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SUMMARY OF DOCTORAL THESIS

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Title: **Modulation of adipogenesis program in cultured preadipocytes transfected stably with cyclooxygenase isoforms in the sense or antisense orientation**

センスあるいはアンチセンス方向にシクロオキシゲナーゼアイソフォームで安定に形質転換した培養前駆脂肪細胞の脂肪細胞形成プログラムの調節

Adipocytes are known to serve as a depot of fuel molecules consisting mainly of triacylglycerol and to regulate the energy homeostasis through the balance between adipogenesis and lipolysis. Obesity is characterized by increase in the size or number of adipocytes with changes in the qualities leading to the insulin resistance and other alterations. With reference to these, adipocytes play crucial roles as endocrine cells to secrete different types of adipocytokines, such as leptin and adiponection. Furthermore, several classes of prostanoids as local hormones are synthesized in adipocytes at different stages of adipogenesis in response to external signaling molecules or nutritional status, which are involved in regulating the differentiation and maturation of adipocytes. The action of prostanoids is complicated because they have the opposing effects depending on the species. Prostanoids are synthesized by the arachidonate cyclooxygenase (COX) pathway in which the formation of prostaglandin (PG) H_2 from free arachidonic acid through the COX reaction is the rate-limiting step. The COX enzyme occurs as two types of isoforms, the constitutive COX-1 and the inducible COX-2. Both COX isoforms have distinct physiological and pathological roles. However, little is known about the specific roles of COX isoforms in adipocytes and the precursor cells.

Here, to manipulate the expression levels of COX isoforms in preadipogenic 3T3-L1 cells and to explore the effect on the adipocyte differentiation, the cultured preadipocytes were transfected stably with the mammalian expression vector harboring the cDNA insert of murine COX-1 or COX-2. The analysis of transcript levels revealed that COX-1 and COX-2 were more highly expressed in the corresponding transfectants. These results were also supported by higher expression levels of COX-1 and COX-2 proteins in the individual transfectants as assessed by Western blot analysis. To evaluate the intracellular COX activity in the transfectants, the cultured cells were incubated with free arachidonic acid. Both COX-1 and COX-2 transfectants exhibited apparently much higher activities to generate PGE_2 than the control cells, consistent with the higher expression levels of COX isoforms in the respective transfectants. The stimulation of PGE_2 synthesis by both transfectants was abolished in the presence of aspirin, a well-known COX inhibitor. Alternatively, I assessed the immediate PGE_2 production from endogenous arachidonic acid by the transfectants or the control cells after stimulation with calcium ionophore A23187. The treatment of both transfectants with COX-1 and COX-2 with A23187 resulted in the significant increases in the synthesis of PGE_2. Thus, cloned transfectants expressing stably either COX-1 or COX-2 were established using cultured 3T3-L1 preadipocytes. Parent 3T3-L1 cells and the transfectants were grown to confluence and allowed to trigger the differentiation program. The resulting cells were then cultured for additional 6 days during the maturation phase. After these cultures, cell growth of transfectants and the control cells was evaluated by counting the cell number of attached cultured cells. The cell numbers of the parent cells and the transfectants with the expression vector only increased nearly by 2-fold from the differentiation phase to the maturation phase, which is characteristic of clonal expansion phase. In sharp contrast, both COX-1 and COX-2 transfectants exhibited about 45% lower cell growth than the parent cells and the transfectants with the vector. In addition, the storage of fats was significantly inhibited in both COX-1 and COX-2 transfectants, indicating the failure of transfectants to forward the adipogenesis program. The transfectants showed spindle-like morphology characteristic of undifferentiated 3T3-L1 preadipocytes. To identify the involvement of endogenous prostanoids in the

inhibition of adipogenesis program in the COX-1 and COX-2 transfectants, the cultured cells at different stages of adipocytes were treated with aspirin, and the accumulation of triacylglycerols was determined after 6 days of the maturation phase. The treatment of the transfectants with aspirin during either phase or the combined phases failed to reverse the reduced storage of fats in both COX-1 and COX-2 transfectants after the maturation phase. The findings indicated that the interference of adipogenesis program by the sustained expression of COX isoforms in preadipocytes occurred through the mechanism independent of the mediation of endogenous prostanoids. Both 15-deoxy-$\Delta^{12,14}$-PGJ_2 and troglitazone as peroxisome proliferator-activated receptor (PPAR)γ agonists stimulated significantly the storage of fats in the parent preadipocytes and the cloned transfectants with COX-2 during the maturation phase. On the other hand, the PPARγ agonists almost failed to reverse the reduced accumulation of fats in the COX-1 transfectants. This observation suggested that some of adipogenesis program was interfered more potently by the stable overexpression of COX-1 in preadipocytes than that of COX-2. The analysis of those mRNA levels revealed that the gene expression of PPARγ, a master regulator of adipocyte differentiation, was much more markedly suppressed in the COX-1 transfectants compared with the COX-2 transfectants. The similar attenuating effects were recognized clearly in the both COX-1 and COX-2 transfectants with respect to the gene expression of typical adipogenesis markers during the terminal differentiation. Hence, the suppression of the PPARγ expression by intracellular COX proteins would partly contributes to the failure of the COX-1 and COX-2 transfectants to continue the adipogenesis program. Taken together, the results suggest the sustained overexpression of either COX-1 or COX-2 resulted in the interference of adipogenesis program through a PG-independent mechanism with a different mode of the action of COX isoforms.

Alternatively, additional attempts were made to unravel the specific role for COX-2 in the control of adipocytes, I tried to block specifically the expression of COX-2 in cultured 3T3-L1 adipocytes as a useful model system by excluding undesirable side effects of COX inhibitors. The present study undertook a novel approach to suppress specifically the expression of COX-2 in cultured preadipocytes transfected stably with the mammalian expression vector having the cDNA insert oriented in the antisense direction. I provided the evidence that the stable expression of antisense mRNA COX-2 is effective in blocking specifically the expression of COX-2 without affecting COX-1, which contributes to the up-regulation of adipogenesis during the maturation phase. Adipocytes and the precursor cells express two types of COX isoforms that are involved in the biosynthesis of different types of PGs exerting opposite effects on adipogenesis. To evaluate the role of the inducible COX-2 isoform in the control of the differentiation and maturation of adipocytes, I made attempts to suppress specifically the expression of COX-2 in adipocytes. Cultured 3T3-L1 preadipocytes were transfected stably with a mammalian expression vector having the full-length cDNA encoding mouse COX-2 oriented in the antisense direction. The cloned transfectants with antisense COX-2 exhibited stable expression of antisense RNA for COX-2, which was accompanied by the suppressed expression of mRNA and protein levels of sense COX-2. However, almost no alteration in the expression of COX-1 was detected. The transfectants with antisense COX-2 showed significant decreases in the delayed synthesis of PGE_2 involving the inducible COX-2 in response to cell stimuli. By contrast, the immediate synthesis of PGE_2 associated with the constitutive COX-1 was not influenced appreciably. The stable expression of antisense mRNA of COX-2 resulted in significant stimulation of fat storage during the maturation phase without affecting the cell proliferation associated with the clonal expansion phase. The gene expression studies revealed higher expression levels of adipocyte-specific markers in the transfectants with antisense COX-2, indicating the mechanism that stimulates adipogenesis program. The up-regulation of fat storage was appreciably prevented by anti-adipogenic prostanoids, such as PGE_2 and $PGF_{2\alpha}$, during the maturation phase. These results suggest that COX-2 is more preferentially involved in the generation of endogenous anti-adipogenic prostanoids during the maturation phase of adipocytes.

Taken together, my results provided novel facets regarding distinct roles of COX isoforms in the control of the differentiation and maturation of cultured adipocytes. The studies would be useful for further studies on the understanding of the arachidonate cascade at different life stages of adipocytes.