Effects of Insulin and Metformin on Glucose Metabolism in Rat Vascular Smooth Muscle

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Glucose metabolism in vascular smooth muscle cells (VSMCs) is characterized by substantial lactate production even in fully oxygenated conditions. Insulin and metformin, an insulin-sensitizing agent, have direct effects on the vascular tissue metabolism. We investigated whether insulin or metformin can induce a switch in VSMC glucose metabolism from lactate production to pyruvate oxidation, by measuring lactate oxidation as determined by the conversion of [1-^{14}C]-D,L-lactate to [1-^{14}C]-pyruvate and subsequent oxidation to acetyl coenzyme A and $^{14}CO_2$ by pyruvate dehydrogenase (PDH). Lactate oxidation was measured in control rat aortic cultured VSMCs incubated for 30 minutes in media with and without additional glucose compared with VSMCs cultured in the presence of insulin or metformin. The addition of glucose to VSMCs decreased lactate oxidation (4.6 ± 1.7 *v* 9.6 ± 2.4 pmol/cell/min, $P < .001$). In the absence of additional glucose, metformin decreased lactate oxidation in VSMCs compared with controls (4.9 ± 1.4 *v* 9.6 ± 2.4 pmol/cell/min, $P < .01$). Metformin in the presence of glucose caused the greatest decline in lactate oxidation (2.5 ± 0.4 pmol/cell/min, $P < .001$). In contrast to the effects of metformin, insulin increased lactate oxidation both with (12.9 ± 1.5 pmol/cell/min, $P < .001$) and without (17.9 ± 4.4, $P < .01$) additional glucose. This suggests that insulin facilitates VSMC utilization of lactate as a source of pyruvate and energy production even during noncontractile periods.

CURRENT INVESTIGATIONS of glucose metabolism in vascular smooth muscle cells (VSMCs) have determined that VSMC glycolysis primarily produces lactate from pyruvate under fully oxygenated conditions,[1-3] rather than pyruvate oxidation via the mitochondrial Krebs cycle, the more common pathway for most tissues. The mechanism involved in this increase in lactate is based on the observation that carbohydrate metabolism in VSM may occur via two functionally independent pathways.[4-6] One pathway involves lactate formation from the catabolism of extracellular glucose, while the other is directed at energy production from the oxidative metabolism of glucosyl units derived from glycogen.[7,8] In support of these two pathways, studies have determined that energy generated during lactate production from extracellular glucose is specifically coupled to sodium-potassium transport in vascular tissues,[9-11] while the energy derived from oxidative metabolism of glycogen and fatty acids is mainly used for active isometric force.[12-14] The evidence describing these pathways in VSMCs, based on in vitro studies of labeled glucose[3-9,12] or measurement of changes in pH,[15-17] is inconclusive. The relative importance of these pathways may be better established by measuring mitochondrial lactate oxidation to determine the amount of pyruvate and lactate used in oxidative metabolism.

Insulin has been reported to increase glucose uptake in VSMCs[18,19] and to decrease intracellular pH by generating lactate. Similarly, metformin has also been associated with an increase in aerobic lactate production and lactic acidosis.[20-22] In VSMCs, metformin increases glucose transport and utilization[23,24] similar to the action of insulin.[25-28] The effects of insulin and metformin on glucose metabolism at the level of mitochondrial oxidation in VSM have not been investigated. The influence of insulin and oral hypoglycemic agents such as metformin on VSMC glucose metabolism is of considerable clinical significance.

The effects of insulin and metformin on glucose metabolism and lactate utilization in VSMCs were investigated in this study by measuring mitochondrial lactate oxidation. We hypothesized that insulin and metformin have different effects on mitochondrial function in VSMCs. Whereas metformin favors lactate production, insulin enhances mitochondrial oxidative phosphorylation and lactate utilization, thereby protecting VSMCs from lactic acidosis.

MATERIALS AND METHODS

Cell Preparation

Four- to 7-week-old male Sprague-Dawley rats (200 to 250 g) were purchased from Charles River Laboratories (Wilmington, MD) and fed standard chow and water ad libitum. Aortic VSMCs were grown from primary cultures as previously described.[18,19] Rats were anesthetized, and the thoracic aorta (from the aortic arch to diaphragm) was removed and transferred to a sterile petri dish containing 2 mL Hanks balanced salt solution (HBSS). The fatty tissue was removed and the vessel washed free of blood. A longitudinal incision was made in the vessel, and the endothelial layer was removed by gentle rubbing. The denuded vessel was minced into small (2- to 4-mm) pieces and placed in 10 mL sterile digestion medium containing 0.5 mg/mL collagenase, 0.3 mg/mL soybean trypsin inhibitor, 0.4 mg/mL papain, 7.5 mg/mL bovine serum albumin, and 0.5 mg/mL dithiothreitol (Sigma Chemical, St Louis, MO) in Dulbecco's modified Eagle's medium ([DMEM] Mediatech, Washington, DC), pH 7.40. The tissue was digested for 90 minutes in a 37°C shaking water bath (300 rpm). The released VSMCs were washed, and the final pellet was resuspended in 3 mL DMEM supplemented with 20% fetal bovine serum ([FBS] Biocell, Carson, CA), 0.2% tylosin, 50 U/mL penicillin G potassium, and 50 μg/mL streptomycin sulfate (GIBCO, Grand Island, NY). Cells were plated into six-well tissue culture dishes and placed in an incubator under 5% CO_2 and 100% humidity at 37°C. After the initial plating in 20% FBS growth medium, cells were passed onto 100-mm tissue culture dishes to obtain an

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adequate number of cells for subsequent experiments. The cells were fed every other day by aspirating the growth medium and replacing it with fresh DMEM containing 10% FBS. Cells were positively identified as VSMCs by treatment of confluent culture dishes with 0.1% trypsin and 1 mmol/L EGTA in Ca^{2+}/Mg^{2+}-free HBSS for 5 minutes at 37°C, followed by immunofluorescent staining specific for smooth muscle–specific actin and myosin.[18]

Tissue Culture

Cells were counted on a Coulter counter (model ZM; Fullerton, CA), subcultured into 25-cm^2 tissue flasks, and grown as monolayer cultures in DMEM supplemented with 10% FBS, 50 U/mL penicillin G potassium, and 50 μg/mL streptomycin sulfate. The cells were grown and maintained at 5% CO_2 in a 37°C humidified incubator. Media were replaced twice per week. Cells were passed during the logarithmic growth phase and transferred for a maximum of 10 passages. Cultures were routinely screened for mycoplasma contamination with Mycotrim TC (Hanna Biologicals, Alameda, CA).

Cells were prepared for the assay soon after they reached confluence. To evaluate the medication effects, metformin (1 mmol/L) was added 24 hours before the assay, whereas insulin (100 μU/mL) was added at the beginning of the assay. Our group has previously reported insulin-like effects of metformin on tyrosine kinase activity and glucose transport after 24 hours in rat VSMCs at a concentration of 1 mmol/L without any evidence of cellular toxicity.[24] The cells remained in the tissue culture flasks for the duration of assay with four flasks per group. The cell number and morphology were used to monitor cellular toxicity. Individual assay results are based on the mean of quadruplicate samples. Assays were performed three times for each group, and cumulative data are expressed as the mean ± SE for all measurements.

Lactate Oxidation Assay

In vitro metabolism of [1-^{14}C]-D,L-lactate was measured in VSMCs grown in normal growth media. At the appropriate time before the assay, the cells were rinsed twice in phosphate-buffered saline (PBS). Glucose-depleted medium containing 0.1 mmol/L glucose and 25 mmol/L potassium phosphate for a final of pH 7.4 was prepared without FBS and sodium bicarbonate. The lactate oxidation assay was performed in cells divided into six groups: (1) glucose-depleted medium, (2) glucose-depleted medium with 100 μU/mL insulin, (3) glucose-depleted medium with 1 mmol/L metformin, (4) glucose-depleted medium with additional glucose (5 mmol/L), (5) depleted medium with additional glucose and 100 μU/mL insulin, and (6) depleted medium with additional glucose and 1 mmol/L metformin. The assays were initiated by the addition of 1 mL glucose-free media with or without additional glucose, containing either insulin or metformin as appropriate, 25 μmol/L L-lactate (Sigma), and 1 μCi/mL [1-^{14}C]-D,L-lactate (American Radiochemical, St Louis, MO). $^{14}CO_2$ was collected on filter paper moistened with 0.2 mL 2.5-mol/L NaOH and suspended directly above the monolayer in each flask with an airtight stopper, and the flasks were slowly shaken at 37°C for 30 minutes. (We previously measured lactate oxidation in VSMCs every 10 minutes for 2 hours and determined that $^{14}CO_2$ accumulation was linear for 60 minutes, and the period of 30 minutes is comparable to our previously reported data for insulin's effects on lactate oxidation in other cell types.[29-31]) Each filter was then transferred to a scintillation vial, and the amount of $^{14}CO_2$ collected was determined. After washing the flasks three times with PBS, the cells were trypsinized and counted. The cells were lysed, and total protein was determined by the bicinchoninic acid assay. Activity is expressed as picomoles of $^{14}CO_2$ released per cell per minute. Background activity was determined using flasks without cells, containing the appropriate glucose concentration, 25 μmol/L L-lactate, and 0.1 μCi/mL [1-^{14}C]-D,L-lactate in 1 mL glucose-free medium and the appropriate concentration of insulin or metformin. Background activity was then subtracted from total radioactivity for each sample.[29-31]

Data Analysis

The effects of insulin and metformin on lactate oxidation in glucose-free and glucose-supplemented conditions were compared by the Student *t* test. The results were considered significant at a *P* level less than .05.

RESULTS

Effect of Insulin and Metformin on Mitochondrial Lactate Oxidation

There was no difference in the cell number or cell morphology as a result of the treatment among any groups (data not shown). Mitochondrial lactate oxidation in VSMCs is presented in Fig 1. The rate of lactate oxidation for group 1 (control VSMCs in glucose-depleted media) was 9.6 ± 2.4 pmol/cell/min. Lactate oxidation for group 2 (insulin-treated VSMCs in glucose-depleted media) increased to 17.9 ± 4.4 pmol/cell/min ($P < .01$ *v* group 1). The rate of lactate oxidation for group 3 (metformin-treated VSMCs in glucose-depleted media) decreased to 4.9 ± 1.4 pmol/cell/min ($P < .01$ *v* group 1 and $P < .001$ *v* group 2).

In group 4 (glucose-treated VSMCs), lactate oxidation decreased to 4.6 ± 1.7 pmol/cell/min ($P < .01$ *v* group 1). In group 5 (insulin and glucose–treated VSMCs), lactate oxidation increased to 12.9 ± 1.6 pmol/cell/min ($P < .01$ *v* group 4), and decreased compared with insulin-treated glucose-depleted VSMCs ($P < .01$ *v* group 2). In group 6 (metformin and glucose–treated VSMCs), the rate of lactate oxidation de-

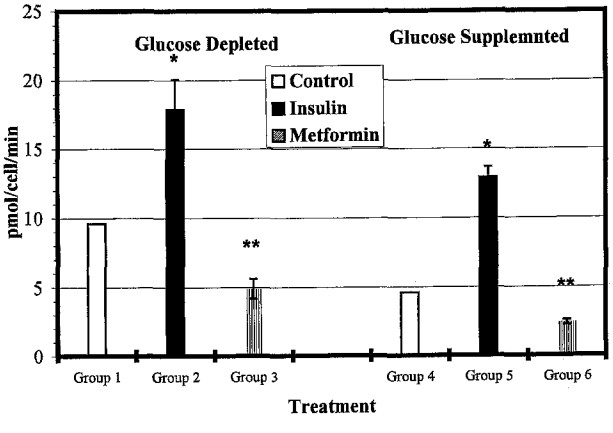

Fig 1. Effect of metformin and insulin on mitochondrial lactate oxidation in VSMCs during glucose depletion or supplementation. Lactate oxidation increased in glucose-depleted VSMC (group 1) *v* glucose-supplemented cells (group 4, *$P < .01$). Insulin treatment increased lactate oxidation, indicating stimulation of PDH activity in glucose-deprived cells (group 1 *v* 2, *$P < .01$), while metformin treatment decreased lactate oxidation (group 1 *v* 3, *$P < .01$; group 2 *v* 3, **$P < .001$). In the presence of glucose, insulin decreased lactate oxidation (group 4 *v* 5, *$P < .01$), while metformin treatment decreased lactate oxidation (group 4 *v* 6, *$P < .01$; group 5 *v* 6, **$P < .001$). Lactate oxidation was determined in VSMCs by determining the amount of 1-$^{14}CO_2$ collected for 30 minutes and is expressed as pmol/cell/min (n = 4 flasks for each condition per assay and 3 assays per determination).

creased to 2.4 ± 0.4 pmol/cell/min ($P < .01$ *v* groups 4, 2, and 1 and $P < .001$ *v* group 5).

DISCUSSION

VSMCs are known to produce a large amount of lactate from glucose.[1-7] The subsequent metabolism of this lactate, the maintenance of lactate/pyruvate balance, and their respective roles in mitochondrial oxidation are important in understanding glucose metabolism. We determined the lactate oxidation activity to better delineate the effects of insulin and metformin on glucose metabolism either toward lactate production or through the mitochondrial processing of pyruvate via the Krebs cycle. We have previously shown that in fibroblasts, adipocytes, and Chinese hamster ovary (CHO) cells, insulin increases glucose uptake and stimulates mitochondrial pyruvate dehydrogenase (PDH), thereby increasing the oxidative metabolism of pyruvate.[29-31] In these cells, the glycolytic pathway activity accounting for lactate production and that accounting for oxidative phosphorylation are regulated by insulin via PDH and can be measured by lactate oxidation activity.[29]

We have previously determined PDH activity by measuring $^{14}CO_2$ directly from cells growing in culture. This method requires a minimum number of cells with minimal handling. As the glucose in the medium is depleted, there is a gradual shift from glucose metabolism to lactate metabolism that is measured by a gradual increase in $^{14}CO_2$ accumulation.[29] We have previously confirmed the accuracy of this method as an indicator of PDH activity in the presence of insulin.[29-33] This is the first time lactate oxidation has been used to study the effects insulin and metformin on glucose metabolism in VSMCs. Therefore, before any similarities can be established between the effects of insulin and metformin on PDH activity in VSMCs, PDH activity must be measured directly.

As glucose is depleted in the medium, lactate oxidation is expected to increase in order to maintain the pyruvate substrate required for oxidative metabolism. We observed that the shift from glucose oxidation to lactate oxidation as a result of a decreased glucose concentration is relatively small in VSMCs (37%) at rest as compared with our observations in other tissues such as a smooth muscle–like cell line (BC_3H1) at rest and CHO cells during growth, where lactate oxidation increases by 96% and 63%, respectively.[29]

The addition of glucose to VSMCs results in glycolysis, increasing the concentration of pyruvate and decreasing the activity of lactate dehydrogenase, shifting the equilibrium of the reaction toward the formation of lactate from pyruvate.[34,35] This shift in equilibrium dilutes the labeled lactate available for conversion to pyruvate. VSMCs, because of the high number of mitochondria and tonic muscle activity, might be expected to have a resting lactate oxidation rate similar to that of BC_3H1 cells. However, we observed that the rate of lactate utilization during aerobic conditions is considerably lower in resting VSMCs compared with our previous findings in BC_3H1 cells at rest.[29] It is unclear whether the pyruvate generated from increased glycolysis is used to increase oxidative metabolism or to increase lactate production. The low lactate oxidation rate of VSMCs in glucose-supplemented media and the merely slight shift to lactate oxidation with glucose depletion do not argue conclusively for or against the utilization of either lactate or pyruvate as an energy source for different cellular functions.

Although insulin has been shown to increase PDH activity in fibroblasts,[29,30,33] its effect on VSMC mitochondria has not been clearly defined. In many cells, the increase in lactate oxidation in response to a decrease in glucose can be accentuated by insulin, which stimulates PDH activity. We have previously shown that treatment with insulin stimulates lactate oxidation in glucose-deprived BC_3H1 cells by 20%, in CHO cells by 0.5%, in fibroblasts by 26%, and in adipocytes by 19%.[32] The present study demonstrates that insulin increases lactate oxidation by 43% in VSMCs deprived of glucose, suggesting that insulin modulates PDH activity.

In the presence of glucose, insulin has been reported to induce intracellular lactic acid production in VSMCs.[17] An increase in aerobic lactate production by VSMCs may result in lactic acidosis in certain vascular diseases such as hypertension and atherosclerosis.[20-22] It has also been argued that diverting glucose metabolism from lactate production to the oxidative pathway has an unfavorable effect on high-energy phosphate metabolism in resting VSMCs.[1] Insulin is known to increase glucose uptake in various tissues, including VSMCs.[24] The effect of insulin on lactate oxidation is different in VSMCs compared with other tissues. In BC_3H1 and CHO cells, insulin increases lactate oxidation with glucose depletion and attenuates lactate oxidation with glucose supplementation. In contrast, insulin increases lactate oxidation in VSMCs in glucose-depleted or -supplemented environments. These observations indicate that insulin regulates the lactic acid pool in VSMCs by increasing lactate utilization and thus protecting the cell from lactic acidosis.

Metformin (dimethyl-biguanide), an insulin-sensitizing agent used in the treatment of type 2 diabetes mellitus, and its precursor phenformin (phenetyl-biguanide) are known to cause lactic acidosis as a potential side effect.[36,37] Although metformin has been reported to stimulate glucose utilization, it is not known whether metformin has insulin-like effects on mitochondria.[24] Metformin decreased the rate of mitochondrial lactate oxidation by 49% in the absence of glucose. This decrease in lactate oxidation is in contrast to the effects of insulin, which increased lactate oxidation in VSMCs by 43%. It has been demonstrated that metformin increases basal and insulin-stimulated glucose transport in isolated adipocytes, skeletal muscle, and VSMCs.[24] However, the mechanism for this process is not completely understood. In contrast to the effects of insulin, metformin significantly decreased lactate oxidation in VSMCs in the presence of glucose. These results may help to explain metformin's propensity to cause lactic acidosis. Unlike insulin, metformin does not appear to have the ability to increase mitochondrial lactate oxidation and regulate the lactate pool. Although metformin exhibits insulin-like effects with respect to glucose uptake in peripheral tissues, unlike insulin, it decreases oxidative metabolism in VSMCs either with or without additional glucose. The action of metformin to selectively increase glucose metabolism to lactate may be harmful to VSMCs.

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