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H$^+$-dependent transport mechanism of nateglinide in the brush-border membrane of

the rat intestine

Shirou Itagaki, Yoshitaka Saito, Sayaka Kubo, Yukio Otsuka, Yuta Yamamoto, Masaki

Kobayashi, Takeshi Hirano and Ken Iseki

Department of Clinical Pharmaceutics & Therapeutics, Graduate School of

Pharmaceutical Sciences, Hokkaido University

Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

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Address correspondence to:

Ken Iseki, Ph. D., Department of Clinical Pharmaceutics & Therapeutics, Graduate

School of Pharmaceutical Sciences, Hokkaido University

Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

Tel/Fax: +81-11-706-3770

e-mail: ken-i@pharm.hokudai.ac.jp

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Abbreviations: Gly-Sar, glycylsarcosine; PEPT, peptide transporter; MCT,

monocarboxylate transporter.

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ABSTRACT

(-)-N-(trans-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (nateglinide) is a novel oral hypoglycemic agent possessing a carboxyl group and a peptide-type bond in its structure. Although nateglinide quickly reaches the maximal serum concentration after oral administration, nateglinide itself is not transported by PEPT1 or MCT1. The aim of this study was to characterize the transporters on the apical side of the small intestine that are responsible for the rapid absorption of nateglinide. The uptake of nateglinide by rat intestinal brush-border membrane vesicles is associated with a proton-coupled transport system. Ceftibuten competitively inhibited H^+-dependent nateglinide uptake. Gly-Sar, cephradine and cephalexin did not significantly inhibit the uptake of nateglinide. The combination of Gly-Sar and nateglinide greatly reduced the uptake of ceftibuten. The effect of the combined treatment was significantly greater than that of Gly-Sar alone. Furthermore, nateglinide competitively inhibited H^+-driven ceftibuten transporter-mediated ceftibuten uptake. Ceftibuten transport occurs via at least two H^+-dependent transport systems: one is PepT1 and the other is the ceftibuten/H^+ cotransport system. On the other hand, we demonstrated that nateglinide transport occurs via a single system that is H^+-dependent but is distinct from PepT1 and may be identical to the ceftibuten/H^+ cotransport system.

Impairment of glucose-induced insulin secretion and insulin resistance are hallmarks of type 2 diabetes (noninsulin-dependent diabetes mellitus). To compensate for defective insulin secretion, sulfonylureas have been widely used for more than 40 years in the treatment of type 2 diabetes. However, sulfonylurea therapy has several disadvantages, such as excess hypoglycemia between meals, due to the long duration of action of these agents.

(-)-N-(trans-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (nateglinide) is a novel oral hypoglycemic agent possessing a carboxyl group and a peptide-type bond in its structure (Fig. 1). Although nateglinide stimulates insulin secretion via the same mechanism as that by which insulin secretion is stimulated by sulfonylureas, it quickly reaches the maximal serum concentration and is eliminated quite rapidly after oral administration (Shinkai et al., 1988; Sato et al., 1991; Ikenoue et al., 1997). Therefore, nateglinide can compensate for impaired insulin secretion to prevent postprandial hyperglycemia without causing prolonged hypoglycemia. These characteristics are expected to be useful in the treatment of type 2 diabetes.

The pharmacokinetic features of nateglinide may be attributable to its rapid intestinal absorption. Because nateglinide is an anionic compound with pKa 3.1, it exists predominantly in ionized form at the intestinal physiological pH of 6.5. Moreover, its chloroform/water partition coefficient is reported to be 0.2 at pH 6.8, indicating that it is scarcely lipophilic. These physicochemical features are incompatible with rapid absorption by passive diffusion, suggesting that nateglinide is

-4-

absorbed via a specific transport system(s) in the intestine. Peptide transporter 1 (PEPT1/*SLC15A1*) mediates the efficient absorption of a wide variety of oral peptide-like drugs in the small intestine (Liang et al., 1995; Katsura and Inui, 2003). Some ß-lactam antibiotics are transported by PepT1 (Wenzel et al., 1995; Han et al., 1999). We previously showed that ceftibuten is absorbed in the intestinal brush-border membrane via at least two H^+-driven transport systems: PepT1 and another H^+-driven transporter (Iseki et al., 1999). Although nateglinide has a dipeptide-type structure, it has been reported not to be transported by PepT1 (Terada et al., 2000). Moreover, nateglinide is not itself transported by monocarboxylate transporter 1 (MCT1/*SLC16A1*) (Okamura et al., 2002). The transport mechanism of nateglinide in intestinal absorption has not been elucidated yet.

The present study was carried out to characterize the transporters on the apical side of the small intestine that are responsible for the rapid absorption of nateglinide from the viewpoint of H^+-driven transport.

Materials and methods

Chemicals. Nateglinide was kindly donated by Yamanouchi (Tokyo, Japan). Cephradine and ceftibuten were kindly supplied by Sankyo (Tokyo, Japan). Glycylsarcosine (Gly-Sar) was purchased from ICN Biomedicals, Ltd. (CA, USA). Cephalexin was purchased from Wako Pure Chemical (Osaka, Japan). [^{14}C] Gly-Sar (110 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All other reagents were of the highest grade available and used without further purification.

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Animals. Male Wistar rats, aged 6 to 7 weeks (300-350 g in weight), were obtained from NRC Haruna (Gunma, Japan). The housing conditions were described previously (Itoh et al., 2004). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals".

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles were prepared from the rat intestine by the calcium precipitation method with some modification as described previously (Kessler et al., 1978). All steps were performed on ice or at 4°C. The intestine was washed with ice-cold saline and cut longitudinally. The mucosa was scraped gently with a glass microscope slide. The scrapings (4 g wet weight) were homogenized in 80 ml of ice-cold solution A (50 mM

D-mannitol, 2 mM Tris / HCl, pH 7.1) with a Warning blender at 16,500 rpm for 4 min. $CaCl_2$ solution (0.5 M) was added to a final concentration of 10 mM, and the homogenate was allowed to stand for 15 min. The homogenate was centrifuged at 6,000 x g for 15 min, and the supernatant was recentrifuged at 27,500 x g for 30 min. The resulting pellet was resuspended in 20 ml of solution B (50 mM D-mannitol, 10 mM HEPES/Tris, pH 7.5) and homogenized in a glass/Teflon Dounce-type homogenizer with 10 strokes. After a final centrifugation at 27,500 x g for 30 min, the brush-border membranes were suspended in a buffer containing 100 mM D-mannitol, 100 mM KCl and 20 mM HEPES/Tris (pH 7.5) or 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5). The level of alkaline phosphatase (a marker enzyme of the brush-border membrane) activity of the brush-border membrane was more than 12-fold higher than that of the initial homogenate. In contrast, the level of Na^+-K^+ ATPase (a marker enzyme of the basolateral membrane) activity of the brush-border membrane was the same as that of the initial homogenate. This means that brush-border membranes were enriched at least 12-fold with respect to basolateral membranes. Measurement of the Na^+-dependent uptake of alanine, a typical substrate for the amino acid transport system, demonstrated the functional integrity of the membrane (Iseki et al., 1999).

Uptake experiments. The uptake of substrates into brush-border membrane vesicles was determined by the rapid filtration technique described previously

(Itagaki et al., 2003). In a routine assay, 40 µl of membrane vesicles (0.4-0.6 mg protein) suspension was added to 200 µl of substrate mixture kept at 37°C. The compositions of the media are described in the figure legends. At selected time intervals, the uptake was stopped by diluting the incubation medium with 5 ml of ice-cold 10 mM MES buffer (pH 5.5) containing 150 mM KCl. The mixture was immediately filtered through a Millipore filter (0.45 µm in pore size, 2.5 cm in diameter; HAWP). The filter was rinsed with 5 ml of the same buffer. Substrate trapped on the filter was extracted with 300 µl of water, and the concentration of substrate was determined. To assay the radiolabelled compounds, substrate trapped on the filter was extracted with 10 ml of ACSII (Amersham International, UK), and the radioactivity was determined.

Analytical procedures. Nateglinide and ceftibuten were determined using an HPLC system equipped with a JASCO 880-PU pump 870-UV UV/VIS detector. The column was a Hitachi ODS Gel #3053 (4 mm i.d. x 250 mm). In the assay for nateglinide, a mobile phase containing of 50 mM H_3PO_4 (pH 2.5): acetonitrile (55:45, v/v) was used. Column temperature and flow rate were 55°C and 0.7 ml/min, respectively. The wavelength for detection of nateglinide was 210 nm. In the assay for ceftibuten, a mobile phase containing acetonitrile/0.05M citric acid buffer with pH adjusted to 2.5 by NaOH (1:9) was used. Column temperature and flow rate were 25°C and 0.7 ml/min, respectively. The wavelength for detection of ceftibuten was

262 nm. Radioactivity was determined using a liquid scintillation counter (Packard, 1600TR). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Student's t-test was used for statistical analysis, and a value of $P < 0.05$ was considered significant. Nonlinear regression analysis and least-squares fitting for the Eadie-Hofstee plot of substrate uptake were performed by using Origin® (version 6.1J).

Results

Effect of proton gradient on nateglinide uptake. The effect of an inwardly directed H^+ gradient on the uptake of nateglinide was investigated by measuring nateglinide uptake into rat brush-border membrane vesicles in the presence (pH_{in}=7.5; pH_{out}=5.5) or absence (pH_{in}=5.5; pH_{out}=5.5) of an inwardly directed H^+ gradient. Fig. 2A shows that typical overshoot uptake of nateglinide was observed in the presence of an H^+ gradient. On the other hand, the uptake of nateglinide was not significantly enhanced in the presence of an inwardly directed Na^+ gradient (Fig. 2B).

Concentration dependence of nateglinide uptake. To characterize the uptake of nateglinide, the concentration-dependence of the uptake of nateglinide was examined. In the absence of an inwardly directed H^+ gradient, the uptake of nateglinide was not saturated up to 350 µM (Fig. 3). Transporter-mediated uptake of nateglinide was saturated at a higher nateglinide concentration. The Km and Vmax values were determined by kinetic analysis to be 0.12 mM and 1.41 nmol/mg protein/15 s, respectively.

Inhibitory effect of ceftibuten on nateglinide uptake. In order to characterize the carrier that is responsible for the uptake of nateglinide in the intestinal brush-border membrane, inhibition experiments were performed in the presence of an inwardly directed H^+ gradient. It has been reported that nateglinide

inhibits the transport activity of PEPT1, although nateglinide itself is not transported by PEPT1 (Terada et al., 2000). We previously found that ceftibuten is absorbed in the intestinal brush-border membrane via at least two H$^+$-driven transport systems: PepT1 and another H$^+$-driven transporter (Iseki et al., 1999). Thus, we focused on this H$^+$-driven ceftibuten transporter.

As shown in Fig. 4, ceftibuten significantly reduced the uptake of nateglinide. In the present study, Gly-Sar, a typical substrate of PepT1, did not significantly inhibit the uptake of nateglinide. Cephradine and cephalexin, which are also substrates of PepT1 (Ganapathy et al., 1995; Chu et al., 2001), also did not significantly inhibit the uptake of nateglinide. Next, the inhibitory effect of nateglinide on the uptake of [^{14}C] Gly-Sar was determined. In the presence of an inwardly directed H$^+$ gradient, [^{14}C] Gly-Sar (20 µM) uptake decreased from 6.15 ± 0.64 pmol/mg protein/20 s (in the absence of nateglinide) to 3.28 ± 0.59 pmol/mg protein/20 s (in the presence of 700 µM nateglinide) (p<0.01).

Dixon plot analysis of nateglinide uptake in the presence of ceftibuten.
Results of Dixon plot analysis of nateglinide uptake in the presence of ceftibuten and an inwardly directed H$^+$ gradient are shown in Fig. 5. Ceftibuten was demonstrated to inhibit the uptake of nateglinide competitively. The regression line obtained from the replot of slopes of the Dixon plot almost coincided with the origin (Fig. 5, inset), indicating that ceftibuten transport is mediated by a common H$^+$/cotransport with

nateglinide. The apparent Ki value calculated from Dixon plots for ceftibuten was 3.20 mM.

Nateglinide/H$^+$ and ceftibuten/H$^+$ cotransporters. In order to clarify whether the nateglinide/H$^+$ cotransport system is identical to the ceftibuten/H$^+$ cotransport system, the inhibitory effect of nateglinide on the uptake of ceftibuten was investigated. Gly-Sar was used as an inhibitor of PepT1 to reduce the contribution of PepT1. As shown in Fig. 6, Gly-Sar significantly reduced the uptake of ceftibuten. This inhibitory effect was saturable at a concentration of more than 10 mM. Moreover, the combination of Gly-Sar and nateglinide greatly reduced the uptake of ceftibuten. The effect of the combined treatment was significantly greater than that of Gly-Sar alone. The inhibitory effect of nateglinide on the H$^+$-driven ceftibuten transporter-mediated ceftibuten uptake was saturable at a concentration of more than 100 µM (data not shown).

We then investigated the kinetics of the inhibitory effect of nateglinide on H$^+$-driven ceftibuten transporter-mediated ceftibuten uptake by brush-border membrane vesicles. Fig. 7 shows the concentration-dependence of H$^+$-driven ceftibuten transporter-mediated ceftibuten uptake in the presence or absence of nateglinide. Eadie-Hofstee plots suggested that nateglinide inhibits H$^+$-driven ceftibuten transporter-mediated ceftibuten uptake in a competitive manner (Ki = 0.17 mM).

-12-

Discussion

Oral drug delivery is generally the most desirable means of administration, mainly because of patient acceptance, convenience in administration, and cost-effective manufacturing. However, there are several problems for the development of oral delivery systems for drugs (Martinez and Amidon, 2002). One of the major problems is the poor permeability through the intestinal mucosa. The use of transporter function offers the possibility of delivering a drug to the target organ, avoiding distribution to other organs (thereby reducing the chance of toxic effects), controlling the elimination process, and improving oral bioavailability (Mizuno and Sugiyama, 2002). Intestinal PEPT1 has been utilized to improve the intestinal absorption of poorly absorbed and pharmacologically active agents by chemically converting them to substrates for PEPT1 (Balimane et al., 1998; Ganapathy et al., 1998).

Since nateglinide is absorbed rapidly form the intestine, it is likely to be absorbed via a specific transporter(s) (Sato et al., 1991; Ikenoue et al., 1997). It has been reported that nateglinide is actively transported in an absorptive direction across Caco-2 cell monolayers, although nateglinide itself is not transported by PEPT1 or MCT1 (Terada et al., 2000; Okamura et al., 2002). The transport mechanism of nateglinide in intestinal absorption has not been elucidated yet.

We previously showed that ceftibuten is absorbed in the intestinal brush-border membrane via at least two H^+-driven transport systems: PepT1 and

-13-

another H^+-driven transporter (Iseki et al., 1999). Therefore, it is likely that the intestinal ceftibuten transporter is involved in the rapid absorption of nateglinide. In the present study, we examined the contribution of a new ceftibuten transporter to the uptake of nateglinide by rat intestinal brush-border membrane vesicles.

It has been reported that the uptake of nateglinide across the apical membrane of Caco-2 cells is H^+-dependent and Na^+-independent (Okamura et al., 2002). To confirm the function of this transport system(s) in rat intestinal brush-border membrane vesicles, we investigated the effects of an inwardly directed H^+ gradient and an inwardly directed Na^+ gradient on the uptake of nateglinide. The nateglinide uptake exhibited an overshoot in the presence of an H^+ gradient. The initial uptake of nateglinide was saturable at higher concentrations. Moreover, an inwardly directed Na^+ gradient had no effect on the uptake of nateglinide. These results suggest that nateglinide is transported across the rat intestinal brush-border membrane via an H^+-coupled transport system.

To examine the contribution of the ceftibuten/H^+ cotransport system to the uptake of nateglinide in the intestinal brush-border membrane, we examined the inhibitory effect of ceftibuten on the uptake of nateglinide. We report here for the first time that a peptide-like drug inhibits the transport of nateglinide. Ceftibuten significantly reduced the uptake of nateglinide. This result suggests that a new ceftibuten transporter, which we have reported previously, may contribute to the uptake of nateglinide. In contrast, Gly-Sar, cephradine and cephalexin, which are also

substrates of PepT1 (Ganapathy et al., 1995; Chu et al., 2001), did not significantly inhibit the uptake of nateglinide. On the other hand, nateglinide inhibited Gly-Sar transport in the presence of an inwardly directed H^+ gradient. These findings indicate that nateglinide interacts with PepT1 but is not transported via PepT1. The results from Dixon plot analysis suggest that ceftibuten and nateglinide share the same transport system at intestinal brush-border membranes.

In the present study, since ceftibuten competitively inhibited H^+-dependent nateglinide uptake by rat intestinal brush-border membrane vesicles, the opposite inhibitory effect of nateglinide on ceftibuten uptake was investigated. In addition to PepT1, ceftibuten uptake by rat intestinal brush-border membrane vesicles also appears to be mediated by a nateglinide-sensitive mechanism. Furthermore, nateglinide competitively inhibited H^+-driven ceftibuten transporter-mediated ceftibuten uptake. Ceftibuten transport occurs via at least two H^+-dependent transport systems: one is PepT1 and the other is the ceftibuten/H^+ cotransport system. On the other hand, we demonstrate that nateglinide transport occurs via a single system that is H^+-dependent system but is distinct from PepT1 and may be identical to the ceftibuten/H^+ cotransport system.

Drug targeting is an effective approach both to increase the pharmaceutical activity of drugs and to reduce their side effects. Utilization of PEPT1 has been considered to be a promising strategy for oral drug delivery. However, drug-drug interactions involving PEPT1 are likely to occur due to the broad substrate specificity

of PEPT1. Changes in pharmacokinetics due to drug-drug interactions can often directly affect the therapeutic safety and efficacy of many important drugs. The present findings should provide important information that will enable improvements in drug absorption or drug design by targeting the nateglinide/H^+ cotransport system. By utilizing the nateglinide/H^+ cotransport system as the target for oral drug delivery, it will be possible to avoid drug-drug interactions involving PEPT1.

In conclusion, ceftibuten transport occurs via at least two H^+-dependent transport systems: one is PepT1 and the other is the ceftibuten/H^+ cotransport system. On the other hand, we have demonstrated that nateglinide transport occurs via a single system that is H^+-dependent but is distinct from PepT1 and may be identical to the ceftibuten/H^+ cotransport system. However, this transport system has not yet been elucidated at the molecular level. Further studies are needed to elucidate the mechanism of rapid intestinal absorption of nateglinide.

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antibiotics by the intestinal peptide transporter. *Br J Pharmacol* 116:3021-3027.

Legends for figures

Fig. 1. Chemical structure of nateglinide.

Fig. 2. Effect of an inwardly directed H^+ gradient (A) and effect of an inwardly directed Na^+ gradient (B) on the uptake of 100 µM nateglinide into rat intestinal brush-border membrane vesicles. (A) Membrane vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5) (control) or 20 mM HEPES/Tris (pH 7.5) (H^+ gradient). The substrate mixture contained 100 mM D-mannitol, 120 µM nateglinide, 100 mM KCl and 20 mM MES/Tris (pH 5.5). Each point represents the mean with S.D. of 4 preparations. **P < .01, significantly different from that in the absence of an inwardly directed H^+ gradient. (B) Membrane vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5). The substrate mixture contained 100 mM D-mannitol, 120 µM nateglinide, 100 mM KCl (control) or 100 mM NaCl (Na^+ gradient) and 20 mM MES/Tris (pH 5.5). Each point represents the mean with S.D. of 3 preparations.

Fig. 3. Concentration dependence of the uptake of nateglinide into rat intestinal brush-border membrane vesicles. Incubation conditions were identical to those described in the legend to Fig. 2A. Uptake of nateglinide was measured for 15 s. The dashed line represents transporter-mediated uptake obtained as the difference between the uptake in the presence of an inwardly directed H^+ gradient and that in the absence

of an inwardly directed H$^+$ gradient. Each point represents the mean with S.D. of 3 to 6 preparations.

Fig. 4. Inhibitory effects of PepT1 substrates on the uptake of nateglinide into rat intestinal brush-border membrane vesicles. Uptake of 100 μM nateglinide into brush-border membrane vesicles was measured for 30 s with or without (control) inhibitors. Membrane vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5) or 20 mM HEPES/Tris (pH 7.5). The substrate mixture contained 100 mM D-mannitol, 120 μM nateglinide, 100 mM KCl and 20 mM MES/Tris (pH 5.5) in the presence of various compounds. Uptake values in the absence of an inwardly directed H$^+$ gradient were subtracted from those in the presence of an inwardly H$^+$ gradient. Each column represents the mean with S.D. of 3 to 9 preparations. The control value for the uptake of nateglinide was 1.16 ± 0.15 nmol/mg protein/30 s. **P < .01, significantly different from the control.

Fig. 5. Dixon plot of the nateglinide uptake into rat intestinal brush-border membrane vesicles in the presence of ceftibuten. Uptake of 50 μM, 75 μM and 100 μM nateglinide was measured for 20 s with ceftibuten. Incubation conditions were identical to those described in the legend to Fig. 4. Each point represents the mean with S.D. of 3 preparations. Inset, replot of the slopes of the Dixon plot. The apparent Ki value was determined to be 3.20 mM by linear regression analysis from the Dixon

plot.

Fig. 6. Additive inhibitory effects of Gly-Sar and nateglinide on the uptake of ceftibuten into rat intestinal brush-border membrane vesicles. Uptake of 100 μM ceftibuten into brush-border membrane vesicles was measured for 30 s with or without (control) inhibitors. Membrane vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5) or 20 mM HEPES/Tris (pH 7.5). The substrate mixture contained 100 mM D-mannitol, 120 μM ceftibuten, 100 mM KCl and 20 mM MES/Tris (pH 5.5) in the presence of various compounds. Uptake values in the absence of an inwardly directed H^+ gradient were subtracted from those in the presence of an inwardly H^+ gradient. Each column represents the mean with S.D. of 3 preparations. The control value for the uptake of ceftibuten was 610 ± 9.88 pmol/mg protein/30 s. **$P < .01$, significantly different from that in the absence of Gly-Sar, ††$P < .01$, significantly different from that in the absence of nateglinide.

Fig. 7. Competitive inhibition by nateglinide of H^+-driven ceftibuten transporter-mediated ceftibuten uptake by rat intestinal brush-border membrane vesicles. Uptake of increasing concentrations of ceftibuten was measured for 20 s in the presence of 20 mM Gly-Sar alone or in the presence of 20 mM Gly-Sar and 100 μM nateglinide. Incubation conditions were identical to those described in the legend

to Fig. 6. Each point represents the mean with S.D. of 3 preparations. The apparent Ki

value was determined to be 0.17 mM

Figure 1
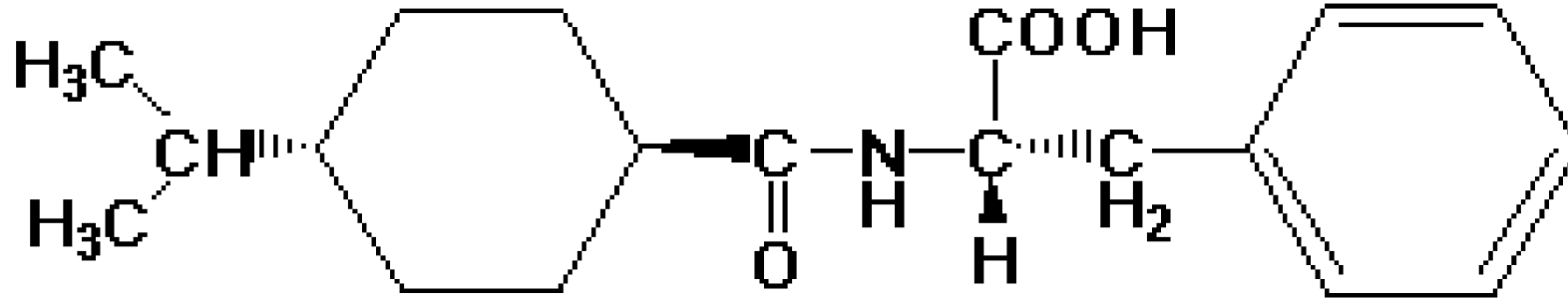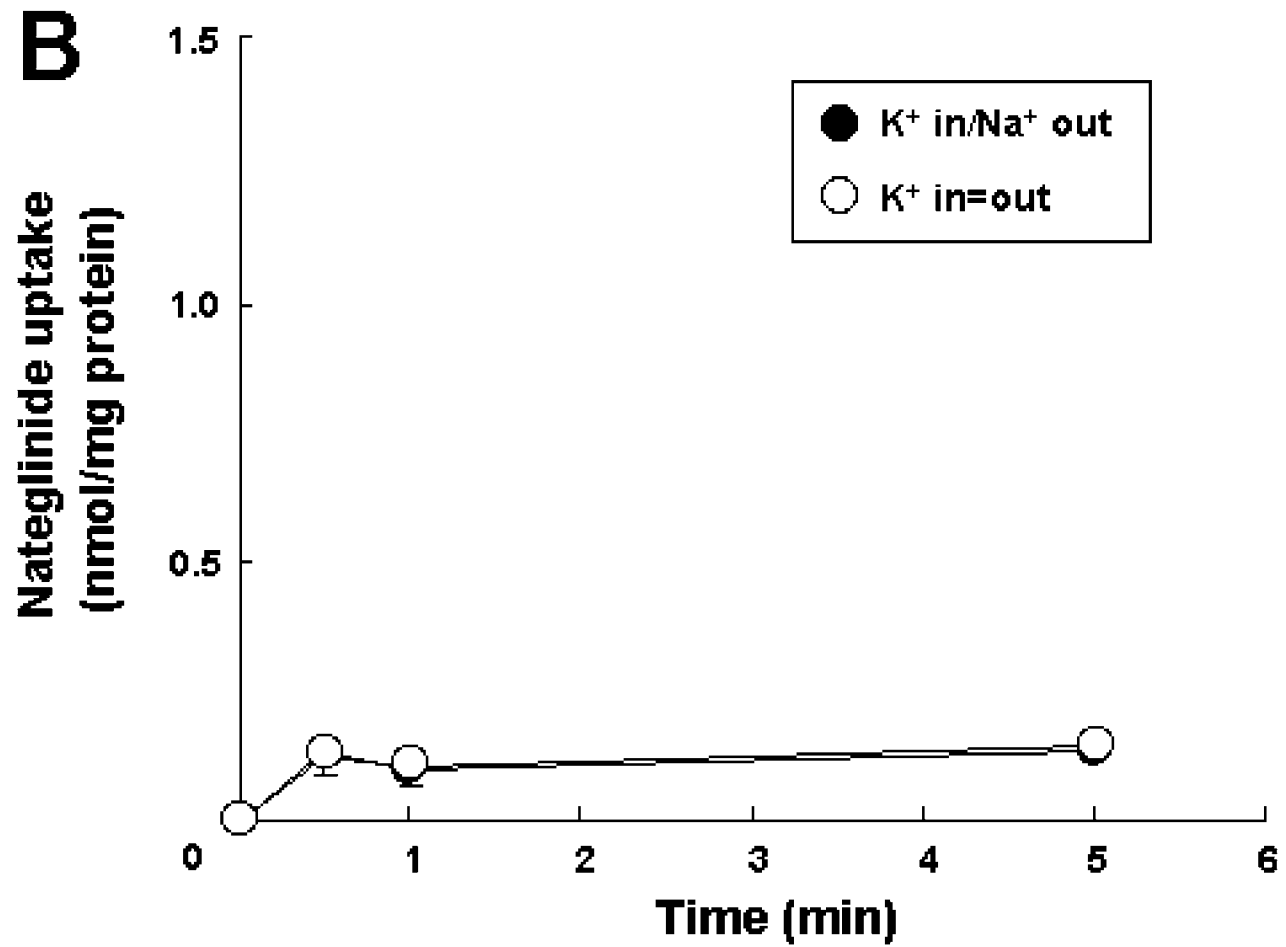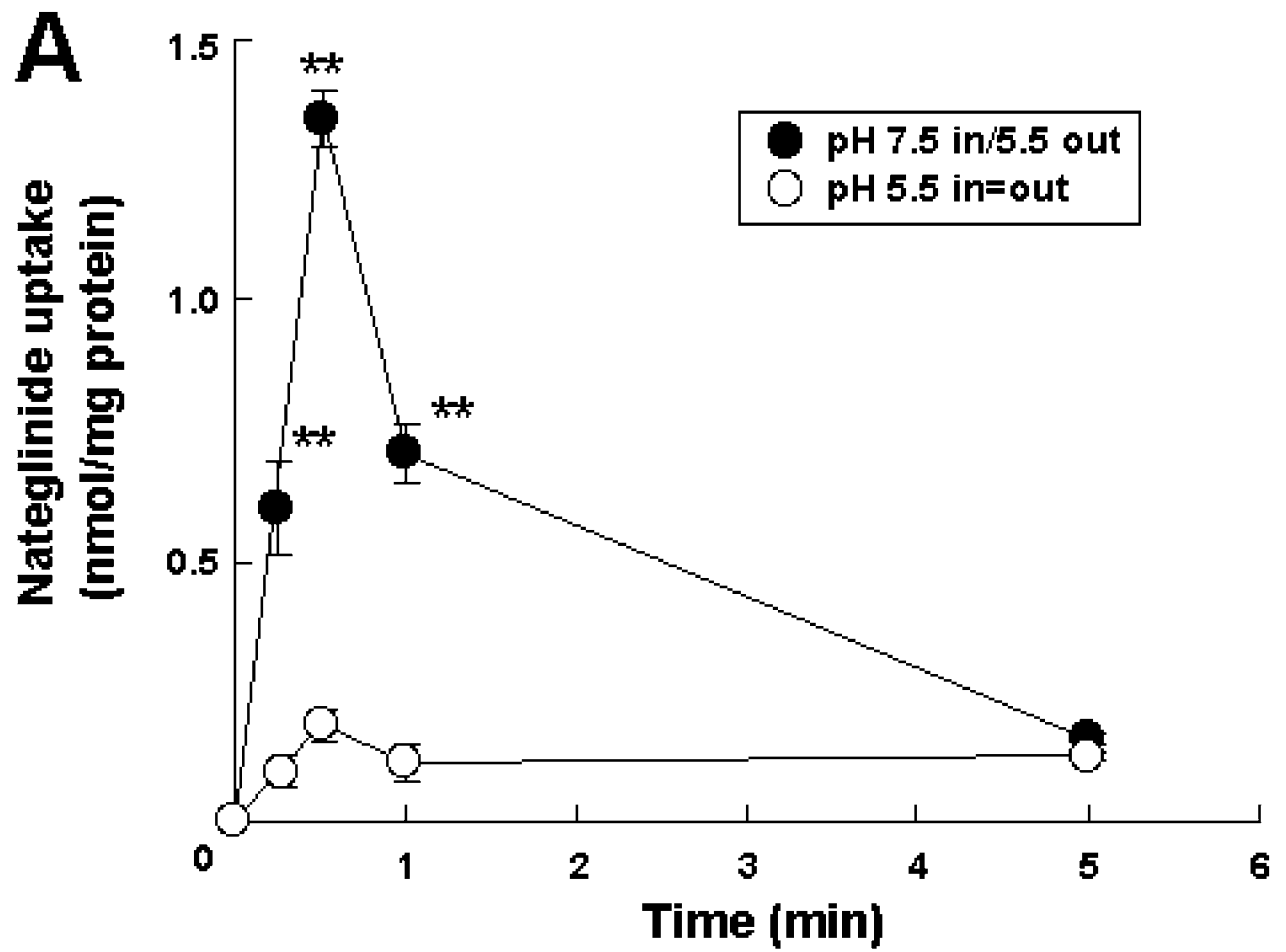
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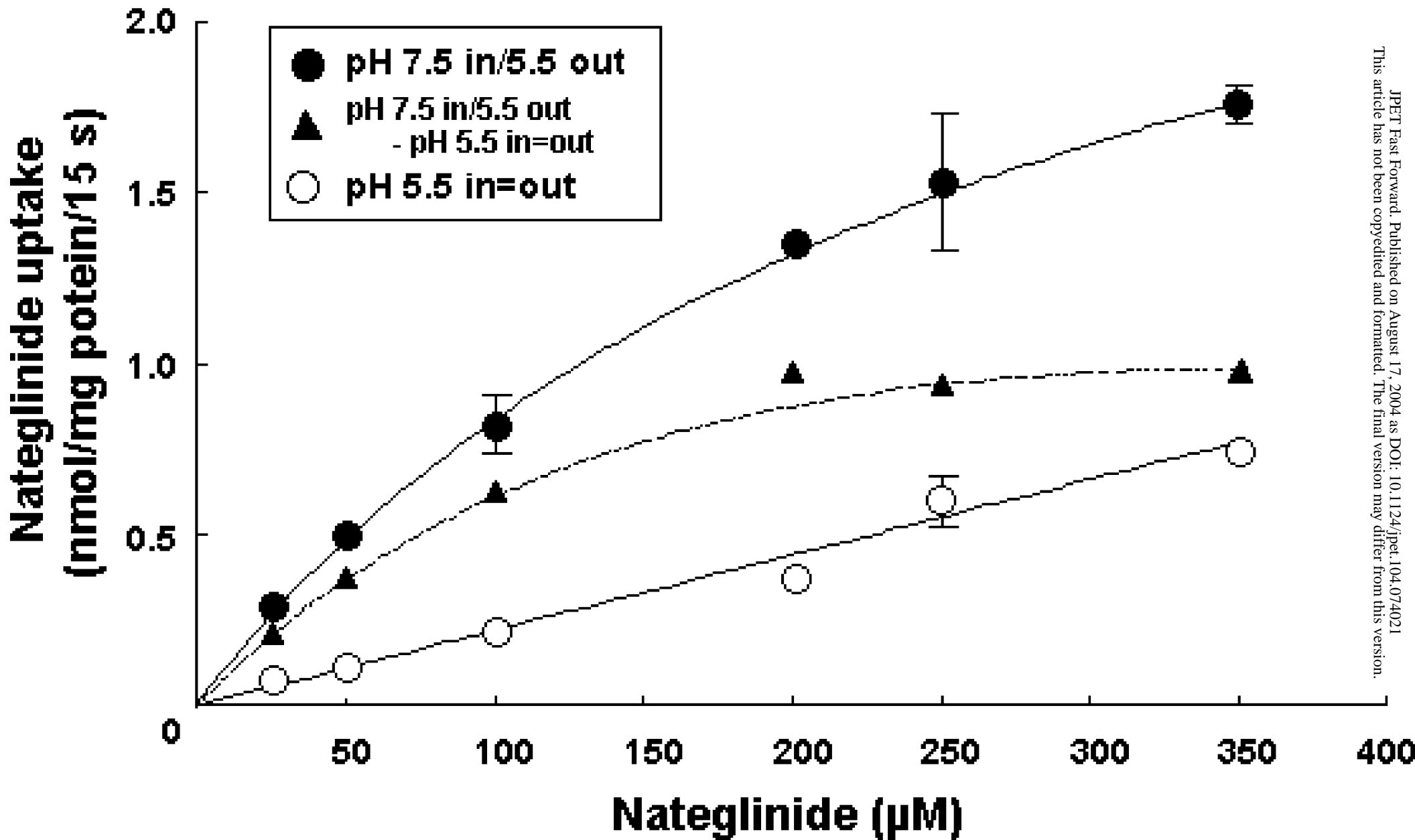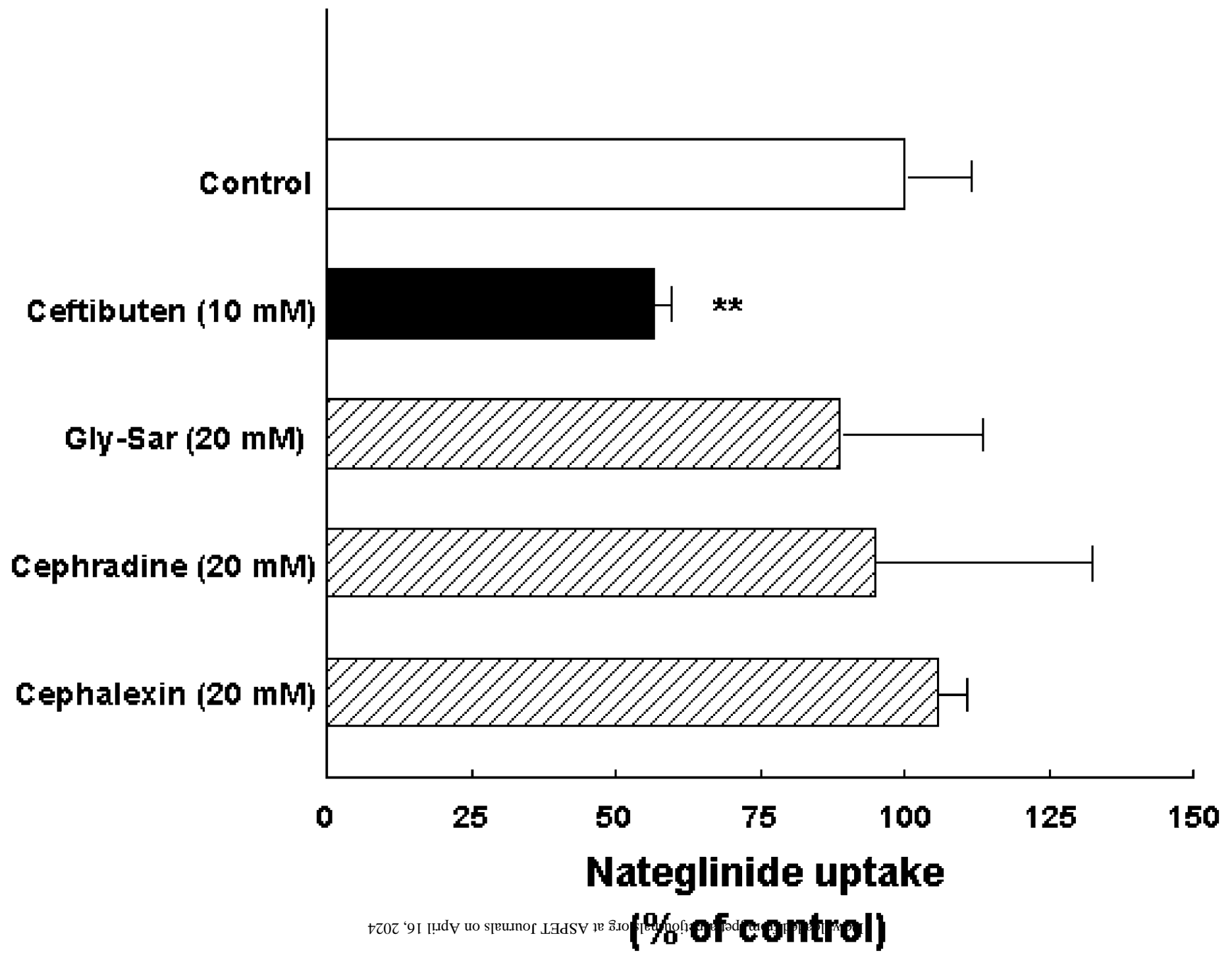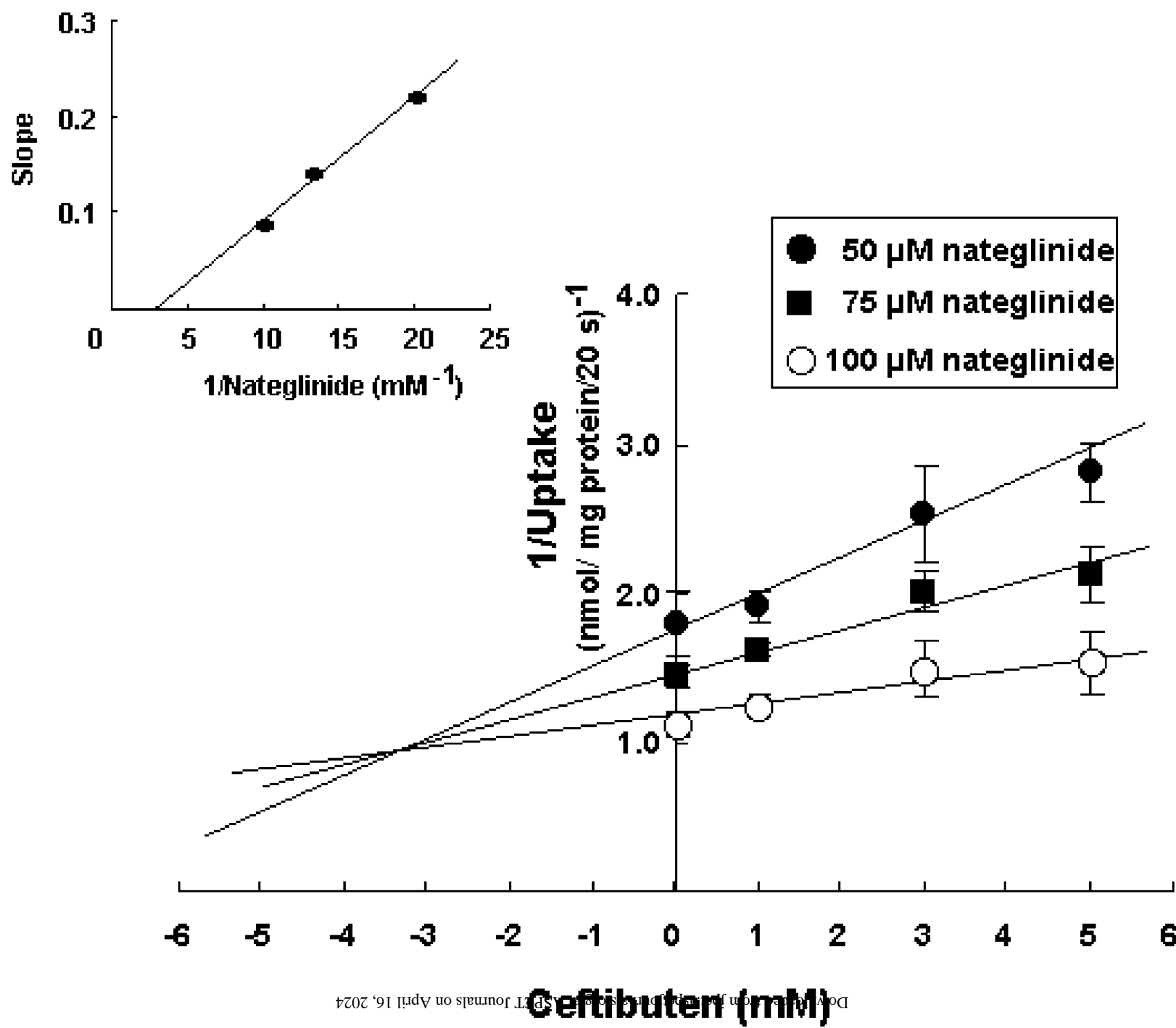
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