Insulin increases NO-stimulated guanylate cyclase activity in cultured VSMC while raising redox potential

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Insulin increases NO-stimulated guanylate cyclase activity in cultured VSMC cells while raising the redox potential. Am J Physiol Endocrinol Metab 278: E627–E633, 2000.—Insulin acutely stimulates cyclic guanosine monophosphate (cGMP) production in primary confluent cultured vascular smooth muscle cells (VSMC) (5, 14, 22, 31). cGMP attenuates the contractile agonist-induced intracellular Ca2+ (Ca2+1) transient in vascular smooth muscle cells (VSMC), thereby decreasing VSMC contraction. cGMP also inhibits VSMC contraction at sites distal to Ca2+1 by affecting contractile protein function (14). In addition, cGMP inhibits VSMC proliferation and migration (5, 22, 31), which are important events contributing to atherosclerosis and restenosis following balloon angioplasty (20).

It has been suggested that insulin stimulates cGMP production in blood vessels. For instance, insulin acutely stimulates nitric oxide (NO) production in cultured human umbilical vein endothelial cells (32), and insulin-induced vasodilation in humans is blocked by an inhibitor of nitric oxide synthase (NOS) (23, 27). It has been proposed that insulin-induced vasodilation is mediated, at least in part, by the hormone’s stimulation of NO production by endothelial cells causing inhibition of contraction of the underlying VSMC, in part, by stimulating VSMC guanylate cyclase activity. In addition, it has been reported that insulin directly increases cGMP production in cultured VSMC (29) and inhibits VSMC contraction in the absence of endothelial cells (12). We reported that physiological concentrations of insulin acutely increased cGMP production (9) and lowered serotonin [5-hydroxytryptamine (5-HT)-induced Ca2+ influx and the Ca2+1 transient in primary confluent cultured cells from canine femoral artery in a glucose-dependent manner (11). Immunoblots of these cells identified inducible NOS (iNOS) protein but not the endothelial constitutive NOS protein (10). The cells had NOS activity (9), and insulin’s stimulation of cGMP production was blocked by Nω-monomethyl-L-arginine (L-NMMA) (9).

A major route of glucose metabolism in vascular smooth muscle (VSM) is to lactate via aerobic glycolysis (19), which increases the redox potential of cells by generating NADH (17). Previous studies with VSMC and other cell types have shown that reducing and oxidizing agents increase and decrease nitrovasodilator-stimulated guanylate cyclase activity, respectively (2, 4, 13, 15).
The purpose of the present study was to determine the mechanism of insulin-induced stimulation of cGMP production in these primary confluent cultured VSMC. We found that insulin did not stimulate iNOS activity but that insulin increased NO-stimulated cGMP production, regardless of whether the NO was derived from VSMC iNOS or an exogenous source. We found that insulin's increase in NO-stimulated cGMP production was associated with an insulin-induced increase in aerobic glycolysis and the cell redox potential.

METHODS

Cell culture. Adult mongrel dogs of both sexes were killed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. Freshly dispersed VSMC and primary confluent VSMC cultures were prepared as previously described (8). Endothelia and adventitia were stripped away, and the media of the arteries were minced and incubated at 37°C in a solution containing elastase (type V, Sigma, St. Louis, MO) and collagenase (typel, Worthington Biochemical, Freehold, NJ). After 2 h, the enzyme solution was discarded and replaced with fresh solution, and the tissue was incubated for an additional 2 h. The dispersed cells were pelleted and washed three times in Hank's balanced salt solution and used for immunoblot studies or for cell culture, where they were suspended to a density of $2 \times 10^5$ cells/ml in DMEM that contained 1% glucose, 10% FCS, 1% penicillin-streptomycin solution (10,000 U/ml penicillin, 10 mg/ml streptomycin). One milliliter of this suspension was placed in 35-mm plastic culture dishes. After seeding, cells were incubated in a humidified tissue culture incubator maintained at 37°C and equilibrated with 5% CO2-95% air. After 72 h and every 72 h thereafter, the media were replaced with 1 ml of the same fresh medium. The cells became confluent in 2 wk when they were used. Gel electrophoresis and immunoblotting. Proteins (10–20 µg) obtained from freshly dispersed VSMC lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose paper by electrophobbing as previously described (10). Subsequent probing of the blots was performed with a site-directed rabbit polyclonal anti-iNOS antibody. Protein was calculated after subtracting basal values by use of authentic NO2- standards. Each experiment was performed in triplicate. cGMP assay. Dishes of primary confluent cultured VSMC were incubated for 30 min at 37°C in PSS plus 0.1% BSA and 0.5 mM of isobutylmethylxanthine to inhibit phosphodiesterase in the presence and absence of 0.1 mM L-NMMA, with or without 5-nitroso-N-acetyl-D,L-penicillamine (SNAP) and/or 1 mM of insulin. In some cases, 10 µM 5-HT were added to the incubation solution for the final 5 min. The solution was removed, and the reaction was stopped by adding 1 ml of ice-cold ethanol acidified with 0.8% 12 M HCl. The extract was centrifuged at 10,000 g for 15 min, the supernatant was evaporated to dryness in a Speed Vac SC100 (Savant, Holbrook, NY). For [14C]arginine and [14C]citrulline measurements, the residue was resuspended in 25 µl of deionized water containing 2 mg/ml L-arginine and 2 mg/ml L-citrulline and applied to silica gel-60 plates, 20 x 20 cm, (Whatman, Clifton, NJ) as previously described (9). A solvent system containing CHCl3-CH3OH-NH4OH-H2O, 1:4:2:1 (vol/vol) was used. Relative fronts for arginine and citrulline were 0.49 and 0.81, respectively, as determined by autoradiography of authentic [14C]arginine and [14C]citrulline standards (Amersham). The location of arginine and citrulline was confirmed by staining with ninhydrin spray. The amounts of [14C]arginine and [14C]citrulline associated with the incubated cells were determined by scraping the silica gel at the appropriate regions of the chromatograph into liquid scintillation vials, vortexing with scintillation cocktail, and counting. The percentage of [14C]arginine that was converted to [14C]citrulline was calculated and corrected for the percentage of the [14C]arginine stock contaminated with [14C]citrulline (~1%, determined in each experiment by TLC as described above). Each experiment was performed in triplicate. For combined NO2/NO3 measurements, the dried residue of the supernatant of centrifuged homogenized cells plus incubation media was suspended in 80 µl of phosphate assay buffer (20 mM, pH 7.4), preincubated with nitrate reductase plus cofactors, and incubated with Griess reagent, and absorbance was measured in 80-well microplates at 540 nm as described in the Cayman's nitrate/nitrite assay kit (Alexis, San Diego, CA). NO2 production per milligram of protein was calculated after subtracting basal values by use of authentic NO2 standards. Each experiment was performed in triplicate.

Fig. 1. Immunoblot showing presence of inducible nitric oxide synthase (iNOS) in freshly dispersed vascular smooth muscle cells (VSMC) and RAW 264.7 cells (RAW) induced with lipopolysaccharide. Cell lysates (20–30 µg protein) were electrophoresed on SDS-polyacrylamide gels and immunoblotted with anti-iNOS antibody.
measured by the method of Lowry. Although basal cGMP production varied from preparation to preparation, the relative effects of specific perturbations on cGMP production were consistent among different preparations. Thus cGMP production was calculated as a percentage of the amount produced under control conditions.

Lactate and pyruvate measurements. Dishes of primary confluent cultured VSMC were preincubated for 30 min in 1 ml of physiological salt solutions (PSS) + 5 mM L-[14C]arginine and 0.1% BSA at 37°C for 30 min with or without 0.1 mM l-NMMA or 1 mM insulin. Cells were incubated with or without additional 10 µM 5-HT for another 5 min, media were removed, cells were homogenized, and %[14C]arginine taken up that was converted to [14C]citrulline was determined by thin-layer chromatography. Data are expressed as % citrulline production under control conditions, which averaged 2.1% of arginine taken up. Data are results of 4 separate experiments performed in triplicate. *P < 0.05 vs. control.

RESULTS

iNOS in freshly dispersed VSMC. We reported previously that primary confluent cultured VSMC from canine femoral artery contain iNOS protein (10) and NOS activity (9), and that l-NMMA blocks insulin-stimulated cGMP production (9). To determine whether the freshly dispersed VSMC from this source contain iNOS, protein extracts from these cells were subjected to electrophoresis and immunoblotted with rabbit polyclonal anti-iNOS antibody. Protein extracts from the mouse macrophage cell line, RAW 264.7, in which iNOS had or had not been induced by previous exposure to lipopolysaccharide, were used as positive and negative controls, respectively. As shown in Fig. 1, immunoblots of lysates from freshly dispersed VSMC and induced RAW cells contained a single band stained positively by iNOS antibody, and it had the expected molecular mass for iNOS (131 kDa) (24). Noninduced RAW cells did not stain with iNOS antibody. Thus iNOS protein is present in freshly dispersed VSMC from canine femoral artery.

Effect of insulin on NOS activity. Because insulin inhibits 5-HT-induced contraction of primary cultured nonproliferated VSMC in an l-NMMA-sensitive manner (9), we determined whether insulin and/or 5-HT increases NOS activity in primary cultured confluent cells. To measure NOS activity, the percentage of conversion of [14C]arginine taken up by the cells to [14C]citrulline was measured. The 30-min uptake of [14C]arginine by l-NMMA-, 5-HT-, insulin-, and 5-HT + insulin-treated cells was 93 ± 9, 104 ± 11, 108 ± 12,

Fig. 2. Graph showing effects of N⁵-monomethyl-L-arginine (l-NMMA), insulin and 5-hydroxytryptamine (5-HT) on citrulline production by primary cultured VSMC. Dishes of VSMC were preincubated in physiological salt solutions (PSS) + 5 mM L-[14C]arginine and 0.1% BSA at 37°C for 30 min with or without 0.1 mM l-NMMA or 1 mM insulin. Cells were incubated with or without additional 10 µM 5-HT for another 5 min, media were removed, cells were homogenized, and %[14C]arginine taken up that was converted to [14C]citrulline was determined by thin-layer chromatography. Data are expressed as % citrulline production under control conditions, which averaged 2.1% of arginine taken up. Data are results of 4 separate experiments performed in triplicate. *P < 0.05 vs. control.

Lactate and pyruvate measurements. Dishes of primary confluent cultured VSMC were preincubated for 30 min in 1 ml of physiological salt solution (PSS) in room air at 37°C and then incubated in 1 ml of PSS, with or without glucose, plus the desired agents for an additional 3 min. The reaction was stopped with 0.08 ml of 60% perchloric acid, and the cells were scraped and homogenized. An aliquot was saved for lactate and pyruvate measurements.

Effect of insulin on NOS activity. Because insulin inhibits 5-HT-induced contraction of primary cultured nonproliferated VSMC in an l-NMMA-sensitive manner (9), we determined whether insulin and/or 5-HT increases NOS activity in primary cultured confluent cells. To measure NOS activity, the percentage of conversion of [14C]arginine taken up by the cells to [14C]citrulline was measured. The 30-min uptake of [14C]arginine by l-NMMA-, 5-HT-, insulin-, and 5-HT + insulin-treated cells was 93 ± 9, 104 ± 11, 108 ± 12,
and 97 ± 7% of control cells, respectively, which averaged 410 pmol arginine/mg protein \([n = 4, P = \text{not significant (NS)}]\). As shown in Fig. 2, citrulline production was inhibited by L-NMMA, an inhibitor of NOS (24), but neither 5-HT nor insulin, nor 5-HT + insulin affected citrulline production. NOS activity was also determined by measuring combined NO\(_3^-\)/NO\(_2^-\) production. The 30-min production of NO\(_3^-\) + NO\(_2^-\) by cells under control conditions, incubated with 10 µM 5-HT, 1 nM insulin, or insulin + 5-HT, were 17.5 ± 2.4, 14.4 ± 2.1, 13.7 ± 1.9, and 16.0 ± 2.1 pmol/mg protein, respectively \((n = 3; P = \text{NS})\). These data demonstrate that insulin, with or without 5-HT, does not acutely affect NOS activity in these cells.

Effects of SNAP and insulin on cGMP. To test whether the NO produced by iNOS in primary confluent cultured VSMC acted in a permissive way to allow insulin’s stimulation of cGMP production, cells were preincubated with or without 0.1 mM L-NMMA and/or 1 nM insulin and/or 0.1 µM SNAP for 30 min. Figure 3 shows that insulin increased cGMP production in the absence but not presence of L-NMMA, as we have previously reported (9). SNAP (0.1 µM) increased cGMP production relative to control, and SNAP + insulin increased cGMP production in an additive manner. When endogenous NOS activity was blocked by L-NMMA, the basal production of cGMP fell, and SNAP increased it to near its control value without L-NMMA. However, insulin failed to increase cGMP production when iNOS activity was blocked by L-NMMA, but the provision of enough SNAP to raise cGMP production to near the normal basal value enabled insulin to increase cGMP production further by 69% \((P < 0.05)\).

A dose-response relationship for SNAP is shown in Fig. 4. In the presence of L-NMMA, 0.01 µM of SNAP did not affect cGMP production, and 1 µM SNAP increased it fourfold. Under these conditions, insulin did not affect cGMP production but did so at an intermediate concentration of SNAP (0.1 µM). The data in Figs. 3 and 4 show that insulin alone cannot stimu-
late cGMP production, but when a moderate amount of NO is present, whether it comes from iNOS or 0.1 µM of SNAP, cGMP production is increased, and insulin stimulates it further.

Lactate-pyruvate measurements. Increasing and decreasing the redox potential in various cell types, including VSM, increases and decreases nitrovasodilator-induced guanylate cyclase activity, respectively (2, 4, 13, 15). To examine the mechanism of insulin’s stimulation of NO-induced cGMP production, we investigated the effect of insulin on aerobic glycolysis and the resultant effect on the cell redox potential. Primary confluent cultured VSMC were incubated in the presence or absence of 5 mM glucose with or without 1 nM insulin, and lactate levels were measured. As shown in Fig. 5A, basal lactate levels were greater in the presence (control) than in the absence of glucose, and insulin increased the lactate level, but only in the presence of glucose. In the presence of the glycolysis inhibitor, 2-deoxyglucose (2-DOG), the lactate level was decreased, and insulin was unable to increase it. These data indicate that these VSMC perform aerobic glycolysis, which has been described in VSM from other sources (19), and that insulin, which increases glucose uptake by these cells (11), stimulates aerobic glycolysis. As also shown in Fig. 5A, 0 glucose or 2-DOG tended to lower the pyruvate levels, but the results were not statistically significant. Insulin plus glucose did not affect the pyruvate levels significantly under these conditions, but importantly, as shown in Fig. 5B, insulin plus glucose increased the lactate-to-pyruvate ratio (LPR) compared with glucose alone (control). In the absence of glucose or in the presence of 2-DOG, the LPR was less than control and not stimulated by insulin. Taken together, the data in Fig. 5 indicate that insulin increases aerobic glycolysis, which increases the redox potential of the cells.

If insulin increases NO-stimulated cGMP production by raising the cell redox potential, then other maneuvers that raise the redox potential should also increase NO-stimulated cGMP production. To increase the redox potential in the absence of insulin, VSMC were incubated with β-hydroxybutyrate for 3 min. The lactate and pyruvate levels were measured and the LPR was calculated. As shown in Fig. 6A, β-hydroxybutyrate, a reducing fuel that is oxidized to acetoacetate, raised and lowered lactate and pyruvate levels, respectively. As shown in Fig. 6B, β-hydroxybutyrate increased LPR, reflecting its ability to increase the redox potential of the cell.

Redox potential and cGMP production. SNAP-stimulated cGMP production was measured in VSM cells exposed to the same conditions as those in Figs. 5 and 6. As shown in Fig. 7, in the presence of L-NMMA and SNAP, 1 nM of insulin increased cGMP production, in
agreement with the data in Figs. 3 and 4. In the absence of glucose or in the presence of 2-DOG, basal cGMP production was not different from control, and insulin did not increase it. As is also shown in Fig. 7, \( \beta \)-hydroxybutyrate, which increased the LPR, increased cGMP production in the absence of insulin. Thus, in the presence of a permissive amount of NO, insulin’s ability to increase cGMP production is associated with the hormone’s increase in the redox potential and can be duplicated by increasing the redox potential in the absence of insulin.

**DISCUSSION**

cGMP inhibits VSMC proliferation, migration, and contraction (5, 14, 22, 31). We have reported previously that insulin acutely increased cGMP production in primary confluent cultured VSMC from canine femoral artery (9). These cells contained iNOS protein (the endothelial constitutive NOS protein was not detected by immunoblot) (10), and had NOS activity (9), and insulin-stimulated cGMP production was blocked by \( L \)-NMMA (9). The present study was performed to determine the mechanism of insulin-stimulated cGMP production in these cells. We predicted that insulin would stimulate NOS activity; this was not the case.

In the present study, we showed that insulin did not acutely affect iNOS activity in these cells. We showed that insulin increased guanylate cyclase activity if guanylate cyclase was moderately stimulated by NO, regardless of whether the NO came from VSM iNOS or from an exogenous source (SNAP). We do not know whether insulin would increase guanylate cyclase activity in these cells if initially activated by agents other than NO. We also showed that iNOS in primary cultured VSMC from canine femoral artery was not merely an artifact of the culture process, because immunoblots of lysates of freshly dispersed VSMC from this source also stained positively for iNOS protein. This finding is consistent with previous reports that iNOS protein has been found in unstimulated VSM from kidney (16, 28).

In this study, we also examined how insulin increased NO-stimulated guanylate cyclase activity. We had previously shown that insulin stimulated glucose uptake and that insulin’s inhibition of VSM contraction required glucose transport (11). Because VSM performs aerobic glycolysis (19), we reasoned that insulin might stimulate aerobic glycolysis, which could increase the redox potential of the cell. This might enhance NO-stimulated guanylate cyclase activity because previous studies in VSM and other cell types showed that reducing and oxidizing agents increased and decreased nitrovasodilator-stimulated guanylate cyclase activity, respectively (2, 3, 13, 15).

In the present study, we demonstrated that in primary confluent cultured VSMC from canine femoral artery, insulin increased aerobic glycolysis and the redox potential of the cell by showing that insulin increased lactate accumulation and the LPR, respectively. This was accompanied by increased NO-stimulated cGMP production. When insulin-induced aerobic glycolysis was blocked by omitting glucose from the incubation media or by adding 2-DOG, insulin could increase neither LPR nor NO-stimulated guanylate cyclase activity. In the absence of insulin, the reducing fuel, \( \beta \)-hydroxybutyrate, increased both the redox state of the cell and NO-stimulated guanylate cyclase activity. Thus, with or without insulin, an increased redox potential is associated with increased NO-stimulated guanylate cyclase activity.

It is possible that NO from endothelial cells is normally responsible for sensitizing VSM guanylate cyclase to stimulation by insulin. The present studies suggest that, wherever its source, moderate levels of NO stimulate VSM cGMP more in the presence of insulin. It has been reported that iNOS is present at sites of athersclerosis or balloon angioplasty and in some sources of unstimulated VSMC (3, 16, 21, 28). We speculate that under these conditions moderate amounts of NO from the VSM cells themselves, without contribution from endothelial cells, may stimulate VSM guanylate cyclase activity, thereby allowing insulin to increase additional cGMP production, possibly resulting in decreased VSMC contraction, proliferation, or migration.

It is possible that insulin’s increase of the VSM redox potential reduces thiol groups on the catalytic region of soluble guanylate cyclase, thereby increasing enzyme activity when the regulatory region has been activated by NO. It is also possible that the insulin-increased redox potential protects a finite amount of NO from being degraded within the cell by endogenous oxidizing agents, thereby increasing cGMP production. The present studies, however, show only an association between the insulin-induced increase in the cell redox potential and insulin’s stimulation of NO-induced cGMP production. Future studies are needed to determine whether this association is due to a cause-and-effect relationship.

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