Effect of Insulin on the Proliferation of Cultured Primate Arterial Smooth Muscle Cells

By Robert W. Stout, Edwin L. Bierman, and Russell Ross

ABSTRACT
Smooth muscle cells were grown from thoracic aortas of 1-year-old monkeys (Macaca nemestrina). The effect of insulin on the proliferation of these cells was studied by comparing the growth of cells in culture medium to which insulin had been added with that of cells in basal (1% monkey serum) medium and in growth-promoting 5% monkey serum. Insulin in concentrations of 10, 100, 1,000, and 10,000 μunits/ml resulted in successively greater stimulation of growth which was highly significant (P < 0.001) by analysis of variance. There was a significant linear relationship between the logarithm of the insulin dose and cell growth. However, the highest concentration of insulin produced only 50% of the effect of 5% monkey serum. Serum from which insulin had been removed stimulated growth less well (P < 0.05) than did untreated serum at the same concentration (5%) but had significant (P < 0.05) stimulating properties compared with whole serum at a lower concentration. Cells that were older in culture life (eight or nine passages) did not show a growth response to insulin and had an attenuated response to 5% serum. The effect of insulin (100 μunits/ml) was inhibited by dibutyryl cyclic adenosine monophosphate (db-cAMP) (5 × 10⁻⁵ M), although there was a latent period of 3 days before inhibition occurred; db-cAMP had no effect on cell counts in the absence of insulin. The electron microscopic appearance of the cells was unaltered by insulin.

KEY WORDS
monkey serum  
cell culture  
cell growth  
cyclic adenosine monophosphate  
atherosclerosis  
electron microscopy

Although atherosclerosis is predominantly a disease of the arterial intima, it is now apparent that the cells of the lesion are smooth muscle cells (1, 2). Whether these cells migrate from the media or proliferate from cells already in the intima is not clear. Nevertheless, it has been shown that in the early lesions of both experimental atherosclerosis in animals (3) and spontaneous atherosclerosis in humans (4) proliferation of smooth muscle cells in the intima is a prominent feature; it precedes the appearance of the lipid, mineral, and protein deposition which characterizes the fully developed atheromatous lesion (5). As the lesion develops, the smooth muscle cells become laden with lipid (1), and at least some of the foam cells of the advanced lesions are probably derived from smooth muscle cells. The factors that stimulate proliferation of these cells have not been identified. However, a technique for growing arterial smooth muscle cells in culture has recently been described (2, 6), and this development allows the growth and the metabolism of these cells to be studied in detail.

Simultaneously with the advent of this tissue culture technique, studies have been performed evaluating hormonal and metabolic control mechanisms in atherosclerotic individuals. In general, many atherosclerotic individuals have abnormal glucose tolerance tests (7), and many diabetic subjects seem to have accelerated atherosclerosis (8). The association of abnormal glucose tolerance tests with atherosclerosis has prompted studies of insulin secretion in this condition. Several studies have indicated that atherosclerotic subjects develop elevated insulin levels in response to oral glucose loads (9–13). The subjects studied have been neither diabetic nor obese and they have included individuals with atherosclerosis of the coronary (11), cerebral (12), and peripheral (13) arteries. Moreover, several other metabolic abnormalities often found in atherosclerotic subjects, including hypertriglyceridemia (14) and obesity, with normal or abnormal glucose tolerance tests (15), are also associated with elevated insulin levels. The interaction between abnormalities of insulin secretion and other abnormalities of carbohydrate and lipid metabolism is unresolved and is clearly complex. However, it has been suggested that insulin may...
have a role in the pathogenesis of atherosclerosis when it is present in high concentrations or in moderate concentrations at inappropriate times (16). In support of this hypothesis, studies have shown that insulin can regulate lipid synthesis in the arterial wall (17, 18) and that chronic insulin administration results in the appearance of lipid-laden aortic lesions in experimental animals (19).

It has been known for many years that insulin is required for complete growth and development (20). There is also evidence that insulin may stimulate the proliferation of certain cells in culture (21). It has been suggested that the early atherosclerotic lesion results from exposure of the subintimal layers of the arterial wall to serum which enters through an altered endothelium and that the serum contains factors that have the property of stimulating smooth muscle cell proliferation (2). To test whether insulin is one of the serum factors involved in this process, the growth-promoting effect of insulin on cultured arterial smooth muscle cells was studied.

**Methods**

**CELL CULTURE TECHNIQUES**

Segments of the thoracic aorta were obtained from 1-year-old pigtail monkeys (*Macaca nemistrina*) by careful dissection under sterile conditions. The segments of aorta were placed in Petri dishes, and the intima and the inner media were carefully removed under a dissecting microscope. The intima and inner media segments were cut into small pieces approximately 1 mm²; 50-100 of these pieces were placed in 250-ml Falcon tissue culture flasks and just covered with tissue culture medium containing 5% monkey serum. The flasks were capped loosely and placed in a tissue culture incubator at 37°C in an atmosphere of 95% air and 5% CO₂. Two days later, a small volume of medium was added gently so as not to disturb the segments that were starting to adhere to the flask. Cells started to grow out from the tissue in 4-7 days and became confluent in about 4 weeks. Smooth muscle cells were distinguished from fibroblasts by their longer lag period before outgrowth from explants, their slower growth rate, their pattern of growth, and their morphology. Any contamination of the cultures by adventitial fibroblasts became apparent soon after explantation, and the fibroblasts rapidly overgrew the smooth muscle cells. All such cultures were discarded.

Confluent cells were washed with 5 ml of a Versene buffer solution and subsequently trypsined with a solution of trypsin in Versene buffer (Difco Bactotrypsin I: 250-0.05% solution, Difco Laboratories Inc.) by incubating them in 5 ml of this solution for 10 minutes at 37°C. The resulting suspension of cells was transferred to a centrifuge tube, and 10 ml of medium with 5% monkey serum was added to inactivate the trypsin. The solution was then centrifuged at room temperature for 5 minutes at 125 g, and the supernatant fluid was discarded. The cells were resuspended in culture medium, and samples containing approximately 7.5 × 10⁶ cells were pipetted into 250-ml Falcon tissue culture flasks. The cells were grown in tissue culture medium containing 5% monkey serum, which was replaced three times a week. When the cells became confluent, in about 2-4 weeks, they were passed again in the same way. The cells used in the experiments to be described had been passed four to six times since explantation; all cells in each experiment were from the same passage. In some experiments, cells that had been passed nine times and had thus undergone 35 or more doublings were used.

For the growth experiments, the cells from six or seven flasks of confluent cells of the same passage from the same monkey were suspended in tissue culture medium containing 1% monkey serum, and samples containing identical numbers of cells were pipetted into 6-cm Falcon Petri dishes. The volume of medium in the dishes was 4 ml, and the number of cells was approximately 100,000/dish. The dishes were placed in a moist tissue culture incubator at 37°C in an atmosphere of 95% air and 5% CO₂, and the cells were fed three times a week.

**CELL COUNTING**

For cell counting, the cells were trypsized as described in the preceding section, using 2 ml of trypsin solution, centrifuged, and resuspended in a known volume of fresh medium. Samples were counted in a Fuchs-Rosenthal counting chamber. For each cell count, the cells in paired plates were counted in quadruplicate. The mean count was expressed as the number of cells per plate.

**BIOCHEMICAL TECHNIQUES**

After counting, the remaining cells were centrifuged and washed. Protein was measured by the Lowry method (22) and deoxyribonucleic acid (DNA) by the diphenylamine method (23). To obtain enough tissue for these measurements, all of the cells from each treatment group were combined. In some experiments, DNA was measured by a fluorometric method (24) on paired individual plates.

**TISSUE CULTURE MEDIA**

The Dulbecco-Vogt modification of Eagle’s medium (25) was modified in that it contained 3 ml of 7.5% sodium bicarbonate, 1.0 ml of a 100 mM solution of sodium pyruvate, 450 mg of dextrose and 5,000 units of penicillin per 100 ml, and 1% or 5% monkey serum.

**SERUM**

The serum was pooled homologous monkey serum; a fresh batch was used for each experiment. The serum was heated to 56°C for 30 minutes to inactivate complement and sterilized by filtration using a Millipore filter with combined 0.22μ, 0.45μ, and prefILTER pads. It was then stored in a refrigerator and used in concentrations of 1% or 5% in the tissue culture medium.

In two experiments, endogenous insulin was removed from the serum by passing the serum through an affinity column of Sepharose 2B (Pharmacia) that had been treated with cyanogen bromide and activated with guinea pig anti-insulin serum of high specific activity (26). Radioimmunoassay of the serum at the end of this procedure confirmed that all of the insulin had been removed. The treated serum was tested against serum
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from the same batch that had not been run through the column in one experiment, and in another experiment it was tested against serum from the same batch that had been run through a column with no antibodies. Both treated and control serum was tested at a concentration of 5% in tissue culture medium.

Radioimmunoassay of insulin in the serum was performed by a modification of the double antibody technique of Morgan and Lazarow (27).

INSULIN

Recrystallized porcine insulin (25 units/mg) was dissolved in Krebs-Ringer's phosphate buffer containing 1% albumin at a concentration of 0.5 units/ml and was sterilized by Millipore filtration using a plastic Swinex-25 Millipore filter. Appropriate dilutions were made with Krebs-Ringer's phosphate buffer which had been similarly sterilized, and the final concentration was made by adding 2 ml of dilute insulin in 100 ml of tissue culture medium. Control media had 2 ml of Krebs-Ringer's phosphate buffer containing 1% albumin alone in 100 ml of tissue culture medium. All manipulations were performed with plastic pipettes.

DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE

N\textsubscript{4}, O\textsubscript{6}-dibutyryl adenosine 3',5'-cyclic monophosphate (db-cAMP) (Calbiochem) was dissolved in Krebs-Ringer's phosphate buffer containing 1% albumin so that 2 ml of the solution in 100 ml of tissue culture medium gave concentrations of 10\textsuperscript{-8} M or 5 \times 10\textsuperscript{-9} M. Sterilization was by Millipore filtration as previously described. Solutions of butyric acid (Eastman Kodak) and adenosine monophosphate (AMP) (Calbiochem), both 5 \times 10\textsuperscript{-5} M, were prepared in the same way as the db-cAMP. Control media had 2 ml of Krebs-Ringer's phosphate buffer containing 1% albumin alone in 100 ml of tissue culture medium.

ELECTRON MICROSCOPY

Cells for examination by electron microscopy were grown in Falcon plastic tissue culture dishes as described previously (6). Electron microscopy was performed on cells from two experiments in which the effect of insulin on cell proliferation was tested; cells grown in basal (1% serum) medium were compared with cells that had been exposed to insulin.

EXPERIMENTAL DESIGN

Samples containing identical numbers of cells were pipetted into 6-cm Falcon plastic Petri dishes and grown in 4 ml of tissue culture medium containing 1% monkey serum. Cells were counted in paired plates on the following day and on alternate days for the first week after the start of the experiment. The culture medium was replaced by fresh medium three times a week. After the test solutions had been added, the cells were counted in paired plates three times a week, that is on 4–7 occasions during the succeeding 10–14 days.

Results

Preliminary experiments showed that provided at least 10\textsuperscript{6} cells in 4 ml of medium were pipetted into each plate at the start of the experiments the differences between the replicate plates during the...
experiments were small and were insignificant compared with the differences between the treatment groups. The cell counts on eight plates of cells pipetted at the same time and grown in 1% serum for 4 days showed a coefficient of variation of 2.5%. The coefficients of variation of ten replicate cell counts of each of two plates were 3.6% and 3.7%, indicating acceptable precision for the counting techniques. The correlation coefficient (r) between the total number of cells in each treatment group and the protein in these samples was 0.928 and between cell count and DNA content was 0.738, both of which are highly significant. The DNA content, measured by the fluorometric method, of arterial smooth muscle cells in the early phase of culture life (three or four passages) was 5.00 ± 0.37 (SE) pg/cell (N = 48); in the late phase (eight or nine passages) it was 2.60 ± 0.27 pg/cell (N = 35) (P < 0.001). No difference could be detected in the immunoassayable insulin concentrations of the growth medium before and after exposure to the cells for 2 or 3 days.

The cell populations were homogenous, and their culture characteristics and morphology were identical to those previously described for monkey smooth muscle cells (2) and for smooth muscle cells cultured from guinea pig (6) and rat (30) aortas by the same technique. All cells contained numerous myofilaments. There were various amounts of plasmalemmal vesicles, and the cells contained different amounts of endoplasmic reticulum, glycogen deposits, and lipid droplets.

### EFFECT OF INSULIN ON CELL PROLIFERATION

In the initial experiments, the effects of three concentrations of insulin (100, 1,000, and 10,000 μunits/ml) added to tissue culture medium containing 1% serum were compared with the effects of the 1% serum medium alone and with the growth-promoting effects of medium containing 5% serum (Table 1, early passage). Increasing concentrations of insulin in the medium resulted in successively greater stimulation of cell proliferation (Fig. 1). The differences among the effects of the different insulin concentrations (0–10,000 μunits/ml) were statistically significant (Table 1). The correlation coefficient (r) between the total number of cells in each treatment group and the protein in these samples was 0.928 and between cell count and DNA content was 0.738, both of which are highly significant. The DNA content, measured by the fluorometric method, of arterial smooth muscle cells in the early phase of culture life (three or four passages) was 5.00 ± 0.37 (SE) pg/cell (N = 48); in the late phase (eight or nine passages) it was 2.60 ± 0.27 pg/cell (N = 35) (P < 0.001). No difference could be detected in the immunoassayable insulin concentrations of the growth medium before and after exposure to the cells for 2 or 3 days.

### TABLE 1

<table>
<thead>
<tr>
<th>Passage</th>
<th>Early passage</th>
<th>Late passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey 1</td>
<td>Monkey 2</td>
<td>Monkey 3</td>
</tr>
<tr>
<td>Proliferative response*</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(A) 1% serum</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(B) 100 μunits/ml of insulin</td>
<td>140</td>
<td>134</td>
</tr>
<tr>
<td>(C) 1,000 μunits/ml of insulin</td>
<td>143</td>
<td>142</td>
</tr>
<tr>
<td>(D) 10,000 μunits/ml of insulin</td>
<td>143</td>
<td>146</td>
</tr>
<tr>
<td>(E) 5% serum</td>
<td>160</td>
<td>445</td>
</tr>
<tr>
<td>Mean response ± SE to 5% serum</td>
<td>383 ± 92</td>
<td>192 ± 36</td>
</tr>
</tbody>
</table>

Significance†

- Analysis of variance (A vs. B vs. C vs. D vs. E)
- Linear trend
- A vs. B
- A vs. C
- A vs. D
- A vs. E
- B vs. C
- B vs. D
- C vs. D
- D vs. E

*Mean of cell counts in the last week of the experiment expressed in terms of the cell count in the basal (1% serum) medium.
†s = significant at least at the 5% level, based on statistical analysis of the total number of cells in each treatment group (see text); ns = not significant.
†NS because of excessive interaction between treatment and days.

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μunits/ml) were significant at the 1% level by the F-test, and for most comparisons in early-passage cells there was a significant difference, at least at the 5% level, between the effects of each of the concentrations of insulin (Table 1). The differences were greater in the later experiments in which larger numbers of cells were used. There was also a significant linear relationship between the mean cell counts in each treatment group and the corresponding insulin concentration. It is notable that even the highest concentration of insulin only resulted in about half as much proliferation as 5% serum.

The effect of a smaller concentration of insulin was then studied in three experiments comparing the proliferation of cells in medium containing 1% serum alone with that of cells exposed to 10 μunits/ml or 100 μunits/ml of added insulin. Cells exposed to 10 μunits/ml showed a growth response intermediate between the base line and the effect of 100 μunits/ml of insulin (Fig. 2). The differences among treatments were significant by the F-test. However, it was difficult to identify a significant effect of 10 μunits/ml of insulin compared with 1% serum alone, although in two experiments a significant difference was observed between 10 μunits/ml and 100 μunits/ml of added insulin. In the third experiment, there was a significant linear relationship between the aggregate cell counts and the insulin concentrations. Therefore, the threshold effect of added insulin can be assumed to lie between concentrations of 10 μunits/ml and 100 μunits/ml.

In cells which had been passed eight or nine times and were thus considerably older in culture life, no consistent effect of insulin could be demonstrated (Table 1, late passage). In these experiments, the cell responses to the different concentrations of insulin could not be differentiated among each other and from 1% serum alone. The proliferative response to 5% serum in these cells was less than that in cells in the earlier phase of culture (Table 1).

Electron microscopic examination showed that insulin administration did not alter the morphological appearance of the aortic smooth muscle cells.

EFFECT OF REMOVING INSULIN FROM SERUM

Serum from which insulin had been removed by passage through an insulin-antibody-coated affinity column was tested against untreated serum from the same batch, both at 5% concentration, and against 1% untreated serum in two experi-
TABLE 2

<table>
<thead>
<tr>
<th>Passage</th>
<th>Proliferative response*</th>
<th>Monkey 3</th>
<th>Monkey 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1% Serum</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>B Insulin-free serum (5%)</td>
<td>312</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>C Whole serum (5%)</td>
<td>369</td>
<td>233</td>
<td></td>
</tr>
</tbody>
</table>

Significance:
- Analysis of variance
  - A vs. B
  - B vs. C
  - A vs. C

*Mean of cell counts in the last week of the experiments expressed in terms of the cell count in the basal (1% serum) medium.
†s = significant at least at the 5% level; NS = not significant.

ments (Table 2). The insulin-free serum stimulated cell proliferation less well than did the whole serum, and the mean differences, though small, were statistically significant by the F-test in both experiments. In the second experiment, the control serum was run through a column which had no antibodies, so that the only difference between the sera was in the insulin. Radioimmunoassay confirmed that the treated serum contained no insulin, whereas the untreated serum had 40 and 58 μunits/ml of insulin, respectively. Thus, at 5% concentration, the effect of no insulin was compared with the effect of 2 or 3 μunits/ml. Even without any insulin, serum at 5% concentration stimulated cell proliferation to a considerable extent compared with 1% whole serum.

EFFECT OF db-cAMP ON THE PROLIFERATIVE RESPONSE TO INSULIN

db-cAMP was added to medium containing 100 μunits/ml of insulin in two concentrations (10^{-5} M and 5 × 10^{-5} M), and the proliferative response was compared with the response to 100 μunits/ml of insulin alone. The results (Fig. 3) showed that the initial response to insulin, the first 3 days after addition of the test solutions, was unaltered by the lower concentration of db-cAMP and a little attenuated by the higher concentrations. However, thereafter the cell count in the db-cAMP-treated cells gradually fell; the fall was much more rapid with the higher concentration of the nucleotide. Ten days after addition, the number of cells in the group treated with 10^{-5} M db-cAMP was essentially the same as that in the 1% serum control group, although the cell count in the group treated with the higher concentration of db-cAMP was considerably below the control count.

In the next experiment, 5 × 10^{-5} M db-cAMP was again added to medium containing 100 μunits/ml of insulin. Once again an initial proliferation of cells occurred followed by a fall in cell count, although in this experiment the cell count in the group exposed to db-cAMP did not fall greatly below the control count. The other group of cells was exposed to db-cAMP (5 × 10^{-5} M) alone for 3 days and then to the same concentration of db-cAMP plus insulin (100 μunits/ml). Prior exposure of the cells to the nucleotide considerably blunted the proliferative response to insulin, and there was no difference between the cell counts of this group and those of the control group (t = 1.2, P > 0.2).

Finally, cells were exposed to db-cAMP for 1 week; for a second week, insulin (100 μunits/ml) without db-cAMP was substituted (Fig. 4). The nucleotide had no effect on the basal cell counts. When insulin was substituted for the db-cAMP, there was a slight but severely attenuated proliferative response; the mean cell count after insulin was added was significantly higher than that when the cells were exposed to db-cAMP (t = 8.80, P < 0.001).

Addition of both butyric acid and AMP in concentrations of 5 × 10^{-5} M to medium containing 100 μunits/ml of insulin resulted in attenuation of
**Effect of db-cAMP on the proliferative response to insulin of cultured arterial smooth muscle cells.** Each point is the mean cell count in two replicate plates. Day 0 is the day insulin or db-cAMP was added to the 1% serum medium in which the cells were undergoing stationary growth. On day 7, the medium in one group of plates was changed as indicated by the change of symbols. The initial period of 1 week when all of the cells were exposed to the basal medium has been omitted. Open circles = medium with 1% serum, solid circles = medium with 1% serum and 100 μunits/ml of insulin, and open triangles = medium with 1% serum 5 × 10⁻⁸ M db-cAMP. Cells are from the same monkey as those used in Figure 2; the cells were used after four passages.

Discussion

The experiments described in the present paper show that insulin stimulates proliferation of aortic smooth muscle cells in culture. The consistent effect of insulin, the relationship between the insulin concentration and the cellular response, and the diminished response to serum when insulin has been extracted from it all confirm that insulin acted as a growth-promoting factor in these experiments. This investigation is the first direct demonstration of an insulin effect on smooth muscle cells in culture. It has been reported, however, that infusion of insulin directly into the femoral artery of a dog results in medial hypertrophy of the artery (31).

**An increase in cell number occurred very soon after exposure to insulin, but stationary growth was achieved after a few days, despite continued exposure to insulin.** The facts that the insulin concentrations in the growth media did not change during 2 or 3 days of exposure to the cells and that the media were changed three times a week indicate that depletion of insulin from the medium was not responsible for this pattern of cell growth. Since the cells were grown in basal medium for 7-10 days before exposure to insulin and the medium was changed three or four times during this period, it is highly unlikely that the addition of insulin merely unmasked a proliferative effect of prior trypsinization.

The present experiments suggest that insulin has growth-promoting properties, but they also show that insulin is not the only growth-promoting factor in serum. The facts that 10,000 μunits/ml of insulin did not stimulate growth to the same degree as serum which contained only 1-3 μunits/ml of insulin and that insulin-free serum had potent growth-promoting properties suggest that there must be other potent growth factors in serum. There is little information available about the identity of these other factors which stimulate proliferation of smooth muscle cells, although recently lipoproteins (2) and platelets (32) have been found to have this property. The growth-promoting activity of serum may thus be the result of the combined activities of a number of factors.

Smooth muscle cells which had been passed eight or nine times and had thus undergone 35 or more doublings did not proliferate in response to insulin and responded atypically to serum. Diploid cells in culture have a finite life-span, which in cells from humans is about 50 doublings (33); the life-span appears to be about the same for aortic smooth muscle cells from *Macaca nemistrina* (34). Thus, 35 doublings in cells from 1-year-old monkeys must represent a major portion of the cell life in culture, and indeed it proved impossible to culture cells for much longer than 12 passages. However, more detailed studies are required before definite conclusions can be drawn on the relationship between insulin action and cellular aging in vitro.

The exact relationship between insulin action on tissues and the intracellular metabolism of cAMP remains unclear, but, in general, the action of insulin is opposite to the action of cAMP (35, 36). Recently it has been suggested that cAMP is intimately concerned with cell proliferation (37-39). The present preliminary experiments suggest that db-cAMP has an effect that opposes the action of insulin on smooth muscle cells. The action of the nucleotide seemed to have a latent period of about 3 days and was related to the concentrations of db-cAMP in the medium. When the nucleotide was added 3 days before the insulin, the proliferative response to insulin was abolished. The fact that exposure of the cells to db-cAMP without insulin for 1 week had no effect on the cell count suggests
that an interaction of cAMP and insulin was involved rather than a nonspecific cytotoxicity.

Addition of butyric acid or AMP also interfered with the proliferative response to insulin. The effects were somewhat different to the effect of db-cAMP in that the initial proliferative response was attenuated and the cell count fell 7 days after addition instead of 3 days as with the nucleotide. It appears, therefore, that, as has been reported in other cell culture systems (40, 41) the breakdown products of db-cAMP have an effect that has similar features to the effect of the whole molecule and may contribute to the observed effects of db-cAMP. Thus, the relationship of the observed effects of db-cAMP to the effect of cAMP in vivo is not clear.

The present experiments show that smooth muscle cells grown from explants of monkey aortas proliferate in response to very small concentrations of insulin similar to those found in plasma under physiological conditions and that this effect of insulin is opposed by db-cAMP. Although proliferation of arterial smooth muscle cells is a prominent feature of early atherosclerosis and elevated insulin levels are commonly found in subjects with this condition, experiments of this type can only be indirectly related to the pathogenesis of atherosclerosis.

Acknowledgment

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