Insulin Reduces Contraction and Intracellular Calcium Concentration in Vascular Smooth Muscle

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Resistance to insulin-induced glucose disposal is associated with hypertension, in accord with recent reports that insulin-induced vasodilation is impaired in men with resistance to insulin-induced glucose disposal. Nevertheless, the mechanism of insulin-induced vasodilation is not known. We wished to determine whether a physiological concentration of insulin inhibits agonist-induced contraction at the level of the individual vascular smooth muscle cell, and if so, how. Dispersed vascular smooth muscle cells from dog femoral artery were grown on collagen gels for 4 to 8 days. Contraction and intracellular Ca^{2+} concentration of individual cells were measured by photomicroscopy and fura 2 epifluorescence microscopy, respectively. Serotonin and angiotensin II contracted cells in a dose-dependent manner. Preincubation of cells for 20 minutes (short-term) or 7 days (long-term) with insulin (40 μU/mL) inhibited serotonin- and angiotensin II-induced contractions by approximately 50%. Insulin (10 μU/mL) acutely inhibited serotonin-induced contraction by 34%. The maximal effect of high extracellular K^{+}-induced contraction was not affected by short-term insulin exposure, but the ED_{50} for extracellular K^{+}-induced contraction was increased from 7.6±2.5 to 16.0±3.9 mmol/L (P<.05). Short-term insulin exposure also attenuated the peak rise of the serotonin-induced intracellular Ca^{2+} transient and increased the rate constant for intracellular Ca^{2+} decline. Verapamil and ouabain completely blocked the attenuation of agonist-induced contraction by short-term insulin exposure, indicating the importance of voltage-operated Ca^{2+} channels and the Na^{+}-K^{+} pump for this effect. We conclude that a physiological insulin concentration inhibits extracellular K^{+} and agonist-induced contractions at the level of the vascular smooth muscle cell and attenuates the intracellular Ca^{2+} transient in agonist-stimulated cells. Insulin may stimulate Na^{+}-K^{+} pump activity, which hyperpolarizes the cell, thereby decreasing Ca^{2+} influx via voltage-operated channels. (Hypertension. 1993;22:735-742.)

**KEY WORDS** • serotonin • angiotensin II • calcium channels • verapamil • ouabain • ion transport • fura-2

Hypertension is associated with obesity and non-insulin-dependent diabetes. These two clinical conditions are marked by resistance to insulin-induced glucose disposal, which has also been found in some groups of patients with essential hypertension. The mechanisms responsible for the link between increased blood pressure and resistance to insulin-induced glucose disposal are unknown.

Previous studies have shown that insulin causes vasodilation in humans and dogs. Insulin-induced vasodilation may be a manifestation of attenuation of tonic neural and humoral mediator-induced vasoconstriction that normally contributes to vascular tone. It is noteworthy that in men with essential hypertension, non-insulin-dependent diabetes, and obesity, euglycemic insulin clamp studies have revealed an association between resistance to insulin-induced glucose disposal and insulin-induced vasodilation. These data support the recently proposed hypothesis that hypertension in insulin-resistant states may be caused in part by resistance to the normal vasodilator effects of insulin. Because the sites of action or mechanisms of insulin-induced attenuation of vasoconstriction in normal arteries are not yet known, the present studies were undertaken to determine whether a physiological concentration of insulin inhibits contraction at the level of the individual vascular smooth muscle cell, and if so, how.

**Methods**

**Cell Culture**

Adult mongrel dogs of either sex were killed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. Vascular smooth muscle cells were cultured as previously described. In brief, the media of the arteries were minced and incubated at 37°C in a solution containing elastase (type V, Sigma Chemical Co, St Louis, Mo) and collagenase (type I, Worthington Biochemical Corp, Freehold, NJ). After 2
centrations of serotonin were added. Dark horizontal line and D, respectively) 10 minutes after the indicated con-

FIG 1. Photomicrographs show effect of serotonin on contraction of individual vascular smooth muscle cell. Cells were preincubated for 20 minutes at 37°C in HEPES physiological salt solution plus 4% bovine serum albu-
imin, and a baseline photomicrograph of a representative cell was obtained (A). Serotonin was serially added (10⁻⁴, 10⁻³, and 10⁻² mol/L) to the dish at 10-minute intervals, and subsequent photomicrographs were obtained (B, C, and D, respectively) 10 minutes after the indicated concentra-
tions of serotonin were added. Dark horizontal line in D represents 50 μm.

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 and washed three times in Hanks' balanced salt solution (GIBCO, Grand Island, NY) and suspended to a density of 2×10⁶ cells per milliliter in Dulbecco's modified Eagle's medium (DMEM) that contained 0.5% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin solution (10 000 U/mL penicillin, 10 mg/mL strep-
tomycin, Sigma). One milliliter of this suspension was placed on the heated (37°C) stage of a Nikon Diaphot

Cell Contraction

After 5 to 8 days, agonist-induced contraction of individual cells was measured, as previously described.⁸ We have previously shown that the cells in this prepa-
ration are contracted by serotonin in a dose-dependent, inhibitable, and reversible manner.⁸ The dishes were placed on the heated (37°C) stage of a Nikon Diaphot

inverted phase-contrast microscope, and the culture medium was replaced with a physiological salt solution that contained (mmol/L) NaCl, 140; KCl, 4; CaCl₂, 2; MgCl₂, 1; glucose, 5; and HEPES-Tris, 10, pH 7.4 (HEPES PSS) plus 4% bovine serum albumen (BSA), with or without bovine insulin (40 μU/mL) (Sigma) (Figs 2 through 4 and 6 through 8). After 20 minutes, a field of at least 6 to 10 cells was photographed at ×200 to obtain baseline images. The medium was replaced with the desired experimental solution, and after 10 minutes another photograph was taken of the same field. The lengths of the longest axes of 6 to 10 arbi-
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for the decline of \([\text{Ca}^{2+}]\) to its steady-state value after the serotonin-induced \([\text{Ca}^{2+}]\) peak was calculated by fitting the data to a single exponential function by regression analysis. The 5-minute area under the \([\text{Ca}^{2+}]\) time curve (AUC) after serotonin exposure was determined by calculating and summing the areas of 10 consecutive 30-second segments after serotonin exposure, assuming that each segment was a rectangle whose height equaled the average of the \([\text{Ca}^{2+}]\) values at the beginning and end of that segment. The 5-minute serotonin-induced contraction of the cell was determined by calculating the percent decrease in its axial length.

Statistical analysis was performed on unpaired data with the Student’s t test.

**Results**

Fig 1 shows photomicrographs of a representative cell in response to increasing doses of serotonin. Note that serotonin contracted the cell in a dose-dependent manner. To determine the effect of a physiological concentration of insulin on agonist-induced contraction, we preincubated cells with or without 40 \(\mu\)U/mL insulin for 20 minutes and determined the cumulative dose-response relations for angiotensin II (Ang II) or serotonin-induced contractions. As shown in Fig 2, Ang II and serotonin contracted cells in a dose-dependent manner. As also shown, insulin inhibited Ang II- and serotonin-induced contractions. Insulin alone did not affect cell length (unpublished data). The maximal contraction achieved with Ang II or serotonin was inhibited approximately 50% by insulin.

In normal individuals, fasting insulin concentration is approximately 10 \(\mu\)U/mL and rises to approximately 40 \(\mu\)U/mL after an oral glucose load. To see whether an insulin concentration in the low physiological range also inhibits agonist-induced contraction, we measured the short-term (20 minutes) effect of 10 and 40 \(\mu\)U/mL insulin on serotonin (10\(^{-7}\) mol/L)-induced contraction. As shown in Fig 3, 10 and 40 \(\mu\)U/mL insulin inhibited contraction by 34% and 57%, respectively. Thus, even an insulin concentration in the fasting range (10 \(\mu\)U/mL) inhibited agonist-induced contraction.

To determine if long-term insulin exposure affected insulin-induced inhibition of agonist-induced contraction, we preincubated cells with 3 mL of media with and without 40 \(\mu\)U/mL insulin for 1 week. Media was replaced every 24 hours. On the day of the experiment, media was removed, the cells washed with HEPES PSS, and the contraction assay performed. As shown in Fig 4, preincubation with insulin (40 \(\mu\)U/mL) for 1 week also...
proximately 75%, but insulin did not inhibit contraction of vascular smooth muscle. Cells were preincubated with 0, 10, and 40 μU/mL insulin for 20 minutes as described in Fig 2 legend. Baseline photomicrograph was obtained; 10⁻⁶ mol/L serotonin was added to the dish and another photomicrograph obtained after 10 minutes. Data represent mean±SEM of mean contractions from baseline lengths of 6 to 10 cells in each of six dishes. *P<.05 vs zero insulin.

To determine if the inhibition by insulin of serotonin-induced contraction was associated with modulation of Ca²⁺ metabolism, we measured [Ca²⁺]ᵢ in single cells by fura 2 epifluorescence microscopy. The cells were preincubated for 20 minutes with or without insulin (40 μU/mL) and then exposed to serotonin (10⁻⁵ mol/L). Fig 5 shows that in the absence of insulin, serotonin induced a short-term rise in [Ca²⁺]ᵢ from a basal value of 78±12 to a peak of 358±46 nmol/L in 30 seconds. [Ca²⁺]ᵢ returned to a stable value of approximately 168 nmol/L at 3 minutes in the continued presence of serotonin. Insulin did not significantly affect basal [Ca²⁺]ᵢ, which was 62±10 nmol/L, but attenuated the serotonin-stimulated [Ca²⁺]ᵢ peak, which was 232±32 nmol/L at 30 seconds (P<.01). Within 2 minutes [Ca²⁺]ᵢ had returned to a stable value of approximately 161 nmol/L in the continued presence of serotonin. The rate constant for the decline of [Ca²⁺]ᵢ to its steady-state value after the serotonin-induced [Ca²⁺]ᵢ peak was 2.58±0.26 and 3.93±0.41 min⁻¹ (P<.05) in the absence and presence of insulin, respectively. The 5-minute AUC after serotonin exposure was decreased from 1042±131 to 738±95 nmol/L·min by insulin (P<.01), and insulin decreased the 5-minute serotonin-induced contraction of these cells from 14.1±2.2% to 4.1±0.8% (P<.01).

To evaluate whether insulin attenuated agonist-induced contraction by inhibiting Ca²⁺ influx via voltage-operated Ca²⁺ channels (VOCs), we examined the effect of insulin (40 μU/mL) on serotonin (10⁻⁵ mol/L)-induced contraction in the presence and absence of verapamil (10⁻⁶ mol/L). As shown in Fig 6, verapamil alone inhibited serotonin-induced contraction by approximately 75%, but insulin did not inhibit contraction further. Similar results were obtained when 10⁻⁶ mol/L Ang II was used instead of serotonin (unpublished observations). These data suggest that insulin inhibits Ca²⁺ influx via VOCs.

To determine whether insulin inhibits depolarization-induced contraction, we examined the effect of short-term insulin exposure on contraction induced by extracellular K⁺ concentration ([K⁺]₀). Fig 7 shows that K⁺-induced contraction of vascular smooth muscle cells in a concentration-dependent manner. The maximal effect of high [K⁺]₀-induced contraction was not affected by insulin, but the ED₅₀ for [K⁺]₀-induced contraction was increased from 7.6±2.5 to 16.0±3.9 nmol/L by insulin (P<.05). The data are consistent with the possibilities that insulin inhibits Ca²⁺ influx via VOCs by shifting the voltage sensitivity of these channels to more depolarized values or that insulin hyperpolarizes the cell, thereby decreasing the sensitivity of the cell to [K⁺]₀-induced depolarization.

Certain experimental manipulations that stimulate Na⁺-K⁺ pump activity in vascular smooth muscle can hyperpolarize the cell and inhibit agonist-induced contraction. Because insulin stimulates Na⁺-K⁺ pump activity in other cell types, we tested whether the inhibition by insulin of agonist-induced contraction was dependent on a functioning Na⁺-K⁺ pump. As shown in Fig 8, the inhibitory effect of insulin on Ang II-induced contraction was blocked in the presence of 10⁻⁶ mol/L ouabain. As also shown in Fig 8, ouabain alone caused a small contraction that was not inhibited by insulin. Similar results were obtained when 10⁻⁵ mol/L serotonin was used instead of Ang II (unpublished observations). Ouabain (10⁻⁵ mol/L) should effectively inhibit the Na⁺-K⁺ pump in these cells, because we have previously shown that the Kᵦ value for ouabain binding to cultured canine vascular smooth muscle cells is 5.8×10⁻⁶ mol/L.¹⁷

**Discussion**

Previous studies have shown that physiological concentrations of insulin induce vasodilation in humans and dogs⁵⁻⁶ and attenuate agonist-induced contraction of isolated rat aortic strips. The locus of this action of insulin has not been determined. Vascular smooth muscle cells, neurons, endothelial cells, interstitial cells, or blood cells could be involved in this inhibition of contraction. The goal of this study was determine whether insulin inhibits agonist-induced contraction at the level of the individual vascular smooth muscle cell. We have developed a vascular smooth muscle cell model to study contraction and ion transport in individual cells. Vascular smooth muscle cells are isolated and cultured in the presence of 0.5% fetal calf serum on top of a collagen gel. Cells do not proliferate under these conditions and remain viable for more than 2 weeks. Their identity as vascular smooth muscle cells is likely because Ang II, serotonin, and high [K⁺]₀ contract the cells, and they were from the same dispersed cell preparation of canine femoral artery that we have previously shown contained muscle myosin heavy chains but not nonmuscle myosin heavy chains.¹⁸ We have previously shown that serotonin contracted these cells in a dose-dependent, reversible, and inhibitable manner.² We have extended these findings in the present study by showing that Ang II and K⁺ also contract these cells in a concentration-dependent manner.
Fig 4. Line graphs show effect of long-term insulin exposure on agonist-stimulated contraction of vascular smooth muscle. Cells grown for 5 days were preincubated in tissue culture incubator for an additional 7 days with or without 40 μU/mL insulin in Dulbecco’s modified Eagle’s medium plus 4% bovine serum albumin. Media (3 mL) were changed daily during this latter period. Dishes were then preincubated for 20 minutes at 37°C in HEPES physiological salt solution plus 4% bovine serum albumin, and a baseline photomicrograph was obtained. Cumulative concentrations of angiotensin II (All) (top) and serotonin (bottom) were added to the bathing media and sequential photomicrographs obtained as described in Fig 1 legend. Data points represent mean±SEM of mean contractions from baseline lengths of 6 to 10 cells in each of six dishes. *P<.05.

When cells were preincubated for 20 minutes with a physiological concentration of insulin (40 μU/mL), Ang II- and serotonin-induced contractions were attenuated. A fasting insulin concentration (10 μU/mL) also inhibited agonist-induced contraction. Thus, these studies demonstrate that insulin inhibits contraction at the level of the individual vascular smooth muscle cell in the absence of any other cell types.

The present studies demonstrate that insulin (40 μU/mL)-attenuated contraction of serotonin-stimulated vascular smooth muscle cells was associated with a diminished serotonin-induced intracellular Ca²⁺ transient (Fig 5). Contraction and the height of the intracellular Ca²⁺ transient were both inhibited by approximately 50%. Insulin inhibited the peak height of the intracellular Ca²⁺ transient and increased the rate constant for [Ca²⁺], decline to its steady-state value. Although the Ca²⁺ transport systems affected by insulin cannot be determined by these data, several inferences can be made regarding possible mechanisms by which physiological concentrations of insulin inhibit agonist-induced contraction. Fig 6 shows that when verapamil inhibited agonist-induced contraction, insulin did not inhibit the contraction further. Agonist-induced contraction that persists despite VOC blockade by verapamil is presumably mediated by the cell membrane receptor-initiated signal transduction system, including G proteins, phospholipase C, polyphosphoinositide turnover, inositol triphosphate, diacylglycerol, protein kinase C, and sarcoplasmic reticular Ca²⁺ release. The data in Fig 6, which show that insulin did not affect agonist-induced contraction in the presence of verapamil, suggest that insulin does not block the above-mentioned components of the signal transduction system. The data also infer that insulin does not potentiate sarcoplasmic reticular Ca²⁺ uptake or sarcolemmal Ca²⁺ efflux mechanisms. These data are consistent with the possibility that insulin directly or indirectly interferes with Ca²⁺ influx via VOCs. It is important to point out, however, that our inability to detect a difference between small contractions does not prove that such differences do not exist.
The data in Fig 7, which show that insulin increased the $E_D^{50}$ for $[K^+]_o$-induced contraction from 7.6 to 16.0 mmol/L, are consistent with the possibility that insulin shifted the sensitivity of VOCs to more depolarized values. Standley et al.\(^6\) have reported that such is the case in a7r5 cells. Alternatively, in the present study, insulin may have hyperpolarized the cell by another mechanism, requiring a higher $[K^+]_o$ to depolarize the cells to values that permit Ca\(^{2+}\) influx via VOCs.

Prior studies have shown that the Na\(^+\) ionophore monensin or prepulses of serotonin in concentrations below the contraction threshold attenuate norepinephrine-induced contraction in intact canine vessels. The data showed that these maneuvers increased Na\(^+\) influx, stimulated Na\(^+\)-K\(^+\) pump activity, and decreased verapamil-sensitive Ca\(^{2+}\) influx.\(^{12,13}\) It has been demonstrated that inhibiting the Na\(^+\)-K\(^+\) pump in vascular smooth muscle with ouabain or zero $[K^+]_o$ depolarizes and contracts the tissue. Returning normal K\(^+\) concentration to the bathing media of a zero $[K^+]_o$-contracted vessel reactivates the Na\(^+\)-K\(^+\) pump, hyperpolarizes the cells, and promptly relaxes the tissue.\(^{12,13,20}\) Finally, it
Fig 8. Bar graph shows effect of ouabain, insulin, and angiotensin II (All) on contraction of individual vascular smooth muscle cells. Cells were preincubated for 20 minutes at 37°C in HEPES physiological salt solution plus 4% bovine serum albumin with or without 40 μU/mL insulin, and a baseline photomicrograph was obtained. All (10⁻⁶ mol/L), ouabain (10⁻⁸ mol/L), or All (10⁻⁶ mol/L) plus ouabain (10⁻⁸ mol/L) was added to the bathing media and another photomicrograph obtained 10 minutes later. Data represent mean±SEM of mean contractions of 6 to 10 cells in each of five dishes. *P<.05 vs All alone.

has been reported that insulin stimulates Na⁺-K⁺ pump or Na⁺,K⁺-ATPase activities in several cell types including vascular smooth muscle.14-16,21 These prior studies are consistent with the hypothesis that insulin may increase the activity of the electrogenic Na⁺-K⁺ pump in agonist-stimulated vascular smooth muscle, causing relative hyperpolarization of the cell membrane, resulting in decreased Ca²⁺ influx via VOCS and attenuation of contraction. The data in Fig 8 are consistent with this possibility because insulin did not affect either agonist-induced contraction when the Na⁺-K⁺ pump was blocked by ouabain or the contraction induced by ouabain alone.

Previous studies have examined the effects of insulin on Ca²⁺ mobilization in vascular smooth muscle cells from several sources. It has recently been reported that insulin (100 μU/mL) inhibited vasopressin-induced and depolarization-induced inward current (presumably Ca²⁺ current) in a7r5 cells and attenuated the peak height of the vasopressin-induced intracellular Ca²⁺ transient.19 The present study is in accord with these data, but it extends their physiological significance because the present study used physiological concentrations of insulin and vascular smooth muscle cells that had maintained the contractile phenotype. Other studies have reported that 100 μU/mL insulin inhibited contraction in phenylephrine-stimulated aortic strips from lean and obese Zucker rats,7 and 1.3 μU/mL insulin stimulated Ca²⁺ efflux from unstimulated rat aortic vascular smooth muscle cells.22 In other studies, 100 μU/mL insulin inhibited inositol triphosphate–induced Ca²⁺ efflux from permeabilized cultured rat aortic vascular smooth muscle cells.23 The results of these studies suggest that insulin stimulates a Ca²⁺ efflux mechanism and attenuates Ca²⁺ mobilization from intracellular stores in rat vascular smooth muscle. The results of the present study cannot rule out that such mechanisms exist in canine cultured vascular smooth muscle cells, but they do not support those conclusions. Our finding that insulin did not inhibit Ang II–induced contraction in the presence of 10⁻⁶ mol/L verapamil does not support the notion that insulin stimulates a Ca²⁺ efflux mechanism or inhibits intracellular Ca²⁺ mobilization. It is important to point out, however, that we did not examine Ca²⁺ fluxes through specific transport systems in the present study, and thus, conclusions regarding these points are speculative. In addition, the source of vascular smooth muscle, concentrations of insulin, and choice of contractile agonists were different between the present study and the previous reports.

In the present study, we report that a physiological concentration of insulin inhibits agonist-induced contraction of vascular smooth muscle. This finding is consistent with the hypothesis that the hypertensive conditions associated with resistance to insulin-induced glucose disposal are also associated with resistance to the attenuation by insulin of agonist-stimulated vascular smooth muscle contraction.1-7 However, proposing that insulin-resistant vessels are chronically overcontracted compared with normal vessels requires demonstrating that long-term insulin exposure inhibits agonist-induced contraction in normal vessels. In this regard, it is noteworthy that in the present study we found that exposure of vascular smooth muscle cells to a physiological concentration of insulin for 1 week still inhibited agonist-induced contraction.

In summary, we found that a physiological concentration of insulin inhibited Ang II– and serotonin-induced contractions of individual canine cultured vascular smooth muscle cells and increased the ED₅₀ for [K⁺]-induced contraction. Insulin also attenuated the serotonin-induced intracellular Ca²⁺ transient. Insulin did not inhibit contraction if VOCS or the Na⁺-K⁺ pump was blocked. These data are consistent with the hypothesis that insulin stimulates the Na⁺-K⁺ pump, thereby hyperpolarizing the cells, decreasing Ca²⁺ influx via VOCS, and reducing agonist-induced contraction. Additional studies are required to test the validity of this hypothesis.

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References


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