Cinnamomum osmophloeum extracts inducing ghrelin gene expression and preventing D-Ribose caused HepG

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ABSTRACT

Diabetes patients need long-term uptake hypoglycemic medications or injections of insulin to overcome problems of glucose metabolism dysfunction which, their bodys ’ often accompany fatty liver injury. In this report, the HepG2 cells were culture with 30 mM D-ribose to mimic high oxidative stress as liver in a diabetes patient environment. Three sources of extracts from different chemical types, which are G2, P3 and TFA represent cinnamaldehyde- cinnamylacetate, mixed and uncharacterized chemical structure, respectively, were added into culture medium. The administration of those cinnamon extracts protected HepG2 cells from D-ribose administration and increased around 20% of cell-surviving. These protections were even better than the addition of aminoguanidine hemisulfate salt, a chemical used in treatment of diabetes. In addition, the expressions of ghrelin gene and two ghrelin-related genes expression were also monitored by RT-PCR. The transcript variant 1 of ghrelin gene was induced by the addition of cinnamon while transcript variant 3 remained about the same level. The membrane bound O-acyltransferase domain containing 4 mRNA, transcribed by the gene of ghrelin modify enzyme, and furin mRNA, the transcribed by the gene of ghrelin process enzyme, were all detected in HepG2 cells and cinnamon treated cells. Both ghrelin transcript variant 1 and 3 were cloned into a vector that is drive under the CMV promoter. These constructed plasmids were transfected into HepG2 cells. We found that both transcript variants 1 and 3 protected HepG2 cells from the addition of D-ribose. We conclude that the cinnamon avoids HepG2 cell damage from D-ribose induced oxidative stress by inducing ghrelin expression, and through the ghrelin signaling increases cell surviving.

Keywords : Cinnamomum osmophloeum、ghrelin、D-ribose

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REFERENCES

1.王振瀾與尹華文。1992。土肉桂精油萃取、組成、季節性變化與葉部精油組成之天然變異。土肉桂專論－林業叢刊（38）:47-61。台北，台灣。 2.行政院衛生署統計98年國人癌症十大統計數據:

http://www.doh.gov.tw/CHT2006/DM/DM2_2_p02.aspx?class_no=440&now_fod_list_no=11397&level_no=-1&doc_no=76512 3.林讚標。1992。土肉桂專論－林業叢刊（38）。台北，台灣。 4.胡家僖。2008。肉桂萃取液對細胞生長的影響及其在化妝品上的應用。大葉大學。分子生物科技學系。彰化。台灣。 5.張上鎮。2002。土肉桂葉精油的抗細菌活性與應用。林業研究專訊9（3）:23-28。 6.張上鎮。2003a。「傑出的生物化學家」－台灣鄉土林木。科學發展（366）:6-11。 7.張上鎮。2003b。土肉桂葉子精油的生物活性與應用。台灣林業28（6）:31-35。 8.張上鎮，鄭森松，王升陽。2009。土肉桂飄香－葉子的神奇功效及應用。台灣林業35（1）: 116-122。 9.許文馨。2010。土肉桂葉精油在STZ-糖尿病大鼠之降血糖作用。中山醫學大學。營養學研究所。台中。台灣。 10.Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M et al: Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Endocrinology 2002, 143（9）: 3341-3350. 11.Ariyasu H, Takaya K, Iwakura H, Hosoda H, Akamizu T, Arai Y, Kangawa K, Nakao K: Transgenic mice overexpressing des-acyl ghrelin show small phenotype. Endocrinology 2005, 146（1）:355-364. 12.Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F: Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 2002, 159（6）:1029-1037. 13.Bataller R, Brenner DA: Liver fibrosis. J Clin Invest 2005, 115（2）:209-218. 14.Baynes JW: Role of oxidative stress in development of complications in diabetes. Diabetes 1991, 40（4）:405-412. 15.Bierman EL, Baker EM, Plough IC, Hall WH: Metabolism of d-ribose in diabetes mellitus. Diabetes 1959, 8:455-458. 16.Brenner DA, Waterboer T, Choi SK, Lindquist JN, Stefanovic B, Burchardt E, Yamauchi M, Gillan A, Rippe RA: New aspects of hepatic fibrosis. J Hepatol 2000, 32（1 Suppl）:32-38. 17.Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005, 54（6）:1615-1625. 18.Camina JP, Campos JF, Caminos JE, Dieguez C, Casanueva FF: Obestatin-mediated proliferation of human retinal pigment epithelial cells: regulatory mechanisms. J Cell Physiol 2007, 211（1）:1-9. 19.Carlini VP, Schioth HB, Debarioglio SR: Obestatin improves memory performance and causes anxiolytic effects in rats. Biochemical and biophysical research communications 2007, 352（4）:907-912. 20.Chang ST, Chen PF, Chang SC: Antibacterial activity of leaf essential oils and their constituents from Cinnamomum osmophloeum. J Ethnopharmacol 2001, 77（1）:123-127. 21.Chou JY, Mansfield BC: Gene therapy for type I glycogen storage diseases. Curr Gene Ther 2007, 7（2）:79-88. 22.Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, Kim H, Park S: Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen-glucose deprivation. Endocrinology 2007, 148（1）:148-159. 23.D'Amico G, Pagliaro L, Bosch J: The treatment of portal hypertension: a meta-analytic review. Hepatology 1995, 22（1）:332-354. 24.Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci U S A 2000, 97（22）:12222-12226. 25.Fuller CJ, Chandalia M, Garg A, Grundy SM, Jialal I: RRR-alpha-tocopheryl acetate supplementation at pharmacologic doses decreases low-density- lipoprotein oxidative susceptibility but not protein glycation in patients with diabetes mellitus. Am J Clin Nutr 1996, 63（5）:753-759. 26.Ghosh K: Thrombohaemorrhagic balance in diabetes mellitus. J Indian Med Assoc 2002, 100（7）:428, 430, 432-423. 27.Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, Destefanis S, Annunziata M, Martinetti M, Catapano F: Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3',5'-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-Kinase/Akt signaling. Endocrinology 2007, 148（2）:512-529. 28.Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E: Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. Diabetes 2008, 57（4）:967-979. 29.Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE: Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci U S A 2008, 105（17）:6320-6325. 30.Horie Y, Yamagishi Y, Kato S, Kajihara M, Kimura H, Ishii H: Low-dose ethanol attenuates gut ischemia/reperfusion-induced liver injury in rats via nitric oxide production. J Gastroenterol Hepatol 2003, 18（2）:211-217. 31.Iseri SO, Sener G, Saglam B, Ercan F, Gedik N, Yegen BC: Ghrelin alleviates biliary obstruction-induced chronic hepatic injury in rats. Regul Pept 2008, 146（1-3）:73-79. 32.Kajimoto Y, Matsuoka T, Kaneto H, Watada H, Fujitani Y, Kishimoto M, Sakamoto K, Matsuhisa M, Kawamori R, Yamasaki Y: Induction of glycation suppresses glucokinase gene expression in HIT-T15 cells. Diabetologia 1999, 42（12）:1417-1424. 33.Kamegai J, Tamura H, Shimizu T, Ishii S, Tatsuguchi A, Sugihara H, Oikawa S, Kineman RD: The role of pituitary ghrelin in growth hormone （GH） secretion: GH-releasing hormone-dependent regulation of pituitary ghrelin gene expression and peptide content. Endocrinology 2004, 145（8）:3731-3738. 34.Kapica M, Zabielska M, Puzio I, Jankowska A, Kato I, Kuwahara A, Zabielski R: Obestatin stimulates the secretion of pancreatic juice enzymes through a vagal pathway in anaesthetized rats - preliminary results. J Physiol Pharmacol 2007, 58 Suppl 3:123-130. 35.Khan A, Safdar M, Ali Khan MM, Khattak KN, Anderson RA: Cinnamon improves glucose and lipids of people with type 2 diabetes. Diabetes Care 2003, 26（12）:3215-3218. 36.Kiefer MC, Tucker JE, Joh R, Landsberg KE, Saltman D, Barr PJ: Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. DNA Cell Biol 1991, 10（10）:757-769. 37.Kim SH, Hyun SH, Choung SY: Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice. J Ethnopharmacol 2006, 104（1-2）:119-123. 38.Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999, 402（6762）:656-660. 39.Kui L, Weiwei Z, Ling L, Daikun H, Guoming Z, Linuo Z, Renming H: Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway. Regul Pept 2009, 155（1-3）:62-69. 40.Lebrec D: Pharmacological treatment of portal hypertension: hemodynamic effects and prevention of bleeding. Pharmacol Ther 1994, 61（1-2）:65-107. 41.Liang TJ, Heller T: Pathogenesis of hepatitis C-associated hepatocellular carcinoma. Gastroenterology 2004, 127（5 Suppl 1）:S62-71.

42.McGlynn KA, Hunter K, LeVoyer T, Roush J, Wise P, Michielli RA, Shen FM, Evans AA, London WT, Buetow KH: Susceptibility to aflatoxin B1-related primary hepatocellular carcinoma in mice and humans. Cancer Res 2003, 63（15）:4594-4601. 43.Meszarosova M, Sirotkin AV, Grossmann R, Darlak K, Valenzuela F: The effect of obestatin on porcine ovarian granulosa cells. Animal reproduction science 2008, 108（1-2）:196-207. 44.Moller P, Wallin H, Knudsen LE: Oxidative stress associated with exercise, psychological stress and life-style factors. Chem Biol Interact 1996, 102（1）:17-36. 45.Moreno M, Chaves JF, Sancho-Bru P, Ramalho F, Ramalho LN, Mansego ML, Ivorra C, Dominguez M, Conde L, Millan C: Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans. Hepatology 2010, 51（3）:974-985. 46.Muller TD, Tschop MH, Jarick I, Ehrlich S, Scherag S, Herpertz-Dahlmann B, Zipfel S, Herzog W, de Zwaan M, Burghardt R: Genetic variation of the ghrelin activator gene ghrelin O-acyltransferase （GOAT） is associated with anorexia nervosa. J Psychiatr Res 2011, 45（5）:706-711. 47.Nelson DL. and Cox MM.: Lehninger principles of biochemistry. Fourth Edition edition. W. H. Freeman 2005 48.Niemela O, Parkkila S, Bradford B, Iimuro Y, Pasanen M, Thurman RG: Effect of Kupffer cell inactivation on ethanol-induced protein adducts in the liver. Free Radic Biol Med 2002, 33（3）:350-355. 49.Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000, 404（6779）:787-790. 50.Park JM, Kakimoto T, Kuroki T, Shiraishi R, Fujise T, Iwakiri R, Fujimoto K: Suppression of intestinal mucosal apoptosis by ghrelin in fasting rats. Exp Biol Med （Maywood） 2008, 233（1）:48-56. 51.Pazos Y, Alvarez CJ, Camina JP, Casanueva FF: Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin. Growth Factors 2007, 25（6）:373-381. 52.Rehermann B: Immune responses in hepatitis B virus infection. Semin Liver Dis 2003, 23（1）:21-38. 53.Ren AJ, Guo ZF, Wang YK, Wang LG, Wang WZ, Lin L, Zheng X, Yuan WJ: Inhibitory effect of obestatin on glucose-induced insulin secretion in rats. Biochemical and biophysical research communications 2008, 369（3）:969-972. 54.Roebroek AJ, Schalken JA, Leunissen JA, Onnekink C, Bloemers HP, Van de Ven WJ: Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. EMBO J 1986, 5（9）:2197-2202. 55.Romero A, Kirchner H, Heppner K, Pfluger PT, Tschop MH, Nogueiras R: GOAT: the master switch for the ghrelin system? Eur J Endocrinol 2010, 163（1）:1-8. 56.Ryman BE: The glycogen storage diseases. J Clin Pathol Suppl （R Coll Pathol） 1974, 8:106-121. 57.Samson WK, White MM, Price C, Ferguson AV: Obestatin acts in brain to inhibit thirst. American journal of physiology Regulatory, integrative and comparative physiology 2007, 292（1）:R637-643. 58.Schafer T, Scheuer C, Roemer K, Menger MD, Vollmar B: Inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death. FASEB J 2003, 17（6）:660-667. 59.Schuppan D: Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis 1990, 10（1）:1-10. 60.Segal S, Foley J: The metabolism of D-ribose in man. The Journal of clinical investigation 1958, 37（5）:719-735. 61.Stengel A, Goebel M, Wang L, Tache Y, Sachs G, Lambrecht NW: Differential distribution of ghrelin-O-acyltransferase （GOAT） immunoreactive cells in the mouse and rat gastric oxyntic mucosa. Biochem Biophys Res Commun 2010, 392（1）:67-71. 62.Stickel F, Brinkhaus B, Krahmer N, Seitz HK, Hahn EG, Schuppan D: Antifibrotic properties of botanicals in chronic liver disease. Hepatogastroenterology 2002, 49（46）:1102-1108. 63.Szentirmai E, Krueger JM: Obestatin alters sleep in rats. Neuroscience letters 2006, 404（1-2）:222-226. 64.Takahashi T, Ida T, Sato T, Nakashima Y, Nakamura Y, Tsuji A, Kojima M: Production of n-octanoyl-modified ghrelin in cultured cells requires prohormone processing protease and ghrelin O-acyltransferase, as well as n-octanoic acid. J Biochem 2009, 146（5）:675-682. 65.Thomson WH, Maclaurin JC, Prineas JW: Skeletal muscle glycogenosis: an investigation of two dissimilar cases. J Neurol Neurosurg Psychiatry 1963, 26:60-68. 66.Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S: Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochem Biophys Res Commun 2001, 281（5）:1220-1225. 67.Vinik A, Flemmer M: Diabetes and macrovascular disease. J Diabetes Complications 2002, 16（3）:235-245. 68. Wise RJ, Barr PJ, Wong PA, Kiefer MC, Brake AJ, Kaufman RJ: Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. Proc Natl Acad Sci USA 1990, 87（23）:9378-9382. 69. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ: Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 2005, 310（5750）:996-999. 70. Zhang Y, Ying B, Shi L, Fan H, Yang D, Xu D, Wei Y, Hu X, Zhang X, Wang T: Ghrelin inhibit cell apoptosis in pancreatic beta cell line HIT-T15 via mitogen-activated protein kinase/phosphoinositide 3-kinase pathways. Toxicology 2007, 237（1-3）:194-202.