[67] Shimizu Y, Nagaya N, Isobe T, *et al.* Increased plasma ghrelin level in lung cancer cachexia. Clin Cancer Res 2003; 9: 774-8.
[68] Hiura Y, Takiguchi S, Yamamoto K, *et al.* Fall in plasma ghrelin concentrations after cisplatin-based chemotherapy in esophageal cancer patients. Int J Clin Oncol 2011; [Epub ahead of print]
[69] Hiura Y, Takiguchi S, Yamamoto K, *et al.* Effects of ghrelin administration during chemotherapy with advanced esophageal cancer patients: A prospective, randomized, placebo-controlled phase 2 study. Cancer 2012. doi: 10.1002/cncr.27430.
[70] Liu YL, Malik NM, Sanger GJ, Andrews PL. Ghrelin alleviates cancer chemotherapy-associated dyspepsia in rodents. Cancer Chemother Pharmacol 2006; 58: 326-33.
[71] Rudd JA, Ngan MP, Wai MK, *et al.* Anti-emetic activity of ghrelin in ferrets exposed to the cytotoxic anti-cancer agent cisplatin. Neurosci Lett 2006; 392: 79-83.
[72] Yakabi K, Sadakane C, Noguchi M, *et al.* Reduced ghrelin secretion in the hypothalamus of rats due to cisplatin-induced anorexia. Endocrinology 2010; 151: 3773-82.
[73] Malik NM, Moore GB, Kaur R, *et al.* Adaptive upregulation of gastric and hypothalamic ghrelin receptors and increased plasma ghrelin in a model of cancer chemotherapy-induced dyspepsia. Regul Pept 2008; 148: 33-8.
[74] Wouters MM, Farrugia G, Schemann M. 5-HT receptors on interstitial cells of Cajal, smooth muscle and enteric nerves. Neurogastroenterol Motil 2007; 19 (Suppl 2): 5-12.
[75] Giorgetti M, Tecott LH. Contributions of 5-HT 2C receptors to multiple actions of central serotonin systems. Eur J Pharmacol 2004; 488: 1-9.
[76] Neary NM, Small CJ, Wren AM, *et al.* Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. J Clin Endocrinol Metab 2004; 89: 2832-6.
[77] Liu Y, Hamaue N, Endo T, Hirafuji M, Minami M. 5-hydroxytryptamine (5-HT) concentrations in the hippocampus, the hypothalamus and the medulla oblongata related to cisplatin-induced pica of rats. Res Commun Mol Pathol Pharmacol 2003; 113-114: 97-113.
[78] Heisler LK, Jobst EE, Sutton GM, *et al.* Serotonin reciprocally regulates melanocortin neurons to modulate food intake. Neuron 2006; 51: 239-49.
[79] Morley JE. Decreased food intake with aging. J Gerontol A Biol Sci Med Sci 2001; 56: 81-8.
[80] Morley JE. Anorexia, sarcopenia, and aging. Nutrition 2001; 17, 660-3.
[81] Morley JE Anorexia of aging: physiologic and pathologic. Am J Clin Nutr 1997; 66: 760-73.
[82] Di Francesco V, Fantin F, Omizzolo F, *et al.* The anorexia of aging. Dig Dis 2007; 25: 129-37.
[83] Hays NP, Roberts SB. The anorexia of aging in humans. Physiol Behav 2006; 88: 257-66.
[84] Gruenewald DA, Marck BT, Matsumoto AM. Fasting-induced increases in food intake and neuropeptide Y gene expression are attenuated in aging male brown Norway rats. Endocrinology 1996; 137: 4460-7.
[85] Kowalski C, Micheau J, Corder R, Gaillard R, Conte-Devolx B. Age-related changes in cortico-releasing factor, somatostatin, neuropeptide Y, methionine enkephalin and beta-endorphin in specific rat brain areas. Brain Res 1992; 582: 38-46.
[86] McShane TM, Wilson ME, Wise PM. Effects of lifelong moderate caloric restriction on levels of neuropeptide Y, proopiomelanocortin, and galanin mRNA. J Gerontol A Biol Sci Med Sci 1999; 54: B14-21.
[87] Sohn EH, Wolden-Hanson T, Matsumoto AM. Testosterone (T)-induced changes in arcuate nucleus cocaine-amphetamine-regulated transcript and NPY mRNA are attenuated in old compared to young male brown Norway rats: contribution of T to age-related changes in cocaine-amphetamine-regulated transcript and NPY gene expression. Endocrinology 2002; 143: 954-63.
[88] Gruenewald DA, Matsumoto AM. Age-related decrease in proopiomelanocortin gene expression in the arcuate nucleus of the male rat brain. Neurobiol Aging 1991; 12: 113-21.
[89] Kaneda T, Makino S, Nishiyama M, Asaba K, Hashimoto K. Differential neuropeptide responses to starvation with ageing. J Neuroendocrinol 2001; 13: 1066-75.
[90] Kappeler L, Gourdji D, Zizzari P, Bluet-Pajot MT, Epelbaum J. Age-associated changes in hypothalamic and pituitary neuroendocrine gene expression in the rat. J Neuroendocrinol 2003; 15: 592-601.
[91] Chapman IM. The anorexia of aging. Clin Geriatr Med 2007; 23: 735-56,
[92] Rigamonti AE, Pincelli AI, Corra B, *et al.* Plasma ghrelin concentrations in elderly subjects: comparison with anorexic and obese patients. J Endocrinol 2002; 175: R1-5.
[93] Schutte AE, Huisman HW, Schutte R, van Rooyen JM, Malan L, Malan NT. Aging influences the level and functions of fasting plasma ghrelin levels: the POWIRS-Study. Regul Pept 2007; 139: 65-71.
[94] Englander EW, Gomez GA, Greeley GH,Jr. Alterations in stomach ghrelin production and in ghrelin-induced growth hormone secretion in the aged rat. Mech Ageing Dev 2004; 125: 871-5.
[95] Sun Y, Garcia JM, Smith RG. Ghrelin and growth hormone secretagogue receptor expression in mice during aging. Endocrinology 2007; 148: 1323-9.
[96] Broglio F, Benso A, Castiglioni C, *et al.* The endocrine response to ghrelin as a function of gender in humans in young and elderly subjects. J Clin Endocrinol Metab 2003; 88: 1537-42.
[97] Schneider SM, Al-Jaouni R, Caruba C, *et al.* Effects of age, malnutrition and refeeding on the expression and secretion of ghrelin. Clin Nutr 2008; 27: 724-31.
[98] Di Francesco V, Zamboni M, Zoico E, *et al.* Unbalanced serum leptin and ghrelin dynamics prolong postprandial satiety and inhibit hunger in healthy elderly: another reason for the anorexia of aging. Am J Clin Nutr 2006; 83: 1149-52.
[99] Serra-Prat M, Palomera E, Clave P, Puig-Domingo M. Effect of age and frailty on ghrelin and cholecystokinin responses to a meal test. Am J Clin Nutr 2009; 89: 1410-7.
[100] Englander EW, Gomez GA, Greeley Jr., GH. Alterations in stomach ghrelin production and in ghrelin-induced growth hormone secretion in the age rat. Mech Ageing Dev 2004; 125: 871-5.
[101] Wolden-Hanson T. Mechanisms of the anorexia of aging in the Brown Norway rat. Physiol Behav 2006; 88: 267-76.
[102] Takeda H, Muto S, Hattori T, *et al.* Rikkunshito ameliorates the aging-associated decrease in ghrelin receptor reactivity via phosphodiesterase III inhibition. Endocrinology 2010; 151: 244-52.
[103] Ariyasu H, Iwakura H, Yamada G, Nakano K, Kangawa K, Akamizu T. Efficacy of ghrelin as a therapeutic approach for age-related physiological changes. Endocrinology 2008; 149; 3722-8.
[104] Barazzoni R, Zanetti M, Stebel M, Biolo G, Cattin L, Guarnieri G. Hyperleptinemia prevents increased plasma ghrelin concentration during short-term moderate caloric restriction in rats. Gastroenterology 2003; 124: 1188-92.
[105] Kohno D, Nakata M, Maekawa F, *et al.* Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase-andphosphodiesterase 3-mediated pathway. Endocrinology 2007; 148: 2251-63.
[106] Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers Jr MG, Schwartz MW. Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 2001; 413: 794-5.
[107] Zhao AZ, Huan JN, Gupta S, Pal R, Sahu A. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. Nat Neurosci 2002; 5: 727-8.
[108] Sahu A. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol 2003; 24: 225-53.
[109] Geliebter A., Aversa A. Emotional eating in overweight, normal weight and underweight individuals. Eat Behav 2003; 3: 341-47.
[110] Stone A., Brownell K. The stress-eating paradox: multiple daily measurements in adult males and females. Psychol Health 1994; 9: 425-36.
[111] Lutter M, Sakata I, Osborne-Lawrence S, *et al.* The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci 1998; 11: 752-3.
[112] Ochi M, Tominaga K, Tanaka F, *et al.* Effect of chronic stress on gastric emptying and plasma ghrelin levels in rats. Life Sci 2008; 82: 862-8.
[113] Zheng J, Dobner A, Babygirija R, Ludwig K, Takahashi T. Effects of repeated restraint stress on gastric motility in rats. Am J Physiol Regul Integr Comp Physiol 2009; 296: R1358-65.
[114] Asakawa A, Inui A, Kaga T, *et al.* A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology 2001; 74: 143-7.

[115] Kristenssson E, Sundqvist M, Astin M, *et al.* Acute psychological stress raises plasma ghrelin in the rat. Regul Pept 2006; 134: 114-17.
[116] Basa NR, Wang LX, Arteaga JR, Heber D, Livingston EH, Tache Y. Bacterial lipopolysaccharide shifts fasted plasma ghrelin to postprandial levels in rats. Neurosci Lett 2003; 343: 25-8.
[117] Hataya YJ, Akamizu T, Hosoda H, *et al.* Alterations of plasma ghrelin levels in rats with lipopolysaccharide-induced wasting syndrome and effects of ghrelin treatment on the syndrome. Endocrinology 2003; 144: 5365-71.
[118] Wang LX, Basa NR, Shaikh A, *et al.* LPS inhibits fasted plasma ghrelin levels in rats: role of IL-1 and PGs and functional implications. Am J Physiol 2006; 291: G611-20.
[119] von Meyenburg C, Langhans W, Hrupka BJ. Evidence for a role of the 5-HT2C receptor in central lipopolysaccharide-,interleukin-1 beta-, and leptin-induced anorexia. Pharmacology Biochem Behav 2003; 74: 1025-31.
[120] von Meyenburg C, Langhans W, Hrupka BJ. Evidence that the anorexia induced by lipopolysaccharide is mediated by the 5-HT2C receptor. Pharmacol Biochem Behav 2003; 74: 505-12.
[121] Stengel A, Goebel-Stengel M, Wang L, *et al.* Abdominal surgery inhibits circulating acyl ghrelin and ghrelin-O-acyltransferase levels in rats: role of the somatostatin receptor subtype 2. Am J Physiol Gastrointest Liver Physiol 2011; 301: G239-48.
[122] Raspopow K, Abizaid A, Matheson K, Anisman H. Psychosocial stressor effects on cortisol and ghrelin in emotional and non-emotional eaters: influence of anger and shame. Horm Behav 2010; 58: 677-84.
[123] Rouach V, Bloch M, Rosenberg N, *et al.* The acute ghrelin response to a psychological stress challenge does not predict the poststress urge to eat. Psychoneuroendocrinology 2007; 32: 693-702.
[124] Tomasik PJ, Sztefko K, Pizon M. The effect of short-term cold and hot exposure on total plasma ghrelin concentrations in humans. Horm Metab Res 2005; 37: 189-90.
[125] Zimmerman US, Buchmann A, Steffin B, Dieterle C, Uhr M. Alcohol administration acutely inhibits ghrelin secretion in an experiment involving psychological stress. Addict Biol 2006; 12: 17-21.
[126] Shiiya T, Ueno H, Toshinai K, *et al.* Significant lowering of plasma ghrelin but not des-acyl ghrelin in response to acute exercise in men. Endocr J 2011; 58: 335-42.
[127] Richard D, Lin Q, Timofeeva E. The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. Eur J Pharmacol 2002; 440: 189-97.
[128] Koob GF, Heinrichs SC. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res 1999; 848: 141-52.
[129] Spina M, Merlo-Pich E, Chan RK, *et al.* Appetite-suppressing effects of urocortin, a CRF-related neuropeptide. Science 1996; 273: 1561-4.
[130] Smagin GN, Howell LA, Ryan DH, De Souza EB, Harris RB. The role of CRF2 receptors in corticotropin-releasing factor- and urocortin-induced anorexia. Neuroreport 1998; 9: 1601-6.
[131] Hotta M, Shibasaki T, Arai K, Demura H. Corticotropin-releasing factor receptor type 1 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats. Brain Res 1999; 823: 221-5.
[132] Reyes TM, Lewis K, Perrin MH, *et al.* Urocortin II: a member of the corticotropinreleasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci USA 2001; 98: 2843-8.
[133] Inoue K, Valdez GR, Reyes TM, *et al.* Human urocortin II, a selective agonist for the type 2 corticotropin-releasing factor receptor, decreases feeding and drinking in the rat. J. Pharmacol. Exp. Ther 2003; 305: 385-93.
[134] Davis ME, Pemberton CJ, Yandle TG, *et al.* Urocortin-1 infusion in normal humans. J Clin Endocrinol Metab 89: 1402-1409: 2004.
[135] Tanaka C, Asakawa A, Ushikai M, *et al.* Comparison of the anorexigenic activity of CRF family peptides. Biochem Biophys Res Commun 2009; 390: 887-91.
[136] Yakabi K, Noguchi M, Ohno S, *et al.* Urocortin 1 reduces food intake and ghrelin secretion via CRF(2) receptors. Am J Physiol Endocrinol Metab 2011; 301: E72-82.
[137] Fone KC, Shalders K, Fox ZD, Arthur R, Marsden CA. Increased 5-HT2C receptor responsiveness occurs on rearing rats in social isolation. Psychopharmacology (Berl) 1996; 123: 346-52.
[138] Miura H, Qiao H, Ohta T. Influence of aging and social isolation on changes in brain monoamine turnover and biosynthesis of rats elicited by novelty stress. Synapse 2002; 46: 116-24.
[139] Saegusa Y, Takeda H, Muto S, *et al.* Decreased plasma ghrelin contributes to anorexia following novelty stress. Am J Physiol Endocrinol Metab 2011; 301: E685-96.
[140] Tisdale MJ. Biology of cachexia. J Natl Cancer Inst 1997; 89: 1763-73.
[141] Inui A. Cancer anorexia-cachexia syndrome: current issues in research and management. CA Cancer J Clin 2002; 52: 72-91.
[142] Tisdale, M.J. Cachexia in cancer patients. Nat. Rev. Cancer 2002; 2: 862-71.
[143] Barber, M.D., Ross, J.A., Fearon, K.C. Changes in nutritional, functional, and inflammatory markers in advanced pancreatic cancer. Nutr. Cancer 1999; 35: 106-10.
[144] Kotler DP. Cachexia. Ann Intern Med 2000; 133: 622-34.
[145] Loprinzi CL, Schaid DJ, Dose AM, Burnham NL, Jensen MD. Body-composition changes in patients who gain weight while receiving megestrol acetate. J Clin Oncol 1993; 11: 152-4.
[146] Gagnon B and Bruera E. A review of the drug treatment of cachexia associated with cancer. Drugs 1998; 55: 675-88.
[147] DeBoer MD. Ghrelin and cachexia: will treatment with GHSR-1a agonists make a difference for patients suffering from chronic wasting syndromes? Mol Cell Endocrinol 2011; 340: 9 7-105.
[148] Smagin GN, Dunn AJ. The role of CRF receptor subtypes in stress-induced behavioura responses. Eur J Pharmacol 2000; 405: 199-206.
[149] Inui A. Feeding and body-weight regulation by hypothalamic neuropeptides-mediation of the actions of leptin. Trends Neurosci 1999; 22: 62-7.
[150] Legakis I, Stathopoulos J, Matzouridis T, Stathopoulos GP. Decreased plasma ghrelin levels in patients with advanced cancer and weight loss in comparison to healthy individuals. Anticancer Res 2009; 29: 3949-52.
[151] Takahashi M, Terashima M, Takagane A, *et al.* Ghrelin and leptin levels in cachectic patients wit cancer of the digestive organs. Int J Clin Oncol 2009; 14: 315-20.
[152] Nagaya N, Uematsu M, Kojima M, *et al.* Elevated circulating level of ghrelin in cachexi associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors Circulation 2001; 104: 2034-8.
[153] Hanada T, Toshinai K, Kajimura N, *et al.* Anti-cachectic effect of ghrelin in nude mice bearing human melanoma cells. Biochem. Biophys. Res Commun 2003; 301: 275-9.
[154] Wang W, Andersson M, Ireşjo BM, Lonnroth C, Lundholm K. Effects of ghrelin on anorexia in tumor-bearing mice with eicosanoid-related cachexia. Int J Oncol 2006; 28: 1393-400.
[155] DeBoer MD, Zhu XX, Levasseur P, *et al.* Ghrelin treatmen causes increased food intake and retention of lean body mass in a rat model o cancer cachexia. Endocrinology 2007; 148: 3004-3012.
[156] Neary NM, Small CJ, Wren AM, *et al.* hrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlle trial. J Clin Endocrinol Metab 2004; 89: 2832-6.
[157] Strasser F, Lutz TA, Maeder MT, *et al.* Safety, tolerability and pharmacokinetics of intravenous ghrelin for cancer-related anorexia/cachexia: a randomised, placebo-controlled, double-blind, doublecrossover study. Br J Cancer 2008; 98: 300-8.
[158] Garcia JM, Polvino WJ. Effect on body weight and safety of RC-1291, a novel, orally available ghrelin mimetic and growth hormone secretagogue: results of a phase I, randomized, placebocontrolled, multiple-dose study in healthy volunteers. Oncologist 2007; 12: 594-600.
[159] Garcia JM, Graham C, Kumor K, Polvino W. A Phase II, randomized, placebo-controlled, double blind study of the efficacy and safety of RC-1291 for the treatment of cancer-cachexia. J Clin Oncol 2007; 25: 9133. Abstract.
[160] Wang W, Danielsson A, Svanberg E, Lundholm K. Lack of effects by tricyclic antidepressant and serotonin inhibitors on anorexia in MCG 101 tumor bearing mice with eicosanoid-related cachexia. Nutrition 2003; 19: 47-53.
[161] Makarenko IG, Meguid MM, Gatto L, *et al.* Hypothalamic 5-HT1B-receptor changes in anorectic bearing rats. Neurosci Lett 2005; 376: 71-5.

[162] Laferrere B, Abraham C, Russell CD, Bowers CY. Growth hormone releasing peptide-2 (GHRP-2), like ghrelin, increases food intake in healthy men. J Clin Endocrinol Metab 2005; 90: 611-4.
[163] Ueda N, Yoshimura R, Shinkai K, Sakata Y, Nakamura J. Higher plasma 5-hydroxyindoleacetic acid levels are associated with SSRI-induced nausea. Neuropsychobiology 2003; 48: 31-4.
[164] Fujitsuka N, Asakawa A, Hayashi M, *et al.* Selective serotonin reuptake inhibitors modify physiological gastrointestinal motor activities via 5-HT2c receptor and acyl ghrelin. Biol Psychiatry 2009; 65: 748-59.
[165] De Vriese C, Gregoire F, Lema-Kisoka R, Waelbroeck M, Robberecht P, Delporte C. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology 2004; 145: 4997-5005.

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CHAPTER TWENTY-ONE

Rikkunshito as a Ghrelin Enhancer

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Contents

Abstract

Rikkunshito is a kampo herbal medicine which is widely used in Japan for the treatment of the upper gastrointestinal symptoms of patients with functional dyspepsia, gastroesophageal reflux disease, dyspeptic symptoms of postgastrointestinal surgery patients, and chemotherapy-induced dyspepsia in cancer patients. Recently, very unique characteristics of rikkunshito have been unveiled; oral administration of rikkunshito potentiates orexigenic action of ghrelin through several different mechanisms. In addition, several lines of evidence obtained from both animal and human studies indicate that

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rikkunshito can be an attractive and promising therapeutic option for the anorectic conditions including cisplatin-induced dyspepsia, anorexia of aging, stress-induced hypophagia, and cancer cachexia–anorexia syndrome. In this chapter, we highlight the orexigenic effect of rikkunshito with a special focus on its interaction with ghrelin signaling system.

1. INTRODUCTION

Rikkunshito is a kampo herbal medicine which is prepared by compounding eight herbal medicines: *Atractylodis Lanceae Rhizoma, Ginseng Radix, Pinelliae Tuber, Hoelen, Zizyphi Fructus, Aurantii Nobilis Pericarpium, Glycyrrhizae Radix*, and *Zingiberis Rhizoma*. Rikkunshito is widely used in Japan for the treatment of the upper gastrointestinal symptoms of patients with functional dyspepsia and gastroesophageal reflux disease, chemotherapy-induced dyspepsia in cancer patients, and dyspeptic symptoms of postgastrointestinal surgery patients (Mochiki et al., 2010; Suzuki et al., 2009; Takeda et al., 2012).

Ghrelin is a peripherally active orexigenic gut hormone consisting of 28 amino acids, and the third N-terminal amino-acid serine (Ser) residue is octanoylated (Kojima and Kangawa, 2005; Kojima et al, 1999). Ghrelin is involved in the hypothalamic regulation of energy homeostasis by increasing food intake and reducing fat utilization (Nakazato et al., 2001; Tschop et al., 2000). Plasma levels of ghrelin rise during fasting, and fall upon eating, which has led to the suggestion that ghrelin is a meal-initiating hormone (Cummings et al., 2001). Plasma levels of ghrelin are inversely correlated with body weight in humans and rise after weight loss (Cummings et al., 2002). Besides the regulation of energy homeostasis, ghrelin mediates an increase in gastric motility, induces a positive inotropic effect on the heart, and causes vasodilatation (Chen et al., 2009; Kojima and Kangawa, 2005; Leite-Moreira and Soares, 2007).

Recently, it was shown that oral administration of rikkunshito stimulates secretion of ghrelin in rodents and humans (Hattori, 2010; Matsumura et al., 2010; Takeda et al., 2008). More recent evidence suggests that rikkunshito enhances ghrelin's orexigenic effect by several additional mechanisms (Fujitsuka et al., 2011; Sadakane et al., 2011; Yakabi et al., 2010a,b). In this chapter, we discuss the currently available evidence on the orexigenic effect of rikkunshito with a special attention to its interaction with the ghrelin signaling system.

2. CISPLATIN-INDUCED ANOREXIA

2.1. Cisplatin-induced anorexia and ghrelin

Patients with cancer being treated with cytotoxic drugs such as cisplatin often experience a number of undesirable side effects which include acute and delayed nausea, vomiting, anorexia, dyspepsia, and disrupted gastrointestinal function.

Recent evidence has demonstrated the relationship between chemotherapy-induced gastrointestinal disorders and ghrelin in both clinical and animal studies. In human studies, one report has demonstrated that an increase in plasma ghrelin concentrations was observed after the start of anticancer chemotherapy (Shimizu et al., 2003), but more recent studies revealed that the plasma concentration of acylated ghrelin was decreased during the treatment with anticancer drugs (Hiura et al., 2011; Ohno et al., 2011). In animal studies, we and others reported that circulating ghrelin concentrations were reduced in cisplatin-treated rats until 6 h during the early stage of anorexia (Takeda et al., 2008; Yakabi et al., 2010a).

Intraperitoneal injection of 5-HT decreased 24-h food intake as well as plasma acylated-ghrelin level in a dose-dependent manner (Takeda et al., 2008). This result suggests that the cisplatin-induced reduction in the plasma level of acylated ghrelin may be mediated via a release of 5-HT from the gastrointestinal tract mucosa triggered by cisplatin. Indeed, a 5-HT2B receptor agonist BW723C86 and a 5-HT2C agonist *m*-chlorophenylpiperazine HCl (mCPP) markedly decreased the acylated-ghrelin levels in plasma and increased intragastric ghrelin content, suggesting that 5-HT2B/2C receptor stimulation inhibits the release of gastric ghrelin into the circulation (Takeda et al., 2008). In contrast, 5-HT3 and 5-HT4 agonists had no effect on ghrelin dynamics. 5-HT2B and 5-HT2C antagonists suppressed the cisplatin-induced decrease of plasma acylated-ghrelin level and food intake. These results strongly imply that activation of 5-HT2B and 5-HT2C receptors, but not 5-HT3 and 5-HT4 receptors, plays an important role in the decrease in plasma ghrelin level in cisplatin-induced anorexia. Of note, granisetron used in this study clearly inhibited delayed gastric emptying after cisplatin treatment, but it failed to improve cisplatin-induced anorexia (Takeda et al., 2008; Yakabi et al., 2010a).

Peripheral administration of exogenous ghrelin ameliorates anorexia (Liu et al., 2006; Takeda et al., 2008) and vomiting (Rudd et al., 2006) induced by cisplatin. Administration of exogenous ghrelin has been shown to have

the potential to reduce each of these symptoms in relevant animal models treated with cisplatin as a cytotoxic agent: emesis in the ferret (Rudd et al., 2006) and anorexia in the rat and mouse (Liu et al., 2006).

Yakabi et al. (2010a) examined the changes of hypothalamic ghrelin secretion in cisplatin-treated rats to elucidate the mechanism underlying chemotherapy-induced delayed anorexia. Although ghrelin secretion in the hypothalamus did not decrease within 24 h of cisplatin administration, it started to decline significantly after 24 h and continued to decrease at least until 48 h, while their plasma ghrelin levels were comparable (Liu et al., 2006; Takeda et al., 2008). Yakabi et al. (2010a) also showed that hypothalamic 5-HT2C receptor gene expression increased significantly in cisplatin-treated rats and the administration of mCPP inhibited hypothalamic ghrelin secretion. Intracerebroventricularly (icv) administered 5-HT2C antagonist SB242084 prevented a decrease in secretion of hypothalamic ghrelin in cisplatin-treated rats, but granisetron, a 5-HT3 antagonist, did not (Yakabi et al., 2010a). These results indicate that the reduced ghrelin secretion in the hypothalamus secondary to 5-HT2C receptor activation may be involved in cisplatin-induced anorexia.

In another study, Yakabi et al. (2010b) demonstrated that hypothalamic GHS-R1a gene expression was significantly reduced after cisplatin or mCPP treatment and this change was reversed by the treatment with the 5-HT2C receptor antagonist SB242084 but not with 5-HT3 receptor antagonists. 5-HT2C receptor antagonist also suppressed cisplatin-induced delayed anorexia. Injection (icv) of GHS-R1a antagonist to saline- or cisplatin-treated rats significantly reduced food intake compared with those injected with saline alone, and this inhibitory effect was abolished by the coadministration of the 5-HT2C receptor antagonist. From these results, it was suggested that delayed-onset anorexia induced by cisplatin may be partly mediated by the activation of the hypothalamic 5-HT2C receptor and the resultant suppression of hypothalamic GHS-R1a gene expression as well as decreased ghrelin secretion in the hypothalamus.

2.2. Effect of rikkunshito on cisplatin-induced anorexia

Rikkunshito ameliorated the decrease in circulating ghrelin concentration and this effect was abolished by coadministration of a GHS-R1a antagonist [D-Lys^3]-GHRP-6 (Takeda et al., 2008). This finding suggests that the mechanism of improvement of anorexia by rikkunshito may involve ghrelin receptor activation. Moreover, Yakabi et al. (2010a) found that rikkunshito

reversed the decrease in hypothalamic ghrelin secretion and the decrease in GHS-R1a gene expression 24 h after cisplatin treatment. Injection (icv) of the GHS-R1a antagonist impedes the rikkunshito-mediated improvement in cisplatin-induced anorexia (Yakabi et al., 2010a). Hence, it seems likely that rikkunshito ameliorates cisplatin-induced anorexia by restoring ghrelin secretion and GHS-R1a expression in the hypothalamus. Collectively, rikkunshito suppressed cisplatin-induced anorexia by improving ghrelin signal transduction system by both the peripheral and the central mechanisms.

The induction of ghrelin secretion by rikkunshito is supposed to be based on the 5-HT2B/2C receptor antagonism due to multiple active ingredients. We screened 33 compounds contained in rikkunshito and found that 13 showed antagonistic activity against binding to any of 5-HT 1A, 1B/D, 2A, 2B, 2C, 3, 4, 6, and 7 receptors (Takeda et al., 2008). Among them, 3,3′,4′,5,6,7,8-heptamethoxyflavone (HMF), nobiletin, and tangeretin contained in *Aurantii nobilis pericarpium* had potent 5-HT2B receptor antagonistic activity. The inhibitory activity of hesperidin against the 5-HT2B receptor was weak, but the concentration of hesperidin in rikkunshito is the highest among the ingredients tested. In addition, isoliquiritigenin, which is an ingredient of *Glycyrrhizae radix*, had the most potent activity against the 5-HT2C receptor binding. Our study indicated that the administration of HMF, isoliquiritigenin, and hesperidin attenuated the decrease in plasma ghrelin level, while tangeretin, nobiletin, and 8-shogaol did not. This suggested that the ingredients that inhibit 5-HT2B/5-HT2C-receptor binding are likely to be effective *in vivo*.

2.3. Experimental methods

2.3.1 Animals

Male SD rats aged 7 weeks (Charles River Laboratories Japan, Inc, Tokyo, Japan) were used. During the study period, the rats were kept in an animal room under the following conditions: controlled temperature and humidity, four to five rats in each cage, with a 12-h light/darkness cycle (7:00 a.m.–7:00p.m.), and food and water given *ad libitum*. All experiments were performed between 9:00 a.m. and 6:00 p.m..

2.3.2 Effects of cisplatin, various 5-HT receptor agonists, and antagonists on plasma ghrelin level

Rats were administered cisplatin (2, 6, 10, or 14 mg/kg) and 5-HT (1, 2, 4, or 8 mg/kg) and subjected to blood collection by decapitation at 120 min (cisplatin) or 30 min (5-HT) after administration. Other groups of rats were

fasted for 24 h and administered BW723C86 (4 or 16 mg/kg), mCPP (3 or 9 mg/kg), 1-(3-chlorophenyl) biguanide HCl (CPB, 1.25 or 6 mg/kg), 2-methylserotonin (1 or 4 mg/kg), cisapride (1.25 or 5 mg/kg), SB215505 (6 mg/kg), or SB242084HCl (10 mg/kg) and subjected to blood collection by decapitation at 60 or 120 min after administration. The stomach was removed from the animals of the treated groups in which the plasma ghrelin level was decreased. All drugs were given intraperitoneally as solutions in physiologic saline.

2.3.3 Effects of rikkunshito and active herbal components on plasma acylated-ghrelin level in rats administered cisplatin

The rats were orally administered rikkunshito (500 or 1000 mg/kg) dissolved in 1 ml of distilled water per 100 g of body weight. After 16 h, the rats were administered cisplatin intraperitoneally and at the same time a second dose of rikkunshito (which was the same size as the first dose) was administered orally. A single dose of HMF (0.8, 4.0, or 20 mg/kg), hesperidin (4.0 or 20 mg/kg), or isoliquiritigenin (4.0 mg/kg) was given orally simultaneously with the administration of cisplatin. At 2 h after the last dose, the rats were subjected to blood collection by decapitation, and plasma samples were obtained by the method detailed later.

2.3.4 Determination of ghrelin level

The plasma samples were promptly centrifuged at 4 °C, and the supernatant was acidified with 1 mol/l HCl (1/10 volume). The tissue samples were boiled in water for 7 min and acidified with 1 mol/l HCl after cooling. The samples then were homogenized and centrifuged at 10,000 rpm for 15 min. The supernatants were stored at −80 °C until use. The ghrelin level was determined using Active Ghrelin or Desacyl Ghrelin Enzyme-Linked Immunoassay Kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

2.3.5 Effects of rat ghrelin, rikkunshito, and 5-HT2B/2C receptor antagonists on food intake in rats administered with cisplatin

Cisplatin was administered intraperitoneally, and after 2 h acylated ghrelin (1 or 5 nmol/rat) was administered intravenously via the tail vein of rats. Rikkunshito (500 or 1000 mg/kg) was administered orally, and after 16 h cisplatin was administered intraperitoneally, and at the same time, a second dose of rikkunshito was administered orally (which was of the same size as the first dose). SB215505 or SB242084HCl was administered intraperitoneally 30 min before the administration of cisplatin. The food intake and body

weight gain were recorded in the subsequent 24 h. In the second experiment, after following the procedures described earlier, physiologic saline or a solution of [D-Lys^3]-GHRP-6 (0.4 μmol/rat) was administered intraperitoneally simultaneously with cisplatin and the second dose of rikkunshito (1000 mg/kg); the food intake in the subsequent 6 h was recorded.

2.3.6 Radioligand binding assays

CHO-K1 cells expressing human recombinant 5-HT receptor were used to prepare membranes in modified Tris–HCl buffer. A membrane protein was incubated with 1.2 nmol/l [^{3}H]LSD for 60 min at 37 °C. Nonspecific binding was estimated in the presence of 10 μmol/l 5-HT. The filters were then counted for radioactivity to determine the amount of specifically bound [^{3}H] LSD (Wolf and Schuts, 1997). CHO-K1 cells stably transfected with a plasmid encoding the human 5-HT2C receptor were used to prepare membranes in modified Tris–HCl buffer. A membrane protein was incubated with 1.0 nmol/ml [^{3}H]-Mesulergine for 60 min at 25 °C. Nonspecific binding was estimated in the presence of 1 μmol/l Mianserin. The filters then were counted for radioactivity to determine the amount of specifically bound [^{3}H]-Mesulergine. Likewise, membranes prepared from cells stably transfected with human recombinant 5-HT3, 5-HT6 receptors, and guinea pig striatum (5-HT4) were incubated with radiolabeled ligands with a high affinity for the given receptor, that is, [^{3}H]LSD, [^{3}H]-GR-65630, and [^{3}H]-GR-113808, respectively. Nonspecific radioligand binding was defined by 5-HT or MDL-72222.

3. ANOREXIA OF AGING

3.1. Anorexia of aging and ghrelin

In elderly subjects, the reduction in energy intake often exceeds energy expenditure, resulting in weight loss and protein energy malnutrition (Morley, 2001). Protein energy malnutrition in the elderly is a frequent and clinically important problem, which leads to increased morbidity, mortality, disability, and health costs in this growing population. One of the most important causes of the reduction in energy intake is anorexia. The causes of the anorexia of aging have not yet been fully defined; they are probably multifactorial and include sensory impairment, social isolation, and psychological and physiologic factors, in addition to the presence of disease (Di Francesco et al., 2007; Morley, 2001).

Although many peripheral anorexigenic hormones including cholecystokinin, leptin, and insulin have been found to rise with increased age, findings for ghrelin are controversial (Takeda et al., 2012). Several lines of animal studies also have revealed mixed results. The reason for these conflicting data seems to be due to the differences in their experimental conditions under which the plasma ghrelin concentration was measured. Indeed, our group found that plasma ghrelin in aged C57BL/6 mice does not increase under fasted conditions but is higher than that in young mice under freely fed conditions (Takeda et al., 2010). This suggests that regulation of ghrelin secretion from the stomach may be disturbed in older mice. In agreement with this, recent clinical studies have suggested that disturbance of regulation of ghrelin secretion and reduced production during hunger and satiety may cause "anorexia of aging" in elderly people (Takeda et al., 2012).

In a previous study, Ariyasu et al. (2008) reported that subcutaneous injection of ghrelin (360 μg/kg twice a day) enhanced food intake in 72-h-fasted and aged mice and restored the decrease in body weight caused by fasting. Contrary to their data, we recently found that much lower dose of ghrelin (33 μg/kg) failed to increase food intake in 75-week-old mice, whereas the same dose of ghrelin had an orexigenic effect in young mice (Takeda et al., 2010), suggesting that aging is associated with attenuation of endogenous ghrelin signaling. Collectively, it seems that dysregulation of ghrelin secretion as well as ghrelin resistance in the appetite control system is occurring in aged mice.

Although the detailed mechanisms of disturbed ghrelin dynamics remain unclear, one of the possible causes seems to be leptin. We have found that plasma leptin and insulin levels in aged mice are significantly higher than in young ones (Takeda et al., 2010). Leptin and insulin are reported to inhibit ghrelin secretion from the stomach into the circulation (Barazzoni et al., 2003); hence elevated leptin and insulin in the elderly may contribute to the inhibition of secretion of ghrelin during fasting, resulting in prolonged satiety and inhibition of hunger sensation. Moreover, the activation of the phosphoinositide 3-kinase (PI3K)–phosphodiesterase 3 (PDE3) pathway was recently proposed as a mechanism by which leptin blocks ghrelin signaling in neuropeptide Y (NPY) neurons, and it may counteract the adenylate cyclase–cAMP–protein kinase A system implicated in the effect of ghrelin (Kohno et al., 2007). Other studies showed that the effect of leptin was abolished by the administration of either PDE3 inhibitor (Niswender et al., 2001) or PI3K inhibitor (Zhao et al., 2002). We demonstrated that the plasma leptin level in aged mice was greatly increased under both feeding and fasting conditions. Furthermore, we found that administration of either

a PI3K inhibitor LY-294002 or the PDE3 inhibitor cilostamide improved anorexia in aged mice (Takeda et al., 2010). These results suggest that plasma leptin, which increases with age, may induce resistance to ghrelin reactivity via camp downregulation.

3.2. Effect of rikkunshito on hypophagia of aged mice

Recently, we demonstrated that the administration of rikkunshito improves anorexia of aging. In addition, we found that rikkunshito increased the reactivity of ghrelin by inhibiting PDE3 activity. The components of rikkunshito (nobiletin, isoliquiritigenin, and HMF) had inhibitory effects against PDE3 activity (Takeda et al., 2012). These results suggest that dysregulation of ghrelin secretion and ghrelin resistance in the appetite control system occurred in aged mice and that rikkunshito ameliorated aging-associated anorexia via inhibition of PDE3.

3.3. Experimental methods

3.3.1 Animals

Male C57BL/6J mice aged 6 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan) and were maintained until 75 weeks of age in Tsumura Research Laboratories (Tokyo, Japan). Male B6.V-Lepob/J homozygous, ob/ob and heterozygous, ob/+ mice aged 6 weeks were purchased from Charles River Japan (Tsukuba, Japan). All animals were fed a normal chow diet and had free access to water and food. Mice were housed five animals per cage in plastic cages on a 12-h light (07:00–19:00 h), 12-h dark cycle. All experiments were carried out between 09:00 and 18:00 h.

3.3.2 Food intake

Food intake was measured by subtracting uneaten food from the initially premeasured food 1 and 24 h after administration. Rat ghrelin (Peptide Institute, Inc., Osaka, Japan) was dissolved in physiological saline, and 33 μg/kg was administered intravenously (i.v.) to conscious mice from the tail vain. Cilostamide (PDE3 inhibitor) or LY-294002 (PI3K inhibitor) was dissolved in distilled water and administered orally at 30 mg/kg. Rikkunshito was dissolved in 10 ml of DW and orally administered at 1000 mg/kg.

3.3.3 Extraction of total RNA, reverse transcription, and real-time RT-PCR analysis

Mice were decapitated and their brains and stomachs removed immediately. The hypothalamus and gastric bodies were dissected out, frozen in liquid nitrogen, and kept at −80 °C until they were processed for RNA extraction.

Isolated tissue homogenization and total RNA extraction were performed according to the manufacturer's protocol using an RNeasy universal tissue kit (QIAGEN Co., Valencia, CA). The OD of the total RNA solution was determined using an ND-1000 UV/vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA), and each sample was diluted to100 ng/μl. The diluted total RNA was incubated at 70 °C for 5 min and then cooled on ice. Total RNA (1000 ng) was reverse-transcribed using TaqMan RT reagents (Applied Biosystems Co., Foster City, CA) according to the manufacturer's protocol. Quantitative PCR assays were performed using the TaqMan universal PCR master mix (Applied Biosystems) on a Prism 7900HT sequence detection system (Applied Biosystems). mRNA expression was normalized to ribosomal protein S29 expression, which served as an endogenous control to correct for differences in the amount of total RNA added to each reaction as expressed by the delta threshold cycle (dCt) value: dCt value $= 2^{(-|A-B|)}$, where A is the number of cycles of the control gene and B is the number of cycles of the target gene. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were obtained from Applied Biosystems: ribosomal protein S29, Mm02342448_gH; preproghrelin, Mm00445450_m1; GHS-R, Mm00616415_m1; NPY, Mm00445771_m1; AGRP, Mm00475829_g1; POMC-α, Mm00435874_m1.

3.3.4 Hormone assay

Blood was collected from the caudal vena cava in tubes containing aprotinin and EDTA-2Na. The blood samples were promptly centrifuged at 4 °C, and the plasma was acidified with 1 mol/l HCl (1:10 plasma volume). The ghrelin level was determined using an active ghrelin or des-acyl ghrelin ELISA kit. The leptin and GH levels were determined using the mouse leptin ELISA kit and rat/mouse GH ELISA kit (Millipore Corp., Billerica, MA). The insulin level was measured by Bio-plex using a Milliplex mouse serum adipokine panel (Millipore).

3.3.5 Assay for PDE3 activity

PDE3 activity was measured using 1 μmol cyclic nucleotides as a substrate in a two-step radioassay procedure adapted from Hidaka and Asano (Hidaka and Asano, 1976). The 5′-[^{3}H]AMP formed by PDE was converted to [^{3}H]adenosine by the activation of nucleotidase. The radioactivity of the product that was isolated by cation exchange resin was counted in a liquid

scintillation counter. Appropriate dilutions of the enzyme were incubated in 50 m*M* Tris–HCl (pH 7.5), 5 m*M* $MgCl_2$, 50 μg BSA, and 1 μmol [^{3}H] AMP. After 15 min at 25 °C, the reaction was terminated by boiling for 5 min. Next, snake venom was added, and an additional incubation was performed for 20 min at 25 °C. Water was then added, and the mixture was applied to a small ion exchange resin column. After the column was washed with DW, adenosine or guanosine was eluted with NH_4OH. The components of rikkunshito, including hesperetin, naringenin, HMF, nobiletin, tangeretin, isoliquiritigenin, liquiritigenin, glycycoumarin, 8-shogaol, 10-shogaol, 10-dehydrogingerdion, 10-gingerdion, and 8-gingerol, were also subjected to a PDE assay.

3.3.6 Assay for PI3K activity

The test compound was preincubated for 5 min at room temperature with the PI3K enzyme (6.5 ng) in a buffer containing 50 m*M* HEPES/NaOH (pH 7.4), 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 40 μ*M* Na_3VO_4, and 0.005% Tween 20. The reaction was then initiated by the addition of 35 n*M* substrate, biotin-KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK, and 11 μ*M* ATP. The mixture was then incubated for 90 min at room temperature. After incubation, the reaction was stopped by the addition of 33 m*M* EDTA. The fluorescence acceptor (XL665-labeled streptavidin) and the fluorescence donor (antiphospho-S6-rib antibody labeled with europium cryptate) were then added. After 60 min, the fluorescence transfer was measured at $\lambda_{ex} = 337$ nm, $\lambda_{em} = 620$ and 665 nm using a microplate reader (Zhao et al., 2005).

4. STRESS

4.1. Stress and ghrelin

Stress and negative emotions have been associated with both increased and decreased food intake. The mechanism underlying this opposed behavioral responses to similar stressors has not been determined, but high stress levels appear to lead to decreased eating (Stone and Brownell, 1994).

Conflicting data are available regarding the effect of stress on ghrelin secretion (Takeda et al., 2012). In animal studies, elevations in plasma ghrelin have been observed in response to various psychological/environmental stressors, including tail-pinch stress, water-avoidance stress, chronic exposure to cold, repeated restraint stress, and chronic social defeat stress. In contrast, exposure to immune, visceral, or strenuous physical stressors causes

reduction of plasma ghrelin level. In humans, acute psychosocial stress or cold exposure increased plasma ghrelin levels. However, there are several reports showing that plasma ghrelin level did not change or even decreased by an exposure to stresses. Collectively, these findings support the idea that acute or severe stress causes a reduction of circulating ghrelin level, resulting in the suppression of appetite, whereas mild or chronic repeated stress causes an upregulation of ghrelin secretion as an adaptation to stress (Takeda et al., 2012). In support of this notion, Lutter et al. (Lutter et al., 1998) found that increased ghrelin levels produced anxiolytic and antidepressant responses in mice, suggesting that increased ghrelin in response to stress protects against depressive reactions to stress and helps them cope with stress.

Corticotropin-releasing factor (CRF) and its family peptides urocortin1 (Ucn1), urocortin2 (Ucn2), and urocortin3 (Ucn3) play an important role in the control of food intake (Richard et al., 2002). Among the CRF family peptides, Ucn1 was shown to have the most potent inhibitory effect on the food intake in mice. Ucn1 has been identified in the brain and has a higher affinity for CRF2 receptors (CRFR2) than for CRF1 receptors (CRFR1), and hence it is believed that CRFR2 plays the major role in satiety.

Activation of CRFR1 in the brain can suppress feeding independently of CRFR2-mediated mechanisms. CRF1 and CRF2 receptor-mediated anorexia appear to exhibit different time courses; in rats, i.c.v. administration of CRFR1 agonists elicited rapid-onset anorexia with short duration, while CRFR2 agonists caused delayed-onset, prolonged anorexia (Richard et al., 2002).

There are several reports showing that administration of Ucn1 to humans and rodents reduces plasma ghrelin concentrations (Tanaka et al., 2009; Yakabi et al., 2011). In addition, Ucn1-induced reduction of plasma ghrelin and food intake was restored by CRFR2 but not CRFR1 (Yakabi et al., 2011). However, much less information is available on the relationship between ghrelin and CRFR1.

The novelty stress model, where animals are removed from their home cage and placed somewhere they have never been before, has been used to estimate the levels of anxiety and depression (Fone et al., 1996; Miura et al., 2002). Using this stress model, we found that 3 h after the novelty stress, appetite reduction was associated with decreased plasma ghrelin level, reduced levels of NPY/agouti-related peptide mRNA, and increased levels of pro-opiomelanocortin mRNA in the hypothalamus (Saegusa et al., 2011). Administering a CRFR1 selective antagonist, but not a

CRFR2 antagonist, resolved the reduction in food intake 3 h after the novelty stress by enhancing circulating ghrelin concentrations. Interestingly, 5-HT1B/2CR antagonist and melanocortin-4 receptor (MC4R) antagonist alleviated the novelty-stress-induced hypophagia and the reduction in the circulating ghrelin level (Saegusa et al., 2011). We hypothesized that the acute appetite suppression due to CRFR1 activation after a novelty stress is caused by a chain reaction of appetite control mechanisms mediated by 5-HT1B/2CR in ARC to MC4R system in paraventricular nucleus, causing lowered peripheral ghrelin secretion.

4.2. Effect of rikkunshito on the novelty stress model

Oral administration of rikkunshito inhibited the reduction of food intake at 1 and 3 h in mice exposed to the novelty stress, and coadministration of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 with rikkunshito abolished this effect (Saegusa et al., 2011). Rikkunshito also increased plasma acyl-ghrelin concentrations at 1 and 3 h after the novelty stress, suggesting that blocking the decrease in endogenous peripheral ghrelin in mice exposed to the novelty stress also acts to sustain the feeding behavior. We found that the oral administration of glycycoumarin and isoliquiritigenin inhibited the reduction in food intake in mice exposed to novelty stress (Saegusa et al., 2011). We have previously shown that glycycoumarin and isoliquiritigenin potently inhibit 5-HT2C receptor ligand binding and that orally administering rikkunshito abolishes the decrease in food intake in mCPP-treated rats (Takeda et al., 2008). These findings support the notion that rikkunshito improved hypophagia and decreased plasma ghrelin levels via 5-HT2C receptor antagonism-like action in mice exposed to the novelty stress.

4.3. Experimental methods

4.3.1 Animals

Male C57BL/6J mice aged 6 weeks (Japan Charles River, Tokyo, Japan) were used. Before the experiment, five mice per cage were maintained in a room with controlled temperature and humidity under a 07:00–19:00 h light cycle with free access to food and water. For the novelty stress, each mouse was transferred from group-housed cages to individual cages. Control mice were housed in individual cages for 7 days before the experiment was initiated. Mice in each group were handled in the same way. All experiments were performed between 09:00 and 18:00 h.

4.3.2 Intracerebroventricular infusion

A 26-gauge stainless steel indwelling cannula was implanted 2.6 mm below the skull surface into the lateral ventricle (1.1 mm lateral to the bregma) of the mice. Injections were performed using a 26-gauge stainless steel injector attached to PE-10 tubing fitted to a 10-μl microsyringe.

4.3.3 Food intake

All protocols were performed under a 24-h-fasted condition. The time course evaluation of the effect of the novelty stress on food intake in 24-h-fasted mice was undertaken 1, 3, and 6 h after the novelty stress and was calculated as the difference between the food weights before and after the feeding period at each time interval. Subsequently, to clarify the role of the CRFR or 5-HT1B/2CR on food intake in control or stressed mice, we investigated the effects of the icv administration of the CRFR1 antagonist NBI27914 (10 μg/mouse), the CRFR2 antagonist astressin2B (10 μg/mouse), the 5-HT2CR antagonist SB-242084 (0.5 or 1.5 μg/mouse), or the 5-HT1BR antagonist SB-224289 (0.5 or 1.5 μg/mouse) on the novelty stress-induced decrease in food intake during a 24-h-fasted condition. Each drug was immediately administered after the novelty stress, and subsequently, each mouse was placed in a single housing cage with access to preweighed mouse chow from the group housing cage.

In another experiment, icv administration of CRF, a CRFR1 agonist, was performed in 24-h-fasted mice (0.1, 0.5, and 1.0 μg/mouse). In addition, the 5-HT1B/2CR agonist mCPP (2, 10, and 50 μg/mouse) in 24-h-fasted mice was administered (icv) 3 h after the novelty stress. This is because icv administration of the 5-HT1B/2CR antagonist significantly suppressed decreased food intake for 3 h after the novelty stress was introduced in the present results. CRF (0.1 μg/mouse) was administered icv to mice 15 min before the mCPP (10 μg/mouse icv), and food intake was evaluated 3 h after the mCPP administration. Administration (icv) of the MC4R antagonist HS014 (0.15 μg/mouse) was conducted immediately in 24-h-fasted mice after the novelty stress.

Rat ghrelin (3.3 and 33 μg/mouse) was administered iv in 24-h-fasted mice, and food intake was measured to clarify the contribution of peripheral ghrelin. Rikkunshito (250 or 500 mg/kg) was administered orally in 24-h-fasted mice 1 h before the novelty stress, in combination with the GHS-R antagonist [D-Lys^3]-GHRP-6 (0.12 mg/mouse i.v.) or saline. In another experiment, two components contained in rikkunshito, glycycoumarin (4 mg/kg po) and isoliquiritigenin (4 mg/kg p.o.), which have 5-HT2CR antagonistic-like activity, were administered.

4.3.4 Determining plasma levels of ghrelin or serum levels of corticosterone

Blood was collected from mice by ether anesthesia 0.5, 1, 3, and 6 h after the novelty stress to investigate changes in plasma corticosterone during the novelty stress. The blood collection to determine plasma ghrelin was performed between 10 a.m. and 12 p.m.. We first investigated the effects of NBI27914 (10 μg/mouse), astressin2B (10 μg/mouse), SB-242084 (1.5 μg/mouse), SB-224289 (1.5 μg/mouse), and HS014 (0.15 μg/mouse) on plasma ghrelin concentration in mice exposed to the novelty stress to clarify the role of 5-HT1B/2CR or MC4R on ghrelin secretion 3 h after the novelty stress. Each test drug was administered, the mice were isolated simultaneously, and blood was collected 3 h after the novelty stress. The results of our evaluation of the post-novelty stress time course revealed that plasma ghrelin decreased significantly after 3 h. We collected blood samples 3 h after administration to clarify the relationship between this change in plasma ghrelin level and improved food intake.

CRF (0.2 μg/mouse) or mCPP (50 μg/mouse) was administered alone, CRF was coadministered with SB-242084 (1.5 μg/mouse) or SB-224289 (1.5 μg/mouse), and blood was collected 1 h later. To clarify MC4R for the decrease in food intake after the novelty stress, icv administration of THIQ (1.5 μg/mouse), an MC4R agonist, or α-MSH (1.5 μg/mouse), an MC3R agonist, was performed, and blood was collected 1 h later. RKT (500 mg/kg) was administered orally 1 h before the novelty stress, and blood was collected 1 or 3 h after the stress.

To determine the serum corticosterone concentration after the novelty stress, blood was collected in a laboratory dish by severing the carotid artery. Ghrelin levels were measured using the Active Ghrelin ELISA kit/Desacyl-Ghrelin ELISA kit (Mitsubishi Chemical Medience), and the corticosterone levels were measured using the Correlate-EIA Corticosterone kit (Enzo Biochem, New York, NY).

4.3.5 Extraction of total RNA for RT-PCR

The hypothalamus and stomach were rapidly removed and immediately frozen by placing them in a tube on dry ice. Homogenization of the isolated tissue and total RNA extraction were performed according to the protocol from the RNeasy Universal Tissue Kit (QIAGEN), following which each sample was diluted to 100 ng/μl. The diluted total RNA was incubated at 70 °C for 5 min and then cooled on ice. Total RNA (1000 ng) was reverse-transcribed using TaqMan Reverse Transcription Reagents

(Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems), using a Prism 7900HT Sequence Detection System (Applied Biosystems). To correct the differences in the amount of total RNA added to each reaction, mRNA expression was normalized using ribosomal protein S29 (RPS29) as an endogenous control. These differences were expressed by the dCT value: $dCT = 2^{(-|A-B|)}$, where A is the number of cycles needed to reach the threshold for the housekeeping gene (CT: threshold cycle) and B is the number of cycles needed for the target gene. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were manufactured by Applied Biosystems (Rps29: Mm02342448_gH; Npy: Mm00445771_m1; Agrp: Mm00475829_g1; Pomc: Mm00435874_m1; ghrelin: Mm00445450_m1).

5. GHRELIN-DEGRADING ENZYME

5.1. Ghrelin-degrading enzyme and rikkunshito

The acylated-ghrelin level in plasma is regulated by both the secretion from stomach and the elimination from circulation which includes degradation of acylghrelin by deacylating enzymes. Acylated ghrelin in plasma is rapidly deacylated in a process that is believed to be primarily mediated by carboxylesterase (CES) in rodents and butyrylcholinesterase (BuChE) in humans (De Vriese et al., 2004).

Rikkunshito administration has been shown to stimulate food intake and peripheral and central ghrelin secretion in rodents (Matsumura et al., 2010; Saegusa et al., 2011; Takeda et al., 2008; Yakabi et al., 2010a) and humans (Matsumura et al., 2010). Interestingly, it was shown that oral administration of rikkunshito enhanced circulating acyl-ghrelin level without a significant effect on the plasma level of des-acyl ghrelin in both normal-fed and cisplatin-treated rats, leading to the increase in the acyl- to des-acyl-ghrelin (A/D) ratio (Takeda et al., 2008). Furthermore, in a human study, it was demonstrated that plasma acyl-ghrelin level and A/D ratio increased significantly after rikkunshito administration, whereas des-acyl-ghrelin level showed a decreasing trend (Matsumura et al., 2010). These results raise the possibility that rikkunshito increases circulating acyl-ghrelin level by inhibiting its degradation.

To test the hypothesis that some components of rikkunshito contribute to the inhibition of ghrelin degradation, 48 components in rikkunshito were screened for inhibitory activity against ghrelin-degrading enzymes such as

CES and BuChE (Sadakane et al., 2011). It was found that eight compounds exhibited inhibitory activity against CES or BuChE. Among them, 10-gingerol exhibited the highest inhibitory activity against CES (5.2 μ*M* inhibition constant) in a competitive manner. In addition, pachymic acid and glycycoumarin were shown to be competitive inhibitors of BuChE. Furthermore, rikkunshito and its component 10-gingerol inhibited the decrease in plasma acyl-ghrelin level of exogenously administered ghrelin, and the CES inhibitor BNPP inhibited cisplatin-induced decreases in food intake (Sadakane et al., 2011). On the basis of these findings, it is conceivable that rikkunshito may enhance plasma acyl-ghrelin level, at least in part, by inhibiting the circulating ghrelin-degrading enzyme.

5.2. Experimental methods

5.2.1 Animals

During the experimental period, 7-week-old male Sprague–Dawley rats (Charles River Japan, Yokohama, Japan) were housed in each cage in a room with controlled temperature and humidity under a 12-h light cycle (07:00–19:00 h) with *ad libitum* access to food and water. All experiments were performed between 09:00 and 18:00 h.

5.2.2 Food intake

The rats were administered saline or cisplatin 1 h after administration of BNPP (20 mg/kg) to 24-h-fasted rats. Food intake was defined as the difference between the weight of food before examination and that recovered subsequent to the test session at 2 and 24 h after cisplatin administration. Food spilled from the food cage was collected, combined with the remaining food, and added to the total weight.

5.2.3 Enzyme assay

CES activity was determined by measuring the hydrolysis of α-naphthyl acetate (Duysen et al., 2001). After preincubating sample solutions for 20 min with 0.01 U CES (Sigma–Aldrich) in 100 m*M* phosphate buffer (pH 7.0), 10 μl of 20 m*M* α-naphthyl acetate was added. Absorbance was measured at 321 nm every 15 s for 10 min. The rate of the absorbance increase (Δ321 nm/s/ml) was calculated as the amount of enzyme hydrolysis activity. BuChE activity was measured by the method developed by Ellman et al. (1961). Briefly, after preincubating the samples with purified human BuChE (0.01 U, Sigma–Aldrich) in 50 m*M* Tris–HCl (pH 7.4), 100 n*M* butyrylthiocholine iodide and 0.25 m*M* 5,5′-dithiobis-2-nitrobenzoate in