Responsiveness to Insulin of Glucose Metabolism in Cultured Rat and Human Arterial Smooth Muscle Cells

YOSHIKU MARUHAMA, SHIN-ICHI OIKAWA, HIROSHI SUZUKI, RYUZO ABE, FUMINOBU OKUGUCHI, TAKASHI KOBAYASHI and YOSHIO GOTO

The Third Department of Internal Medicine, Tohoku University School of Medicine, Sendai 980

Smooth muscle cells growing in the primary culture derived from outgrowths of the intimal-medial explants of both rat and human arteries were used. The 72-hr sequential glucose uptake by the cells of both species in culture dishes was enhanced only slightly with time by the addition of insulin to culture medium, and this enhancement was statistically not significant. The glucose conversions to CO2 and lipids by the rat and human cells dissociated for tracer study were not affected significantly during the 2-hr incubation by the insulin addition in vitro. The smooth muscle cells of both species cultured for a week in medium enriched with insulin and then dissociated revealed the significantly increased glucose conversion to lipids, while the increase in the glucose conversion to CO2 was not significant in these cells. Thus, the smooth muscle cells of both rat and human seem to show significant metabolic response to chronic, but not acute, exposure to insulin. Therefore, it is likely that the persistent change in the insulin level may lead to abnormal metabolic state in the artery.

Smooth muscle cells of the artery, the major constituent of the tissue, are known to play an important role through their early proliferation and lipid deposition in the development of atherosclerosis (Ross and Glomset 1973; Goldstein et al. 1977). It is also well-known that insulin has multiple influences on metabolic pathways of almost all tissues by enhancing the membrane transport, and activating and synthesizing the key enzymes. Thus, insulin may also affect arterial smooth muscle cells through one or more of the above mechanisms, maintaining the normal cell activity and, under the altered level of the hormone, leading to abnormal metabolism. However, some controversy seems to remain concerning the metabolic responsiveness of arterial cells to insulin. Urrutia et al. (1962) reported no immediate responsiveness to insulin of the glucose metabolism in...
isolated rat aortic tissue in vitro. Similar results were obtained in the in vitro experiment on rabbit aortic tissue by Mulcahy and Winegrad (1962), and on pig aortic tissue by Somer and Schwartz (1976). On the contrary, Stout (1971, 1975) reported a significant acceleration of lipid synthesis by aorta in vivo within a few hr after insulin injection in the rat. Although the reason for the discrepancy between the above authors could not be clarified, the in vitro and in vivo studies may well yield the different results. However, there have been few studies on the responsiveness to insulin of human arterial smooth muscle cells. Such studies in the human may have clinical implication since arterial metabolism in the human might be different from that in other species (Lindsay and Chaikoff 1963).

Recently, the cell culture system has been widely adopted to evaluate a direct and chronic interaction between cells and selected substance. In the present study, we aimed at observing the responsiveness to insulin of cultured human arterial smooth muscle cells in comparison with that of rat cells using glucose metabolism as an indicator.

**MATERIALS AND METHODS**

The explants for the culture of rat arterial smooth muscle cells were obtained from the intimal-medial segments of the total aorta of 2 male rats of the Wistar strain, weighing 215 g and 310 g, respectively, which were kept on normal laboratory chow. The preparation of the explants was conducted under sterile conditions. The explants for human arterial smooth muscle cells were obtained from the intimal-medial segments of the iliac artery and the renal artery. The former segments were obtained from a 15-year-old male patient during transplantation of an artificial artery because of the iliac arterial embolism after cardiac surgery. Macroscopically normal parts of the artery were used for the explants. The latter arterial segments were obtained from a 30-year-old male patient with cystic tumor of the kidney during nephrectomy. The intimal-medial segments of both rat and human were prepared after the removal of adherent tissue and the adventitia. Approximately 50 explants of 1–2 mm in diameter from each donor were placed on several separate Petri dishes (Falcon plastic culture dish, 60 mm; Div. Decton, Dickinson and Co., Oxnard, Calif., USA). After the explants attached to the bottom wall of the dishes, each 3 ml of growth medium was added to the dishes. The growth medium consisted of Basal Medium of RPMI 1640 (Nissui Seiyaku Co., Tokyo), supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin, 1.25 mg/ml of sodium bicarbonate and 20% (v/v) of fetal calf serum (FCS: Lot No. R560617, Grand Island Biological Co., New York, USA). FCS of the same lot was used throughout the experiments. The culture dishes were incubated in a humidified incubator (Type B CO₂ Incubator, Ikemoto Co., Tokyo) at 37°C with 5% CO₂ and 95% air. After the explantation, the initial outgrowth of the smooth muscle cells was observed through phase contrast microscopy in a week for the rat preparations and in 1–2 weeks for the human preparations. A half part of the medium was changed with the fresh medium every other day. When the cells became confluent, the old explant segments were removed from the dishes. The cells in the dishes were dissociated with addition of 0.2 mg/ml of protease (protease from Bacillus polymyxa, 5,000 protease units/mg; commercially available as Dispase™ from Godoshusei Co., Tokyo). This protease is widely used in Japan and is effective in medium containing serum. It is also known to give little cell damage. When the insulin effect on glucose uptake by rat adipocytes dispersed by trypsin, collagenase and this protease at their optimum effective dose were compared, the insulin effect was maximum in the adipocytes treated with the last (Maruhama and Abe, unpublished). The smooth muscle cells thus dissociated were washed with the growth
medium by centrifugation (800 rpm) and then diluted with the growth medium. Approximately $5 \times 10^4$ cells in 3 ml of the medium were seeded in each 60 mm Petri dish. This subculture was repeated until the amounts of the cells became sufficient for experiment. The experiment was carried out using the cells of the 3rd to the 5th subculture. The cells from 4 different sources, 2 from the rats and the other 2 from the humans, were cultured separately. The cells from each source were observed to grow predominantly in multiple overlapping layers with hills and hollows and showed the morphology characteristic of the smooth muscle cell as described by Ross (1971). However, the homogeneity of the cells in each cell series had not been proven to be established despite the several subculture. The culture dishes with an apparent contamination by fibroblasts were excluded.

The growth medium contained 194±1 mg/100 ml (mean±S.E.) of glucose and 6.0±0.2 μU/ml of insulin that was derived from FCS by triplicate measurements. This medium was also used for the experiments as standard medium. The half of the medium was changed with the fresh medium every other day. The insulin-enriched culture medium was prepared in the following manner. Bovine crystal insulin (23 units/mg, Carbiochem., La Jolla, Calif., USA) was dissolved in 1/400 N HCl solution in the amount of 1 unit/ml and then diluted up to 25 times by physiological saline. The standard medium was supplemented with this insulin solution to bring the final concentration of 200 μU/ml. The volume of the insulin solution added was negligible (0.6%) and pH of the medium did not change.

The first experiment was carried out on a total of 8 culture dishes of the rat cells - each 4 dishes from the 2 rat series - and on 6 dishes of one human series (the cells from the 15 year-old patient). In these dishes the cells had grown equally near confluency. For the half sets of these culture dishes, the medium that was changed fresh in half part 2 days before the experiment was replaced again by the standard medium, but for the other half sets the medium was replaced by the insulin-enriched medium. After sampling 0.05 ml of each medium for glucose determination (Huggett and Nixon 1957), the dishes were placed in the CO₂ incubator, and glucose concentration was determined 24, 48 and 72 hr after the start of the experiment. At the end of the experiment, the cells in each dish were dissociated as described above and the cell number was counted.

The second experiment was performed to observe the acute effect of insulin on the metabolism of glucose-U-14C in the dissociated cells from the 2 rat series and the 2 human series. The cells of each series grown to confluency in 4 dishes in the standard medium were dissociated as described above and the cells gathered in a plastic tube were washed 3 times by centrifugation with Krebs-Ringer bicarbonate buffer of pH 7.4 containing 3% of bovine serum albumin (fraction V, fatty acid free; Miles Lab., Elkhart, Indiana, USA) and no glucose. The cells suspended in Krebs-Ringer bicarbonate buffer were transferred to a 10 ml volume of plastic incubation tube. After sampling of a small portion for counting the cell number, the cell suspension was centrifuged and the buffer solution was carefully aspirated off. Each 8 tubes containing the smooth muscle cells from one series (32 tubes in total) were prepared in that manner. 2 ml of oxygenated Krebs-Ringer bicarbonate buffer of pH 7.4 containing 3% of bovine serum albumin, 200 mg/100 ml of glucose and 0.1 μCi of glucose-U-14C (16.7 μCi/mg, Radiochemical Centre, Amersham, England) were added to the half sets of the incubation tubes. To the other half sets the same incubation buffer but supplemented with insulin (200 μU/ml) was added. The incubation tube was capped with a rubber stopper from which was suspended a small plastic chamber for CO₂ collection. The incubation was carried out at 37°C for 2 hr in a metabolic shaker at 80 oscillations/min. After the incubation 0.5 ml of hyamine hydrochloride was injected into the small chamber and 0.2 ml of 10 N H₂SO₄ injected into the medium. The tubes were then incubated for another 1 hr. Hyamine solution aspirated from the chamber was placed to a counting vial. The smooth muscle cells were homogenized and the total lipids were extracted and purified (Folch et al. 1957). The chloroform phase containing the lipids was placed to a counting vial and chloroform was evaporated under nitrogen. Insta-Gel® (aqueous and non-aqueous scintillant, Packard Instrument
Co., Downers Grove, Ill., USA) was added to the vials containing hyamine hydrochloride and total lipids. The radioactivity of CO₂ and lipids was measured by an Aloka liquid scintillation spectrometer (Model PSM 601Z, Nihonmusen Co., Tokyo). Quenchings were corrected for efficiency by an automatic external standardization.

The third experiment was carried out in order to observe chronic cell responsiveness to insulin also using the 4 cell series. The cells from each series previously cultured with the standard medium and grown to near confluency were exposed to the insulin-enriched medium (200 µU/ml, 3 ml/dish) and cultured for 7 days. The half part of the medium was also changed with the fresh insulin-enriched medium every other day. At the end of this culture, the in vitro incubation studies were carried out in the same manner as those in the second experiment. The third experiment was done simultaneously with the second experiment and the latter results were used as controls.

In a separate series the smooth muscle cells from the 4 cell series were cultured with the standard medium and the insulin-enriched medium for 7 days. The cells dissociated were washed 5 times with physiologic saline. After a small portion of the cell suspension was taken for counting the cell number, the cells were homogenized and the cell contents of total lipids (Frings and Dunn 1970) and protein (Lowry et al. 1951) were measured. The unpaired t-test was applied to examine statistical significance.

RESULTS

The 72-hr sequential changes in glucose concentrations of the culture medium of the rat and human cells are shown in Fig. 1. Mean±S.E. of the cell number/dish counted at the end of this experiment was 2.0±0.1 (×10⁶) for the rat cells cultured with the standard medium, 2.2±0.2 (×10⁶) for the rat cells cultured with the insulin-enriched medium, 1.9±0.2 (×10⁶) for the human cells cultured with the standard medium and 2.0±0.3 (×10⁶) for the human cells cultured with

![Fig. 1. Sequential changes in medium glucose concentrations in standard medium without cells in culture dish (●—●), in culture of rat and human arterial smooth muscle cells with standard medium (○—○), and in culture of rat and human cells with insulin-enriched medium (Δ—Δ). Mean values±S.E. of each 8 experiments for rat cells and 6 experiments for human cells are shown. No significant difference was noted in the glucose concentrations between the cultures with standard medium and insulin-enriched medium at each time of culture for either rat or human cells. Cell numbers of the 2 series at 72-hr were similar for both species (see text).](image-url)
TABLE 1. Acute and chronic effects of insulin on glucose oxidation to CO₂ in cultured rat and human arterial smooth muscle cells

<table>
<thead>
<tr>
<th></th>
<th>Cells cultured without insulin addition</th>
<th>Cells cultured with insulin addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro insulin</td>
<td>In vitro insulin</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Rat</td>
<td>32.6 ± 6.6a1</td>
<td>40.6 ± 7.6a2</td>
</tr>
<tr>
<td>Human</td>
<td>46.9 ± 9.9b1</td>
<td>52.6 ± 14.1b2</td>
</tr>
</tbody>
</table>

Figures express mean±s.e. of each 8 experiments.

a1 vs. a2, a3; a3 vs. a4: not significantly different. b1 vs. b2, b3; b3 vs. b4: not significantly different.

TABLE 2. Acute and chronic effects of insulin on glucose conversion to lipids in cultured rat and human arterial smooth muscle cells

<table>
<thead>
<tr>
<th></th>
<th>Cells cultured without insulin addition</th>
<th>Cells cultured with insulin addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro insulin</td>
<td>In vitro insulin</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Rat</td>
<td>145.0 ± 22.6a1</td>
<td>177.8 ± 56.8a2</td>
</tr>
<tr>
<td>Human</td>
<td>103.9 ± 16.1b1</td>
<td>120.9 ± 21.4b2</td>
</tr>
</tbody>
</table>

Figures express mean±s.e. of each 8 experiments.

a1 vs. a3: significantly different (p<0.02). b1 vs. b3: significantly different (p<0.05). a1 vs. a2; a3 vs. a4: not significantly different. b1 vs. b2; b3 vs. b4: not significantly different.

the insulin-enriched medium. Since no differences were noted between the above cell numbers, the rates of decrease in the medium glucose concentrations shown in Fig. 1 can be compared as the rates of cell glucose uptake in these dishes. Both rat and human cells were shown to take up glucose avidly at a fairly constant rate during the 72-hr period. No apparent increase in the rate of glucose uptake was noted in rat or human cells cultured with the insulin-enriched medium as compared to those cultured with the standard medium.

The results of the second and the third experiments are summarized together in Tables 1 and 2. Table 1 shows the acute and chronic effects of insulin on glucose oxidation to CO₂ in the cells. The in vitro insulin addition to the incubation medium containing the cells previously cultured with the insulin poor medium produced only insignificant increases (p>0.05) in the glucose conversion to CO₂ either in the rat series (a1 vs. a2, 25%) or in the human series (b1 vs. b2,
TABLE 3. Chronic effects of insulin on lipid and protein contents of cultured rat and human arterial smooth muscle cells

<table>
<thead>
<tr>
<th></th>
<th>Cell number/dish (×10⁴)</th>
<th>Total lipids (mg/10⁴ cells)</th>
<th>Protein (mg/10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insulin addition in culture medium</td>
<td>Insulin addition in culture medium</td>
<td>Insulin addition in culture medium</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Rat</td>
<td>2.1±0.1</td>
<td>2.2±0.1</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Human</td>
<td>2.3±0.2</td>
<td>2.6±0.1</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

Figures express mean ± S.E. of each 5 dishes. Approximately 5×10⁴ cells were seeded into each Petri dish. When the cells grew to near confluency in the dishes, the cells were then cultured in the medium with or without insulin addition (200 μU/ml). Effect of insulin addition on the cell number, or cell lipid or protein content was not statistically significant (p>0.05).

12%), during the 2-hr incubation. When the rat and human cells were cultured for a week in the insulin-enriched medium, the in vitro glucose conversion to CO₂ during the 2-hr incubation also showed only insignificant increases both in the rat cells (a1 vs. a3, 45%) and in the human cells (b1 vs. b3, 45%). The in vitro insulin addition to the incubation medium did not accelerate the glucose oxidation to CO₂ in the cells of either species cultured for a week with the insulin-enriched medium (a3 vs. a4, b3 vs. b4). No significant differences were found between the two species in the acute or chronic cell responsiveness to insulin measured by the glucose conversion to CO₂ as indicator.

Table 2 shows the acute and chronic insulin effects on the glucose conversion to total lipids in the cultured cells. The in vitro insulin addition to the cells cultured previously with the insulin poor medium showed no apparent effects on the glucose conversion to lipids either in the rat cells (a1 vs. a2, 23%) or in the human cells (b1 vs. b2, 16%). The cells cultured for a week with the insulin-enriched medium revealed an increased conversion of glucose to lipids, and the increase was significant both in the rat cells (a1 vs. a3, 64%, p<0.02) and in the human cells (b1 vs. b3, 91%, p<0.05). The in vitro insulin addition did not accelerate the glucose conversion to lipids either in the rat or human cells cultured for a week with the insulin-enriched medium (a3 vs. a4, b3 vs. b4). No significant differences were noted between the rat and human cells in the acute or chronic responsiveness to insulin measured by the glucose incorporation into lipids as indicator.

As shown in Table 3, the cells cultured in the medium enriched with insulin for a week showed no increase in cell number and only insignificant increases in cell lipid and protein contents for both species.

DISCUSSION

In the present study, insulin showed no apparent acceleration of glucose uptake
during the 72-hr period by the arterial smooth muscle cells of either rat or human in the culture dishes. The dissociated cells of either species showed no immediate response to insulin in vitro when glucose conversion to CO₂ or lipids was adopted as indicator. Thus, the glucose transport into the arterial smooth muscle cells of both species may possibly be insulin independent. Our results on the rat cells were essentially similar to those of Urrutia et al. (1962) and different from those of Stout (1971, 1975). No immediate effect of insulin on glucose metabolism was found in the aortic tissue of the rabbit (Mulcahy and Winegrad 1962) or the pig (Somer and Schwartz 1976). In addition, the present study showed no prompt response to insulin of glucose metabolism of the human arterial cells. To be noted is that the glucose conversions to lipids were significantly increased after the cells were cultured with the insulin-enriched medium for a week. Although the control cells were cultured with the medium containing the small amount of insulin derived from FCS, the observed increase in the cell response to the insulin-enriched medium may be accounted for by the increased activity of the enzymes related to glucose metabolism that was caused by the insulin addition. However, no difference was noted between the two species in the responsiveness of cell glucose metabolism to the chronic exposure to insulin. The lipid content of the rat and human cells cultured with the insulin-enriched medium was increased only slightly despite the significantly increased rate of lipid synthesis. This might suggest that the turnover of lipids was also accelerated in such cells. The reason for the suggestive increase in the protein content of the arterial smooth muscle cells of humans as compared to that of rats during the culture with the insulin-enriched medium remains obscure.

Finally, it is presumable that the long-lasting hyperinsulinism in certain human obesity as well as insulinopenia in diabetes mellitus may lead to metabolic disorders in the artery.

Acknowledgment

We are grateful to Dr. T. Nishihira for expert help in tissue culture and to Drs. H. Ohuchi and A. Sugita for supply of the resected human arteries.

References


