論文の内容の要旨

論文題目　Cross-enhancement of ANGPTL4 by HIF1 alpha and PPAR beta/delta is the result of the conformational proximity of two response elements

（ANGPTL4 は、HIF1α および PPARβ/δ の結合領域が立体構造上近接関係をきたすことによって協調的に誘導される）

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[Introduction]

The vascular system sits at the center of oxygen delivery in mammals, and its inner layer endothelial cells (ECs) play an essential role in network formation. In addition to the physiological angiogenesis which occurs in wound healing and aerobic exercise, hypoxia is involved in various pathological conditions, e.g. cardiovascular disease, diabetic complications, inflammatory diseases, cancer and kidney disease. Poor perfusion of vital organs, including the brain, heart, liver and kidney, can result in hypoxia and critical loss of function. In the core of solid tumors, oxygen demand surpasses the capacity feeding arteries and the cells are exposed to hypoxia, sometimes with deleterious effects on the progress of the disease. In both contexts, the endothelium is the first cell layer which senses hypoxia as well as changes in hemodynamic forces and blood-borne signals, and this evokes the first step in response to hypoxia, namely angiogenesis. Responding to a demand for more oxygen, endothelial cells migrate and proliferate to form solid endothelial cell sprouts into the stromal space through the induction of a series of gene transcriptional events required for an increased oxygen supply. In the gene regulation that takes place under hypoxia, hypoxia-inducible factor (HIF)1 is regarded as one of the master gene regulators and we previously reported genome-wide analysis of HIF1 in endothelial cells. Angiogenesis is enhanced by HIF, and it is further orchestrated by various other angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietins and angiopoietin-like proteins (ANGPTL). In addition to transcription factors (TF), recent studies revealed the involvement of nuclear receptors (NR), among which the peroxisome proliferator-activated receptors (PPAR) β/δ are reported to participate in angiogenesis. PPARs are known to be important in the regulation of numerous biological processes, including lipid metabolism, adipocyte differentiation, cell proliferation and inflammation. In a recent study it was reported that the PPARβ/δ agonist GW501516 stimulated human umbilical vein endothelial cells (HUVECs) proliferation dose-dependently, promoted endothelial tube formation, and increased angiogenesis. Another PPARβ/δ agonist, GW0742, or muscle-specific overexpression of PPARβ/δ, also promoted angiogenesis in mouse skeletal muscle. Additional evidence further suggested that PPARβ/δ is one of a small number of “hub nodes” in the angiogenic network in ECs. These lines of evidence are strongly

suggestive of a role for PPARβ/δ in angiogenesis. Although several key TFs and/or NRs have been shown to be involved in angiogenesis, the detailed underlying hierarchical or mutual interaction of multiple cascades is only partially understood. To dissect the molecular mechanism of crosstalk in angiogenesis, we selected two important angiogenic stimuli, hypoxia and PPARβ/δ ligand stimulation, and investigated the molecular mechanism by which these two signals in concert are able to enhance a common angiogenesis-related target gene.

[Material and method]

HUVECs are used for all the experiments. Basic molecular biology techniques are used in the experiments. In addition, we used epigenetic methods including Chromatin Immunoprecipitation (ChIP) assay with deep sequencing (ChIP-Seq) analysis of HIF1α, PPARβ/δ, RNA polymerase II (PolII) and acetylated histone 3 lysine 27 (H3K27ac), Chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) and Chromosome Conformation Capture (3C)-PCR assay to dissect the molecular mechanism of HIF1α dependent gene expression.

[Results]

Endothelial cell migration is synergistically enhanced by hypoxic and PPARβ/δ ligand stimuli.

To confirm the physiological effect of hypoxia and the PPARβ/δ ligands, and to evaluate the physiological crosstalk of these angiogenic stimuli in endothelial cells, we applied PPARβ/δ agonists and hypoxia to HUVECs and studied the effect on cellular migration function by using a monolayer-wound healing assay. By the means of each stimulus, there was a tendency to greater recovery than under normoxia and DMSO, but without statistical significance. However, a simultaneous application of both stimuli resulted in a significant increase in migration of endothelial cells compared to untreated control. This finding suggested that this experimental motif could be applied to elucidate the synergistic activation that is exerted through PPARβ/δ and HIF1α in endothelial cell function. Therefore, we focused on dissecting the molecular mechanism underlying the synergistic effect of the two stimuli.

Genome-wide analysis of PPARβ/δ and/or hypoxia-induced genes in endothelial cells identified angiopoietin-like 4 *(ANGPTL4)* as the common target gene.

To estimate the possible interaction of the PPARβ/δ and HIF1α signaling pathways in a more comprehensive manner, we performed transcriptome analysis using microarrays at 24 hours under treatment with a PPARβ/δ-selective agonist (GW501516, 100 nM) and/or hypoxic (1 % O2) stimulation. In general, the number of genes induced by hypoxia was much larger than that of the genes induced by the PPARβ/δ ligand. To extract the genes responsive to either each of stimulus, the genes which had fold change >= 2.0 were selected, and 288 genes remained. 208 out of 288 genes exhibited induction by hypoxia, and 9 genes were induced by GW501516, with an overlap of only one gene. The overlapped gene was ANGPTL4, exhibiting 35.3 fold induction under PPARβ/δ ligand treatment and hypoxia compared to no stimulation. The gene most highly induced by the PPARβ/δ ligand was also ANGPTL4, which displayed a 7.0 fold induction compared with vehicle treatment. In addition, ANGPTL4 was the gene most highly induced by hypoxia, having a 20.1 fold induction compared to normoxia. Up-regulation of ANGPTL4 by PPARβ/δ ligand treatment and hypoxic stimulation were confirmed by qRT-PCR, with the result showing synergistic activation. Taking these data into account, we focused on ANGPTL4 as a key motif in the elucidation of the molecular crosstalk mechanism.

Synergistic activation of *ANGPTL4* transcription by the PPARβ/δ ligand and hypoxia in endothelial cells, and identification of the functional HRE and PPRE on *ANGPTL4*.

To extract the genes which are directly regulated by PPARβ/δ, we carried out ChIP-Seq using a PPARβ/δ antibody in HUVECs treated with PPARβ/δ ligand stimulation for 24 hours. In total 364 binding regions were identified as PPARβ/δ enrichment sites under PPARβ/δ ligand-treatment. Using previously obtained data on the HIF1α binding sites, the commonly bound genes were extracted, and then the binding of PPARβ/δ and HIF1α at the ANGPTL4 locus was confirmed. The enhanced recruitment of RNA polymerase II to ANGPTL4 under the stimuli together was also observed by ChIP-Seq analysis. Since ANGPTL4 sits downstream of the two transcription cascades and is commonly activated, we tried to dissect the molecular mechanism of the dual enhancement. We therefore set out to identify the functional hypoxia responsive element (HRE) and PPAR responsive element (PPRE) in ANGPTL4. After luciferase activity was up-regulated in the presence of the promoter region under hypoxia, we further made a series of deletion mutant constructs and HRE motif-mutated constructs to identify the hypoxia responsive sites. Using these mutated constructs, HRE located 2.0 kb upstream from the TSS was revealed important for hypoxia responsive induction. With the similar methods, we confirmed that PPRE at the 3rd intron had the most profound effect in the regulation of ANGPTL4 through PPARβ/δ ligand stimulation.

The quantity of PPARβ/δ binding to the third intron of the ANGPTL4 was not changed, but histone acetylation level of the response elements was induced by the stimuli.

As in the case of HIF1α recruitment, we originally hypothesized that PPARβ/δ binding might be increased in the course of the synergistic activation, and PPARβ/δ binding was analyzed by ChIP-Seq under four conditions; no stimulation, PPARβ/δ ligand stimulation, hypoxia, and both PPARβ/δ ligand and hypoxia for 24 hours. Unexpectedly, the locations and distribution patterns of the PPARβ/δ binding at ANGPTL4 did not change under the four conditions. Furthermore, the quantity of PPARβ/δ binding at ANGPTL4 was compared by ChIP-PCR using primers of the PPARβ/δ binding site at the 3rd intron of ANGPTL4, and the level of PPARβ/δ binding under the four conditions was equivalent. Thus, we speculated that PPARβ/δ might be activated without any distribution change, and to test this notion, we determined whether the activity of the enhancer was affected. Previously, CBP/p300-mediated H3K27 acetylation in PPARβ/δ-dependent transcription was reported, so we evaluated the intensity of H3K27ac, a marker of enhancer activity. In terms of ANGPTL4, consistent with general tendency, the binding distributions of H3K27ac in ANGPTL4 did not change depending on the conditions, but the intensity of H3K27 acetylation did change with the different types of stimulation conditions. To compare this quantitatively, we performed ChIP-PCR using the primers designed for the HRE and PPRE sites. The level of H3K27 acetylation around the functional PPRE was 3.7 times more enhanced by the PPARβ/δ ligand, which is consistent with the ChIP-Seq data. Surprisingly, however, even with hypoxic stimulation, the acetylation level around PPRE was 3.0 times up-regulated, and a 5.3 times induction was observed by a combination of hypoxia and PPAR ligand. The same phenomenon was observed around the functional HRE and vice versa. The level of H3K27 acetylation around HRE was 4.2 times more enhanced under hypoxia. In addition, the acetylation around HRE was 2.3 times increased even with the PPARβ/δ ligand alone, and 6.4 times in combination. These results suggest that hypoxia and PPARβ/δ together cross-enhance the intensity of the transcription factor-bound enhancer sites.

The chromatin conformation was changed at the *ANGPTL4* locus by HIF1α and PPARβ/δ.

To dissect the molecular mechanism by which the two different signaling cascades communicate with each other, and with the intention of providing a physical basis for the phenomenon, we considered the possibility that chromatin conformation change might participate in the cross-talk, since the main role of the enhancer is forming chromatin loop through spatial proximity with the TSS. To test whether the two different transcription factors binding regions directly communicate, we first performed whole genome ChIA-PET using active Pol II antibody. Direct interaction between HRE and PPRE of ANGPTL4 was detected, suggesting these regions have a potential to co-exist with promoter in Pol II rich transcription complex. To evaluate the proximity of the two response sites (HRE and PPRE) identified above, 3C assay was performed under the four different stimulations. The functional HRE and PPRE are separated by approximately 5.3 kb, and to perform the 3C assay, we chose Sau3AI, a four base pair cutter, for DNA fragmentation. The primers and TaqMan probes for the 3C target analysis were designed using both of the fragments containing the functional HRE or PPRE. Compared to normoxia and DMSO, in the case of either stimulation, this relative crosslink frequency was observed. This result suggested that both of the single stimulations brought one responsive site into the proximity of the other responsive site. To validate the 3C assay experiment, PCR products were directly sequenced and the conjunction of the HRE and PPRE fragments mediated by the restriction site was confirmed. To determine whether HIF1α or PPARβ/δ binding directly mediates the chromatin conformation change observed at the ANGPTL4 locus, we treated cells with siRNA against HIF1α and/or PPARβ/δ and performed 3C assays under stimulation with both hypoxia and the PPARβ/δ ligand. The frequency was changed by a reduction of either HIF1α or PPARβ/δ, supporting the notion that HIF1α and/or PPARβ/δ were involved in chromatin loop formation at HRE and PPRE of the ANGPTL4 locus.

[Conclusion] To the best of our knowledge, this is the first report of two different TF/NRs cooperating in transcriptional regulation through the conformational change of the target gene. NRs have a potential for cross talk with various other sequence-specific DNA-binding TFs at adjacent sites, resulting in a modification of gene expression. The existence of cross-talk between NRs and other TFs has already been reported, and some of the mechanisms have been elucidated. Our findings imply that chromatin conformational change may underlie of the synergistic gene activation that takes place with different stimuli. In conclusion, ChIA-PET, 3C and ChIP studies clearly identified the mechanism of synergistic ANGPTL4 activation is comprised of DNA looping and histone modification. The mechanism of synergistic ANGPTL4 activation provides an important clue to how different types of stimulation interact with each other.