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METFORMIN INCREASES GLUCOSE TRANSPORTER PROTEIN AND GENE EXPRESSION IN HUMAN FIBROBLASTS

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SUMMARY To investigate the effect of the antihyperglycemic drug metformin on glucose transporter protein and gene expression, skin fibroblasts obtained from normal and diabetic volunteers were grown in culture and incubated with metformin at various concentration for up to 16 days. Metformin caused a dose and time dependent increase in GLUT1 number with a maximum at a concentration of 10 µg metformin given over 4 days. This was accompanied by an increase in GLUT1 mRNA, suggesting that metformin has a stimulating effect on glucose transporter gene expression. No significant difference was observed between cells obtained from type II diabetic patients and those from controls. We conclude that in human fibroblasts GLUT1 de novo synthesis is involved in the long term effect of metformin on glucose transport.

The antidiabetic drug metformin (dimethylbiguanide) is widely used to treat obese type II diabetic patients. Metformin has no hypoglycemic effect in non-diabetic individuals and no effect on insulin secretion, although a certain quantity of endogenous or exogenous insulin is a prerequisite for its therapeutic effect. Reduced hepatic gluconeogenesis and diminished intestinal absorption of carbohydrates may contribute to the glucose lowering effect of the drug, but are not sufficient to explain the antihyperglycemic action. Several studies in vivo and in vitro have indicated that metformin reduces peripheral insulin resistance by stimulating glucose uptake in insulin target tissues, namely skeletal muscle and adipocytes (reviewed in 1 and 2). We have recently shown (3) that incubation of isolated rat adipocytes with metformin for 2 hours increased insulin stimulated glucose transport up to 43 percent. This effect was neither associated with an increase in insulin binding nor in insulin receptor kinase activity. Instead we found a metformin induced potentiation of insulin induced translocation of glucose transporters GLUT1 and GLUT4 from an intracellular pool to the plasma membrane after a preincubation of the fat cells with metformin. Glucose transporter gene expression was not affected by the short term metformin treatment. The aim of this study was therefore: 1) to investigate the cellular mechanism of long term in vitro metformin treatment on glucose transporter protein and mRNA, and 2) to determine whether such a cellular mechanism is different in cells obtained from diabetic patients compared to those from controls.

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MATERIALS AND METHODS

Cell culture

Human fibroblasts were cultured from forearm skin biopsies of normal and diabetic volunteers. The cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 20 mM HEPES, 10 % fetal calf serum, 100 µg/ml streptomycin and 80 units/ml penicillin in a humidified atmosphere of 95 % air and 5 % CO_2 at 37°C. The cells were used between the 6th and 14th passages. For cellular membrane preparation and RNA isolation cells were grown to confluency and monolayers were exposed to 0-100 µg/ml metformin (Lipha, France) for 4 days or to 10 µg metformin for 0-16 days, respectively.

Preparation of total cellular membranes and immunodetection of glucose transporters

Confluent monolayers were washed twice with PBS and then harvested in ice cold TES-PI buffer containing 20 mM Tris-HCl, 1 mM EDTA, 255 mM sucrose, 2.5 µg/ml leupeptin, 2.5 µg/ml pepstatin and 2.5 µg/ml aprotinin. After sonication was performed for 4 x 20 sec, the homogenate was centrifuged at 300,000 x g for 15 min (4°C). Crude membrane pellets were resuspended in 150-200 µl TES-PI and protein content determined using the method described by Bradford (4). 100 µg protein were taken up in SDS sample buffer and analyzed by sodium dodecyl sulfate-9% polyacrylamide gel-electrophoresis (5). The proteins were transfered to nitrocellulose membranes (6) and reacted with GLUT1 antiserum (East Acres Biologicals, Southbridge, MA). Crossreacting protein was visualized using [^{125}I]-protein A (New England Nuclear, Boston, MA) according to the method of Wheeler et al. (7). The filters were then exposed to Kodak X-ray films at -80°C and finally for quantitation the autoradiographs were scanned by laser densitometry.

Isolation of cellular RNA and Northern blot analysis

Confluent monolayers were washed twice with PBS and then total cellular RNA was isolated according to the method of Chomczynski (8). 15 µg aliquots of RNA were separated by 1.2 % agarose gel electrophoresis, blotted to nylon filters and probed with the GLUT1 cDNA (kindly provided by Dr. M. Mueckler, Washington University, St. Louis, MO). Radiolabeling with [^{32}P]-UTP (New England Nuclear, Boston, MA) was performed with the riboprobe kit from Stratagene (La Jolla, CA). The hybridization and washing procedures were as described (9) and autoradiography was performed at -80°C. Finally autoradiographs were quantitated by laser densitometry.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical significance was evaluated by Student's t test.

RESULTS AND DISCUSSION

We obtained the forearm skin biopsies from a group of volunteers which consisted of 8 type II diabetic patients (3 male, 5 female, age 64 ± 3 years) and 7 controls (3 male, 4 female, age 55 ± 3 years). None of the controls had a family history for type II diabetes. The average duration of the disease in the diabetic group was 8 ± 2 years. $HbA1_c$ data were significantly different with 9.2 ± 0.9 % for diabetics vs. 5.5 ± 0.2 % for controls (normal range 4.5 - 6.0 %). Of the 8 type II diabetic patients, 2 patients were on diet alone, 5 patients were on sulfonylurea and one patient was on insulin plus sulfonylurea. None of these patients was treated with metformin.

Fig. 1 demonstrates the effect of metformin on glucose transporter number in human fibroblasts, determined by the Western blotting method using antibodies against the c-

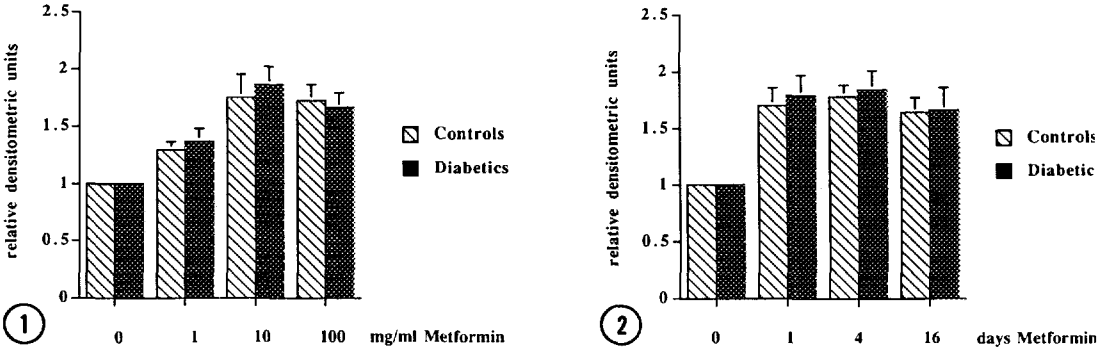

Fig. 1. Dose dependent effect of metformin on GLUT1 protein in cultured human fibroblasts. Cells were incubated with the indicated metformin concentation for 4 days and after crude membranes were prepared, 100 µg of protein from each sample was analyzed by Western blotting. Glucose transporter was identified using an antiserum against the COOH-terminal peptide of GLUT1. Graph illustrates means ± S.E.M of relative increase in GLUT1 protein of 15 independent and in duplicate performed membrane preparations.

Fig. 2. Time dependent effect of metformin on GLUT1 protein in cultured human fibroblasts. Cells were incubated with 10 µg/ml metformin for the indicated duration of time and after crude membranes were prepared, 100 µg of protein from each sample was analyzed by Western blotting. Glucose transporter was identified using an antiserum against the COOH-terminal peptide of GLUT1. Graph shows means ± S.E.M of relative increase in GLUT1 protein of 15 independent and in duplicate performed membrane preparations.

terminal part of GLUT1. Incubation of fibroblasts with increasing concentrations of metformin for 4 days resulted in a dose dependent increase of the immunodetectable number of GLUT1. A significant effect of metformin was already observed at a concentration of 1 µg/ml, which is about half of the maximal plasma concentration after oral administration of the drug (2). The highest effect was found at a metformin concentration of 10 µg/ml with a 1.86 ± 0.16 fold increase in transporter number in cells from control subjects compared to 1.75 ± 0.20 fold in cells from diabetics. At 100 µg/ml metformin's effect was slightly lower. No significant difference in the maximal increase of GLUT1 protein after metformin preincubation was observed between fibroblast strains from type II diabetic patients and those from controls at any of the tested concentrations of the drug.

The following experiments were carried out at 10 µg/ml metformin, since at this concentration we observed the highest increase in GLUT1 protein level. Fig. 2 depicts the result of the subsequent time course experiment. Incubation of fibroblasts with metformin for 24 hours, 96 hours and 16 days caused a significant increase in the amount of GLUT1 protein compared to cells which were not exposed to the drug. The maximal effect appeared after only 24 hours incubation with metformin and it was of no statistically significant difference whether fibroblasts were incubated with metformin for 24 hours or for a longer period of time, respectively. The maximal average increase in GLUT1 protein was 1.82 ± 0.14 fold. Again, immunodetection did not reveal significant

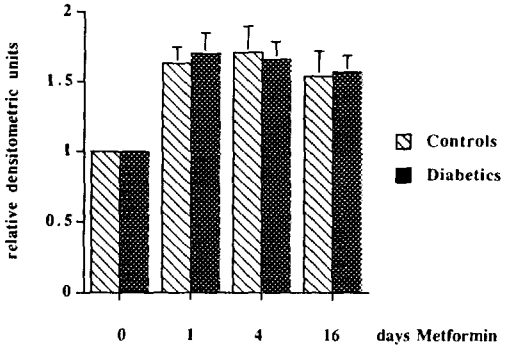

Fig. 3. Effect of metformin on GLUT1 mRNA in cultured human fibroblasts. Cells were preincubated with 10 μg/ml metformin for the indicated duration of time and after total RNA was prepared, 15 μg of RNA from each sample was analyzed by Northern blotting and probed with a riboprobe derived from the GLUT1 cDNA. Graph depicts means ± S.E.M of relative increase in GLUT1 mRNA of 15 independent and in duplicate performed RNA preparations.

differences in GLUT1 protein between fibroblast strains obtained from type II diabetic patients and those from controls.

To examine whether this metformin effect was associated with an increased GLUT1 gene expression, we isolated RNA from fibroblasts which were incubated under the same conditions as in the previous experiments, i. e. with 10 μg/ml metformin for 0, 1, 4 or 16 days, respectively. Northern blot hybridization of RNA from cultured fibroblasts with a [^{32}P]-UTP labeled riboprobe derived from the GLUT1 cDNA exhibited a single band with a size of about 2.8 kb. Fig. 3 demonstrates the average relative GLUT1 mRNA levels determined in 15 strains of fibroblasts from 7 control and 8 diabetic subjects. A significant increase in GLUT1 mRNA was detected after 24 hours of incubation with metformin. This effect was of no significant difference to the effect observed after 96 hours of incubation, while after 16 days the increase in GLUT1 gene expression was slightly lower. Glucose transporter mRNA was maximally induced 1.71 ± 0.19 fold by metformin. The average increase in mRNA levels in cells from diabetics and those from controls were again of no statistically significant difference. However, after different periods of exposure to 10 μg/ml metformin the magnitude of changes in GLUT1 protein amount and GLUT1 mRNA levels were quantitatively concordant. Therefore this effect appears to be independent from possible mechanisms of posttranslational regulation of GLUT1 levels by metformin. We did not observe significant differences in GLUT1 protein or mRNA in cells that were not treated with metformin between diabetics and controls in any of our described experiments. Under none of the described conditions did metformin treatment alter the total amount of protein per cell.

Recent studies suggested that the major effect of metformin is to stimulate glucose utilization in peripheral tissues by enhancing insulin action through effects distal to the insulin receptor kinase, i. e.,increasing insulin stimulated glucose transport (1, 2, 10). In

isolated rat adipocytes and in cultured L6 muscle cells this effect involves potentiating insulin induced translocation of glucose transporters from an intracellular pool to the plasma membrane (3, 11). In several studies the increase in translocation could not fully account for metformin's effect to stimulate 2-deoxyglucose or 3-O-methylglucose uptake. This may be due to an increased intrinsic activity of the glucose transporter or to an enlarged total number of either GLUT1 or GLUT4 transporters, i. e. augmented glucose transporter de novo synthesis. Both possible effects have not been observed so far. In this study we found in cultured human fibroblasts an increase in the total amount of GLUT1 protein after exposure of the cells to various concentrations of metformin for different periods of time. This was correlated to an increase in GLUT1 gene expression, indicating that glucose transporter protein synthesis may partly be responsible for long term effects of metformin on glucose transport. GLUT1 is currently known to be the most important, if not the only glucose transporter to be expressed in fibroblasts. Insulin is able to stimulate glucose uptake in cultured fibroblasts and glucose transport activity corresponds with GLUT1 expression (12). However, fibroblasts are not representative of the major insulin target tissues. The insulin responsiveness of muscle and fat is associated with the presence of GLUT4, which is at least ten times more abundant than GLUT1 in these tissues. Insulin resistance in diabetes is associated with a pretranslational suppression of GLUT4, while GLUT1 mRNA and protein are unchanged (13). The latter finding is consistent with our observations in those cells which were not treated with metformin, in which we did not find a difference between fibroblasts from diabetics and cells from controls. Considering that the role of GLUT1 in normal and diabetic states is not fully understood, one can only speculate on a possible importance of a metformin-induced increase in this transporter species for the improvement of insulin resistance and diabetes. Assuming that basal glucose transport is associated with GLUT1, a metformin-induced increase in GLUT1 protein would consecutively lead to an enhanced glucose disposal into many tissues, not only muscle and fat, where glucose transport is rapidly regulated by insulin. Elevated GLUT1 expression could therefore contribute to the antihyperglycemic action of metformin. However, it is also possible that long term metformin treatment increases as well mRNA and protein abundance of GLUT4 in insulin target tissues. Further studies using other experimental systems have therefore to be undertaken to clarify this issue and are currently underway in our laboratory.

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