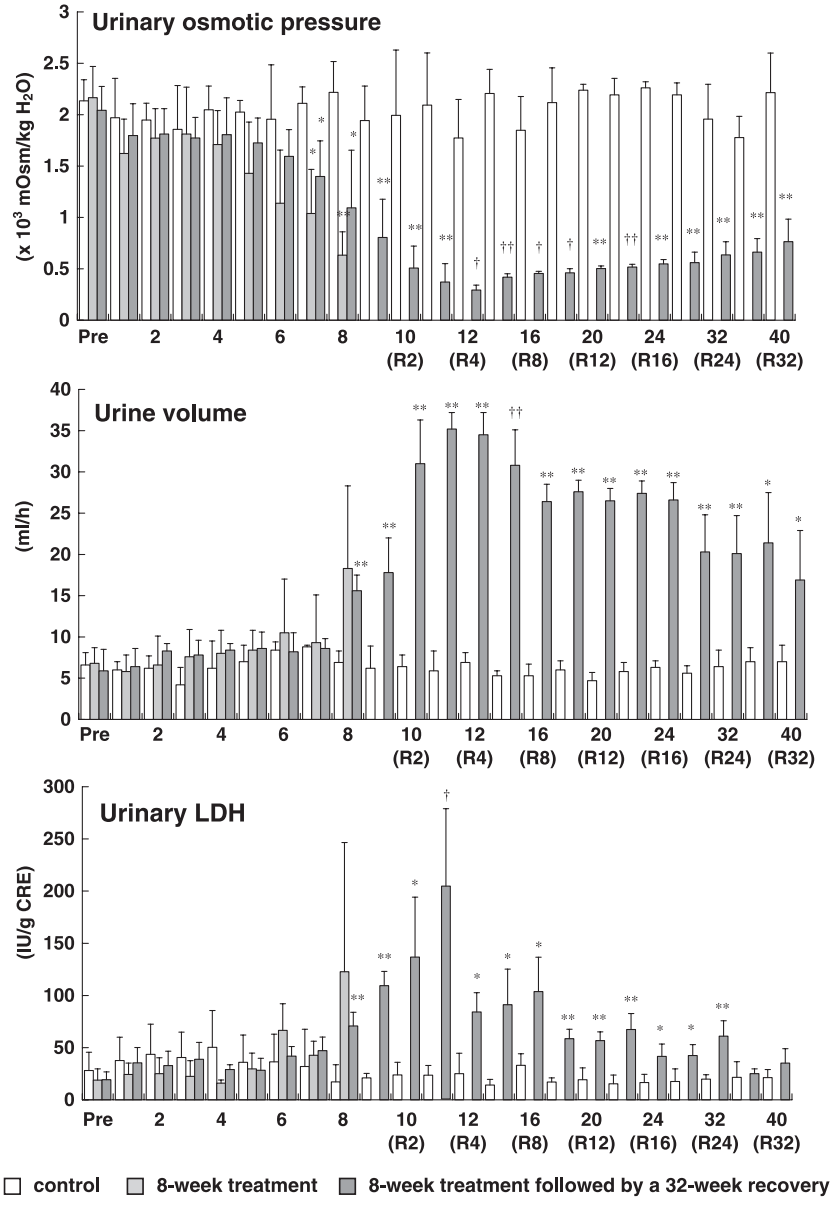

Fig. 2. Urinary osmotic pressure, urine volume, and urinary lactate dehydrogenase (LDH) values in male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day for 8 weeks, followed by a 32-week recovery period. CRE: creatinine. Pre: pre-administration. R: recovery. Values are shown as the means ± S.D. ($n = 3–4$). *$P < 0.05$, **$P < 0.01$, significantly different from controls (Student's *t*-test). †$P < 0.05$, ††$P < 0.01$, significantly different from controls (Aspin–Welch test).

5.10 (Arm, 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 ($n = 1–5$).

3. Results

3.1. 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 (Jindo et al., 1994; unpublished data). Conversely, according to previous data, dogs administered with 180 mg/kg/day of nefiracetam had increases in urine volume and urinary protein and renal papillary necrosis with hemorrhage and cell infiltration of the medulla at the scheduled killing time after a 13-week treatment (Sugawara et al., 1994a).

In the present study, dogs administered with 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

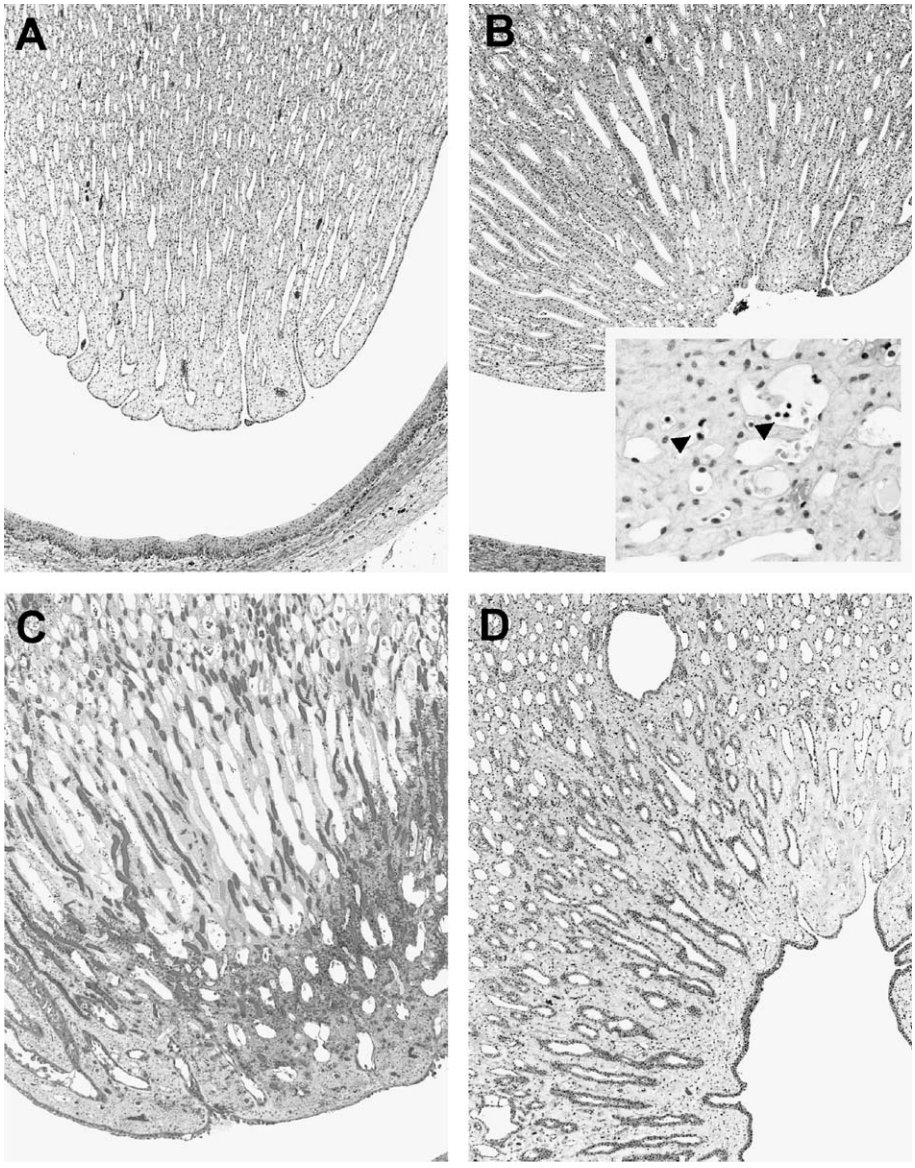

Fig. 3. Microscopic findings for the renal papilla from male dogs receiving repeated oral administration of nefiracetam at 300 mg/kg/day. (A) The papilla from a control dog, hematoxylin and eosin (**H&E**), alternatively, **HE**×4. (B) The papilla from a dog receiving the 8-week treatment, **H&E**, alternatively, **HE**×4. Inset: higher magnification, necrosis of ductal epithelia in the papilla (arrow) was seen, H and E, ×40. (C) The papilla from a dog receiving the 11-week treatment. Severe renal papillary necrosis with hemorrhage was seen, **H&E**, alternatively, **HE**×4. (D) The papilla from a dog receiving the 8-week treatment, followed by a 32-week recovery period. Only slight to moderate renal papillary necrosis with desquamation at the tip of the papilla was seen, **H&E**, alternatively, **HE**×4.

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 lactate dehydrogenase 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 (data not shown).

Small but significant increases in serum urea nitrogen and creatinine were observed from weeks 12 to 40 (recovery week 32). Urinary creatinine, acid phosphatase, γ-glutamyl transpeptidase, and *N*-acetyl-β-D-glucosaminidase fluctuated significantly during the course of the experiment, but these changes were minor and of doubtful toxicological importance (data not shown).

At necropsy, dark red foci in the renal papilla were seen in two of three 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. Microscopically, necrosis of ductal epithelia in the papilla was observed in the dogs killed in week 8 (Fig. 3B). In week 11, hemorrhage and necrosis of ductal epithelia in the papilla were observed in two of three and three of three dogs, respectively. One of these dogs had severe renal papillary necrosis 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 renal papillary necrosis 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).

3.2. 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 four 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 (0.85–2.75 for rats, 0.94–4.02 for dogs, and 1.02–1.40 for monkeys).

3.3. Prostaglandin synthesis in canine renal papillary slices

The metabolite, M-18, 5 mM, significantly decreased both prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ synthesis to 17.2% and 38.5%, respectively, as compared with the vehicle controls (Fig. 5). On the contrary, nefiracetam and its three other metabolites had no significant effects on

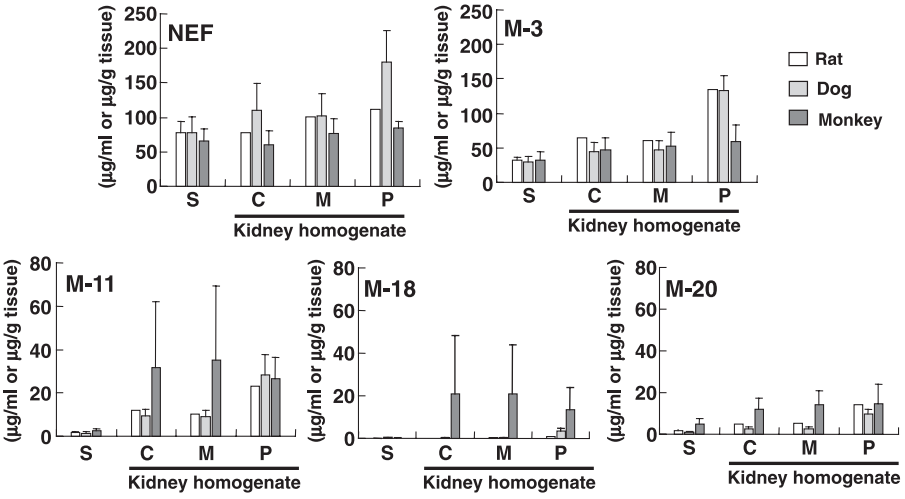

Fig. 4. Concentrations of nefiracetam (NEF) and metabolites (M-3, M-11, M-18, and M-20) in sera (S) and renal cortex (C), medulla (M), and papilla (P) homogenates in rats, dogs, and monkeys. NEF was given orally to 10 male rats, 4 male dogs, and 4 female monkeys at 180 mg/kg/day for 3 weeks. Two hours after the final administration (day 21), the kidneys were divided into three 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.

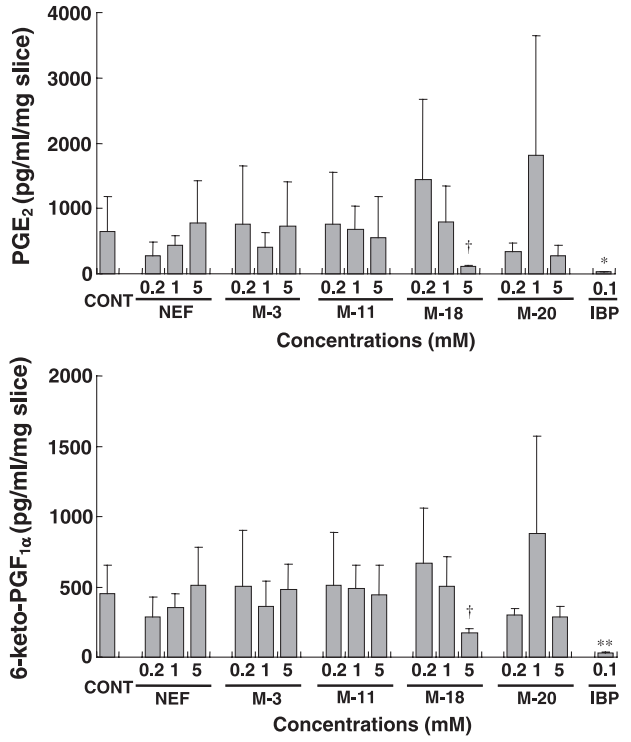

Fig. 5. Effects of nefiracetam (NEF) and metabolites (M-3, M-11, M-18, and M-20) on prostaglandin (PG) E_2 and 6-keto-prostaglandin (PG) $F_{1\alpha}$ synthesis in canine renal papillary slices. NEF, M-3, M-11, M-18, and M-20 (0.2–5 mM), and ibuprofen (IBP, 0.1 mM) as a positive control were dissolved 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 controls (Aspin–Welch test). †$P<0.05$, significantly different from controls (Dunnett's test).

either prostaglandin synthesis, even at 5 mM. Ibuprofen used as a positive control showed potent inhibitory effects on both prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ synthesis up to 3.9% and 6.4%, respectively. No changes were seen in aspartate aminotransferase and lactate dehydrogenase leakage to the medium or in potassium content of all treated slices (data not shown).

There was a great interspecies difference in basal prostaglandin synthesis in renal papillary slices. The rank order of prostaglandin E_2 (pg/ml/mg slice) and 6-keto-prostaglandin $F_{1\alpha}$ (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 prostaglandin synthesis.

4. Discussion

Repeated oral administration of nefiracetam at a high dose caused renal papillary necrosis only in dogs. Briefly, decreased urine osmotic pressure was first noted in week 5; subsequently, urine volume and urinary lactate dehydrogenase increased from week 8. These changes became most severe in week 11 or 12. Histopathologically, necrosis of ductal epithelia in the renal papilla was an initial change observed in week 8 when increased urinary lactate dehydrogenase occurred. Although compounds such as analgesics and NSAIDs have been shown to evoke renal papillary necrosis, there are no specific and sensitive diagnostic biomarkers available by which changes in the papilla can be detected before irreversible lesions emerge. Lactate dehydrogenase, a cytosolic enzyme, has been reported to be relatively specific to the distal tubules (Ross and Guder, 1982; Endoh, 1987). In contrast, urinary creatinine, acid phosphatase, or γ-glutamyl transpeptidase, a brush border enzyme in the proximal tubules, and *N*-acetyl-β-D-glucosaminidase, 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 urea nitrogen and creatinine were observed from week 12. Therefore, urinary lactate dehydrogenase was thought to be a useful biomarker for detecting severe renal papillary necrosis 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 lactate dehydrogenase and that there were no evident morphological changes in the renal papilla at this time (Kashida et al., 1996), we regard the decreased urinary osmotic pressure as an initial functional event in renal papillary necrosis. Though the recovery of decreased urinary osmotic pressure and increased urine volume was very slow, there was a tendency to recuperate 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 renal papillary necrosis with hemorrhage (Kashida et al., 1996). In the present study, severe renal papillary necrosis with hemorrhage was also observed in week 11, and slight to moderate renal papillary necrosis 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 microscopic changes were seen by week 4 (Kashida et al., 1996). In view of these observations, it is considered that if the treatments were stopped when urinary osmotic pressure is decreased, nefiracetam-induced renal papillary necrosis could be preventable.

In our in vitro work, ibuprofen markedly decreased both prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ levels in canine renal papillary slices, in which either of these prostaglandins induces vasodilation (Whelton, 1999; Miyatake et al., 2002). While nefiracetam itself showed inhibitory effects on neither prostaglandin E_2 nor 6-keto-prostaglandin $F_{1\alpha}$ synthesis in papillary slices, of the metabolites, only M-18

decreased the synthesis of both prostaglandins. Evidence thus suggests that M-18 may be a determinant for the induction of renal papillary necrosis in dogs.

Renal papillary necrosis provoked experimentally by analgesics and NSAIDs has been noted mainly in rats, occasionally in rabbits and dogs, and rarely in monkeys (Molland, 1978; Bach and Hardy, 1985; Alden and Frith, 1991). Alden and Frith (1991) have also explained the high susceptibility of rats to renal papillary necrosis 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. (1998) have suggested that the difference in cyclooxygenase distribution within the kidney can also contribute to interspecies differences in the susceptibility to renal papillary necrosis. In the present study, the species difference in basal levels of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ in the renal papillary slices was also confirmed, and dogs had the lowest basal prostaglandin synthesis.

Nefiracetam-induced renal papillary necrosis was not seen in rats and monkeys. It is considered that rats did not develop renal papillary necrosis because of the low concentration (0.78 μg/g) of M-18 in the renal papilla, in spite of the high sensitivity to renal papillary necrosis as mentioned above. In monkeys, higher prostaglandin contents may protect from the onset of renal papillary necrosis, even though M-18, which provokes a decrease in renal prostaglandins, resulted in the highest concentration (13.4 μg/g) in the papilla. In dogs with the lowest basal prostaglandin syntheses, 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 renal papillary necrosis. In fact, renal papillary necrosis in dogs was observed only after repeated administration of nefiracetam for a long period, but not after the single large dose (500 mg/kg, Sugawara et al., 1994b). These phenomena implied that a close relation among basal prostaglandin synthesis, M-18 penetration into the renal papilla with an intrarenal gradient, and inhibitory potential of M-18 on prostaglandin synthesis may explain the species difference. In human clinical trials, there has so far been no report dealing with the occurrence of renal papillary necrosis (unpublished data). 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 prostaglandin 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 aspartate aminotransferase or lactate dehydrogenase leakage or in potassium contents were seen in the canine renal papillary slices exposed to nefiracetam and its four metabolites, suggesting the absence of direct cytotoxic effects.

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

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