Insulin Reduces Contraction and Intracellular Calcium Concentration in Vascular Smooth Muscle

Andrew M. Kahn, Charles L. Seidel, Julius C. Allen, Roger G. O'Neil, Harnath Shelat, Tom Song

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.1 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.1,2 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 humans3-5 and dogs.6 Insulin-induced vasodilation may be a manifestation of attenuation of tonic neural and humoral mediator-induced vasoconstriction that normally contributes to vascular tone.7 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.3,4 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.1,7 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.8 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
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 albumin, and a baseline photomicrograph of a representative cell was obtained (A). Serotonin was serially added (10⁻⁸, 10⁻⁷, and 10⁻⁵ M) to the dish at 10-minute intervals, and subsequent photomicrographs were obtained (B, C, and D, respectively) 10 minutes after the indicated concentrations of serotonin were added. Dark horizontal line in D represents 50 μm.

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 arbitrarily chosen cells were measured in the first photograph, and lengths of the same cells were measured in the subsequent photographs. For each cell, the percent contraction from the baseline length was calculated, and these values were averaged for all cells. This average was taken as the response for that particular culture dish. The average baseline cell lengths were consistent from preparation to preparation. The basal cell length from 60 cells (6 randomly chosen cells per dish from 10 separate preparations) was 92.1 ±5.9 μm (mean ±SEM). The ED₅₀ for K⁺-induced contraction was calculated by a logistic equation that fitted the data to a Michaelis-Menten type of rectangular hyperbola.

Fura 2 Fluorescence

Intracellular Ca²⁺ concentration ([Ca²⁺]) of individual cells was measured by monitoring the fluorescence emissions of fura 2 using a custom-built video monitor-equipped epifluorescence microscope that has been previously described. Cells were grown on collagen gels for 5 to 8 days, as described above, in a 400-μL glass-bottomed chamber. The chamber was placed on the microscope stage and superfused at 1 mL/min with HEPES PSS at 37°C. One cell was centered in the field of view, and autofluorescence of that cell was determined by exciting the cell with alternating 100-millisecond pulses of light at 340 and 380 nm and recording the emission intensity at 510 nm with a photomultiplier tube. The emissions from all other cells had been excluded by adjusting a diaphragm proximal to the photomultiplier tube. The cell was loaded for 45 minutes with 2.4 μmol/L fura 2-AM (Molecular Probes, Inc, Eugene, Ore) that had been sonicated for 20 seconds in DMEM with 0.1% BSA. The chamber was superfused again at 1 mL/min with HEPES PSS plus 4% BSA with or without insulin (40 μU/mL) for 20 minutes. Baseline fluorescence emissions were serially recorded, the cell superfused with HEPES PSS plus 10⁻⁵ mol/L serotonin with or without insulin (40 μU/mL), and fluorescence measurements continued for 20 minutes. The image of the cell was continuously recorded on videotape. The fluorescent emissions were corrected for autofluorescence, and the 340/380 intensity ratios were recorded. [Ca²⁺] values were calculated from the fluorescence ratio recordings using the formula: [Ca²⁺] = Kᵣ(ΔR/ΔR₀) - R₀/Kᵣ. The Kᵣ value was taken as 224 nmol/L, and the symbols in the equation have their usual meaning. R₀, R₀', and S₀'/S₀ were determined at the end of each experiment by measuring the autofluorescence-corrected 510-nm emissions on 340 and 380 nm excitation while superfusing the cells with HEPES PSS containing 4 mmol/L Ca²⁺ plus 2.5 μmol/L ionomycin (Sigma), followed by perfusion with nominally Ca²⁺-free HEPES PSS plus 10 mmol/L EGTA. The rate constant...
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 \(\mu\text{U/mL}\) 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 \(\mu\text{U/mL}\) and rises to approximately 40 \(\mu\text{U/mL}\) 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 \(\mu\text{U/mL}\) insulin on serotonin (\(10^{-8}\) mol/L)-induced contraction. As shown in Fig 3, 10 and 40 \(\mu\text{U/mL}\) insulin inhibited contraction by 34% and 57%, respectively. Thus, even an insulin concentration in the fasting range (10 \(\mu\text{U/mL}\)) 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 \(\mu\text{U/mL}\) 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 \(\mu\text{U/mL}\)) for 1 week also
Ang II was used instead of serotonin (unpublished observations). These data suggest that insulin inhibits Ca^{2+} 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\(^+\)]\(_o\)). Fig 7 shows that K\(^+\) contracted cells in a concentration-dependent manner. The maximal effect of high [K\(^+\)]\(_o\)-induced contraction was not affected by insulin, but the ED\(_{50}\) for [K\(^+\)]\(_o\)-induced contraction was increased from 7.6±2.5 to 16±3.9 mmol/L by insulin (P<.05). The data are consistent with the possibilities that insulin inhibits Ca^{2+} 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\(^+\)]\(_o\)-induced depolarization.

Certain experimental manipulations that stimulate Na\(^+\)-K\(^+\) pump activity in vascular smooth muscle can hyperpolarize the cell and inhibit agonist-induced contraction.12,13 Because insulin stimulates Na\(^+\)-K\(^+\) pump activity in other cell types,14-16 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\(^{-5}\) 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 K\(_o\) 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 dogs3-6 and attenuate agonist-induced contraction of isolated rat aortic strips.3 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 to 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\(^+\)]\(_o\) 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.8 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.
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 Ca\(^{2+}\) transient (Fig 5). Contraction and the height of the intracellular Ca\(^{2+}\) transient were both inhibited by approximately 50%. Insulin inhibited the peak height of the intracellular Ca\(^{2+}\) transient and increased the rate constant for [Ca\(^{2+}\)]\(_i\) decline to its steady-state value. Although the Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) uptake or sarcolemmal Ca\(^{2+}\) efflux mechanisms. These data are consistent with the possibility that insulin directly or indirectly interferes with Ca\(^{2+}\) 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 ED₅₀ for [K⁺]₀-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.¹⁹ have reported that such is the case in a7r5 cells. Alternatively, in the present study, insulin may have hyperpolarized the cells by another mechanism, requiring a higher [K⁺]₀ to depolarize the cells to values that permit Ca²⁺ 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 Ca²⁺ influx.¹²,¹³ It has been demonstrated that inhibiting the Na⁺-K⁺ pump in vascular smooth muscle with ouabain or zero [K⁺]₀ depolarizes and contracts the tissue. Returning normal K⁺ concentration to the bathing media of a zero [K⁺]₀-contracted vessel reactivates the Na⁺-K⁺ pump, hyperpolarizes the cells, and promptly relaxes the tissue.¹²,¹³,²⁰ Finally, it
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 electronegative 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⁺ metabolism in vascular smooth muscle cells from several sources. It has recently been reported that insulin (100 mU/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 mU/mL insulin inhibited contraction in phenylephrine-stimulated aortic strips from lean and obese Zucker rats, **7** and 1.3 mU/mL insulin stimulated [Ca²⁺] efflux from unstimulated rat vascular smooth muscle. **22** 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 and increased the ED₅₀ for [K⁺]o-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.

In summary, we found that a physiological concentration of insulin inhibited agonist-induced contractions of individual canine cultured vascular smooth muscle cells and increased the ED₅₀ for [K⁺]o-induced contraction. Insulin also attenuated the serotonin-induced intracellular Ca²⁺ transient. 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 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⁺]o-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.

**Acknowledgment**

Supported by grants HL-40480 and HL-34280 from the National Heart, Lung, and Blood Institute.

**References**


Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle.

A M Kahn, C L Seidel, J C Allen, R G O'Neil, H Shelat and T Song

Hypertension. 1993;22:735-742
doi: 10.1161/01.HYP.22.5.735

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/22/5/735

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/