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Vascular endothelial cells and smooth muscle cells mediate carbachol-induced hepatocyte proliferation via muscarinic receptors and IP_3/PKC signaling cascades

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Abstract

An acetylcholine (ACh) agonist, carbachol (Cch), causes hepatocytes to proliferate in the presence of hepatic nonparenchymal cells (HNPCs). To identify the HNPCs and ACh receptor subtypes involved in carbachol-induced hepatocyte proliferation (CIHP), we examined two types of vascular cells as candidates for HNPCs mediating CIHP in cocultures of hepatocytes using the Transwell filter insert. In the coculture with vascular smooth muscle cells (VSMCs) or endothelial cells (VECs), but not in the monoculture, 72 h treatment with Cch significantly increased the numbers of hepatocytes. The results suggest that both VSMCs and VECs are involved in CIHP through soluble factors secreted from these cells. Interestingly, coculture with VECs, but not with VSMCs, markedly increased the number of hepatocytes, even in the absence of Cch. Cell proliferation assays using an analogue of thymidine, bromodeoxyuridine (BrdU), demonstrated that the hepatocytes in both cocultures transiently replicated their chromosomes 12 h after Cch administration. Blocking the muscarinic type 1 ACh receptor (M_1), $M_{3/5}$, intracellular inositol triphosphate (IP_3) receptor, or protein kinase C (PKC) pathways inhibited VSMC-mediated CIHP, whereas blocking the $M_{3/5}$, IP_3 receptor, or PKC pathways inhibited VEC-mediated CIHP. Co-culturing hepatocytes with both types of vascular cells markedly increased their albumin content, but addition of Cch had no effect. In conclusion, VSMCs among vascular cells mediate CIHP through M_1, $M_{3/5}$, and IP_3/PKC signal transduction pathways, whereas VECs do so through $M_{3/5}$, and IP_3/PKC pathways.

Keywords: Neural obesity; Obese; Vagus nerve; Cell growth

1. Introduction

The autonomic nervous system plays a crucial role in hepatic regeneration and metabolic regulation in the liver (Bioulac et al., 1990; Kato and Shimazu, 1983; Lamar and Holloway, 1977; Tanaka et al., 1987). In partially hepatectomized rats bilateral cervical vagotomies suppress DNA synthesis in the liver (Lamar and Holloway, 1977), strongly suggesting that vagal nerve activity contributes significantly to hepatic cell proliferation. An animal model of hypothalamic obesity, the ventromedial hypothalamus (VMH)-lesioned rat, markedly gains weight as it rapidly accumulates fat for a few days after VMH lesioning. The animal's abdominal organs (liver, pancreas and gut) also show extensive hyperplasia with enhanced cell proliferation. This largely depends upon autonomic tone, especially vagal tone (Kato and Shimazu, 1983; Kiba, 2002; Kiba et al., 1992, 1993, 1996). Cholinergic innervation of the liver is required for proliferative and functional responses with growth of the biliary duct epithelial cells after extrahepatic obstruction by ligation (Glaser et al., 1997; Kanno et al., 2001; LeSage et al., 1999); however, the cholinergic effects on the growth of hepatocytes are largely unknown.

Abbreviations: CIHP, carbachol-induced hepatocyte proliferation; Co(HH), coculture with HH; Co(SM-3), coculture with SM-3; HNPC, hepatic nonparenchymal cell; PCNA, proliferating cell nuclear antigen; VEC, vascular endothelial cell; VMH, ventromedial hypothalamus; VSMC, vascular smooth muscle cell.

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We have shown that the infusion of carbachol (Cch; ACh agonist) and VMH lesions induce cell proliferation in pancreatic B-cells and in epithelial cells of the duodenal crypts (Yoshimura et al., 2006). More recently, we found that the Cch-induced expression of proliferating cell nuclear antigen (PCNA) in hepatocytes depends on the presence of hepatic nonparenchymal cells (HNPCs) (Yoshimura et al., 2007). The latter report strongly suggests that Cch-induced hepatocyte proliferation (CIHP) is mediated by neighboring HNPCs, and also that cholinergic stimulus-induced hepatic cell proliferation can occur even in the locally closed environment of the liver.

The HNPC subtype that contributes to CIHP remains to be identified. HNPCs including bile duct cells, sinusoidal endothelial cells, mesenchymal cells, and fat storing cells (hepatic stellate cells) contribute to the growth and functions of hepatocytes (Boulton et al., 1997). Abundant blood vessels, such as interlobular arteries, veins and sinusoids, are ubiquitously distributed close to hepatocytes throughout the liver, and have a critical role in its development and metabolism (Matsumoto et al., 2001; McCuskey et al., 2003); therefore, we focused on vascular cells as candidates for HNPCs involved in CIHP.

Identification of the receptor subtypes involved in CIHP is also important. ACh receptors are divided into muscarinic and nicotinic types (mAChRs and nAChRs). The mAChRs are G protein-coupled metabotropic receptors, identified as M_1–M_5 subtypes, and subdivided further into 2 subclasses. The M_1, M_3, and M_5 receptors couple with the $G_{q/11}$ protein family, and stimulate phosphoinositide metabolism, whereas M_2 and M_4 couple with the pertussis toxin (PTX)-sensitive $G_{i/o}$ family, decrease intracellular cAMP production, and can modify K^+ and Ca^{2+} channels (TIPS, 1996). In contrast, nAChRs are ligand-gated ion channels consisting of particular combinations of α_{1-9}, β_{1-4}, γ, δ, and ε subunits, and are divided into at least 4 subclasses: muscle, ganglionic, neuronal CNS, and α_7 neuronal (Lukas et al., 1999; TIPS, 1996). mRNA for M_1–M_3 and M_5 ACh receptors, but not the M_4 receptor, are found human liver homogenates, and the hepatic vagus branches activate the hepatic progenitor cell compartment in the diseased liver, probably through the M_3 type receptor expressed on the cells (Cassiman et al., 2002). However, the involvement of mAChRs in the mechanism of hepatocyte proliferation has not been fully clarified. The expression and function of nAChRs in the liver are also quite obscure, although nAChRs might be related to CIHP.

We have first examined 2 types of vascular cells as candidates for HNPCs that may be responsible for CIHP. We then investigated the ACh receptor subtypes and intracellular transduction pathways involved in CIHP by coculture with vascular cells using agents, including receptor antagonists and a protein kinase inhibitor.

2. Materials and methods

2.1. Cell lines

The following cell lines were used: hepatocytes derived from rat liver, RLN-10 (JCRB0415, Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan) (Sato et al., 1968); vascular smooth muscle cells from rabbit aortic media, SM-3, (Sasaki et al., 1989), donated by Dr Sasaki, Kitasato University School of Pharmaceutical Sciences, Tokyo, Japan; and vascular endothelial cells from bovine carotid arterial intima, HH (JCRB0099, JCRB) (Hagiwara et al., 1984). All these cell lines were subcultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen).

2.2. Coculture of RLN-10 and vascular cells using filter insert

RLN-10 cells were co-cultured with vascular cells using a Transwell insert with a filter (0.4 μm pore) (Fig. 1 A). RLN-10 cells were plated on a 24-well plate at 2×10^4 cells/cm^2. After 24 h culture with serum-free DMEM, an insert with a collagen type IV-coated filter was placed in each well and SM-3 or HH cells were plated on the filter at 2×10^4 cells/cm^2. We cultured RLN-10 cells using the same method, but without vascular cells on the filter.

After 24 h culture at 37 °C in a humidified atmosphere containing 5% CO_2 in air, an ACh receptor agonist, Cch was administered at 0.3 mM. Cells were consequently cultured for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

2.3. Immunocytochemistry for PCNA

After fixation with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), RLN-10 cells cultured in the lower well were permeabilized for 15 min with 0.3% Triton X-100 in PBS, and immunoblocked for 1 h with 1% skim milk in PBS. Cells were incubated overnight at 4 °C with the primary antibody (mouse anti-PCNA antibody; 1:1500; Novotech Ltd., Sydney, Australia) in PBS. After being rinsed, the cells were incubated for 60 min with biotinylated horse anti-mouse IgG antibody (1:200; Vector Laboratories, Inc., Burlingame, CA) as the secondary antibody, followed by avidin-biotinylated horseradish peroxidase complex (ABC) (Vectastain Elite ABC Kit, Vector) for 1 h. Immunoreactivity was visualized with 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.005% dihydroperoxide (H_2O_2) in 0.05 M Tris–HCl buffer (pH 7.6). Cells were weakly counterstained with hematoxylin, and dehydrated.

2.4. Cell Proliferation Assay

5-bromo-2′-deoxyuridine (BrdU; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) 3 μg/ml was administered to RLN-10 cells 2 h before their fixation in each experimental group. RLN-10 cells were fixed with 4% paraformaldehyde in 0.01 M PBS (pH7.4) 0, 2, 4, 12, 24, or 48 h after the administration of Cch. They were permeabilized for 15 min with 0.3% Triton X-100 in PBS, immunoblocked with 1% skim