modified eagle medium (DMEM: Invitrogen®) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo, Japan) in 0.2 µg of each cDNA according to the protocol provided by the manufacturer. Cells were used in confocal microscopy 16–24 h after transfection. cDNA for rat MOR was kindly provided by Dr. Dascal (Tel Aviv University). Venus, a brighter variant of yellow fluorescent protein (Nagai et al., 2002) was obtained from Dr. T. Nagai (Riken, Wako, Japan). Primers (5′-GGG GTA CCC CAT GGA CAG CAG CAC-3′) and (5′-GCG GCC GCG GGG CAA TGG AGC AGT-3′) were engineered to ligate the N-terminus of MOR by using standard molecular approaches with the polymerase chain reaction (PCR). Venus-fused MOR was created by ligating the MOR cDNA sequences into the *NotI* site of the corresponding Venus site. cDNA for transfection in BHK cells was subcloned into pcDNA3.1 (Invitrogen® Life Technologies, CA). cDNA for rat β-arrestin 2 was generously provided by Dr. Y. Nagayama (Nagasaki University, Japan). For the analysis of the agonist-induced internalization of MORs, BHK cells that had been transfected with Venus-fused MORs and β-arrestin-2 were incubated in the absence or presence of 100 nM β-endorphin for 30 min at 37°C, and then treated with 10 µM morphine, 100 nM fentanyl or 10 µM oxycodone. To investigate the resensitization of MORs, the cells were incubated with 100 nM fentanyl or 10 µM oxycodone in the presence or absence of β-endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. The cells were subsequently fixed and examined by confocal microscopy as previously reported (Corbani et al., 2004). Venus was excited by a 488-nm laser was used to detect Venus fluorescence with a 505- to 530-nm band-pass filter, and images were obtained by placing the dish on the stage of an inverted Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). Data were stored on the hard disc with and analyzed with the Zeiss LSM software Zen 2009. For the quantitative analysis of agonist-induced internalization of MORs, BHK cells were fixed with 4% paraformaldehyde in PBS and stored at 4°C. The numbers of cells expressing Venus-fused MORs were counted. For counting cells whether Venus fluorescence was at the plasma membrane or in cytosol (internalization), we basically followed by Corbani et al. (2004). Localization of Venus-fused MORs in BHK cells was categorized as "mainly expressed at the plasma membrane," "not detected in plasma membrane but detected in cytosol," or "not detected" (whose localization was not belong to the former category), separated with a software Zen 2009 equipped with Zeiss LSM510 META confocal microscope, with reference to
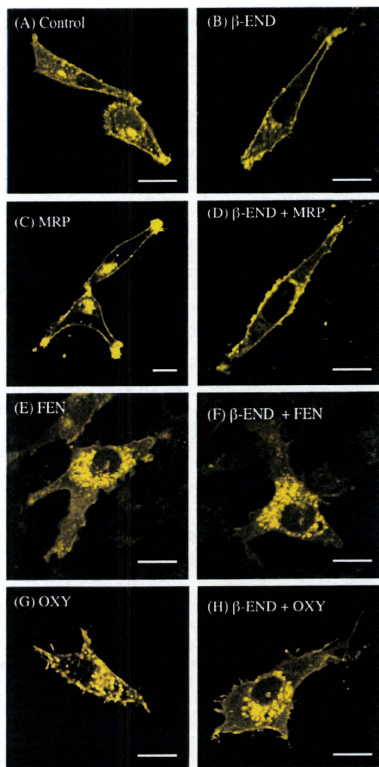

Fig. 1. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. The cells were incubated in the absence (A, C, E, and G) or presence (B, D, F, and H) of 100 nM β-endorphin (β-END) for 30 min at 37°C and then treated with 10 µM morphine (MRP; C, D), 100 nM fentanyl (FEN; E, F), or 10 µM oxycodone (OXY; G, H). The cells were subsequently fixed and examined by confocal microscopy. Yellow fluorescence from Venus indicates the localization of MORs in BHK cells. Scale bars, 10 µm.

the control, not stimulated BHK cells. A total of 100 cells (counted mean 200–250 cells in sum of "the plasma membrane," "in the cytosol," plus "not detected") in six independent each dish. % Internalization was described as cytosol × 100/[plasma membrane + cytosol (total 100 cells)]. The drugs used in this study were fentanyl citrate (Hisamitsu Pharmaceutical, Tokyo, Japan), morphine hydrochloride
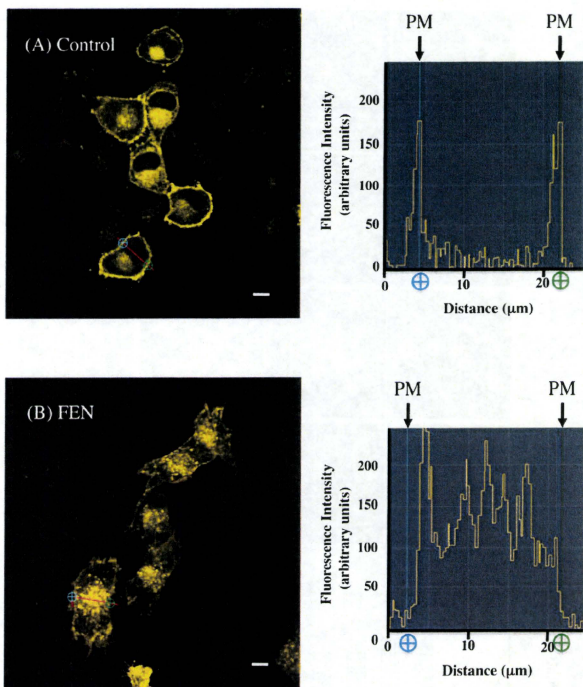
Fig. 2. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. Typical cells where most of MOR-Venus intensity was at the plasma membranes, [A, control cells (Control)] or in the cytosolic fraction [B, 100 nM fentanyl-stimurated for 30 min (FEN)]. PM; plasma membranes in BHK cells. Scale bars, 10 μm.

(Daiichi-Sankyo, Tokyo, Japan), oxycodone hydrochloride (a kind gift from Shionogi Pharmaceutical, Osaka, Japan), and β-endorphin (Sigma-Aldrich, St Louis, MO), which were dissolved in assay buffer.

RESULTS AND DISCUSSION

In this study, we assessed whether β-endorphin could affect the trafficking properties of MORs using immunocytochemical methods in BHK cells with confocal microscope. Confocal imaging of the BHK cells expressing Venus-fused MOR with β-arrestin-2 revealed that the yellow fluorescence was largely confined to the plasma membrane (Figs. 1A and 2A). In both the presence and absence of 100 nM β-endorphin, at which concentration there did not cause any

internalization of MORs (Figs. 1B and 1C), cells expressing MORs treated with 10 μM morphine (Figs. 1C and 1D) showed little internalization of MORs, while the cells treated with 100 nM fentanyl (Figs. 1E, 1F, and 2B) and 10 μM oxycodone (Figs. 1G and 1H) showed robust internalization of the receptor. These findings were consistent with previous reports that fentanyl and etorphine caused partial internalization, while morphine failed to induce detectable MOR endocytosis (Koch et al., 2005). We next investigated the resensitization properties of MORs after the washing-out of agonists. In the absence of β-endorphin, internalized MOR returned to the plasma membrane from 90 min after the washing-out of fentanyl (Figs. 3B–3D). However, in the presence of β-endorphin, the internalized MOR induced by fentanyl
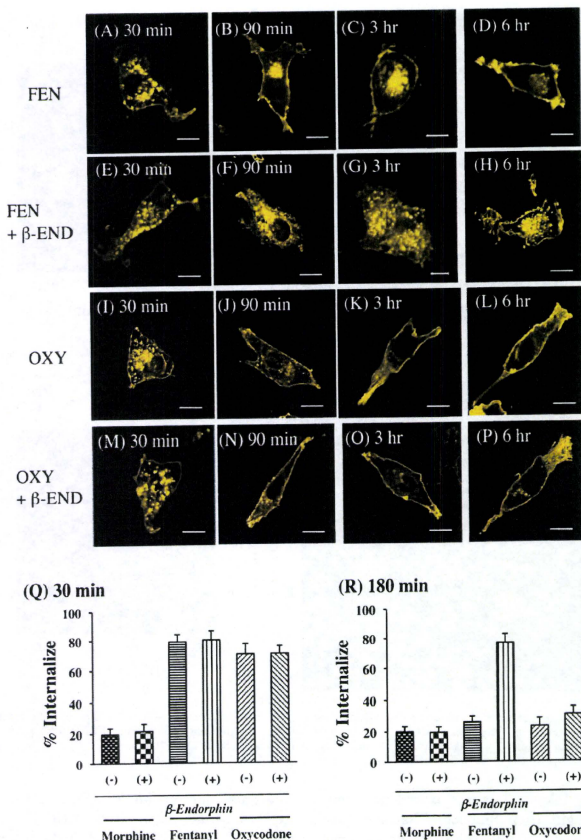
Fig. 3. Confocal imaging of resensitization of MORs in BHK cells expressing Venus-fused MORs. Cells were incubated with 100 nM fentanyl (**A–H**) or 10 μM oxycodone (**I–P**) in the absence (A-D and I-L) or presence (E-H and M-P) of β-endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. The cells were then fixed and counted by confocal microscopy. Yellow fluorescence from Venus indicates the cellular localization of MOR in BHK cells. Scale bars, 10 μm. Quantitative analysis of the % of the internalized cells expressing Venus-fused MORs treated with the drugs for 30 min (**Q**) or 180 min (**R**), respectively. The agonist concentrations represent the dose required to induce the maximal effect on receptor endocytosis for each drug. Each value represents the mean ± SEM of six separate experiments.

remained in the cytosolic fraction at 3–6 h after the washing-out of β-endorphin and fentanyl (Figs. 3F–3H). However, in both the presence and absence of β-endorphin, the internalized MOR induced by oxycodone returned to the plasma membrane after the washing-out of agonist in a time-dependent manner (Figs. 3I–3P). We performed quantitative analysis of the agonist-induced internalization of MORs after the washing-out of each agonist shown in Materials and Methods. At 30 min after the washing-out of agonists,

cells treated with fentanyl or oxycodone showed robust internalization of MORs (fentanyl: 79.0 ± 5.14%, β-endorphin fentanyl: 80.2 ± 3.7%, oxycodone: 70.5 ± 7.09%, β-endorphin oxycodone: 70.7 ± 5.35%), which was not seen in morphine-treated cells (morphine: 19.67 ± 3.93%, β-endorphin morphine: 21.5 ± 4.76%; Fig. 3Q). However, while there was no difference in the degree of oxycodone-induced MOR internalization between the presence and absence of β-endorphin 3 h after washing-out (oxycodone: 23.17 ± 5.12%, β-endorphin oxycodone: 30.5 ± 4.72%), in fentanyl-treated cells, β-endorphin caused the prolonged internalization of MORs and fluorescence was stayed in the cytosolic fraction (fentanyl: 27.67 ± 5.47%, β-endorphin fentanyl: 76.5 ± 6.02%; Fig. 3R).

It has been widely accepted that receptor desensitization, internalization and trafficking appear to play a key role in the development of opioid tolerance (Claing et al., 2002; Gainetdinov et al., 2004). The initial process in these events is the phosphorylation of intracellular domains of MOR. Phosphorylated MORs are mostly internalized via clathrin-coated pits into early endosomes and subsequently dephosphorylated by intracellular protein phosphatases. The dephosphorylated MORs might either be recycled to the plasma membrane or transported to lysosomes for degradation. A growing body (Smalheiser and Lugli) of evidence suggests that among diverse serine/threonine (Thr) residues of the intracellular domain of MOR, the phosphorylation of Ser 375 in the mouse MOR is essential for the internalization of MORs (Schulz et al., 2004). In a previous study, we found that repeated treatment with fentanyl, but not morphine, resulted in an increase in the levels of phosphorylated-MOR (Ser 375) associated with the enhanced inactivation of protein phosphatase 2A and a reduction in Rab4-dependent MOR resensitization in the spinal cord of mice that showed inflammatory pain (Imai et al., 2006). However, several lines of evidence indicate that, in response to pain stimulus, endogenous β-endorphin is released within some brain regions (Zubieta et al., 2001). We previously reported that β-endorphin released in the ventral tegmental area is a key factor in regulating the dysfunction of MOR to negatively modulate opioid reward under a neuropathic pain-like state (Niikura et al., 2008, 2010). Taken together, although further studies are still needed, these findings support the idea that inhi-

bition of the resensitization system of MOR following chronic treatment with fentanyl in the presence of β-endorphin may be associated with antihyperalgesic tolerance to fentanyl under a chronic pain-like state.

In conclusion, we demonstrated here that unlikely morphine, either fentanyl or oxycodone induced a robust MOR internalization and, in turn, its resensitization. In the presence of β-endorphin, the internalized MOR induced by fentanyl, but not oxycodone, remained within the cytosolic fraction even after washing out. These findings strongly support that idea that fentanyl has different pharmacological profile form that of morphine or oxycodone.

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がん患者の症状緩和のために
―がん悪液質の予防,症状改善をめざす基礎医学研究

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上園　保仁

はじめに

2009年より始まった基礎医学セミナーでは,基礎医学研究が臨床医学にどのように結びついているか,そしてがん患者のために活かされているかについて紹介させていただいています。第1回セミナーでは,「がん患者の生活の質(quality of life;QOL)の向上をめざして,基礎医学研究者も積極的にがんの痛みなどの基礎研究に携わり,そこで明らかとなった知見が臨床の現場で活かされるようになれば。その具体的な研究を今後のセミナーでご紹介いたします」と結びました[1]。そのなかで,がん患者のQOLを下げるものは痛みに止まらず疲労感,倦怠感,食思不振,不眠,便秘,嘔気嘔吐などたくさんの症状があることをお伝えしました。倦怠感,衰弱感,食思不振といった一連の症状は,進行がん患者の「悪液質」と呼ばれる症状で多くみられます。

今回は,進行がん患者の約50〜60%にみられる「がん悪液質」について,がん悪液質の病態の説明,そしてがん悪液質の予防ならびにその症状改善のためにどのような基礎‐臨床医学の橋渡し研究が行われているのかについてご紹介いたします。

がん悪液質とは

悪液質は,食思不振,体重減少,特に筋肉量の減少を主症状として,疲労感,倦怠感を伴い,また血中炎症性サイトカインレベルなどに異常がみられる消耗性の疾患です。体重減少に関しては「飢餓」の状態と変わらないのですが,飢餓では基礎代謝,糖代謝が低下しているのに対して悪液質ではむしろ基礎代謝や糖代謝が亢進すること,また飢餓では脂肪組織の減少が主にみられるのに対し,悪液質では筋肉量の低下が著明であることなど,悪液質は単なる食思不振や栄養不足のために体重減少を伴う症状ではないことが知られています。この悪液質は,がん患者以外に慢性呼吸器疾患や慢性心臓病,慢性腎臓病の患者などでもみられます。がん悪液質は終末期のがん患者では50〜60%に認められますが,近年明らかになってきたのは,がん悪液質患者は明らかに予後が悪く,さらにがん悪液質自体が原因で死亡する患者ががん死亡の20%を占めるということです。すべてのがん腫でがん悪液質の報告がみられますが,特に膵臓がん,胃がん患者にがん悪液質の傾向が高く(両がん患者とも約80%),ほかに食道がん,頭頸部がん,大腸がん患者もがん悪液質を伴うことが多いことが報告されています[2]。

がん悪液質の研究報告を調べますと,がん悪液質を予防し症状の改善を行うことは,がん患者のQOLを向上させるのみならず生命予後を長くすることが示されています。つまり,がん悪液質にならない,あるいは発症時期を遅らせる,発症してもできるだけ症状を軽くするという治療は,患者のために有効であるということです。ところが,がん悪液質の予防,治療には決定的なものがないのが現状です。

そもそもがん悪液質の成因，素因といったものは，がん細胞自体が出す何らかのファクターによるもの，がん細胞からのファクターに反応して起こる宿主の免疫，代謝異常などの二次性反応によるものなど，さまざまな原因が報告されてはいるものの，その本質はほとんどわかっていません。したがって，原因がはっきりしないのでそれに対する予防法，治療法もわからないということです。

がん悪液質の診断基準

2008年に悪液質の診断基準が示されました。それによると，12ヵ月以内に5％以上体重が減少し，加えて筋力低下，疲労などの症状の5つの基準のうち3つ以上を満たすこと，と定義されています(図1)[3]。

また，ごく最近新たな分類も提唱されています。それによると，がん悪液質が前悪液質(pre-cachexia)，悪液質(cachexia)，治療不応性悪液質(refractory cachexia)の3種に分類されています[4]。がん患者の悪液質は早期に対応すればするほどその改善が顕著であることを考えると，がん悪液質の早期診断，そして早期介入はとても重要です。前悪液質として定義される基準が導入されると着実な早期診断ができると思われるので，がん患者にとっても介入を行う医療サイドとしても，この基準の導入は望まれるところです。また，がん悪液質への薬効を検討する臨床治験においても，どのがん患者に対してどのタイミングでどのような薬剤の治験を行うかという詳細な基準を設定できることにもなり，正確な薬効評価が生まれるものと期待されます。

がん悪液質治療の具体例 (研究中であるものも含む)

悪液質を有しているがん患者にはその基礎疾患であるがんが存在するのは明白であり，もちろんその基礎疾患が治癒されれば悪液質は消失します。しかし，がん本体の治癒は，手術で取り除けない例や，また抗がん剤治療を含む内科的治療はまだ完璧ではないこともあり，とても困難な課題です。がん悪液質の患者に対しては食思不振の改善や体重減少を防止する薬物療法が行われることになりますが，栄養面での工夫，改善もまた重要な治療介入ポイントです。

がん悪液質とは
がん悪液質とは，食思不振ならびに体重減少(体脂肪量に加え筋肉量が減少した状態)を主徴とする病態です。そのほかに疲労感，筋力低下，虚脱感などを伴います。

2008年の悪液質診断基準では，
1. 12ヵ月以内に5％の体重低下のあることに加え，
2. ①筋力低下，②疲労感，③食欲不振，④除脂肪量低下，⑤血液検査異常(炎症性マーカー上昇(CRP，IL-6)，貧血(Hb<12g/dL)，低アルブミン血症(<3.2g/dL))，のなかで3つ以上を満たすものとなっています。

悪液質診断

12ヵ月以内に少なくても5％の体重低下(もしくはBMI<20kg/m²) ＋ 5つのうち3つ以上

①筋力低下
②疲労感
③食欲不振
④除脂肪量低下
⑤血液検査異常
　炎症性マーカー上昇(CRP，IL-6)
　貧血(Hb<12g/dL)
　低アルブミン血症(<3.2g/dL)

図1．がん悪液質の診断基準
　CRP：C反応性蛋白，IL：インターロイキン，Hb：ヘモグロビン

(文献3より一部引用)

悪液質の症状改善において，現在さまざまな薬剤が用いられています。なかにはまだ確固たるエビデンスがなく，動物実験や臨床試験の最中のものもあります。現在のところ，がん悪液質治療法を開発するためのアプローチとしては，以下の3つが考えられています（図2）。

①食思不振，やせなど，摂食に伴う調節の異常が起こっているのが原因であるという仮説に基づいて，摂食ホルモンアゴニスト，あるいは満腹中枢を刺激しているホルモンの阻害薬を開発することで，食，エネルギー代謝のバランスを取る。あるいは，食思改善を期待して用いられるステロイド薬などを使用する。
②炎症性サイトカインをはじめとした内因性のホルモンや神経伝達物質により栄養，代謝バランスが破綻しているという仮説に基づき，エネルギー代謝異常をきたしているサイトカインなどに焦点を当てて治療を行う。
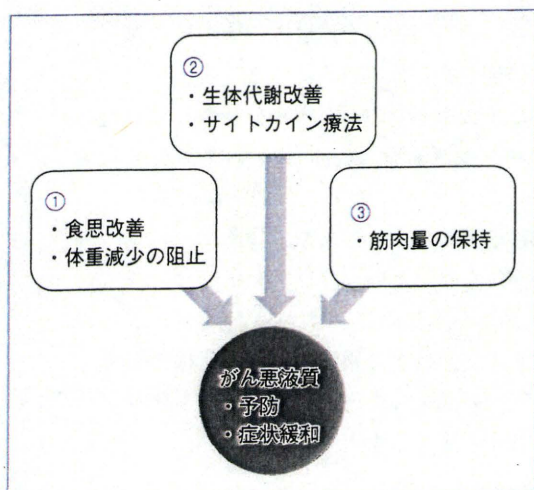③がん悪液質では脂肪量，筋肉量が低下するが，筋肉量の低下こそが悪液質の諸症状を悪化させているという仮説に基づき，筋肉量を増やす，あるいは減らさないための治療を行う。

図2．がん悪液質に対する予防ならびに症状緩和療法

これまでに，さまざまながん悪液質治療法が動物実験，臨床試験などで試されています。現在も新たな治療候補薬が検討され，試されています[5]が，結論をいえば決定的な治療法，症状改善法はいまだ確立されていません。まずはがん悪液質の原因を明らかにして，そこから導かれる治療法を探っていくのが遠回りでも一番確実な方法であると思われます。

上記①～③のカテゴリーに沿って，今まで有用と思われ試されている薬剤を分類し，示します。しかし，まだエビデンスのあるものは少なく，さらに基礎研究，臨床研究が必要です。

①体重減少阻止，食思改善に働く薬剤
●ステロイド薬（プロゲステロン製剤，副腎皮質ステロイド薬（酢酸メゲストロール（本邦未承認），メドロキシプロゲステロン，プレドニゾロンなど））

現在までの基礎ならびに臨床試験を含めて食思改善，体重増加などの効果が最も明らかになっている薬剤で，実際の臨床においても食思不振のがん患者に用いられています。
●カンナビノイド製剤（ドロナビノール（合成経口カンナビノイド製剤，本邦未承認）など）

カンナビノイドは大麻から抽出された物質で，食思改善作用，制吐作用ならびに鎮痛作用を有する薬剤です。大麻より成分を抽出したもの，あるいは有効成分を合成したものが用いられています。現在のところ，本邦ではどちらのカンナビノイド製剤も使用できません。米国では合成製剤のみその使用が認められており，食思不振を改善し，体重減少を抑制したという報告があります。
●セロトニン阻害薬（シプロヘプタジンなど）

セロトニン受容体活性化が中枢における摂食シグナルを阻害することから，セロトニンの阻害薬が用いられています。

●グレリン（摂食ペプチド）（グレリン，RC-1291（経口グレリン模倣薬）など）

グレリンは，1999年に成長ホルモン分泌促進因子受容体の内在性リガンドとして日本人により胃から発見・同定されたペプチド性ホルモンです。また，末梢では唯一の食思改善，体重増加作用を有するホルモンです。したがって，グレリンを体外から与えることで体重増加，食思改善を期待する研究が多くの研究室で進められています。また，グレリン作用をもつ経口製剤も多数開発されており，一部は臨床試験でその効果が試されているところです。がん悪液質の症状を改善する薬剤としてかなり有望視されている薬剤です。

●メラノコルチン阻害薬

メラノコルチン受容体は食欲抑制系神経を活性化させることがわかっています。したがって，その受容体を阻害することで食思改善，体重減少の阻止を期待して用いられます。しかし，まだ動物実験レベルでの研究です。

②生体代謝を調節する薬剤

●サイトカイン製剤を基軸とした治療法

サイトカインは多彩な作用をもつ蛋白性内因性リガンドです。たくさんのサイトカインが種々のサイトカイン受容体に結合し，また多彩な生体反応を引き起こします。そのなかでも特に炎症性サイトカイン（腫瘍壊死因子（tumor necrosis factor；TNF）-α，インターロイキン-1，インターロイキン-6など）は体重減少，筋肉量の減少などの諸症状を引き起こす原因と考えられています。したがって，これらのサイトカインの阻害薬ががん悪液質の治療薬として用いられています。しかし，まだ動物実験，治験段階でのデータしかありません。

●サリドマイド製剤

1957年に発売された睡眠薬で，かつてわが国では妊婦の服用により奇形児の誕生を引き起こし，その使用が中止になった薬剤です。その後，多発性骨髄腫にサリドマイド製剤が有効であることがわかってきました。サリドマイド製剤は動物，ヒトを用いた研究で，食思を改善すること，体重減少を阻止することがわかっています。しかし，患者の生存率の改善がみられておらず，さらに検討が必要な薬剤です。

●抗体製剤，可溶化サイトカイン受容体製剤

炎症性サイトカインががん悪液質を惹起すると報告され，サイトカインの作用をブロックする抗サイトカイン抗体に効果があるのではということで研究が行われています。特に，抗インターロイキン-6抗体などは現在治験が進められているところです。また，抗インターフェロン-γ抗体も，動物実験においてがん悪液質症状を改善したという報告があります。そのほかにも，さまざまな抗サイトカイン抗体が動物レベルで検討されているところです。

③筋肉量の減少を防止する（筋肉量を増やす）薬剤

●ミオスタチン阻害薬

ミオスタチンは，形質転換増殖因子（transforming growth factor；TGF）-βのファミリーに属するサイトカインで，特に筋肉の発達を妨げる作用を有しています。したがって，このミオスタチンを阻害すると，筋肉の増量が期待されます。現在，その阻害薬を用いての筋肉量増加を期待した研究が行われているところです。

2010年，ミオスタチンのシグナルを制御している2型アクチビン受容体（ActRⅡB）シグナルを抑制することでミオスタチンシグナルが抑制され，結果的に筋肉の増量，体重減少の阻止，そして延命効果が得られたというマウスを用いた実験が報告されました。このことは，ActRⅡBシグナルを阻害する薬剤ががん悪液質患者の症状を改善し，また延命効果をもたらす可能性を示すもので，注目に値する研究です[6]。

そのほかにもたくさんの薬剤，たとえばβアドレナリン受容体アゴニスト，不飽和脂肪酸（ω-3-fatty acid），プロスタグランジン阻害薬，エリスロポエチン，アデノシン三リン酸（ATP），ある種のアミノ酸などにおいて，がん悪液質症状改善薬候補として動物実験ならびにヒトを用いた臨床試験が行われています。詳しくは，文献5）をご覧下さい。

国立がん研究センター研究所におけるがん悪液質改善のための基礎から臨床への橋渡し研究

私たちは，平成23年度より国立がん研究センターのプロジェクトとして，実験的に異なる手法を用いて作製した種々の動物悪液質モデルを同じ研究の土俵に上げ，統合的研究を行っていきます。①これらの動物のがん悪液質症状は，何の異常から，あるいは何の破綻から生じているのか，②これらの症状は何を処方すれば回復するのか，③これらの悪液質モデルを比較して，同じものは何か，違っているものは何か，について，共同研究グループと協力して明らかにしていく予定です。動物モデルには現在，大腸がん細胞を腹膜接種して得られるもの，胃がん細胞の皮下投与，膵臓がん細胞を神経内に浸潤させるもの，p53などの遺伝子を操作して得られる悪液質モデルなどがあります。いずれの動物モデルも異なる手法で作製されていますが，体重の減少や食思不振を示すこと，さらに血中サイトカインの異常値（サイトカインの種類はさまざまです）を認めるのは共通です。

がん悪液質の症状改善には，「経験に基づいた治療」としてさまざまなものが考えられ，治療が行われています。しかし，がん悪液質動物モデルにおいて，がん悪液質症状はさまざまながん細胞を植えることで発症すること，症状の表現型は必ずしもすべて一致するものではないこと，また血中で異常値を示すサイトカインなどはかなり異な

ることなどから考えても，がん悪液質はおそらく1つの原因で起こっているものではないと考えられます。したがって，がん患者においても，がんの種類，進行時期，患者一人ひとりそれぞれに異なる遺伝子・ゲノムなどの背景によってさまざまな病態（一見，体重減少，食思不振など同じ症状を示しているようにみえても）が引き起こされているのだと思われます。当然，がん悪液質の症状を改善する薬剤もがん患者一人ひとりで違ってくると思われます。この点についても，臨床の先生方と討論を重ねて情報を共有しながら，症状改善薬の開発を行っていきたいと考えています。

いずれにしても，がん悪液質症状はそれを改善することで終末期患者のQOLを改善するだけでなく，予後についてもそれを延長することができます。国立がん研究センター研究所においては，前述した数種類のがん悪液質モデル動物を用いた統合的基礎研究を行うことで，がん患者に福音となる薬剤の開発が迅速に行えればと期待しています。また，がん悪液質の症状改善に効果的な薬物処方や効果的な手段を一刻も早く開発することが望まれていることは明白であり，それに応えるためにも基礎研究を臨床につなげることができるよう，身を引き締めて研究を行っていきたいと思います。

第6回国際悪液質学会in神戸（6th CACHEXIA CONFERENCE in KOBE）

2011年12月5〜7日の3日間，神戸で第6回国際悪液質学会が開かれます（会長：乾　明夫先生（鹿児島大学大学院医歯学総合研究科心身内科学教授））7）。この学会は，2年おきに世界各地で開かれているもので，2000年に第1回が開かれ，第5回は2009年にスペインのバルセロナで開催されました。

2011年は特に，①悪液質と種々の疾病，②悪液質の病態生理と生化学，③悪液質の治療，④悪液

質ならびに筋肉減少症の定義および診断基準，などのコンセンサスについて発表・討論が行われる予定です。がん悪液質に限らず，さまざまな疾患に伴う悪液質の研究がここ日本で行われることとなりますが，興味のある方は是非当学会について参加，あるいは情報のフォローをいただければと思っています。また，日本での開催を期に私たちも本邦よりの研究成果を世界に発信できるようにがんばっていきたいと思っています。

おわりに

筆者の所属する国立がん研究センター研究所がん患者病態生理研究分野では，がん悪液質の研究を進めていくために，ヒト胃がん細胞をラット皮下に移植することによりヒト胃がん悪液質モデルを作成し，がん悪液質の諸症状を改善する薬剤の研究を行っています。作製したラットは100％の確率でがん悪液質を引き起こします。がん悪液質を起こしている要因は何か，どのような薬剤ががん悪液質の症状を改善するのか，どのような（飼育環境などの）条件ががん悪液質発症を予防するのかなどについて，鋭意研究を重ねているところです。また，さまざまながん腫を用いて作製された他のがん悪液質モデルをもつ研究室とも今年度より共同研究を行いますので，これらの研究を進めていくなかで新たな，がん患者に還元できる，福音となる知見が得られればと思っています。そして，この基礎医学セミナーのシリーズで結果を報告できればと願っています。

がんの痛みに効果を示す薬剤（特に鎮痛補助薬について）や，がん悪液質の症状改善の薬剤は，臨床の先生方の鋭い長年の観察により得られた「経験に基づいた治療」が有効であることが多く認められるのは，皆さんご存じのことと思います。われわれ基礎医学研究者は，その根拠となるデータをしっかりと提供し，治療についてエビデンスのある基盤データを構築しなければならないと考えています。がん患者病態生理研究分野も，国立がん研究センター中央病院緩和医療科・精神腫瘍科と協働して，「基礎から臨床への双方向性トランスレーショナルリサーチ」を掲げて共同研究を続けています。そして，少しでも早く研究結果ががん患者のQOL向上に寄与できればと願っています。

がん患者のQOLを低下させている痛みやがん悪液質以外のさまざまな要因，不眠や口内炎，うつを始めとしたさまざまな精神症状についても，基礎-臨床の双方向性トランスレーショナルリサーチが始まっています。次回は，がん患者のQOLを向上させるための基礎-臨床研究の新たな取り組みについて紹介させていただければと思います。

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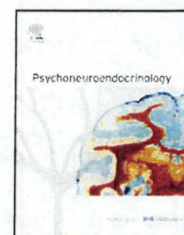7）First Announcement：6th CACHEXIA CONFERENCE.(http://www.lms-events.com/19/Flyer_Kobe.pdf)

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Parathyroid hormone-related protein has an anorexigenic activity via activation of hypothalamic urocortins 2 and 3

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Summary   Cancer cachexia is reported to be a major cause of cancer-related death. Since the pathogenesis is not entirely understood, only few effective therapies have been established. Since myriad tumors produce parathyroid hormone-related protein (PTHrP), plasma concentrations of PTHrP are increased in cancer cachexia. We measured the food intake, gastric emptying, conditioned taste aversion (CTA), and gene expression of hypothalamic neuropeptides in mice after administering PTHrP intraperitoneally. We administered PTHrP intravenously in rats and examined the gastroduodenal motility and vagal nerve activities. We also examined whether chronic administration of PTHrP influenced the food intake and body weight. Peripherally administered PTHrP induced negative energy balance by decreasing the food intake and gastric emptying; however, it did not induce CTA. The mechanism involved the activation of hypothalamic urocortins 2 and 3 through vagal afferent pathways and the suppression of gastroduodenal motor activity. The continuous infusion of PTHrP reduced the food intake and body weight gain with a concomitant decrease in the fat and skeletal muscle. Our findings suggest that PTHrP influences the food intake and body weight; therefore, PTHrP can be considered as a therapeutic target for cancer cachexia.
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1. Introduction

Cancer cachexia syndrome is characterized primarily by anorexia, weight loss, poor mental and physical performance, and a compromised quality of life, none of which are resolved by forced nutrient intake (Toomey et al., 1995; Inui, 2002; Harvey et al., 1979). Cachexia is reportedly responsible for up to 30% of cancer-related deaths overall, and 30–50% of deaths in patients with gastrointestinal tract cancers. However, few effective therapies have been established because its pathogenesis is not entirely understood. Among symptoms of cancer cachexia syndrome, weight loss is a key feature of cachexia (Toomey et al., 1995; Inui, 2002; Harvey et al., 1979). In general, patients lose body weight because of reduced food intake. Numerous studies have reported that hypothalamic neuropeptides and gut motility play a pivotal role in the regulation of food intake and the etiology of eating abnormalities (Schwartz et al., 2000; Inui et al., 2004).

Various humoral mediators including parathyroid hormone-related protein (PTHrP), tumor necrosis factor (TNF)α, interleukin (IL) 1 and IL6 are reportedly produced in cancer cachexia syndrome (Toomey et al., 1995; Inui, 2002; Strewler et al., 1987; Moseley et al., 1987; Burtis et al., 1987; Strewler, 2000). Parathyroid hormone-related protein has been identified and its cDNA was cloned from human tumors associated with the syndrome of humoral hypercalcemia of malignancy. Since then, a substantial amount of investigative effort has specifically addressed the role of PTHrP in calcium metabolism and function (Strewler, 2000). Parathyroid hormone-related protein-knockout mice display a systemic chondrodysplasia that is lethal at birth (Karaplis et al., 1994). Whereas PTH is a classic systemic hormone charged with regulating calcium and phosphorous homeostasis, PTHrP acts exclusively in an autocrine or paracrine manner. However, numerous tumors, including colon, lung, renal, breast, skin, prostate, and ovarian carcinomas, and T-cell leukemia produce PTHrP; consequently, plasma concentration of PTHrP is increased in tumor-bearing animals and cancer cachexia syndrome (Burtis et al., 1990; Gaich and Burtis, 1990; Pardo et al., 2004). So far, whether PTHrP is involved in the regulation of food intake, gut motility, and body weight remains unknown. Herein, we demonstrate that PTHrP influences food intake, gut motility, and body weight and may be a therapeutic target for cancer cachexia syndrome.

2. Materials and methods

2.1. Animals and drugs

We used male ddy mice (34–37 g, 8–9 weeks of age; Japan SLC Inc., Shizuoka, Japan), and male Wistar rats (230–280 g, 8–10 weeks of age; CLEA Japan Inc., Tokyo, Japan). The mice and rats were housed individually in a regulated environment (22 ± 2 °C, 55 ± 10% humidity, 12:12 h light:dark cycle with light on at 7:00 a.m.). Food and water were available *ad libitum* except as indicated. They were used only once each in the experiment. Our university animal care committee approved all experiments. Mouse/rat PTHrP$_{1-34}$ was purchased from Peptide Institute Inc. (Osaka, Japan). The Yanaihara Institute Inc. (Shizuoka, Japan) produced antibodies

against urocortins 1, 2 or 3. Immediately before administration, PTHrP was diluted in physiological saline containing 2% L-cysteine which also served as control solutions. The PTHrP doses were determined on the basis of previous studies (Burtis et al., 1990; Iguchi et al., 2006) and our preliminary experiments on food intake.

2.2. Production of antisera

Production of antisera was carried out against urocortins 1, 2 and 3. Synthetic mouse urocortin 1 (15.0 mg), urocortin 2 (15.0 mg) or urocortin 3 (15.5 mg) and porcine thyroglobulin (Sigma—Aldrich Corp., MO, USA) (54.6 mg) were dissolved in 0.1 M HEPES buffer (6 ml, pH 8.1). To that mixture, dimethyl suberimidate 2HCl (Pierce Chemical Co., IL, USA) (4.4 mg) was added. The mixture was stirred for 2 h at room temperature. The ensuing conjugate (1.5 ml) was emulsified with Freund's complete adjuvant (Calbiochem-Behringer, CA, USA) (1.5 ml) with a mixer for 45 min in an ice bath. The emulsion was injected intradermally into multiple sites of three Japanese white female rabbits. For primary immunization, each rabbit received a portion of the emulsion containing approximately 1.25 mg of peptide. Immunization was performed at 2-week intervals using half the dose of the immunogen used for primary immunization. The rabbits were bled from the ear vein 10 days after each immunizations. After the sixth immunization, one of the three rabbits gave a high titer antiserum. The antisera specificity was characterized by mouse urocortin 1, 2 and 3 specific enzyme immunoassay (EIA) (Supplementary Fig. S1—S3).

2.3. Intracerebroventricular (icv) substance application

For icv administration, the mice were anesthetized with sodium pentobarbital (80–85 mg/kg intraperitoneal (i.p.) administration) and placed in a stereotaxic instrument 7 days before experiments. A hole was made in each mouse's skull using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24-gauge cannula beveled at one end over a distance of 3 mm was implanted into the third cerebral ventricle for icv administration. The cannula was fixed to the skull with dental cement and capped with silicon without an obtruder. A 27-gauge administration insert was attached to a microsyringe using PE-20 tubing.

2.4. Feeding tests

Before feeding tests, mice were deprived of food for 16 h with free access to water, or were given free access to food and water. A standard diet (CLEA Japan Inc., Tokyo, Japan) was used except for the experiment testing the effect of vagotomy on feeding suppression induced by PTHrP, which used a liquid diet (Oriental Yeast Co. Ltd., Tokyo, Japan). Dark-phase feeding studies administrations were done immediately before lights-off (7:00 p.m.) for non-food-deprived mice. For food-deprived mice, PTHrP was administered at 10:00 a.m. Food intake was measured by subtracting uneaten food from initially premeasured food at 20 min, 1 h, 2 h, and 4 h after administration and checking the food spillage.

2.5. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Mice were deprived of food for 16 h with free access to water; then they were treated with PTHrP (300 pmol/mouse) or vehicle every 6 h for 12 h, with the third and final administration at 30 min before the mice were killed by cervical dislocation. Immediately after, the hypothalamic block, stomach and epididymal fat were removed, frozen on dry ice, and stored at −80 °C until preparation of real-time RT-PCR. Using the RNeasy Mini Kit (Qiagen Inc., Tokyo, Japan) RNA was isolated from the hypothalamic block, stomach and epididymal fat. Quantification of mRNA levels was performed with SYBR-green chemistry (Qiagen Inc., Tokyo, Japan) using a one-step RT-PCR reaction on a sequence detection system (ABI PRISM 7700; Applied Biosystems Japan, Tokyo, Japan). The reaction was performed under standard conditions recommended by the manufacturer. We used the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. All expression data were normalized to GAPDH expression level from the same individual sample. The following primers were used for real-time RT-PCR: GAPDH forward, ATGGTGAAGGTCGGTGTGAA; and reverse, GAGTGGAGTCA-TACTGGAAC. Neuropeptide Y (NPY) forward, TTT-CCAAGTTTCCACCCTCATC; and reverse, AGTGGTGGC-ATGCATGGT. Agouti-related protein (AGRP) forward, GAGTTCCCAGGTCTAAGTCTGAATG; and reverse, ATCTAG-CACCTCCGCCAAAG. Orexin A forward, CGTAACTACCACCGCTT-TAGCA; and reverse, TGCCATTTACCAAGAGACTGACAG. Melanin-concentrating hormone (MCH) forward, GGAAGA-TACTGCAGAAAGATCCG; and reverse, ATGAAACCGCTCT-CGTCGTT. Cocaine and amphetamine-regulated transcript (CART) forward, GCAGATCGAAGCGTTGCAA; and reverse, TTGGCCGTACTTCTTCTCGTAGA. Proopiomelanocortin (POMC) forward, GGCTTGCAAACTCGACCTCT; and reverse, TGACCCAT-GACGTACTTCCG. Corticotropin-releasing factor (CRF) forward, CGCAGCCCTTGAATTTCTTG; and reverse, TCT-GTTGAGATTCCCCAGGC. Urocortin 1 forward, ACTGTCCATC-GACCTCACCTTC; and reverse, AAGGCTTTCGTGACCCCATA. Urocortin 2 forward, CCTCAGAGAGCTCCTCAGGTACC; and reverse, GGTAAGGGCTGGCTTTAGAGTTG. Urocortin 3 forward, CGCACCTCCAGATCAAAAGAA; and reverse, GGG-TGCTCCCAGCTCCAT. Ghrelin forward, AGCATGCTCTGGA-TGGACATG; and reverse, GCAGTTTAGCTGGTGGCTTCTT. Leptin forward, CTGTGGCTTTGGTCCTATCT; and reverse, TGATA-GACTGCCAGAGTCTG. Adiponectin forward, TGCTA-CTGTTGCAAGCTCTC; and reverse, AGGACCAAGAAGACCTG-CAT.

2.6. Immunohistochemistry

Mice were deprived of food for 16 h with free access to water, then administered intraperitoneally with PTHrP (300 pmol/mouse) or vehicle. The mice were anesthetized with sodium pentobarbital (80—85 mg/kg i.p.) and perfused with 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer 90 min after administration. Brains were removed and postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The brains were cut into 20-μm-thick coronal sections in a cryostat. Immunohistochemistry for c-fos protein expression was performed

using ABC and DAB methods, whereas immunohistochemistry for urocortins 1, 2 and 3 was performed using immunofluorescence methods according to previous studies (Chen et al., 2005). Positive reaction was observed under light microscopy (Olympus DX51; Olympus Optical Co. Ltd., Tokyo, Japan) or laser scanning microscope (LSM 510; Carl Zeiss Inc. Japan, Tokyo, Japan).

2.7. Gastroduodenal motility

Gastroduodenal motility was measured in conscious, freely moving rats by manometric method. Rats were deprived of food and given free access to water for 16 h before the abdominal operation. They were anesthetized with pentobarbital sodium (50 mg/kg i.p.); then a motility recording device was implanted as follows. Two manometric catheters (3-Fr, 1 mm diameter; Atom Medical Corp., Tokyo, Japan) with side holes were inserted through the fistula at the gastric body and the tips were placed at the gastric antrum and 3 cm distal to the pylorus. Catheters were fixed at the gastric wall by purse-string suture and run subcutaneously to emerge at the crown of the neck and were secured at the animals' skin. A catheter (3-Fr, 1 mm diameter) was placed in the right jugular vein, run subcutaneously to emerge at the crown with the manometric catheter, and used for intravenous (i.v.) administration. The catheter was filled with heparinized saline to prevent obstruction. After 1 week, animals were fasted for 16 h before the experiment. On the day of the experiment, a manometric catheter was connected to a pressure transducer (TP-400T; Nihon Kohden Corp., Tokyo, Japan), and connected to the infusion swivel (dual type, 20-gauge; Instech Laboratories Inc., PA, USA) to allow free movement. The catheter was infused continuously with bubble-free 0.9% saline at a rate of 1.5 ml/h by a low-compliance capillary infusion system using a heavy-duty pump (CVF-3100; Nihon Kohden Corp.). The PTHrP (300 pmol/rat) was dissolved in physiological saline containing 2% L-cysteine in a 300 μl volume for i.v. administration.

2.8. Gastric emptying

Before experiments for gastric emptying, mice were deprived of food for 16 h with free access to water. The fasted mice had free access to pre-weighed pellets for 1 h; they were then administered i.p. with PTHrP (3—300 pmol/mouse) or vehicle. The mice were deprived of food again for 2 h after administration. Food intake was measured by weighing uneaten pellets. Mice were killed by cervical dislocation 3 h after the start of experiments. Immediately after, the stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardia, then removed; then the dry content was weighed. Contents were dried using a vacuum freeze-drying system (Model 77400; Labconco Corp., MO, USA). Gastric emptying was calculated according to the following formula: gastric emptying (%) = {1 − (dry weight of food recovered from the stomach/ weight of food intake)} × 100.

2.9. Truncal vagotomy

Four days before experiments, truncal vagotomy was performed. The mice were anesthetized with sodium pentobar-

bital (80—85 mg/kg i.p.). After a midline incision of the abdominal wall, the stomach was covered with sterile gauze moistened with warm saline. The lower part of the esophagus was exposed and the anterior and posterior branches of the vagal nerve were incised. At the end of the operation, the abdominal wall was sutured in two layers. In sham-operated mice, vagal trunks were similarly exposed, but not cut. Vagotomized and sham-operated mice were maintained on a nutritionally complete liquid diet. Completeness of vagotomy was verified during postmortem inspection. Mice were fixed for enzyme histochemistry. Loss of acetylcholine esterase positive fibers in the gastrointestinal tracts was determined by light microscopic observation.

2.10. Electrophysiological study

Electrophysiological measurements were performed to record changes in activity of gastric and hepatic vagal nerves. Rats were anesthetized with urethane (1 g/kg i.p.), and a tracheal cannula was inserted. Under a dissecting micro-

scope, a nerve filament was dissected from the peripheral cut end of the vagus nerve to record afferent nerve activity via a pair of silver wire electrodes. A rate meter with a rest time of 5 s was used to observe the time course of nerve activity. Administration of PTHrP (0.1—10 µg/rat) was done through a small catheter inserted into the inferior vena cava in a 100 µl volume. The effect of PTHrP on the vagal nerve activity was determined by comparing the mean number of impulses per 5 s over 50 s before and after the administration.

2.11. Conditioned taste aversion

A standard two-bottle taste aversion paradigm was used for conditioned taste aversion (CTA) assay. Mice were accustomed to having access to water for 1 h (10:00—11:00 a.m.) per day for 5 days. On the sixth day, mice received an i.p. administration of PTHrP (300 pmol/mouse) or vehicle following access to a 0.5% (v/v) sodium saccharin solution instead of water. Intraperitoneal administration of
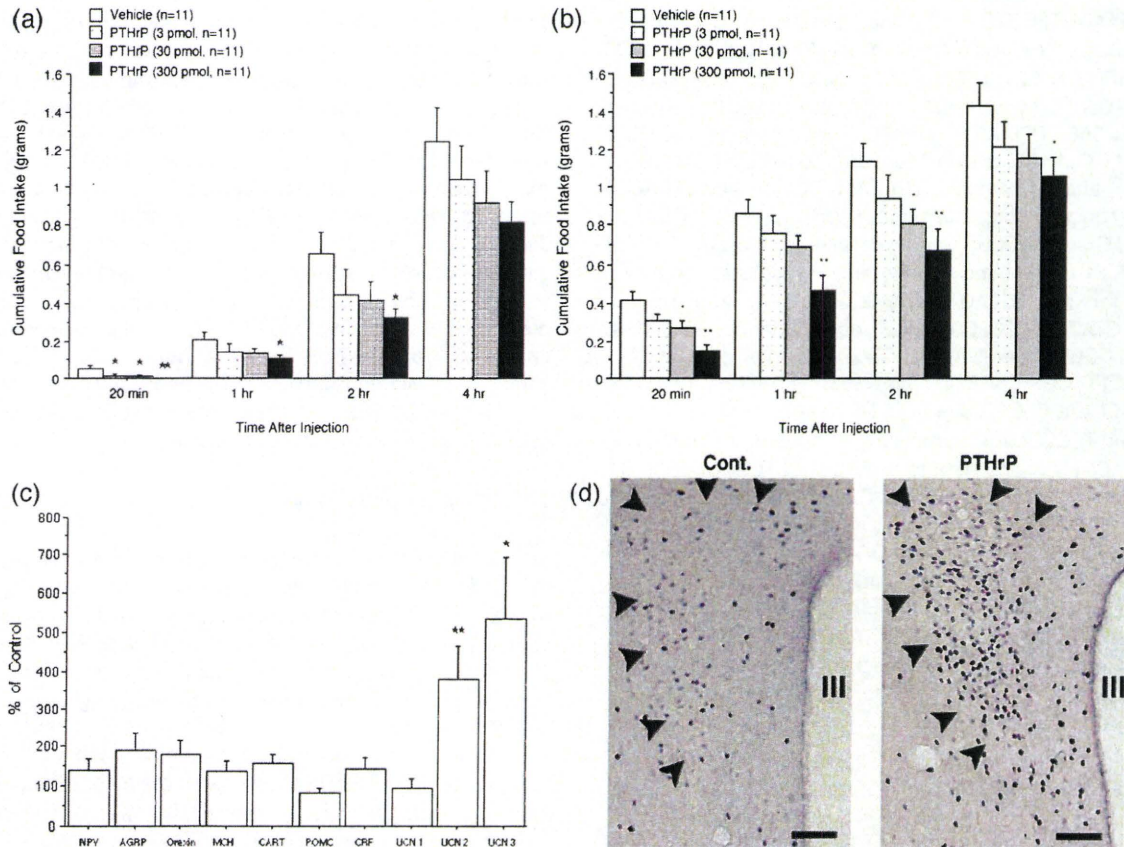

Figure 1   PTHrP has an anorexigenic activity. (A and B) Effects of intraperitoneally administered PTHrP (3—300 pmol/mouse) on cumulative food intake in non-food-deprived mice (A) and food-deprived mice (B). (C) Effects of intraperitoneally administered PTHrP (300 pmol/mouse every 6 h for 12 h) on hypothalamic peptides mRNA levels, as assessed by real-time RT-PCR in food-deprived mice, expressed as a percentage of vehicle treated control ($n = 5$—6). (D) Effects of intraperitoneally administered PTHrP (300 pmol/mouse) on c-Fos expression in the paraventricular nucleus 90 min after administration ($n = 3$—4). Scale bars = 50 µm. (E) Photomicrographs of immunohistochemical demonstration for urocortins 1, 2 and 3 in PVN ($n = 3$—4). Scale bars = 50 µm. (F) Antagonistic effects of either anti-mouse urocortin 2 or 3 antiserum administered icv (4 µl/mouse) on feeding induced by i.p. administration of PTHrP (300 pmol/mouse) in food-deprived mice. Each bar represents the mean ± S.E. n indicates the number of mice used. *$P < 0.05$; **$P < 0.01$ by Bonferroni's t-test.
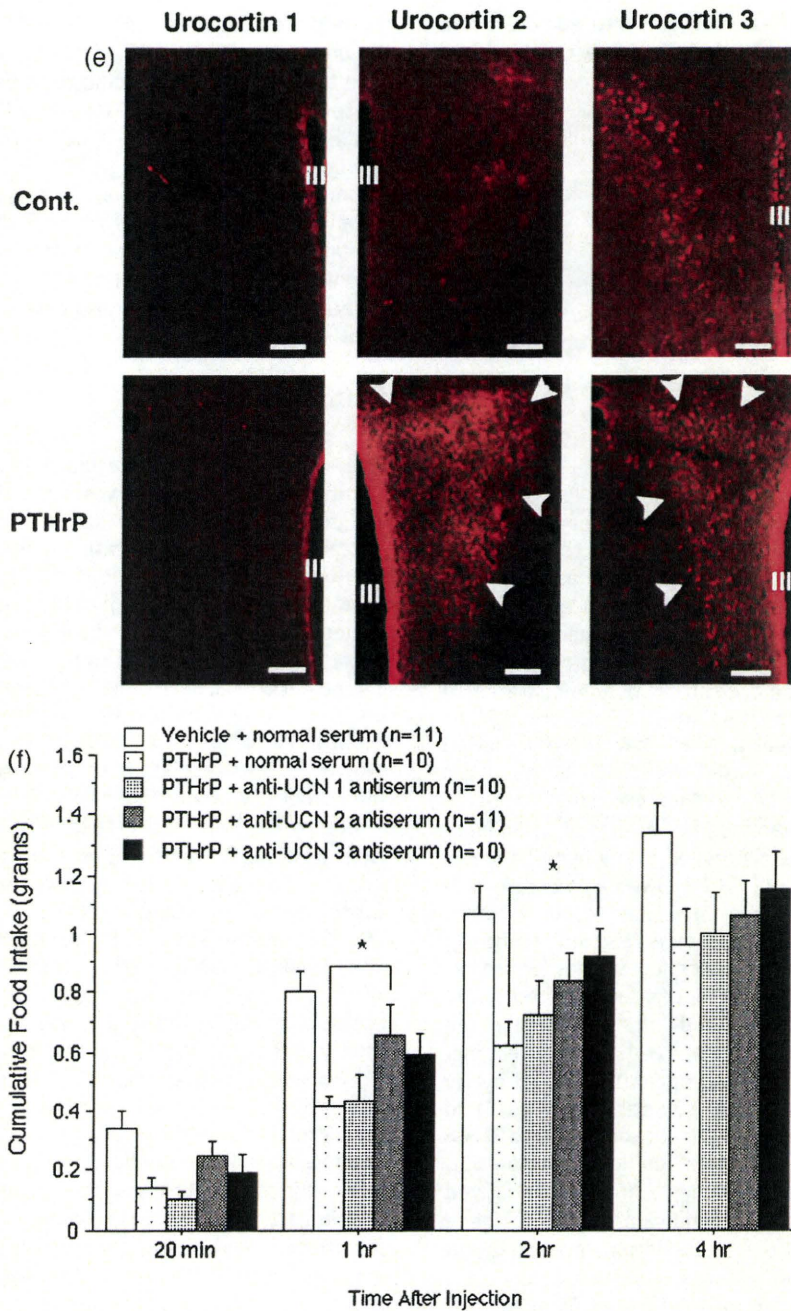
Figure 1. (Continued).

LiCl (2% BW of a 0.15 M solution) or physiological saline served respectively as positive and negative controls. Three days later, mice were offered both water and saccharin solution in separate bottles during their 1-h drinking period. The saccharin preference ratio was calculated according to the following formula: preference ratio (%) = (saccharin intake/total fluid intake) × 100.

2.12. Continuous infusion

Continuous systemic administration of PTHrP was achieved via osmotic minipumps (Alza Corp., CA, USA). Model 1007 mini-osmotic pumps were filled with a 200 μl volume of

control solution or PTHrP (12.5 pmol/h). Mice were anesthetized with sodium pentobarbital (80–85 mg/kg i.p.). The pump was implanted i.p. under sterile conditions after a small midline incision according to the manufacturer's instructions. The abdominal wall was sutured in two layers. The actual mean pumping rate, reservoir fill volume, and duration of the pump are 0.5 μl/h, 100 μl, and 7 days, respectively. Food and water intake and body weight were measured daily. On the final day, serum was separated from blood obtained from the orbital sinus under ether anesthesia at the end of the experiment (6 h after removal of food). The entire sampling procedure was done in less than 2 min. Mice were killed by cervical dislocation. Immediately after, the

epididymal fat pad mass, assessed as white adipose tissue (WAT), and the gastrocnemius muscle were removed and weighed. Blood glucose and serum triglycerides were measured respectively using the glucose oxidase method and enzymatic method (Wako Pure Chemical Industries Ltd., Tokyo, Japan).

2.13. Statistical analysis

Analysis of variance followed by Bonferroni's t-test was used to assess differences among groups. Results are expressed as mean value $\pm$ S.E. $P < 0.05$ was considered to be statistically significant.

3. Results

PTHrP has an anorexigenic activity. We first examined the effects of i.p. administration of PTHrP on feeding in non-food-deprived mice and food-deprived mice to investigate whether or not PTHrP influences feeding behavior. Peripherally administered PTHrP significantly produced inhibitory effects on feeding behavior in a dose-related manner (Fig. 1A and B). We examined gene expression of hypothalamic neuropeptides in food-deprived mice after i.p. administration of PTHrP to evaluate the possibility that PTHrP acts through the hypothalamic pathway. Real-time RT-PCR analysis showed that PTHrP significantly increased expression of urocortins 2 and 3 respectively by 281% and 436% compared with controls (Fig. 1C). We next examined the effects of i.p. administration of PTHrP on c-fos expression in the hypothalamus. Peripheral administration of PTHrP showed an increase in c-fos expression in the paraventricular nucleus (PVN) (56.2 ± 7.59 vs. 23.3 ± 2.30 number/section [control], $P < 0.02$) (Fig. 1D). Immunohistochemical studies for urocortins 1, 2 and 3 showed that PTHrP increased the intensity of immunoreaction for urocortins 2 and 3 in the PVN compared with controls (Fig. 1E). We also observed that the feeding-inhibitory effect of PTHrP was significantly blocked by icv administration of anti-urocortin 2 or 3 antiserum (Fig. 1F). In addition, we investigated whether or not administered PTHrP influences gene expression of ghrelin and leptin in stomach, and adiponectin, leptin and resistin in WAT of food-deprived mice ($n = 7$–8). PTHrP had no significant effects on gene expression of ghrelin ($104.0 \pm 18.7\%$ of control) and leptin ($124.2 \pm 51.9\%$ of control) in stomach, and adiponectin ($67.5 \pm 9.3\%$ of control) and leptin ($130.5 \pm 34.5\%$ of control) in WAT.

PTHrP influences gastric emptying and vagal nerve. Next, we examined whether or not PTHrP influences gastroduodenal motility. Intravenous administration of PTHrP disrupted the fasted motor activity in the antrum and duodenum in food-deprived rats (Fig. 2A). In addition, peripherally administered PTHrP decreased the gastric emptying rate in a dose-related manner (Fig. 2B). We also used mice that had received truncal vagotomy to investigate whether or not the feeding-inhibitory effect of PTHrP is associated with a vagally mediated pathway. Although vagotomy is an invasive operation, vagotomy eliminated inhibitory effects on feeding induced by i.p. administration of PTHrP (Fig. 2C). The electrophysiological study showed that i.v. administration of PTHrP significantly increased afferent activity of the gastric

and hepatic vagal nerves (Fig. 2D and E). The possibility that the anorectic effect of PTHrP is partly or completely attribute to malaise or nonspecific toxic effects was also investigated. Administration of PTHrP at a dose that reduced food intake did not result in a CTA, whereas lithium chloride produced a robust CTA (Fig. 2F). We also investigated the chronic effects of PTHrP on feeding and body weight. A chronic i.p. infusion of PTHrP for 7 days using an osmotic minipump decreased food intake and body weight gain during the infusion period (Table 1 and Fig. 2G). Fat pad mass and skeletal muscle mass were also decreased by PTHrP administration.

4. Discussion

Here, we show that PTHrP regulates feeding behavior via the hypothalamus and vagal nerves. Since the discovery of leptin and ghrelin, much progress has been made in the study of controlling energy homeostasis (Inui et al., 2004; Flier and Maratos-Flier, 1998; Zhang et al., 2005). Until now, hypothalamic neuropeptides, including NPY, AGRP, orexin, MCH, α-melanocyte stimulating hormone (α-MSH), CART, CRF and urocortin, have been shown to be involved in the regulation of food intake (Schwartz et al., 2000; Inui et al., 2004). Urocortin was identified in 1995 as the second ligand for CRF receptor (Vaughan et al., 1995). Whereas CRF is mainly involved in the regulation of stress-related behavior and colonic motility, urocortin is involved mainly in the regulation of feeding behavior and gastric motility (Dautzenberg and Hauger, 2002). Previous studies have shown that both urocortins 2 and 3 potently suppress food intake and delay gastric emptying (Hsu and Hsueh, 2001; Ohata and Shibasaki, 2004; Czimmer et al., 2006). Our results support a mechanism of feeding-inhibitory effect of PTHrP involved in urocortins 2 and 3 in the hypothalamus. Feeding stimulatory peptides such as NPY and ghrelin induce the fasted motor activity in the gastrointestinal tracts, whereas feeding-inhibitory peptides such as urocortin, bombesin and cholecystokinin (CCK) disrupt the fasted motor activity in the gastrointestinal tracts (Fujino et al., 2003). In addition, considerable evidence indicates cumulatively that delayed gastric emptying is closely related to anorexia and cachexia, as rapid gastric emptying is to overeating and obesity (Inui, 2002; Duggan and Booth, 1986). In our study, peripherally administered PTHrP disrupted the fasted motor activity in the stomach and duodenum, and decreased the gastric emptying rate. Moreover, our results in vagotomy study show that anorexigenic effects of PTHrP are mediated, at least in part, by the vagal nerve pathway. Electrophysiologic study results also supports vagal mediation of the anorexigenic activity. Numerous studies have shown that the neural mechanism, supporting and extending the hepatic receptor hypothesis, is important for integrating autonomic regulation of gastrointestinal functions as well as feeding behavior (Geary et al., 1993; Niijima and Meguid, 1995). This study indicates the existence of the peripheral receptor mechanism of PTHrP in hepatic vagal afferents. In addition, the effect of PTHrP on the afferent activity of the gastric vagal nerve is similar to that of anorexigenic peptides CCK and peptide YY $(PYY)_{3-36}$, and contrary to that of orexigenic peptide ghrelin (Asakawa et al., 2001; Koda et al., 2005). Behavioral specificity was
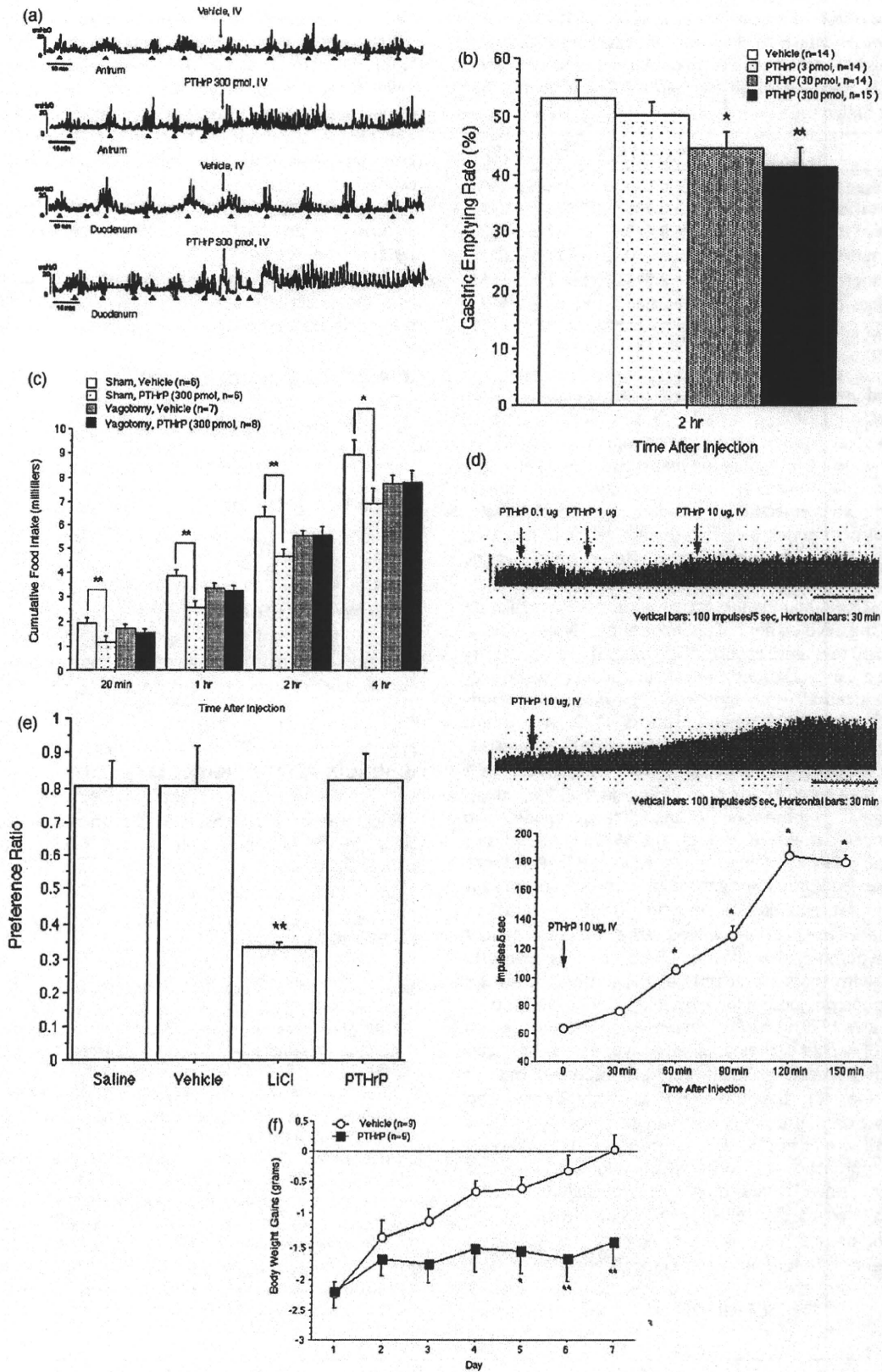
Figure 2   PTHrP influences gastric emptying and vagal nerve. (A) Effects of intravenously administered PTHrP (300 pmol/rat) on the fasted motor activity of the antrum and duodenum (n = 5–6). (B) Inhibitory effects of intraperitoneally administered PTHrP (3–300 pmol/mouse) on the gastric emptying rate 2 h after administration. (C) Loss of feeding-inhibitory effect of intraperitoneally

Table 1 Effects of chronic administration of PTHrP on food intake, water intake, epididymal fat mass, gastrocnemius muscle and concentrations of blood glucose, and triglycerides. Results are expressed as mean ± S.E. n indicates the number of mice used.

	Vehicle (n = 9)	PTHrP (n = 9)
Food intake (g/day)	4.007 ± 0.075	3.716 ± 0.100[*]
Water in take (ml/day)	8.050 ± 0.236	7.874 ± 0.249
Fat pad mass (g)	0.467 ± 0.029	0.362 ± 0.038[*]
Skeletal muscle (g)	0.344 ± 0.010	0.311 ± 0.011[*]
Glucose (mg/dl)	145.7 ± 2.819	142.2 ± 5.666
Triglycerides (mg/dl)	103.3 ± 14.88	96.89 ± 9.119

[*] $P < 0.05$ by Bonferroni's t-test.

determined using a CTA assay where the drug administration is paired with ingestion of a palatable substance (saccharin) (Bernstein et al., 1983). Peripheral administration of PTHrP does not induce a CTA, indicating that the anorectic effect of PTHrP is specific and is not caused by aversion. These observations indicate that PTHrP has an anorexigenic activity with its mechanism of action involving the activation of hypothalamic urocortins 2 and 3 through vagal afferent pathways and the suppression of gastroduodenal motor activity.

The most common manifestation of advanced malignant diseases is the development of anorexia and body weight loss. Plasma PTHrP levels are reportedly increased in the majority of advanced cancers, especially cancer cachexia syndrome, and are reduced after chemotherapeutic or surgical treatment (Burtis et al., 1990; Gaich and Burtis, 1990; Pardo et al., 2004). The plasma PTHrP concentrations after IP administration of the effective minimum dose of PTHrP (3 pmol) appear to be very high compared to those found in healthy humans and are similar to those found in animal cancer models and human cancer patients (Burtis et al., 1990; Iguchi et al., 2006). In our study, chronic administration of PTHrP reduced food intake and body weight gain with a concomitant decrease in fat and skeletal muscle. These observations indicate that PTHrP may be involved in the etiology of cancer cachexia syndrome. The degree of cachexia is inversely correlated with the survival time of the patient: it always implies a poor prognosis (Toomey et al., 1995; Inui, 2002; Harvey et al., 1979). Despite numerous efforts devoted to treatment of cancer cachexia, no effective therapy has been established. Recently, Sato et al. have reported that in tumor-bearing rats, the anti-PTHrP antibody ameliorated cancer anorexia and produced a marked recovery of body weight loss (Sato et al., 2003; Onuma et al., 2005). Various combined approaches have been widely adopted for cancer management. Nevertheless, most therapies including anticancer drugs have more or fewer adverse side effects such as anorexia and nausea. Moreover, cachexia not only worsens a patient's general condition; it also compels abandonment of positive therapies that engender unfavorable side effects (Harvey et al., 1979; Schnell, 2003). On the other hand,

antibody therapy is characterized by its low incidence of side effects and high target-specificity (Forero and Lobuglio, 2003). Therefore, anti-PTHrP therapy might represent a novel treatment strategy for cancer-cachexia syndrome and various cancers treated with combined anticancer therapies. The development of effective anti-PTHrP antibodies as potential therapeutic agents for cancer cachexia syndrome is awaited with great interest.

In conclusion, this study indicates that PTHrP exhibits a novel, anorexigenic activity. Its mechanism of action involves the activation of hypothalamic urocortins 2 and 3 through vagal afferent pathways and the suppression of gastroduodenal motor activity. Further, PTHrP may play an important role in the etiology of the cancer cachexia syndrome.

Role of the funding source

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen. 2010.02.003.

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administered PTHrP (300 pmol/mouse) after vagotomy in food-deprived mice. (D and E) Effects of PTHrP administered into the inferior vena cava on afferent activity of the gastric vagal nerve (D) and hepatic vagal nerve (E) in rats (n = 5). (F) Conditioned taste aversion assay (n = 6–7). (G) Effects of chronic administration of PTHrP on body weight gain. Each bar represents the mean ± S.E. n indicates the number of mice used. *$P < 0.05$; **$P < 0.01$ by Bonferroni's t-test.

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Changes in Acyl Ghrelin, Des-acyl Ghrelin, and Ratio of Acyl Ghrelin to Total Ghrelin with Short-term Refeeding in Female Inpatients with Restricting-type Anorexia Nervosa

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Abstract
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Restricting type of anorexia nervosa (AN-R) is a serious disorder affecting adolescents and young adults and decreases quality of life over a long period. Successful weight restoration is an important prognostic factor for disease outcome; however, the underlying mechanism of refeeding resistance, a core psychopathology relevant to 'ambivalent' eating behaviors, remains unclear in this disorder. Ghrelin plays an important role in the regulation of growth hormone release, appetite, and energy metabolism. However, the early progress of these patients and changes in the levels of acyl ghrelin and des-acyl ghrelin during treatment were not reported. The purpose of this study was to determine the changes in ghrelin levels (acyl and des-acyl) during early treatment.

As a result, des-acyl ghrelin in AN-R patients is higher than in control subjects before the therapy, but it decreases with treatment. The plasma des-acyl ghrelin level in AN-R patients started decreasing more rapidly and in early stage of the hospitalization than ever reported, and after 8 weeks, it is significantly lower than in control subjects. It means that des-acyl ghrelin is sensitive and changeable with their nutrition state. Furthermore, the ratio of the acyl ghrelin to total ghrelin increases with 8 weeks treatment. Eight weeks after, energy intake of the AN-R patients is recovered near the normal range with a daily energy intake of 1700 ± 93.54 kcal. These findings may be valuable for future AN-R treatments in order to increase acyl ghrelin and decrease des-acyl ghrelin, thereby influencing the refeeding outcome.

Introduction
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Anorexia nervosa (AN) is a serious disorder affecting adolescents and young adults, and decreases the quality of life in persons affected for long periods [1]. Restricting-type AN (AN-R) is characterized by severe emaciation with chronic food restriction secondary to an inordinately strong desire for being thin and fear of obesity [2]. Successful weight restoration is an important prognostic factor for disease outcome [1]; however, the mechanism underlying refeeding resistance, a core psychopathology relevant to 'ambivalent' eating behaviors, remains unclear in this disorder [3–7]. Therefore, studies to determine the physiological and psychological effects of AN refeeding are very important.

Ghrelin plays an important role in the regulation of growth hormone release [8,9], appetite [10,11], and energy metabolism [12]. AN-R patients have been found to have increased circulating ghrelin levels [13]. Furthermore, ghrelin is involved in stimulating gastrointestinal motility and emptying [14]. It is secreted mainly by the stomach, and two major molecular forms are found in plasma: acylated ghrelin, with an *n*-octanoylated serine residue in position 3 and des-acyl ghrelin [15]. The acylated form of ghrelin (acyl ghrelin) is quite unstable and rapidly changes to the des-acylated form (des-acyl ghrelin) or smaller fragments [2]. It has been reported that plasma acyl ghrelin levels were not significantly different between AN patients and healthy control subjects, but plasma des-acyl ghrelin levels were significantly higher in AN patients than control subjects [16]. The administration of acyl ghrelin induces gains in body weight (BW) and fat mass via increased food intake and decreased fat oxidation for energy expenditure [17]. In contrast to acyl ghrelin, it has been reported that the peripheral administration of des-acyl ghrelin in mice decreases food intake and disrupts fasted stomach motility [18,19]. Very recently, Inhoff