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SUMMARY OF DOCTORAL THESIS

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Title: **Role of cyclooxygenase-1 in adipogenesis and biosynthesis of prostacyclin at the maturation phase of adipocytes**

（脂肪細胞形成におけるシクロオキシゲナーゼ-1の役割と脂肪細胞の成熟期でのプロスタサイクリンの生合成）

The present study was undertaken to conduct cell science studies regarding role of cyclooxygenase (COX)-1, the constitutive COX isoform, in adipogenesis and biosynthesis of prostacyclin called also prostaglandin (PG) I_2 at the maturation phase of adipocytes using cultured preadipogenic cells with the ability to differentiate into adipocytes. PGs are synthesized through the arachidonate COX pathway in which the enzymatic reaction of PGH synthase alternatively called COX is the rate-limiting step for the generation of PGs after the release of free arachidonic acid. The COX enzyme is known to occur as two types of isoforms, including the constitutive COX-1 and the inducible COX-2. Cultured mouse preadipogenic 3T3-L1 cells have been useful for the studies on the regulation of the arachidonate COX pathway and the role of the products in the control of adipogenesis and other functions of adipocytes because of the established culture conditions consisted of the growth, differentiation, and maturation phases. Therefore, I have been studying the gene expression of the biosynthetic enzymes of the COX pathway and the roles for endogenous and exogenous PGs at different life stages. The delayed synthesis of PGE_2 and $PGF_{2\alpha}$ by cultured preadipocytes can be simulated by the addition of a mixture of active phorbol diester and calcium ionophore, which was accompanied by the induction of COX-2. Alternatively, our study has shown that cultured adipocytes during the maturation phase enhance the ability to generate endogenous PGJ_2 derivatives and contribute to the up-regulation of adipogenesis. Since both specific inhibitors for COX-1 and COX-2 were found to be effective in the inhibition of the generation of endogenous PGJ_2 derivatives, both COX isoforms could be responsible for the production of them. Some of other studies have reported the selective inhibitors for COX-1 and COX-2 similarly accelerated the differentiation of 3T3-L1 adipocytes in the modified differentiation medium without dexamethasone in the presence of a peroxisome proliferator-activated receptor γ (PPARγ) agonist or in another culture medium supplemented with free arachidonic acid. Thus, COX inhibitors sometimes exert different effects on adipogenesis by the use of modified differentiation medium with varied hormone cocktails. As well, it should be noted that some of COX inhibitors by themselves act as active ligands for PPARγ at higher concentrations. On the basis of the findings and uncertainties as described above, I attempted to investigate the contribution of the constitutive COX-1 to adipogenesis during the maturation phase of adipocytes. For this analysis, I undertook the approach to selectively suppress the expression of COX-1 by making use of cultured preadipocytes transfected stably with COX-1 oriented in the antisense direction.

The arachidonate COX pathway is involved in the generation of several types of endogenous PGs with opposite effects on adipogenesis at different life stages of adipocytes. However, specific role of COX isoforms, the rate limiting enzymes for the pathway, remains elusive in the regulation of the endogenous synthesis of PGs. This study was aimed at the selective suppression of the constitutive COX-1 in cultured preadipocytes by the isolation of cloned preadipocytes transfected stably with a mammalian expression vector harboring cDNA encoding mouse COX-1 in the antisense direction. The gene expression analysis revealed that the transcript and protein levels of the constitutive COX-1 were substantially suppressed in the isolated cloned transfectants with antisense COX-1. By contrast, the

expression of the inducible COX-2 was not affected in the stable transfectants with antisense COX-1. All the cloned stable transfectants with antisense COX-1 exhibited the significant reduction in the immediate synthesis of PGE_2 serving as an anti-adipogenic factor. The sustained expression of COX-1 at the antisense direction induced the appreciable stimulation of fat storage in adipocytes during the maturation phase, which was associated with the higher expression levels of adipocyte-specific genes, indicating the positive regulation of adipogenesis program. Moreover, the up-regulation of adipogenesis is accompanied by higher production of J_2 series PGs including 15-deoxy-$\Delta^{12,14}$-PGJ_2 and Δ^{12}-PGJ_2 known as pro-adipogenic factors by the transfectants with antisense COX-1. The results suggest that the inducible COX-2 can contribute to the endogenous synthesis of PGJ_2 derivatives acting as autocrine mediators to simulate adipogenesis during the maturation phase by way of compensation for the suppressed expression of the constitutive COX-1.

Earlier studies have reported that carbaprostacyclin, a stable analogue of prostacyclin also called PGI_2, stimulates terminal differentiation of Ob1771 mouse pre-adipose cells in serum-free hormone-supplemented medium. Furthermore, under the same conditions, they recognized the pro-adipogenic effect of arachidonic acid, which was blocked by COX inhibitors. According to the ability of those cells to 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of unstable parent PGI_2, and $PGF_{2\alpha}$, those prostanoids are considered as autocrine mediators in the process of adipose conversion. Nevertheless, the pro-adipogenic action of endogenous PGI_2 remains obscure due to the instability of PGI_2 and its uncertainty of effective concentration of the parent form. Biologically active PGI_2 is rapidly hydrolyzed to inactive 6-keto-$PGF_{1\alpha}$ in most biological fluids. It should be noted that different cell types and alterations in the culture conditions of cultured adipocytes and precursor cells would gave contradictory results on adipogenesis. For example, $PGF_{2\alpha}$ serves as anti-adipogenic factor in cultured 3T3-L1 cells in contrast to the stimulatory effect on the adipocyte differentiation of cultured Ob1771 pre-adipose cells as described above. In addition, a previous study observed that the repeated addition of exogenous PGI_2 to cultured 3T3-L1 cells inhibited insulin- and indomethacin-mediated adipocyte differentiation. Earlier, cultured 3T3-L1 cells have been described to generate PGI_2 as an immediate response to calcium ionophore A23187 for 5 min or by the incubation with extracellular arachidonic acid. However, until now the biosynthesis of PGI_2, which is usually quantified as the levels of stable 6-keto-$PGF_{1\alpha}$, has not been monitored comprehensively at different life stages of adipocytes through the growth, differentiation, and maturation phases. In this study, I obtained an antiserum specific for 6-keto-$PGF_{1\alpha}$ and used for the development of a sensitive, convenient solid-phase enzyme-linked immunosorbent assay (ELISA) for the quantification of 6-keto-$PGF_{1\alpha}$. After the confirmation of the precision and accuracy of our ELISA, this immunological assay was applied to the determination of 6-keto-$PGF_{1\alpha}$ reflecting the endogenous levels of PGI_2 synthesized by cultured 3T3-L1 cells at different life stages of adipogenesis.

Prostacyclin alternatively called PGI_2 is an unstable metabolite synthesized by the arachidonate COX pathway. Earlier studies have suggested that prostacyclin analogues can act as a potent effector of adipose differentiation. However, biosynthesis of PGI_2 has not been determined comprehensively at different life stages of adipocytes. PGI_2 is rapidly hydrolyzed to the stable product, 6-keto-$PGF_{1\alpha}$, in biological fluids. Therefore, the generation of PGI_2 can be quantified as the amount of 6-keto-$PGF_{1\alpha}$. In this study, I attempted to develop a solid-phase ELISA using a mouse antiserum specific for 6-keto-$PGF_{1\alpha}$. According to the typical calibration curve of our ELISA, 6-keto-$PGF_{1\alpha}$ can be quantified from 0.8 pg to 7.7 ng in an assay. The evaluation of our ELISA revealed the higher specificity of our antiserum without the cross-reaction with other related prostanoids while it exhibited only the cross-reaction of 1.5% with $PGF_{2\alpha}$. The resulting ELISA was applied to the quantification of 6-keto-$PGF_{1\alpha}$ generated endogenously by cultured 3T3-L1 cells at different stages. The cultured cells showed the highest capability to generate 6-keto-$PGF_{1\alpha}$ during the maturation phase of 4-6 days, which was consistent with the coordinated changes in the gene expression of PGI synthase and the IP receptor for PGI_2. Following these events, the accumulation of fats was continuously promoted up to 14 days. Thus, the present immunological assay specific for 6-keto-$PGF_{1\alpha}$ is useful for monitoring the endogenous levels of the unstable parent PGI_2 at different life stages of adipogenesis and for further studies on the potential association with the up-regulation of adipogenesis in cultured adipocytes.