DIFFERENTIATION OF 3T3-L1 FIBROBLASTS TO ADIPOCYTES THE EFFECT OF INDOMETHACIN, PROSTAGLANDIN E_1 AND CYCLIC AMP ON THE PROCESS OF DIFFERENTIATION

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Summary

In the presence of insulin, resting 3T3-L1 fibroblasts differentiate into adipocytes after a lag period of 12 to 20 days. Indomethacin shortens the lag period dramatically and increases the rate of expression of lipogenic activity. In the presence of 1.74×10^{-7} M insulin and indomethacin, cells become fully differentiated before any increases in the lipogenic activities of insulin plates became apparent. Cyclic AMP and Prostaglandin E_1 potentiate the effect of insulin on the induction of lipogenic activities at the very early stages of differentiation. At the later stages both compounds become inhibitory. The extent of inhibition depends both on the concentration of insulin and of PGE_1 or cAMP. PGE_1 (4.32×10^{-6} M) or cAMP (5×10^{-4} M) completely block differentiation beyond the initial stages in the presence of insulin alone but they only slow down the rate of increase of lipogenic activities in the presence of insulin plus indomethacin.

Introduction

Resting 3T3-L1 cells in culture differentiate into adipocytes (1-4) in a process greatly accelerated by the continuous presence of insulin in the medium (1-4). During the course of differentiation the activities of the enzymes of fatty acid biosynthesis increase coordinately with the rate of [^{14}C]acetate incorporation into the cellular triglycerides (4). At least in the case of acetylCoA carboxylase, this increase in activity is due to the synthesis of new enzyme (4).

Various observations made in the authors' laboratory suggested that hormonal and humoral factors other than insulin may be involved in enhancing or counteracting the effect of insulin upon differentiation. A search of the literature revealed that of all the hormones which interact with both cell types - adipocytes isolated from rats or mice, and 3T3 mouse-embryo fibroblasts in culture - prostaglandins[1] had the unique distinction of lowering cyclic AMP levels in isolated adipocytes and raising cAMP levels in 3T3 fibroblasts. This behavior of prostaglandins suggested to us that the process of differen-

[1]Abbreviations used: PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$ prostaglandin E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$; Bt_2cAMP, dibutyryl cyclic AMP.

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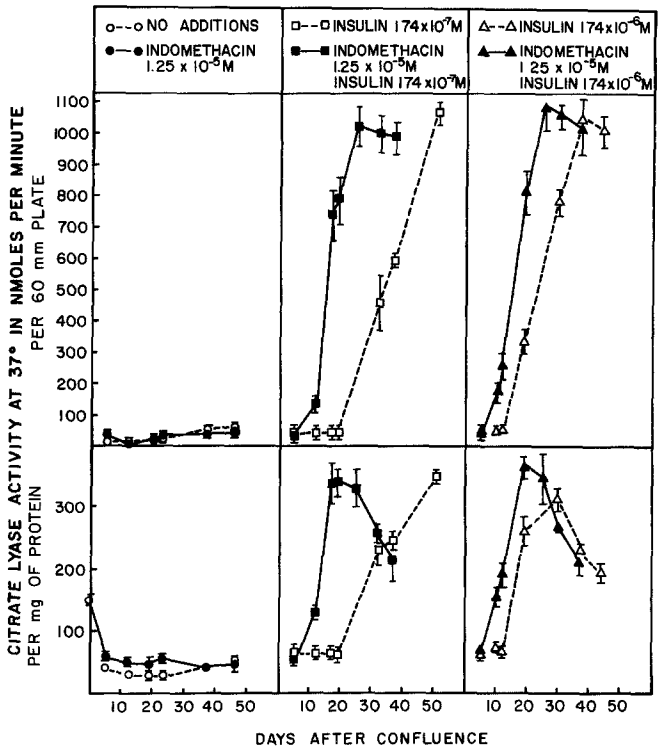

Figure 1. The effect of indomethacin on the induction of ATP-citrate lyase activity during the differentiation of 3T3-L1 fibroblasts into adipocytes. Additions of insulin and indomethacin were made on the day of confluence and in every change of medium thereafter (see Experimental Procedure).

tiation might be accompanied or even be dependent on changes in the nature of prostaglandin receptors. Consequently, the effect of prostaglandins, cAMP, and indomethacin - an inhibitor of prostaglandin biosynthesis - on the process of differentiation of 3T3-L1 cells to adipocytes was examined. The expression of the adipocytic phenotype during differentiation was followed by measurement of the activity of ATP-citrate lyase, the rate of incorporation of [^{14}C]acetate into cellular triglycerides and histochemically by staining for stored triglycerides with Oil Red-O. Some early results of these studies are presented here.

Experimental Procedure

The growth of cells, the preparation of cell-free extracts, the assay of ATP-citrate lyase activity, the [^{14}C]acetate incorporation into cellular triglycerides and protein determinations were carried out essentially as described (4), however, the volume of medium was reduced from 0.3 ml per cm^2 of plating surface area to 0.225 ml/cm^2 (4.7 ml per 60 mm plate). ATP-citrate lyase activity is the average of three determinations plus or minus one standard deviation. [^{14}C]Acetate incorporation is the average of duplicate determinations.

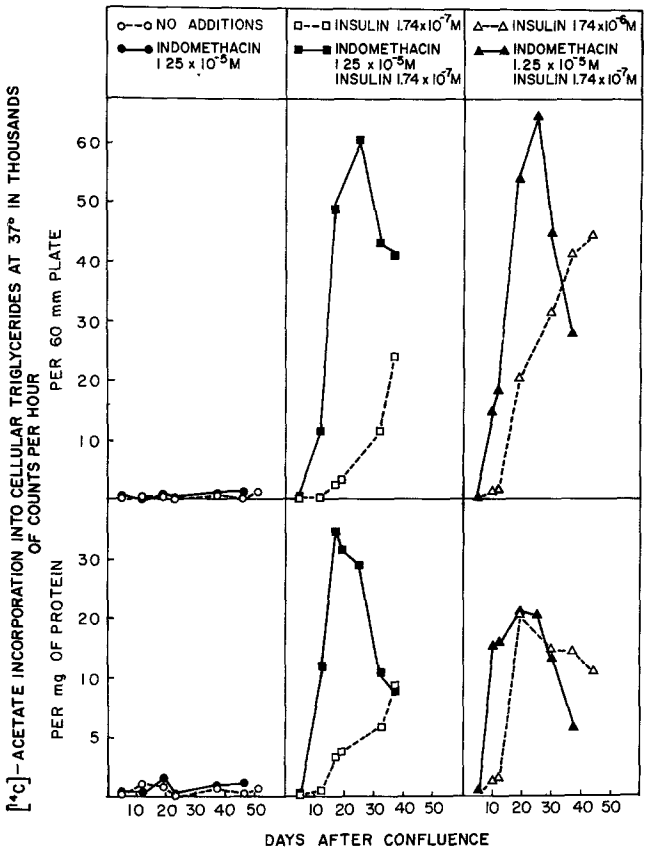

Figure 2. The effect of indomethacin on the rate of triglyceride biosynthesis from [^{14}C]acetate during the differentiation of 3T3-L1 fibroblast into adipocytes (see legend of Fig. 1 and Experimental Procedure).

Results

Effect of Indomethacin on the induction of ATP-citrate lyase and the formation of triglycerides from [^{14}C]acetate during the adipocytic conversion of 3T3-L1 cells. Fig. 1 and 2 show the effects of indomethacin on the induction of ATP-citrate lyase i.e. the first enzyme of the pathway of fatty acid biosynthesis, and on the rate of [^{14}C]acetate incorporation into cellular triglycerides respectively. In the absence of insulin no substantial changes in the levels of the citrate lyase or in the rate of formation of triglycerides were observed. In the presence of a "low" insulin concentration (1.74×10^{-7} M, i.e. 1 µg per ml) induction of citrate lyase and a substantial increase in the rate of formation of triglycerides was not observed till about 17 to 20 days after confluence. However, in the presence of indomethacin (1.25×10^{-5} M)

and "low" insulin the specific activity of citrate lyase reached 50% of the maximal value at day 12 post confluence and peaked at day 17. Between 17 and 25 days post confluence the specific activity of the enzyme did not change appreciably while the total activity per 60 mm plate continued to increase. Between 25 and 37 days post confluence total activity per plate decreased slightly, while the specific activity of the enzyme decreased by almost 40% due to an increase in the protein content of plates. A similar pattern is observed in Fig. 2 where the formation of triglycerides from [^{14}C]acetate is presented. There acetate incorporation per mg of protein reached about 50% of the maximal activity in the plates with indomethacin and "low" insulin by day 12 post confluence, and formation of triglycerides per mg of protein peaked at day 17 and did not change substantially between day 17 and 25. Thereafter both the formation of triglycerides per mg of protein and per plate decreased substantially. By this time most cells in the plates with indomethacin and "low" insulin had accumulated large amounts of fat so that the decreased rate of acetate incorporation may be due to the inability of some cells to store any more fat. By day 17 when indomethacin plus low plates had reached maximal citrate lyase specific activity the plates with low insulin had not shown any increases in the levels or specific activity of the enzyme and only small increases in the rate of [^{14}C]acetate incorporation into triglycerides. At day 51 post confluence the plates on "low" insulin reached the level and specific activity of citrate lyase seen 17 days post confluence in the plates where indomethacin was also present. Inspection of Fig. 1 also reveals that the rate of appearance of citrate lyase activity is greatly accelerated by the addition of indomethacin into plates with "low" insulin. Previous experience (4, 5) suggests that the increase in the activity of ATP-citrate lyase is most probably due to the formation of new enzyme. In summary, the addition of indomethacin into plates with low insulin reduces the lag period before the induction of lipogenic activities and also increases the rate of formation of lipogenic enzymes.

"High" insulin (1.74×10^{-6} M, i.e. 10 μg/ml) increased the rate of formation of lipogenic enzymes (compare in Figs. 1 and 2 low insulin in frame 2 with high insulin in frame 3). The addition of indomethacin into "high" insulin plates had no discernible effect on the rate of induction, but it clearly shortened the lag period preceding induction (Frame 3 figs. 1 and 2).

In the presence of indomethacin the lag period and the rate of formation of ATP-citrate lyase was the same in plates containing either "low" or "high" insulin (compare Figs. 1 and 2 low insulin plus indomethacin in frame 2 with high insulin plus indomethacin in frame 3). In Fig. 3 plates stained with

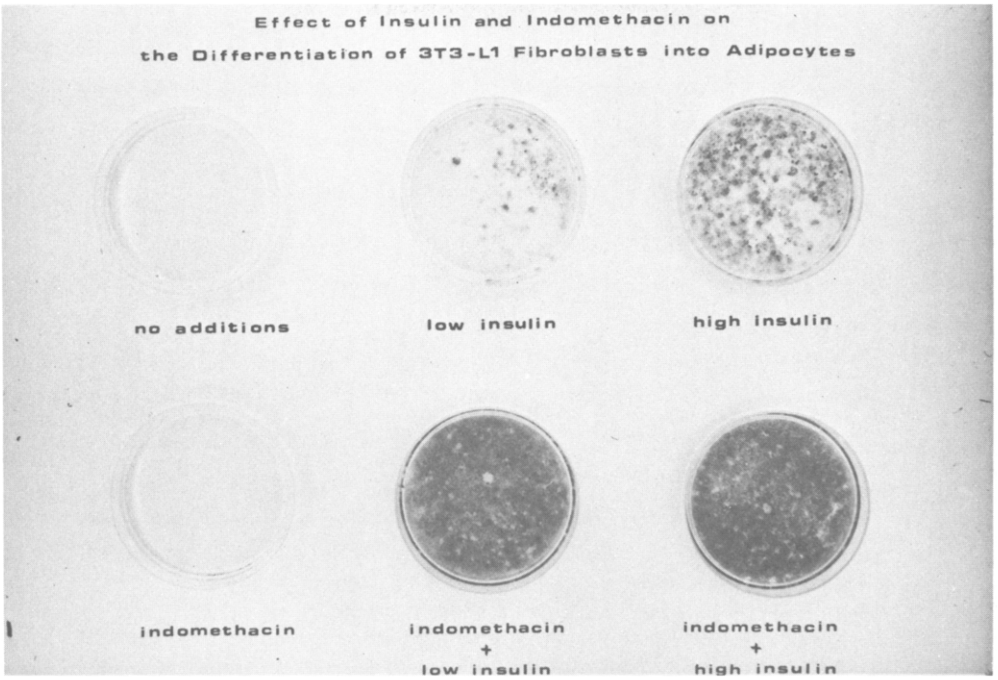

Figure 3. 3T3-L1 cells stained with Oil Red-O, thirty days after confluence.

Oil Red-O 30 days post confluence are shown. This time was chosen so that differentiation on plates with "low" insulin could be obvious and contrasted to the lack of differentiation in plates without insulin. Plates containing insulin plus indomethacin are full of fat cells while in plates with high insulin the accumulation of fat has not yet reached its maximum. The difference between plates containing indomethacin plus low or high insulin and high insulin only is more pronounced at earlier stages.

The effect of prostaglandin E_1 dibutyrylcyclic AMP and cyclic AMP on the process of induction of lipogenic activities. The mode of action of indomethacin in the above described experiments is not known. Nevertheless, indomethacin is known to inhibit prostaglandin biosynthesis in a variety of cells (6). If this is also true in our case we would expect PGE_1 to inhibit the induction process and differentiation. At the very beginning of differentiation PGE_1 (4.32×10^{-6} M) stimulates the induction of citrate lyase by about two-fold, however the specific activity of the enzyme does not increase any further and in fact, the continuous presence of PGE_1 in the medium of differentiating cells completely inhibits the further induction of

TABLE I

The effect of Prostaglandin E_1 and dibutyryl cyclic AMP on the induction of ATP-citrate lyase activity during the differentiation of 3T3-L1 fibroblasts into adipocytes

Additions to the medium	Citrate lyase activity at 37° in nmoles/min/mg of protein		
	Days at confluence		
	11	17	32
Low Insulin	32 ± 1	79 ± 13	321 ± 22
Low Insulin + PGE_1	76 ± 4	74 ± 4	67 ± 7
Low Insulin + Bt_2cAMP	71 ± 11	131 ± 37	188 ± 10

Low Insulin, 1.74×10^{-7} M i.e. 1 µg/ml; PGE_1, 4.32×10^{-6} M; Bt_2cAMP, 5.11×10^{-5} M.

lipogenic activities in the presence of either high or low concentrations of insulin. The effect of PGE_1 in the presence of low insulin is shown in Table 1. PGE_1 does not inhibit induction at the very early stages of differentiation (11 or 17 days post confluence), but it becomes clearly inhibitory at a later stage (32 days post confluence). Similar results were obtained with high insulin and PGE_1. Other prostaglandins (PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$) tested at the same concentration (4.32×10^{-6} M) gave identical results. In general either in the absence or presence of insulin (1.74×10^{-7} - 1.74×10^{-6} M) the continuous presence of prostaglandins in the medium at a concentration of 4.32×10^{-6} M prevents the adipocytic conversion of 3T3-L1 cells and results instead in overgrowth of cells and the formation of multilayers. In the presence of indomethacin however, PGE_1 does not abolish the process of differentiation and the induction of lipogenic activities, although the levels of citrate lyase attained in the presence of PGE_1 are lower than the controls (Table 2). With high insulin and indomethacin (results not shown) the specific activity of citrate lyase in the presence of PGE_1 25 days post confluence was about one-half of the specific activity of the control plates (high insulin plus indomethacin).

TABLE II

The effect of Prostaglandin E_1 and dibutyryl cyclic AMP on the process of differentiation of 3T3-L1 fibroblast into adipocytes in the presence of insulin and indomethacin

Additions to the medium	Citrate lyase activity at 37° nmoles/min/mg of protein	
	Days at confluence	
	17	25
Indomethacin + low Insulin	337 ± 32	328 ± 31
Indomethacin + low Insulin + PGE_1	276 ± 6	204 ± 3
Indomethacin + low Insulin + Bt_2cAMP	372 ± 15	289 ± 9

Indomethacin, 1.25×10^{-5} M; Low Insulin, 1.74×10^{-7} M; PGE_1, 4.32×10^{-6} M; Bt_2cAMP, 5.11×10^{-5} M.

Prostaglandins are believed to exert their effect by modulating the levels of cyclic nucleotides. PGE_1 increases the levels of cAMP in fibroblasts (6) and decreases the levels of cAMP in adipocytes (6). We therefore investigated the effect of cAMP on the process of differentiation. The data of Table I indicate that dibutyryl cyclic AMP enhances the effect of insulin at the early stages of differentiation. 17 days post confluence the activity of citrate lyase in the plates with low insulin plus Bt_2cAMP is 66% higher than the activities of plates with low insulin. Ten days post confluence the activity of the same enzyme in the plates with high insulin and Bt_2cAMP was 65% higher than in plates with high insulin alone. Again ten days post confluence the activity of the lyase in plates with high insulin, indomethacin and Bt_2cAMP was 65% higher than the activity of plates with high insulin and indomethacin. However, at later stages of differentiation (Table 1, 32 days and Table 2, 17 and 25 days post confluence) the effect of Bt_2cAMP is not apparent. Because butyrate formed by hydrolysis of Bt_2cAMP could conceivably accelerate the process of differentiation, the experiment was repeated along with the appropriate controls. Table 3 indicates that butyrate does not enhance the

TABLE III

The effect of butyrate, dibutyryl cyclic AMP and cyclic AMP on the process of differentiation of 3T3-L1 fibroblasts into adipocytes in the presence of insulin

Additions to the medium	Citrate lyase activity at 37° nmoles/min/mg of protein Days at confluence	
	16	21
Low Insulin	72 ± 1	195 ± 26
Low Insulin + Butyrate	76 ± 20	177 ± 6
Low Insulin + cAMP	162 ± 20	287 ± 21
Low Insulin + Butyrate + cAMP	158 ± 1	249 ± 26
Low Insulin + Bt_2cAMP	122 ± 4	265 ± 21

Low Insulin, 1.74×10^{-7} M; Butyrate, 1.022×10^{-4} M; cAMP, 5.11×10^{-5} M; Bt_2cAMP, 5.11×10^{-5} M.

effect of insulin at the early stages of differentiation and that cAMP itself is equally good or better than Bt_2cAMP as a potentiator of the insulin effect.

While this work was in progress Russell and Ho (7) published their experiments on the effect of prostaglandin $F_{2\alpha}$ on the process of differentiation of 3T3-L1 cells into adipocytes. These authors performed their experiments by treating cells for 3-5 days with insulin and prostaglandin $F_{2\alpha}$, cAMP or 1-methyl-3-isobutylxanthine (an inhibitor of phosphodiesterase) followed by insulin alone. They observed that prostaglandin $F_{2\alpha}$, and 1-methyl-3-isobutyl-xanthine were able to trigger the process of differentiation. The results of these studies and our studies are not necessary in conflict since they treated cells with $PGF_{2\alpha}$ only for a limited period of time and with concentrations different than the concentrations used in our experiments. These studies however did reinforce the need for a careful and more detailed study of the effects of various prostaglandins and cyclic AMP on the process of differentiation. We have now studied the effect of various concentration of indomethacin

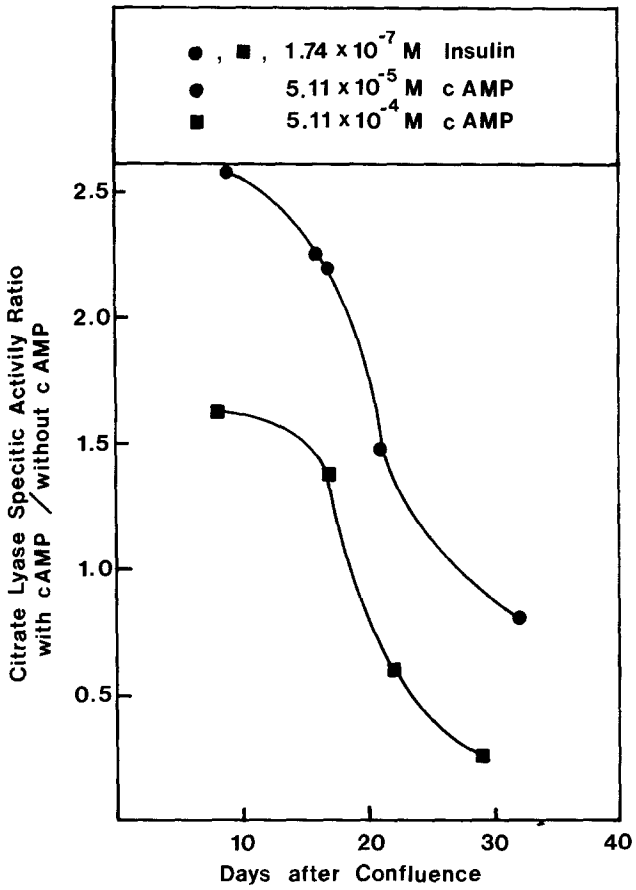

Figure 4. The effect of the continuous presence of cAMP on the process of induction of citrate lyase by insulin.

(2.7×10^{-6} - 1.3×10^{-4} M) cyclic AMP (1.3×10^{-5} - 5.1×10^{-4} M) PGE_1 (8.5×10^{-8} - 4.6×10^{-6} M) and animophylline (8.5×10^{-5} - 1×10^{-3} M) on the process of differentiation and in the presence of three different insulin concentrations (1.74×10^{-8}, 1.74×10^{-7} and 1.74×10^{-6} M). We can state categorically that indomethacin at all concentrations tested potentiates the effect of insulin on differentiation. Furthermore, the minimum concentration of indomethacin required for optimal rate of induction of adipocytic phenotype depends on the concentration of insulin. The higher the insulin concentration the lower the indomethacin concentration required.

In contrast to indomethacin, the effects of cAMP, PGE_1, and aminophylline (an inhibitor of phosphodiesterase activity) are complicated. At low concentrations cyclic AMP (1 - 5 x 10^{-5} M), PGE_1 (2.1 - 4.2 x 10^{-7} M) and aminophylline

($0.1 - 0.4 \times 10^{-3}$ M) all stimulate the rate of induction of citrate lyase. The stimulation is especially noticeable at the very early stages of differentiation. As an example the rate of induction of citrate lyase by 1.74×10^{-7} M insulin in the presence and absence of cAMP is presented in Fig. 4. At the beginning of differentiation the activity of citrate lyase in plates containing 5.11×10^{-5} M cAMP is 2.5 times higher than in plates without cAMP. As differentiation progresses the activity of the enzyme remains higher in the plates with cAMP almost to the end of the process although the ratio of activities in the plates with cAMP to the plates without cAMP is constantly decreasing and becomes equal to 1.0 about 30 days post confluence, suggesting that the cAMP at low concentrations becomes moderately inhibitory at the later stages of differentiation. High concentration of cAMP ($1 - 5 \times 10^{-4}$ M), PGE_1 ($0.9 - 4.6 \times 10^{-6}$ M) and aminophylline ($0.8 - 1.0 \times 10^{-3}$ M) also stimulate the induction of enzyme at the very early stages of differentiation. However the continuous presence of these agents into the medium becomes subsequently severely inhibitory. In the presence of 1.74×10^{-7} M insulin, 5.11×10^{-4} M cAMP stimulates the induction of citrate lyase by 50% in the first few days of differentiation. Subsequently the activity of the enzyme increases very little and as a consequence at the end of the differentiation period the activity of the plates with 5.11×10^{-4} M cAMP is only 1/6 to 1/10 of the activity of the plates without cAMP. The final specific activity of citrate lyase depends both on the concentration of cAMP and insulin. For a given insulin concentration the higher the concentration of cAMP the lower the final specific activity of the enzyme. Also for a given concentration of cAMP the higher the concentration of insulin the higher the specific activity of the lyase. Our data on the effects of PGE_1 and aminophylline are quantitatively and qualitatively identical to the results obtained with cAMP and suggest that the effects of this compound are most probably mediated by the cyclic nucleotide.

Discussion

The two effects of indomethacin on the process of differentiation (Fig. 1, panel 2) are the shortening of the lag period before induction of lipogenic activities and the increase in the rate of induction observed in the presence of indomethacin and low insulin. These effects may or may not be independent of each other, furthermore, they may be due to influences exerted by indomethacin at different levels of the induction process. A high insulin concentration (Fig. 1, panel 3) mimics the effect of indomethacin in that it increases the rate of induction almost to the same level obtained with low insulin and indomethacin. However, the same concentration of insulin (Fig. 1, panel 3) does not reduce the duration of the lag period to the same extent as the

combination of insulin and indomethacin does. These observations support the idea that the two effects of indomethacin may be exerted at different levels of differentiation, although, these effects may be at least partly mediated by common factors. Indomethacin is an inhibitor of prostaglandin biosynthesis. At this stage, it is not possible to say whether the effects of indomethacin on the process of differentiation are in fact due to inhibition of prostaglandin biosynthesis. However, the fact that prostaglandin E_1 does inhibit the process of differentiation in the presence of insulin, suggests that the effect of indomethacin on the induction of lipogenic activities in differentiating cells may be partly due to inhibition of prostaglandin biosynthesis. Although prostaglandin E_1 completely abolishes the process of differentiation in the presence of insulin it does not do so in the presence of both indomethacin and insulin. Although the concentration of PGE_1 (4.32×10^{-6} M) in the medium is high, the possibility that it is not sufficient to completely abolish differentiation, when endogenous synthesis is inhibited by indomethacin, cannot be excluded at present. Alternatively, it is possible that the effects of indomethacin are due to inhibition of thromboxane and endoperoxide synthesis, or only partly due to the inhibition of prostaglandin synthesis, or exerted on a locus not related to the production of prostaglandins, or that endogenously produced and externally supplied prostaglandins do not exactly have the same effects on cells.

Work is now in progress to elucidate the mechanism of action of cyclic AMP, aminophylline and PGE_1 on the process of differentiation. Preliminary experiments indicate that the inhibitory effect of cAMP is exerted directly at the level of synthesis of the lipogenic activities while the very early stimulatory effect of the nucleotide on the induction of the same enzyme could be indirect and due to an increase in the number of insulin receptors induced by the cAMP. This suggestion is also supported by the results of Thomopoulos et al (8) who have shown that cAMP increases the concentration of insulin receptors in cultured fibroblasts and Reed et al (9) who have shown that the number of insulin receptors of 3T3-L1 cells increase during differentiation.

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