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INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on June 29, 2012 to 3 recipients.
The titles of theses and other information are as follows:

Biochemical analyses of proinsulin C-peptide-binding protein and its binding properties

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C-peptide is a connecting segment of proinsulin and is secreted from pancreatic β-cells into the circulation along with insulin after the cleavage of proinsulin. Although it is well established that insulin plays pivotal roles in the regulation of energy metabolism, C-peptide had been considered biologically inert. However, recent studies have suggested that C-peptide possesses several beneficial effects on diabetic complications of patients with type 1 diabetes mellitus. More recently, anti-inflammatory properties of C-peptide have been demonstrated, but there are some reports showing that C-peptide induces chemotaxis for monocytes, neutrophils and T lymphocytes.

Functions of the C-peptide are supposed to be mediated by a specific receptor for C-peptide. However, attempts to demonstrate the interaction between C-peptide and cellular membranes and the possible receptor structure have not been successful so far. The aim of this study was to examine the molecular mechanisms of C-peptide's cell membrane interaction in an attempt to identify and characterize membrane proteins to which C-peptide binds.

Using a human promyelocytic leukemia HL-60 cell line that responded to C-peptide in chemotaxis assay, the candidate proteins were isolated by co-immunoprecipitation with anti-C-peptide antibody after chemical cross-linking of C-peptide to the cell surface proteins at 4°C. There was a protein selectively present only after chemical cross-linking, which had a molecular weight of around 45 kDa and a pI value of 7.0, and the protein of interest was identified as α-enolase by mass spectrometry.

α-enolase is a rate-limiting enzyme in the glycolytic pathway located in the cytosol. but is also expressed on the cell surface of hematopoetic, epithelial and endothelial cells as a plasminogen receptor. Moreover, it has been shown that the lysine residue present at the carboxy (C)-terminal end of α-enolase is important for α-enolase-plasminogen interactions. To investigate the possible involvement of α-enolase in the C-peptide signaling, I examined site-specific phosphorylation of extracellular signal-regulated kinase (ERK) by C-peptide in A31 mouse fibroblastic cells expressing α-enolase on the cell membrane. Treatment of the cells with either C-peptide or plasminogen activated ERK phosphorylation, which was abrogated by both prior treatment of carboxypeptidase B to remove C-terminal amino acid residues and inclusion of e-aminocaproic acid (EACA) as a competitor for lysine residue. These results suggested that cell surface molecule(s) containing basic amino acid(s) at the C-terminal, such as α-enolase, mediated signal transduction of C-peptide.

To further characterize the interaction between C-peptide and α-enolase, the enzyme activity catalyzing conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate was mmeasured either in the absence or presence of C-peptide. Recombinant α-enolase activity was modulated by C-peptide, with a significant decrease in Km for 2-PG without affecting Vmax. Dose-response experiments of C-peptide revealed that an equimolecular amount of C-peptide to α-enolase was sufficient to fully modify the enzyme activity, suggesting that one molecule of C-peptide interacts with one molecule of the enzyme. The enzyme modulation by C-peptide was abolished when C-terminal basic lysine residue (K434) of the enzyme was replaced by neutral alanine (K434A) or acidic glutamate (K434E), although both mutant enzymes exhibited similar catalytic activity to the wild-type enzyme. However, the enzyme modulation was also observed in the arginine-replaced mutant enzyme (K434R). These results indicate that the C-terminal lysine residue of α-enolase is essential for the stimulatory effect of C-peptide.

Human C-peptide is composed of 31 amino acids and the glutamate residue at position 27 (E27) of C-peptide is suggested to be important for C-peptide binding to cell membranes. Next to determine the involvement of E27 in the interaction between C-peptide and α-enolase, the enzyme activity was measured in the presence of C-peptide fragments: N-terminal 26 peptides (F1), C-terminal 5 peptides (F2), N-terminal 5 peptides (F3), C-terminal 26 peptides (F4), C-terminal 4 peptides (F5), the mutant C-terminal 5 peptides in which 27th glutamate was replaced with alanine (F6) and rat C-peptide C-terminal 5 peptides (F7). Among the fragments, peptides containing E27 (F2, F4 and F7) were as effective as the full-length peptide, although peptides without E27 (F5 and F6) did not affect α-enolase activity. The fragments F1 and F3 containing a glutamate residue at positions of 1 and 3 tended to increase α-enolase activity slightly, with a tendency for a decrease in both Km and Vmax values. These results suggested that glutamate residues of C-peptide are involved in the modulation of the enzyme and that the glutamate residue at position 27 is essential for the stimulatory effect of C-peptide on α-enolase.

In conclusion, I have demonstrated that C-peptide modulates α-enolase activity through a specific interaction between E27 of the peptide and K434 of the enzyme. This C-peptide-α-enolase interaction is likely to occur on the cell surface, and might be of importance for signal transduction and the effects of C-peptide.

The original papers of this thesis appeared in *J. Biochem.* (2012); doi: 10.1093/jb/mvs052

Role of neuropeptide Y in the regulation of brown adipose tissue functions and the possible involvement of STAT3, a transcription factor, in the role

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Neuropeptide Y (NPY) is one of the most abundant peptides in the central nervous systems, and plays pivotal roles in the regulation of food intake and emotional behavior. NPY

exhibits its physiological effects through at least four receptors known as Y1, Y2, Y4, and Y5. It is well established that NPY receptors are coupled to the inhibitory G-protein (Gi) and thereby antagonize an agonist-induced activation of adenylyl cyclase. In addition, NPY is known to mobilize Ca^{2+} from intracellular stores and to activate extracellular signal-regulated kinase (ERK), a MAP kinase.

In many peripheral tissues, NPY is co-released with norepinephrine (NE) from sympathetic nerve endings. NPY works synergistically with NE in inducing long-lasting vasoconstrictions and in increasing vascular permeability and re-vascularization after ischemic stress. These synergistic actions of NPY are thought to be due to potentiation of Ca^{2+} signal by α-adrenergic receptor (AR) and/or suppression of β-adrenergic action.

Acute cold exposure and feeding significantly stimulate thermogenesis in the brown adipose tissue (BAT). The sympathetic nervous system controls this adaptive thermogenesis through the activation of βARs, especially β3AR. It is well established that βARs are coupled to the stimulatory G-protein (Gs) and thereby sequentially activate adenylyl cyclase, cAMP formation, and cAMP-dependent protein kinase (PKA). It is also established that active PKA leads to activation of lipolytic enzymes and enhanced expression of the genes encoding uncoupling protein-1 (UCP1). Furthermore, thermogenesis in the BAT is principally dependent on the activation of UCP1, which facilitates proton leakage across the inner mitochondrial membrane to dissipate the electrochemical gradient as heat.

Co-existence of NPY in sympathetic nerve endings has been demonstrated in BAT. It is simply expected that NPY suppresses NE-induced cAMP production and thermogenesis because NPY receptors are coupled to Gi protein. However, direct effects of NPY on BAT thermogenesis have not been elucidated yet. Therefore, in the doctoral dissertation, to elucidate the role of NPY in the BAT function, I first examined the effects of NPY on brown adipocytes expressing Y1 and Y5 NPY receptor mRNA. I also examined the effects of NPY on stromal vascular cells (SVC) expressing Y1 NPY receptor mRNA.

Treatment of isolated brown adipocytes with NE increased rates of oxygen consumption (an index of thermogenesis) and cAMP accumulation, although NPY did not influence the basal and NE-enhanced rates of oxygen consumption and cAMP accumulation. Treatment with NPY also failed to induce ERK (Thr202/Tyr204) phosphorylation, an index of activation, in the brown adipocytes. In contrast, treatment with NPY increased ERK phosphorylation in cultured SVC from the BAT. Treatment with NPY in SVC also increased STAT3 (Ser727) phosphorylation and STAT3-responsible gene (early growth response 1: *Egr-1*) expression, while STAT3 (Tyr705) phosphorylation occurred constitutively. Since both STAT3 (Ser727) phosphorylation and *Egr-1* mRNA expression were abrogated by an ERK kinase inhibitor, these results suggest that NPY mainly acts on SVC in BAT and regulates gene transcription through ERK and STAT3 pathway, while NPY does not affect the thermogenic function of brown adipocytes.

STAT is a transcription factor that is activated upon an activation of cytokine receptor-associated Janus-activated kinase (JAK) and a cellular non-receptor type tyrosine kinase, c-Src. To further analyze the involvement of STAT3 in the NPY signaling as seen in SVC in BAT, I examined the effect of NPY on cellular signaling in LEII mouse lung microvascular endothelial cells expressing Y1 NPY receptor mRNA. Similar to SVC, treatment with NPY increased ERK phosphorylation, STAT3 (Ser727) phosphorylation and *Egr-1* mRNA expression, while STAT3 (Tyr705) was constitutively phosphorylated. Interestingly, NPY increased phosphorylation of JAK2 in LEII cells in a dose- and time-dependent manner, and that was abrogated by AG490, a JAK2 inhibitor. However, AG490 failed to inhibit NPY-induced phosphorylation of ERK and STAT3 at Ser727 and Tyr705. Moreover, a

Src inhibitor was also ineffective on these phosphorylation. These results suggest that NPY enhances gene transcription through ERK and STAT3 pathway that is independent on JAK2 and c-Src.

I have demonstrated for the first time that NPY induces phosphorylation of JAK2 and STAT3 at Ser727. However, further study is necessary to elucidate the precious roles of these signals in the physiological relevance of NPY.

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Novel regulation mechanisms of aryl hydrocarbon receptor by carotenoid and retinoic acid

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Aryl hydrocarbon receptor (AHR), a well conserved transcription factor, can be activated in both ligand-dependent and -independent manners. Previous reports have revealed that AHR requires AHR nuclear translocator (ARNT) to interact with its cognate DNA sequence, xenobiotic responsive element (XRE). Then, the transcription of AHR battery genes, e.g., cytochrome P450 (CYP) 1A1, is activated. However, the physiological function of AHR is still unclear. Previous reports have indicated that carotenoids, precursors of retinoid, induce CYP1A activity through unknown mechanism. In addition, AHR transcriptionally regulates various genes which are related to retinoic acid metabolism, suggesting a possibility that there is an interaction between the function of AHR and retinoic acid regulation. I hence investigated the effects of astaxanthin (Ax), one of xanthophyll carotenoids, and *all-trans* retinoic acid (atRA) on AHR-regulated gene transcription.

In the present study, oral administration of Ax (100 mg/kg body weight/day, 3 days) increased CYP1A-dependent activity through induction of CYP1A1 mRNA and protein expression levels in liver of male Wistar rats. On the other hand, NADPH P450 reductase activity, which supplies electrons to CYP, was decreased in liver microsomes from the treated group. In conclusion, Ax altered CYP1A-dependent activities through induction of CYP1A1 mRNA and protein expression, and inhibition of the electron supply for the enzyme.

Next, in order to cralify a mechanism of CYP1A1 induction by Ax, I investigated how AHR-regulated transcription cascade was affected by the addition of Ax in H4IIE rat hepatoma cells. I found that Ax activated AHR to translocate from cytoplasm to nuclei, and to bind to XRE sequence in gel shift assay. Moreover, a reporter assay showed that Ax increased the transactivating ability in CYP1A1 enhancer/promoter region in a dose dependent manner. However, Ax needed tyrosine kinase cascade for transactivation of CYP1A1, unlike typical AHR ligands. These results indicate that Ax is a novel ligand-independt AHR agonist.

Finally, I focused on effects of atRA on AHR-regulated gene transcription in HepG2 human hepatoma cells. Treatment with atRA significantly reduced transactivation and expression level of CYP1A1 mRNA to less than half of control value, and this inhibitory effect was mediated by retinoic acid receptor α and/or

retinoid X receptor α. The result of chromatin immunoprecipitation assay indicated that treatment with atRA at 1–100 nM drastically inhibited the recruitment of ARNT to DNA regions containing XRE sequences. In conclusion, atRA at physiological concentrations could reduce AHR/ARNT-mediated gene transcription via the inhibition of recruitment of ARNT to relevant DNA regions.

The present study has shown that AHR-dependent gene transcription is up-regulated by carotenoid and down-regulated by retinoic acid. Some of retinoic acid synthesising enzymes, including CYP1A subfamily, are transcriptionally regulated by AHR. Here, I propose a hypothesis that AHR is one of determinants for intracellular retinoic acid concentration through the alteration of CYP1A subfamily expressions. This study will contribute to the further understanding of AHR and its physiological function.

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