Insulin Inhibits Migration of Vascular Smooth Muscle Cells With Inducible Nitric Oxide Synthase

Andrew M. Kahn, Julius C. Allen, Charles L. Seidel, Sui Zhang

Abstract—Vascular smooth muscle cell (VSMC) migration participates in atherosclerosis and arterial restenosis after balloon angioplasty. Because these processes are enhanced in insulin-resistant states, our goal was to determine whether insulin affects VSMC migration and, if so, how. The migration of primary cultured VSMCs from canine femoral artery was measured with the use of a wound migration assay and related to cGMP levels. Insulin (1 nmol/L) did not affect migration or cGMP production in control cells. When inducible nitric oxide synthase (iNOS) was induced by 24-hour preincubation with lipopolysaccharide and interleukin-1β, basal migration decreased, cGMP production increased, and insulin inhibited migration by >90% and stimulated cGMP production by 3-fold. The nitric oxide synthase inhibitor Nω-monomethyl-L-arginine blocked the affect of insulin on the migration of VSMCs with iNOS. 8-Bromo-cGMP inhibited VSMC migration in control cells, and 1-[[1,2,4]oxadiazolo-[4,3-a]quinoxolin-1-one, a selective inhibitor of guanylate cyclase, blocked the inhibition by insulin of migration of cells with iNOS. We conclude that insulin does not normally affect cGMP production or the migration of these VSMCs. However, after the induction of iNOS, insulin stimulates cGMP production and inhibits migration via an NOS-and a cGMP-dependent mechanism. (Hypertension. 2000;35[part 2]:303-306.)

Key Words: cyclic 3′,5′guanosine monophosphate ■ muscle, smooth, vascular ■ nitric oxide ■ insulin

Insulin resistance, which occurs in obesity, non–insulin-dependent diabetes mellitus, and essential hypertension, is associated with increased atherosclerosis and restenosis after balloon angioplasty.1–3 But the reasons for this are obscure. The migration of vascular smooth muscle cells (VSMCs) from the arterial media through the internal elastic membrane into the neointima is an integral part of the pathogenesis of atherosclerosis and restenosis.4 The insulin-resistant conditions noted are marked by hyperinsulinemia as the pancreas attempts to maintain plasma glucose concentration at a normal level.1

Because hyperinsulinemia has been thought to aggravate atherosclerosis or restenosis,5 several investigators have studied the effects of insulin on VSMC migration.6–8 Although some studies have shown a small stimulation of VSMC migration with insulin, the results are controversial. In some studies, the concentrations of insulin that were used were supraphysiological,8 such that the insulin-like growth factor-I pathway may have been activated. Insulin-like growth factor-I is known to stimulate VSMC migration.9 In other studies, insulin alone had no effect on VSMC migration but increased it when it was stimulated by another agent.7

We have reported that physiological concentrations of insulin stimulated cGMP production in primary cultured VSMCs from canine femoral artery.10,11 These cells were cultured under conditions such that they expressed the inducible isoform of nitric oxide synthase (iNOS),11 and an inhibitor of NOS blocked the stimulation by insulin of cGMP production.10 Because cGMP is known to inhibit VSMC migration,12 our goal was to determine whether insulin inhibits the migration of these VSMCs via a cGMP-dependent mechanism and whether this process is dependent on the presence of iNOS.

Methods

Cell Culture

Adult mongrel dogs of either gender were killed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. The 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 Chemical Co) and collagenase (type I; Worthington Biochemical). After 2 hours, the enzyme solution was discarded and replaced with fresh solution, and the tissue was incubated for an additional 2 hours. The dispersed cells were pelleted, washed three times in Hank’s balanced salt solution, and suspended to a density of 2×105 cells/mL in DMEM (<0.1 ng/mL endotoxin; Sigma Chemical Co), which contained 1% glutamine, 10% FCS, and 1% penicillin/streptomycin solution (10 000 U/mL penicillin and 10 mg/mL streptomycin), and 1 mL of this suspension was placed into 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

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From the Division of Nephrology and Hypertension (A.M.K., S.Z.), Department of Medicine, The University of Texas Health Science Center (Houston); and Department of Medicine (J.C.A., C.L.S.), Baylor College of Medicine, Houston, Tex.

Correspondence to Andrew M. Kahn, MD, 4.107 MSB, University of Texas Health Science Center, PO Box 20708, Houston, TX 77225. E-mail akahn@heart.med.uth.tmc.edu

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hours and every 72 hours thereafter, the media were replaced with 1 mL of the same fresh medium. The cells became confluent within 2 weeks, when they were used.

cGMP Assay
Dishes of VSMCs were incubated for 30 minutes at 37°C in PSS plus 0.1% BSA, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) was added to inhibit phosphodiesterase. The solution was removed, and the reaction was stopped by the addition of 1 mL of ice-cold ethanol acidified with 0.8% of 12 mol/L HCl. The extract was centrifuged at 10 000g for 15 minutes, the supernatant was evaporated to dryness in a Speed Vac SC100, and the cGMP content of acetylated samples was measured with the use of the cGMP \([125\text{I}]\) Assay System (Amersham), as previously described. Each experiment was performed in triplicate. Although basal cGMP production varied among preparations, the relative effects of specific perturbations on cGMP production were consistent among different preparations. Thus, cGMP production was calculated as a percent of the amount produced under control conditions.

**Cell Migration**

Dishes of cells were washed twice with PBS, and a wound was made through the monolayer with a stylet, producing a cell-free area 0.3 mm wide, as previously described. Then, 1 mL serum-free DMEM was placed in the dish, and a video image was made with the use of a charged-couple device video camera attached to a Nikon inverted phase-contrast microscope at \(\times200\) magnification. With the use of image-processing software (Optimus Bioscan), the area of the wound was determined. The dish of cells was returned to a 95% CO\(_2\)/5% O\(_2\) humidified incubator at 37°C; repeat images of the wound closure could be due to cell migration, proliferation, or both. In other experiments (data not shown), it was determined with bromodeoxyuridine labeling that cell proliferation was minimal during a 12-hour period.

Bovine insulin, lipopolysaccharide (E. coli O26:B6), and recombinant interleukin-1\(\beta\) were obtained from Tocris Cookson. 

**Results**

**Induction of iNOS**

We previously found that insulin stimulated cGMP production in VSMCs with iNOS,\(^{10,11}\) and it has also been reported that cGMP inhibits VSMC migration.\(^{12}\) For these reasons, we used cultured VSMCs before and after iNOS induction in the present study to determine the effects of insulin on VSMC migration. Dishes of primary cultured VSMCs were serum starved for 24 hours and preincubated with or without 100 ng/mL lipopolysaccharide and 10 ng/mL interleukin-1\(\beta\) for an additional 24 hours. Protein extract from the cells was subjected to electrophoresis and immunoblotted with rabbit polyclonal anti-iNOS antibody. Protein extract from the mouse macrophage cell line RAW 264.7, in which iNOS had been induced through a 24-hour preincubation with 10 \(\mu\)g/mL lipopolysaccharide, was used as a positive control. As shown in Figure 1, immunoblots from VSMCs preincubated with lipopolysaccharide and interleukin-1\(\beta\) and macrophages preincubated with lipopolysaccharide had single identical bands stained positively with iNOS antibody at the expected molecular mass for iNOS (131 kDa). Lysates from control noninduced VSMCs lacked iNOS protein. A similar procedure with the use of anti-constitutive NOS (cNOS) antibody, which yielded positive immunoblots with lysates from endothelial cells, did not reveal cNOS protein in lysates from VSMCs preincubated with or without lipopolysaccharide and interleukin-1\(\beta\) (data not shown).

**cGMP Measurements**

Both induced and noninduced VSMCs were incubated with 0.5 mmol/L IBMX in the presence or absence of 1 mmol/L insulin for 30 minutes, and the cGMP content of the cells was measured. As shown in Figure 2, insulin did not affect cGMP levels in noninduced cells. cGMP was increased in induced cells, and insulin further increased cGMP levels in those cells by 3-fold. These data indicate that although insulin had no effect on cGMP production in cells without iNOS, after iNOS induction, cGMP production was stimulated and insulin further stimulated it.

**Migration Measurements**

The effects of insulin on migration of VSMCs before and after iNOS induction were determined. As shown in Figure 3, 1 mmol/L insulin had no effect on the migration of noninduced cells. Induced VSMCs migrated slower than control cells at 6 and 9 hours, and insulin further inhibited migration of those cells at 3, 6, and 9 hours by \(\approx90\%\). To determine
whether the inhibition by insulin of VSMC migration in induced cells was dependent on NOS activity, the effect of insulin on the migration of induced cells was determined in the presence and absence of L-NMMA, an NOS inhibitor. Insulin (1 nmol/L) decreased the migration of induced VSMCs by 95±6%, 91±6%, and 87±5% at 3, 6, and 9 hours, respectively (all P<0.05, n=4). In the presence of 0.1 mmol/L L-NMMA, insulin decreased the migration of induced cells at 3, 6, and 9 hours by only 1±4%, 2±5%, and 3±4%, respectively (all P=NS versus 0, n=4). Thus, L-NMMA totally blocked the inhibition by insulin of the migration of induced VSMCs, indicating that NOS activity was required for this effect of insulin.

We tested whether the inhibition by insulin of the migration of VSMCs with iNOS was dependent on cGMP production. Insulin (1 nmol/L) decreased the migration of induced VSMCs by 95±6%, 91±6%, and 87±5% at 3, 6, and 9 hours, respectively (all P<0.05, n=4). In the presence of 0.1 mmol/L L-NMMA, insulin decreased the migration of induced cells at 3, 6, and 9 hours by only 1±4%, 2±5%, and 3±4%, respectively (all P=NS versus 0, n=4). Thus, L-NMMA totally blocked the inhibition by insulin of the migration of induced VSMCs, indicating that NOS activity was required for this effect of insulin.

Figure 2. Effect of insulin and iNOS on cGMP production. Primary confluent cultured VSMCs were preincubated for 24 hours with (Induced) or without (Non-induced) lipopolysaccharide and interleukin-1β to induce iNOS, as described in legend to Figure 1. Cells were incubated with 0.1 mmol/L IBMX with or without 1 mmol/L insulin for 30 minutes, and cGMP content of cells was determined. Data are expressed as a percent of cGMP level in noninduced cells in absence of insulin, which averaged 48.8 fmol/mg protein. Values are mean±SEM of 4 separate experiments. *P<0.05 vs Non-Induced. **P<0.05 vs Induced.

Figure 3. Effect of insulin and iNOS on VSMC migration. Primary confluent cultured VSMCs were preincubated for 24 hours with (Induced) or without (Non-Induced) lipopolysaccharide and interleukin-1β to induce iNOS, as described in legend to Figure 1. Cells were wounded with a linear scratch and incubated with or without 50 μmol/L 8-bromo-cGMP or 1 nmol/L insulin or insulin plus 10 μmol/L ODQ, and migration of cells into the injured area was measured during a 9-hour period. Data represent percent of initial injured area that remains open and are expressed as mean±SEM of 4 separate experiments. *P<0.05 vs Non-Induced. **P<0.05 vs Induced + Insulin.

Discussion

Atherosclerosis and restenosis occurring after balloon angioplasty are aggravated in insulin-resistant states. 1-3 Because these conditions are marked by hyperinsulinemia, 1 it has been thought that insulin stimulates atherosclerosis and restenosis. 5 It has been reported that insulin deficiency protects animals from experimental atherosclerosis and that this protection is lost with insulin treatment. 16 VSMC migration from the tunica media to the neointima is an integral part of atherosclerosis and restenosis. 4 Several authors have examined the effect of insulin on VSMC migration, but the results are controversial. Nakao et al 7 reported that physiological concentrations of insulin (<1 nmol/L) do not affect the acute migration of cultured rat aortic VSMCs. However, if the cells were pretreated with insulin for 5 days, the migration of cells stimulated by 12-l-hydroxy-5,8,10,14 eicosatetraenoic acid was increased. 7 Gockerman et al 8 reported that insulin (≈300 nmol/L) stimulated the migration of porcine aortic cultured VSMCs. This concentration of insulin can stimulate insulin-like growth factor-1 receptors, which are known to mediate VSMC migration. 9 Grotendorst et al 10 reported that insulin (≈150...
nmol/L) did not affect the migration of cultured VSMCs from bovine aorta.

In the present study, we found that 1 nmol/L insulin did not affect the migration of primary cultured confluent VSMCs from canine femoral artery but that when iNOS had been induced, migration was inhibited by \( \approx 90\% \). In addition, insulin did not affect cGMP production in noninduced cells but stimulated it 3-fold in induced cells. The present data show that the inhibition by insulin of migration of VSMCs with iNOS was dependent on cGMP production, because block of guanylate cyclase with ODQ eliminated this effect of insulin. We also showed that 8-bromo-cGMP alone inhibited the migration of noninduced VSMCs. This has been shown by others with different cultured VSMCs.\(^2\) We did not use ODQ with noninduced cells because insulin did not affect cGMP production or migration in those cells, and it has been reported that ODQ per se does not affect VSMC migration.\(^3\) These data indicate that the inhibition by insulin of the migration of induced VSMCs is dependent on the stimulation by the hormone of cGMP production. The findings that, in the absence of insulin, cells with iNOS have increased basal cGMP levels and lower migration rates compared with noninduced cells indicate that basal cGMP production induced by iNOS-derived NO inhibited the migration of these VSMCs. We also showed in the present study that L-NMMA completely blocked the inhibition by insulin of the migration of induced VSMCs, indicating that NOS activity was responsible for the effects of insulin in induced cells. This conclusion is consistent with our previous finding that the stimulation by insulin of cGMP production in cells with iNOS was also blocked by L-NMMA.\(^1\)

The present finding that insulin only inhibited the migration of induced VSMCs may have pathophysiological implications. After the induction of experimental atherosclerosis or balloon angioplasty in animals, it has been reported that iNOS was present in the underlying medial and neointimal VSMCs.\(^18\)\(^,\)\(^19\) According to the present results, the migration of these cells with iNOS may be inhibited with insulin. If clinical conditions marked by resistance to insulin-induced glucose disposal (eg, non–insulin-dependent diabetes mellitus, obesity, essential hypertension) also have resistance to the inhibition by insulin of migration of VSMCs with iNOS, aggravation of atherosclerosis and restenosis would be expected.

It is controversial whether hyperinsulinemia or insulin resistance is responsible for advanced cardiovascular disease in insulin-resistant states.\(^5\)\(^,\)\(^2\)\(^0\)\(^,\)\(^2\)\(^1\) We showed only an inhibitory effect of insulin on VSMC migration, but the present study did not address the possible role of hyperinsulinemia in the acceleration of cardiovascular disease through the stimulation of VSMC migration. Further studies are necessary to determine the effects of insulin on VSMCs from other vessels and species, with and without iNOS.

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