

Structure-Activity Relationships of GPR120 Agonists Based on a Docking Simulation^S

Qi Sun, Akira Hirasawa, Takafumi Hara, Ikuo Kimura, Tetsuya Adachi, Takeo Awaji, Masaji Ishiguro, Takayoshi Suzuki, Naoki Miyata, and Gozoh Tsujimoto

Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan (Q.S., A.H., T.H., I.K., T.Ad., G.T.); Department of Pharmacology, Saitama Medical University, Saitama, Japan (T.Aw.); Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan (M.I.); and Graduate School of Pharmaceutical Sciences, Nagoya City University, Aichi, Japan (T.S., N.M.)

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ABSTRACT

GPR120 is a G protein-coupled receptor expressed preferentially in the intestinal tract and adipose tissue, that has been implicated in mediating free fatty acid-stimulated glucagon-like peptide-1 (GLP-1) secretion. To develop GPR120-specific agonists, a series of compounds (denoted as NCG compounds) derived from a peroxisome proliferator-activated receptor γ agonist were synthesized, and their structure-activity relationships as GPR120 agonists were explored. To examine the agonistic activities of these newly synthesized NCG compounds, and of compounds already shown to have GPR120 agonistic activity (grifolic acid and MEDICA16), we conducted docking simulation in a GPR120 homology model that was developed on the basis of a photoactivated model derived from the crystal structure of bovine rhodopsin. We calculated the

hydrogen bonding energies between the compounds and the GPR120 model. These energies correlated well with the GPR120 agonistic activity of the compounds ($R^2 = 0.73$). NCG21, the NCG compound with the lowest calculated hydrogen bonding energy, showed the most potent extracellular signal-regulated kinase (ERK) activation in a cloned GPR120 system. Furthermore, NCG21 potently activated ERK, intracellular calcium responses and GLP-1 secretion in murine enteroendocrine STC-1 cells that express GPR120 endogenously. Moreover, administration of NCG21 into the mouse colon caused an increase in plasma GLP-1 levels. Taken together, our present study showed that a docking simulation using a GPR120 homology model might be useful to predict the agonistic activity of compounds.

Introduction

FFAs are not only essential nutritional components, but they also function as signaling molecules. Multiple receptors for FFAs have been successfully identified using a GPCR deorphanizing strategy. GPR120, which is activated by medium- to long-chain fatty acids, is expressed in the human and mouse intestinal tract and in adipose tissue, and is also abundantly expressed in the murine enteroendocrine STC-1 cells (Hirasawa et al., 2005; Gotoh et al., 2007; Miyauchi et al., 2009). GPR120 mediates FFA-promoted secretion of in-

cretin hormones (GLP-1 and cholecystokinin) in mouse, rat, and STC-1 cells (Sidhu et al., 2000; Hirasawa et al., 2005; Tanaka et al., 2008). GPR120 couples to G_q family proteins and mediates the $[Ca^{2+}]_i$ responses induced by FFAs in STC-1 cells (Hirasawa et al., 2005). Besides GPR120, another receptor for which endogenous ligands are medium- to long-chain FFAs is FFAR1 (free fatty acid receptor 1; previously known as GPR40). FFAR1 is abundantly expressed in the pancreatic β -cell, where it mediates FFA-enhanced glucose-stimulated insulin secretion (Briscoe et al., 2003; Itoh et al., 2003; Poitout, 2003; Steneberg et al., 2005; Feng et al., 2006; Stoddart et al., 2008). Because both GPR120 and FFAR1 promote glucose-stimulated insulin secretion, they have received increasing attention as attractive drug targets for diabetes (Miligan et al., 2006; Hirasawa et al., 2008; Suzuki et al., 2008).

Compared with the increasing number of reported FFAR1 ligands (Briscoe et al., 2003; Tikhonova et al., 2007; Bharate et al., 2008; Davi and Lebel, 2008; Hirasawa et al., 2008;

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ABBREVIATIONS: FFAs, free fatty acids; GLP-1, glucagons-like peptide-1; FFAR1, free fatty acid receptor 1; NCG21, 4-{4-[2-(phenyl-2-pyridinylamino)ethoxy]phenyl}butyric acid; α -LA, α -linolenic acid; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; ANOVA, analysis of variance; SAR, structure-activity relationship.

TABLE 1

Structures of compounds, relative maximal ERK activity and hydrogen bonding energy between compounds and the homology model of GPR120
The relative maximal ERK response was calculated as percentage of agonistic response elicited by 100 μM compounds with respect to the response evoked by 100 μM α -LA in GPR120-expressing cells. Values are means of at least three experiments.

Compound	Structure	Relative Maximum ERK response	H-Bonding Energy	Compound	Structure	Relative Maximum ERK response	H-Bonding Energy
		%	au			%	au
NCG21		1.54	-5.92	NCG40		0.62	-2.73
NCG20		1.38	-4.04	NCG41		0.96	-2.61
NCG22		0.93	-5.07	NCG42		0.54	-2.92
NCG23		1.20	-4.10	NCG43		0.70	-3.10
NCG25		1.25	-4.73	NCG44		0.64	-3.10
NCG26		1.02	-4.54	NCG45		0.76	-3.39
NCG27		0.55	-2.23	NCG46		1.20	-5.41
NCG28		0.80	-3.66	NCG48		0.92	-3.98
NCG29		1.46	-3.56	NCG49		0.75	-2.61
NCG30		1.45	-5.57	NCG50		1.14	-4.93
NCG31		1.40	-4.06	NCG51		1.32	-4.77
NCG32		1.03	-3.86	NCG52		0.29	-2.70
NCG33		0.39	-2.88	NCG53		1.27	-4.56
NCG34		1.37	-5.36	NCG56		1.14	-3.82
NCG35		1.48	-4.75	NCG57		1.09	-4.11
NCG37		1.48	-5.37	MEDICA16		0.21	-1.73
NCG38		1.24	-4.87	grifolic acid		0.41	-2.31
NCG39		1.23	-4.75	α -LA		1.00	-3.03
				α -LA methyl ester		0.00	-1.38

au, arbitrary units.

Compounds. We examined NCG21 (4-{4-[2-(phenyl-2-pyridinylamino)ethoxy]phenyl}butyric acid) (Suzuki et al., 2008), together with 32 other NCG compounds derived from a peroxisome proliferator-activated receptor γ agonist (Table 1). α -Linolenic acid (α -LA) and MEDICA16 were purchased from Sigma (St Louis, MO). Grifolic acid was a gift from Drs. Toshihiro Hashimoto and Yoshinori Asakawa (Tokushima Bunri University, Tokushima, Japan). All compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and stored at -20°C .

Cell Lines. We used a stable cell line Flp-in GPR120 that was established previously (Hara et al., 2009b). Flp-in GPR120 cells and murine enteroendocrine STC-1 cells were cultured as described previously (Hirasawa et al., 2005; Hara et al., 2009b). In brief, Flp-in GPR120 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 100 μ g/ml hygromycin (Invitrogen, Carlsbad, CA). STC-1 cells were maintained in Dulbecco's modified Eagle's medium containing 15% horse serum and 2.5% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air.

ing sites of GPR120 protein were calculated using the Molegro cavity detection algorithm. The hydrogen bonding energy, which is considered to be one of the important parameters in characterizing the interaction between GPCRs and their ligands (Shim et al., 2003; Xhaard et al., 2006), was estimated in arbitrary units using the Molegro program.

[Ca²⁺]_i Measurement. [Ca²⁺]_i was monitored by a Ca²⁺ imaging method using an image processor (Argus 50; Hamamatsu Photonics, Hamamatsu, Japan) as described previously (Hara et al., 2009a). To measure [Ca²⁺]_i, STC-1 cells were loaded with 2 μM fura-2 acetoxymethyl ester (Dojindo, Tokyo, Japan) by incubation with this compound for 30 min at 37°C. [Ca²⁺]_i measurement was performed at 30°C in Tyrode's solution. Fluorescence of fura-2 was measured by illuminating samples with UV light at 340 and 380 nm alternately; emitted light then passed through a 505-nm dichroic mirror (DCLP; Omega Optical, Brattleboro, VT) and the fluorescence was detected using a single-pass detection–charge-coupled device camera (MC681APD-R0B0; Texas Instruments, Dallas, TX). Ca²⁺ images were acquired at intervals of 20 s and processed to calculate F_{340}/F_{380} later using the NIH Image program (<http://rsbweb.nih.gov/nihi-image/>).

GLP-1 Secretion. GLP-1 secretion from STC-1 cells was measured as described previously (Hirasawa et al., 2005). In brief, STC-1 cells were seeded in 24-well culture plates and allowed to reach 60 to 80% confluence by incubating for 48 h at 37°C. On the day of the experiment, STC-1 cells were washed three times with Hanks' balanced salt solution (Invitrogen), and then transferred to growth medium and incubated for 60 min at 37°C in Hanks' balanced salt solution containing various concentrations of compounds that were sonicated just before use with a probe sonicator (Tomy Seiko, Japan).



Fig. 1. Sequence alignment of rhodopsin and GPR120. Amino acid sequences corresponding to rhodopsin and GPR120 were aligned using the ClustalW algorithm (Chenna et al., 2003). TM, seven transmembrane domains; *, residues of rhodopsin and GPR120 in the sequence alignment are identical; †, conserved residues observed; ‡, semiconserved residues observed.

After incubation, conditioned medium was collected and the concentration of GLP-1 was determined by enzyme immunoassay using a GLP-1 enzyme-linked immunosorbent assay kit (Wako Pure Chemical, Osaka, Japan).

Data Analysis. In the present study, we investigated the relationship between calculated hydrogen bonding energy and relative ERK efficacy (not affinity) for each compound. The hydrogen bonding energy is considered to be directly related to the affinity of the different molecules and not to their efficacy at a single dose. Our preliminary series of experiments, however, showed that the NCG series of compounds contained partial and full agonists and that we could not obtain the full dose-response curves for all compounds. Because the theoretical simulation based on the two-state model of receptor activation (Leff, 1995) showed that hydrogen bonding energy can be well related to the efficacy at a single dose of the different compounds when their EC_{50} values are within a relatively narrow range (~ 100 -fold), we surrogated the relationship between the hydrogen bonding energy and ERK activity in the present study.

One-way analysis of variance (ANOVA) was used to evaluate treatment effects. If the ANOVA value was significant, comparisons between the control and treatment groups were performed using ANOVA followed by Dunnett's t test to localize the significant difference. $P < 0.05$ was considered statistically significant.

Results

Docking Simulation of GPR120 Agonists using a GPR120 Homology Model. To develop GPR120 agonists and explore the SARs of GPR120 agonists, a series of NCG compounds were synthesized, and their ERK activities in cells stably expressing GPR120 were examined. All of these NCG compounds stimulated an ERK response, but the potency of their activities differed according to their structure. The ERK activity was distinctly dependent on the length of methylene chain between the phenyl and carboxyl groups (Table 1). Among the NCG compounds, NCG21, which has a three-carbon methylene chain between the phenyl and carboxyl groups, showed more potent ERK activation than NCG23 and NCG25, which has methylene chains of length four and two, respectively. The activity of the NCG compounds also varied dependent on the conversion of *N*-phenyl-2-pyridinamine structure (Table 1). Although the conversion of the 2-pyridine ring of NCG21 to a 3-pyridine (NCG26) or thiazole ring (NCG46) retained the ERK activity to some extent, replacement with a pyrimidine ring (NCG44) significantly reduced the activity. In the 6-substituted 2-pyridine derivatives, compounds with a relatively large substituent (NCG40 and NCG42) were much less potent than NCG21, whereas analogs bearing a small substituent (NCG37, NCG38, and NCG39) showed the ERK activity only slightly less than that of NCG21. A similar SAR was observed in 4-substituted series (NCG43, NCG34, and NCG35). As for the conversion of the phenyl ring of NCG21, NCG20, in which the phenyl group of NCG21 was replaced with *n*-butyl group, sustained the ERK activity. In addition, the introduction of a relatively small electron-donating group to the 4-position of the phenyl ring (NCG50 and NCG51) tended to maintain the activity. On the other hand, compounds having an electron-withdrawing group or a large substituent at the 4-position of the phenyl ring (NCG48, NCG49, and NCG52) decreased the potency. The ERK activity of the analogs with a substituent at the 3-position of the phenyl ring (NCG53, NCG56, and NCG57) was a little less than that of NCG21. Because the ERK activity seems to be dependent on the electron density

on the phenyl ring, the interaction (such as π - π interaction and CH- π interaction) between the phenyl ring and hydrophobic amino acid residues (Met 115, Leu 187, Phe 202) located around the phenyl ring may be important for the GPR120 agonistic activity.

A docking simulation of these NCG compounds was carried out next, together with α -LA, an endogenous ligand for GPR120 (Hirasawa et al., 2005); grifolic acid, known as a selective partial agonist for GPR120; and MEDICA16, known as a selective agonist for FFAR1 (Hara et al., 2009b). A homology model of GPR120 was constructed on the basis of a photoactivated model derived from the crystal structure of bovine rhodopsin. Thirty-seven compounds were then docked individually into the GPR120 model using the Molegro Virtual Docker subroutine. An inspection of the stimulated receptor-ligand complexes (for example, the GPR120/ α -LA complex and the GPR120/NCG21 complex) showed that there seemed to be hydrogen bonds between the oxygen of the carboxylate on both of these compounds and the guanidine of Arg99. The distances between the oxygen of the carboxylate of NCG21 and α -LA and nitrogen of guanidine in Arg99 were 2.63 and 3.07 Å, respectively (Fig. 2, A and B). In addition, α -LA methyl ester, which constituted inactive molecules of GPR120, also docked with GPR120 (Fig. 2C). The distance between the oxygen of the carboxylate of α -LA methyl ester and nitrogen of the guanidine was 7.01 Å. In silico-calculated hydrogen-bonding energies of these compounds docked into the GPR120 model are shown in Table 1. A plot of relative ERK activity versus calculated hydrogen bonding energy (Fig. 3) showed a high correlation between the hydrogen bonding energy and ERK activity ($R^2 = 0.73$). The rank order of these predicted hydrogen bonding energies (NCG21 < α -LA < grifolic acid < MEDICA 16 < α -LA methyl ester) was consistent with the experimental ERK activity data.

As mentioned above, the in silico calculated hydrogen

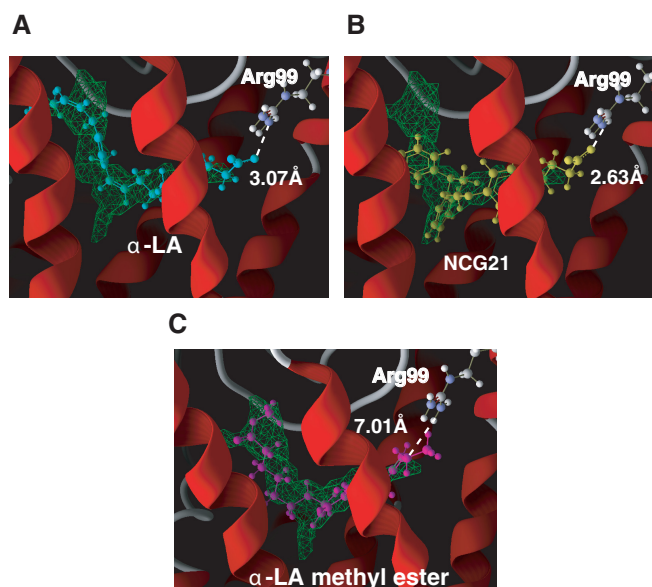


Fig. 2. GPR120 homology model docked with α -LA, NCG21, and α -LA methyl ester. α -LA (A), NCG21 (B), and α -LA methyl ester (C) were docked into the binding pocket of GPR120. Cyan, α -LA; yellow, NCG21; pink, α -LA methyl ester; green: the predicted binding pocket by Molegro cavity detection algorithm. The dashed lines indicate the distance between the oxygen of the carboxylate of compounds and the guanidine nitrogen of Arg 99.

bonding energies of NCG compounds are consistent with the experimental results of ERK activation. Among these NCG compounds, NCG21, which most potently activated the ERK response, showed the lowest hydrogen bonding energy to the GPR120 homology model (-5.92). In contrast, the hydrogen bonding energy of grifolic acid (-2.31), which is a selective partial agonist for GPR120, was higher than that of NCG21. An endogenous ligand for GPR120, α -LA, showed much lower hydrogen bonding energy (-3.03) than α -LA methyl ester (-1.38), which did not activate GPR120. On the other hand, the selective FFAR1 agonist MEDICA16 showed a high calculated hydrogen bonding energy (-1.73) to the GPR120 model.

Pharmacological Effects of NCG21 In Vitro and In Vivo. The docking simulations predicted that NCG21 had the lowest hydrogen bonding energy among the 37 compounds examined, indicating that it may have the most potent agonistic activity. To test the agonistic activity and the selectivity of NCG21, together with grifolic acid and MEDICA16, we examined the $[Ca^{2+}]_i$ responses induced by these compounds in human embryonic kidney 293 cells expressing GPR120 or FFAR1 (Supplemental Fig. 1). We found that NCG21 and grifolic acid more potently activated the

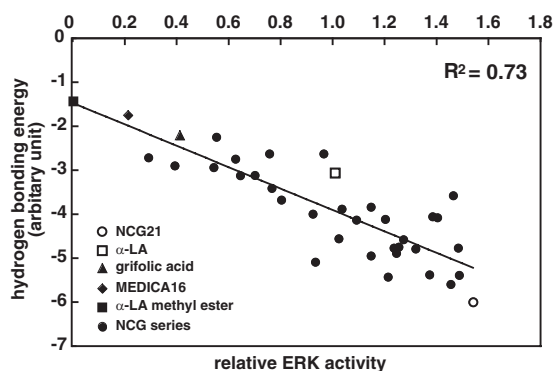


Fig. 3. Structure-activity relationships of GPR120 ligands. The relative ERK activity (x-axis) versus the calculated energy of interaction based on modeling (y-axis) was plotted. The straight line represents the line $y = -2.44 \times -1.47$. The coefficient of determination ($R^2 = 0.73$) reflects a high correlation between the hydrogen bonding energy and relative ERK activity.

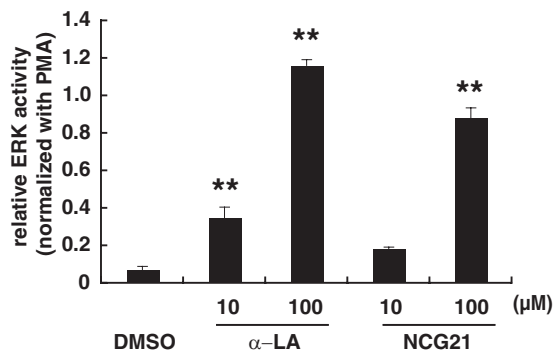


Fig. 4. Effect of NCG21 on ERK response in STC-1 cells. Cells were serum-starved for 2 h and then treated with various compounds at 10 and 100 μ M. Cell lysates were analyzed by immunoblotting using anti-phospho- and anti-total-kinase antibodies. The amount of phosphorylated ERK was normalized to the amount of total ERK. The data were then presented as the fold difference relative to the amount of ERK phosphorylation that was obtained in the presence of phorbol 12-myristate 13-acetate (PMA). Results represent means \pm S.E.M of three independent experiments. Significant differences were shown (**, $p < 0.01$) between treatment with the control (DMSO) and with the indicated compounds.

$[Ca^{2+}]_i$ response in GPR120-expressing cells than in FFAR-expressing cells. Then we examined the pharmacological properties of these compounds by using the STC-1 murine enteroendocrine cell line. As shown in Fig. 4, NCG21 and α -LA increased ERK responses in a dose-dependent manner. In addition, as shown in Fig. 5, NCG21, α -LA, and grifolic acid promoted $[Ca^{2+}]_i$ in STC-1 cells. NCG21 had greater potency than the other two compounds. In contrast, MEDICA16 did not stimulate a $[Ca^{2+}]_i$ response (Fig. 5, A and B). The results seemed to be in good agreement with the relationship of calculated hydrogen bonding energy and ERK activity as shown in Fig. 3. Furthermore, NCG21 and α -LA potently stimulated GLP-1 secretion in STC-1 cells (Fig. 6). The in vivo effect of NCG21 was also examined. As shown in Fig. 7, similar to α -LA, administration of NCG21 directly into the colon increased the plasma GLP-1 level in the mouse.

Discussion

In this study, we showed that a docking simulation using a GPR120 homology model might be useful to predict the agonistic activity of compounds. A series of NCG compounds derived from a peroxisome proliferator-activated receptor γ agonist were synthesized, and the SARs of these compounds were explored by carrying out docking simulations. Those NCG com-

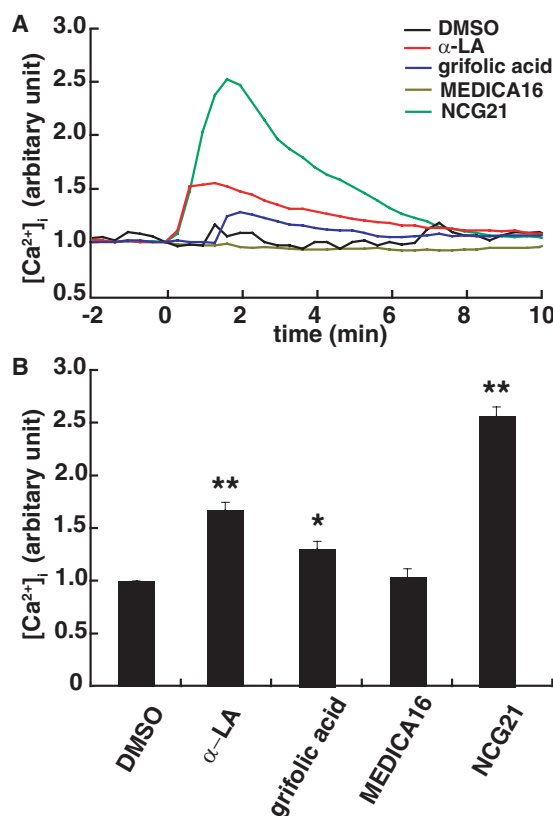


Fig. 5. Effect of NCG21 on $[Ca^{2+}]_i$ in STC-1 cells. STC-1 cells were stimulated with each compound (1 μ M). A, representative results were shown with values, expressed as means of measurement from five to six cells, obtained from one of three independent experiments. Two additional experiments gave similar results. The time point when indicated compounds were administered was considered 0 s. B, the maximum $[Ca^{2+}]_i$ response induced by the indicated compounds between 0 and 10 min was shown. Results are means \pm S.E.M of three independent experiments. The data were normalized against the maximum response observed from DMSO. Significant differences were indicated (*, $P < 0.05$; **, $P < 0.01$) between treatment with the control (DMSO) and with the compound.

pounds, together with the selective GPR120 partial agonist grifolic acid and the selective FFAR1 agonist MEDICA16 (Hara et al., 2009b), were then docked individually into the GPR120 homology model. The simulations showed that the calculated hydrogen bonding energy for the compounds docked into GPR120 model correlated well with their GPR120 agonistic activities, suggesting that this method could be useful to explore the SARs of GPR120 agonists. The structural features of NCG21 allowed its carboxylate group to dock into a position closer to Arg 99 in the binding site of GPR120, thus allowing it to form a strong interaction with this residue. Mutation studies of the residue confirmed that Arg 99 is most critical for GPR120 activation (T. Hara, manuscript in preparation). The reason that the substituent introduction to the pyridine or the phenyl ring of NCG21 reduced the ERK activity in many cases was unclear. However, the calculation results in this study suggested that the substituent on the pyridine or phenyl ring alters the conformation of the compound in the ligand binding domain by repulsive or attractive interaction with the hydrophobic amino acid residues (Met 115, Leu 187, Trp 189, Phe 202, Ile 247, and Phe 270) located around the pyridine and phenyl group, which affected the interaction between its carboxylate anion and Arg 99. On the basis of this docking simulation, NCG21 was predicted to be the most potent ligand with agonistic activity among NCG compounds. This prediction was then validated by biological assays.

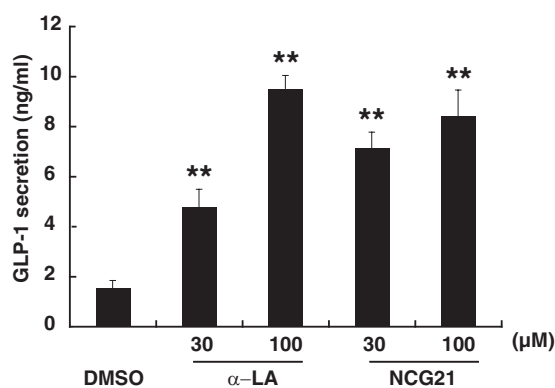


Fig. 6. Effect of NCG21 on GLP-1 secretion in STC-1 cells. GLP-1 secretion in STC-1 cells was measured after treatment with 30 and 100 μ M each compound for 60 min at 37°C in HBSS. Results represent means \pm S.E.M of three independent experiments. Significant differences (**, $P < 0.01$) between treatment with the control (DMSO) and with the compound were indicated.

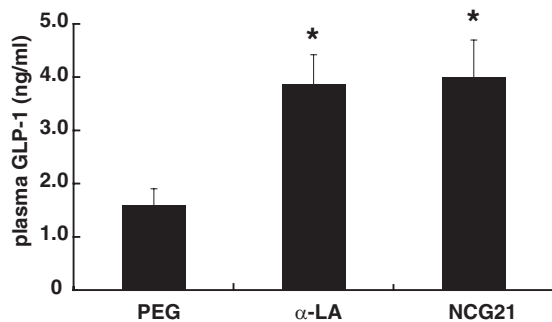


Fig. 7. Effect of NCG21 on GLP-1 secretion in vivo. α -LA, NCG21, and the vehicle PEG (polyethylene glycol) were administered into the mouse colon experiments. Significant differences indicated. Using an intestinal cannulation method. Results represent means \pm S.E.M of 11 independent (*, $P < 0.05$) compared with vehicle administration were.

The pharmacological properties of NCG21 were further characterized in STC-1 cells, which express GPR120 endogenously (Hirasawa et al., 2005). The results showed that NCG21 potently stimulated ERK, $[Ca^{2+}]_i$ responses, and GLP-1 secretion in STC-1 cells. The selective partial agonist for GPR120, grifolic acid, could also induce a $[Ca^{2+}]_i$ response and GLP-1 secretion (Hara et al., 2009b), but the selective FFAR1 agonist MEDICA16 showed no effect on STC-1 cells. These results indicate that NCG21 could potently and selectively activate GPR120 not only in a cloned GPR120 system but also in STC-1 cells. Moreover, administration of NCG21 into the mouse colon caused an increase in plasma GLP-1 levels, a finding consistent with the result of in vitro assays.

In conclusion, we report here the SARs of a series of NCG compounds with GPR120-agonistic activities that correlated with hydrogen bonding energies calculated using docking simulations. The SARs indicated that, among the NCG compounds, NCG21 was predicted to have the most potent agonistic activity. Therefore, our present study showed that a docking simulation using a GPR120 homology model might be useful to predict the agonistic activity of compounds. In addition, NCG21, which was confirmed to be a potent agonist, would become an important pharmacological tool to investigate the biological functions of GPR120.

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Address correspondence to: Gozoh Tsujimoto, M.D., Ph.D., Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501. E-mail: gtsuji@pharm.kyoto-u.ac.jp
