Insulin Attenuates Agonist-Evoked Calcium Transients in Vascular Smooth Muscle Cells

Rhian M. Touyz, Barbara Tolloczko, Ernesto L. Schiffrin

Abstract  Insulin may decrease the contractile response of vascular smooth muscle to vasoactive agents. This could be due to interactions of insulin with the effects of vasoactive agonists on intracellular free calcium transients in vascular smooth muscle cells. This study assesses the effects of physiological doses of insulin (70 μU/mL) on calcium responses in cultured vascular smooth muscle cells (primary unpassaged and passaged) to angiotensin II (1 nmol/L), arginine vasopressin (10 nmol/L), and norepinephrine (10 μmol/L). Intracellular free Ca²⁺ concentrations in single cells were measured microphotometrically using fura 2-AM. Insulin, angiotensin II, arginine vasopressin, and norepinephrine significantly increased calcium (to 115±7, 183±20, 184±15, and 168±12 nmol/L, respectively, from basal calcium of 90±10 nmol/L). Insulin significantly attenuated the agonist-induced calcium responses. The effects of insulin were almost completely inhibited by diltiazem, staurosporine, calphostin C, and thapsigargin. In conclusion, insulin stimulates calcium transients but blunts agonist-mediated calcium rises in vascular smooth muscle cells. These responses are related to regulatory effects of insulin on cellular calcium homeostasis and may explain how insulin modulates vascular smooth muscle contraction. (Hypertension. 1994;23[suppl 1]:I-25-I-28.)

Key words • insulin resistance • blood vessels • calcium • muscle, smooth, vascular

Insulin has been implicated in the pathogenesis of hypertension. Chronic hyperinsulinemia has been associated with changes to small blood vessels and increased peripheral resistance and blood pressure, whereas acute hyperinsulinemia may result in lowering of blood pressure.1-4 In isolated mesenteric rat resistance arteries and rabbit femoral vessels, insulin attenuated vasoconstrictor responses to norepinephrine, angiotensin II (Ang II), serotonin, and K⁺.5,6 In vessels from insulin-resistant and insulin-resistant rats, vasoconstrictor responses to vasoactive agents were increased.7-8 Insulin appears to decrease vascular contractile responses to both receptor- and voltage-mediated stimuli. The underlying cellular mechanisms responsible for these insulin-associated phenomena remain unclear but may be related to the effects of this peptide on transmembrane cation transport systems. Insulin affects intracellular sodium and proton metabolism by stimulating Na⁺,K⁺-ATPase, Na⁺-H⁺ exchange, and Na⁺-coupled amino acid transport and by increasing cell membrane permeability.5,9 In addition, it modulates intracellular free calcium concentrations ([Ca²⁺]) by influencing Ca²⁺-ATPase and Na⁺-Ca²⁺ exchange.10,11 Because calcium is an important determinant of vascular smooth muscle tone and contractility, insulin may play a role in blood pressure control by influencing intracellular calcium.12 Several vasoactive agents that may be important in hypertension increase vascular smooth muscle [Ca²⁺].13,14 Among others, Ang II, arginine vasopressin, and norepinephrine increase [Ca²⁺], by mobilizing intracellular calcium stores via stimulation of the phosphoinositide system and by increasing calcium influx via protein kinase C-mediated mechanisms.15 Insulin may alter vascular smooth muscle function by modulating agonist-induced calcium transients. In cultured vascular smooth muscle cells (VSMCs), supraphysiological concentrations of insulin decreased the magnitude of vasopressin-induced calcium responses.16 This study assesses the effects of physiological doses of insulin on agonist-mediated calcium responses in cultured VSMCs from rat mesenteric arteries and rabbit femoral vessels. The underling mechanisms underlying the relations between insulin and agonist-stimulated calcium responses.

Methods

Cultured Vascular Smooth Muscle Cells

Primary unpassaged and serially passaged cultured VSMCs (one to six passages) were derived from the mesenteric arteries of adult Sprague-Dawley rats weighing 250 g. The cells were prepared according to previously described techniques.17 Mesenteric arteries were cleaned of all adipose and connective tissue. VSMCs were dissociated by digestion of arteriolar arcades with 0.12 mg/mL elastase, 2 mg/mL collagenase (type I), 0.36 mg/mL soybean trypsin inhibitor, 2 mg/mL bovine serum albumin, and 100 μg/mL gentamycin in Ham's F-12 medium for 60 minutes at 37°C. The tissue was filtered through a 100-μm nylon mesh, and the cell suspension was centrifuged at 200g for 5 minutes in Dulbecco's modified Eagle medium containing 10% heat-inactivated calf serum, 2 mmol/L l-glutamine, 20 mmol/L HEPES (pH 7.4), 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin. Cells were grown on round glass coverslips (25 mm diameter) in plastic wells and maintained at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂. When confluent, the cells were subcultured by trypsinization. Mesenteric artery smooth muscle cells were passaged between one and six times. VSMCs take 7 to 8 days to reach confluence after plating; at confluence, they have the typical "hill and valley" pattern and test positive for smooth muscle cell α-actin. Cells were used for analysis on reaching confluence.

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Preparation of Cells for Fluorescence Measurements

The cells on the glass coverslips were washed three times with 2 mL. Hanks' buffered saline solution containing (mmol/L) NaCl, 137; NaHCO₃, 4.2; Na₂HPO₄, 3; KCl, 5.4; KH₂PO₄, 0.4; CaCl₂, 1.3; MgCl₂, 0.5; glucose, 10; and HEPES, 5 (pH 7.4). Fura 2-AM, dissolved in dimethyl sulfoxide (DMSO) and pluronic, was added to obtain a final concentration of 4 μmol/L fura 2-AM and 0.01% DMSO. Cells were incubated for 30 minutes at 37%. The loaded cells were washed three times with 2 mL. Hanks' buffer. Three to four glass rings (4 to 5 mm diameter) were placed on the coverslip, and a vacuum seal formed between the ring and the coverslip. Each ring was filled with 50 μL. Hanks' buffer. This method allowed examination of three to four single cells per coverslip. The coverslip containing cells and rings was placed in a Leiden chamber (Medical Systems Corp, Greenville, NY) atop the stage of an inverted microscope equipped for epifluorescence with a x40 oil immersion objective (Nikon, Montreal, Quebec, Canada). Fluorescence measurements were made using double excitation wavelength measurements (345/380 nm) and a single emission wavelength (510 nm) with a PTI D401 microfluorometer (Photon Technology International Inc, Princeton, NJ). For single cell calibration, background fluorescence was determined for every cell studied, and this was subtracted before ratio measurements were obtained. Maximum and minimum fluorescence intensities were obtained for each cell by exposure to ionomycin (10 μmol/L) and EGTA (3 mmol/L), respectively. [Ca²⁺], was calculated according to the formula of Grynkiewicz et al., 19 with a binding constant (Kᵣ) of Ca²⁺ to fura 2 of 224 nmol/L.

Protocol for Insulin, Angiotensin II, Arginine Vasopressin, and Norepinephrine

Basal and agonist-stimulated [Ca²⁺], were measured. The effects of insulin (70 μU/mL), Ang II (1 nmol/L), arginine vasopressin (10 nmol/L), and norepinephrine (10 μmol/L) on [Ca²⁺], were determined by evaluating the maximal responses to various vasoactive agents. For the determination of the effects of insulin on calcium responses to various vasoactive agents, cells were preincubated with insulin (70 μU/mL), and once the calcium responses had returned to baseline or plateau levels, the agonist was added. Single experiments were performed on single cells. The effects of diltiazem (0.1 μmol/L), staurosporine (0.1 μmol/L), calphostin C (0.1 μmol/L), and thapsigargin (0.1 μmol/L) on insulin-stimulated calcium responses were each determined in six different cells. Responsiveness of the cells to the various agonists was greater than 98%.

Materials

Insulin (human recombinant), diltiazem, staurosporine, calphostin C, and thapsigargin were from Sigma Chemical Co, St Louis, Mo. Fura 2-AM, DMSO, and pluronic were from Molecular Probes Inc, Eugene, Ore. Human Ang II, arginine vasopressin, and norepinephrine were from Peninsula Laboratories, Inc, Belmont, Calif.

Analysis of Data

Data are presented as mean±SD. Statistical analyses were performed by Student's t test or one-way analysis of variance followed by a post hoc Bonferroni correction, as appropriate, and differences were considered significant at a value of P<.05.

Results

The basal [Ca²⁺], of VSMCs was 90±10 nmol/L in primary unpassaged cells (n=17) and 82±9 nmol/L in passaged cells (one to six passages, n=29). There were no significant differences in [Ca²⁺], between unpassaged and passaged cells. Insulin, Ang II, arginine vasopressin, and norepinephrine all caused a significant transient rise in [Ca²⁺],. Insulin (70 μU/mL) increased [Ca²⁺], by 25±3 nmol/L. Ang II (1 nmol/L) by 93±8 nmol/L, arginine vasopressin (10 nmol/L) by 94±5 nmol/L, and norepinephrine (10 μmol/L) by 46±7 nmol/L (Fig 1). The stimulated [Ca²⁺], for all four agonists was significantly greater than basal [Ca²⁺], (P<.01) (Fig 1). Maximal responses to the agonists were achieved within 150 seconds, and baseline or plateau levels were obtained within 300 seconds. In cells prestimulated with insulin, the increases in [Ca²⁺], by the vasoactive agents were significantly decreased (Figs 1 and 2). The differences in [Ca²⁺], in the absence and presence of insulin were 59±8 nmol/L for Ang II, 60±4 nmol/L for arginine vasopressin, and 32±5 nmol/L for norepinephrine (Fig 1). As a control experiment to assess whether these effects were due to a "Ca²⁺ tachyphylaxis," multiple studies were performed in which cells were prestimulated with one agonist (Ang II, norepinephrine, or arginine vasopressin) and the calcium response to a second agonist was determined. Prestimulation of cells had no effect on calcium transients induced by various agonists (including Ang II, arginine vasopressin, norepinephrine, and endothelin-1, data not shown).

Insulin-induced calcium responses were almost completely abolished by diltiazem, staurosporine, and calphostin C (Table). Thapsigargin increased [Ca²⁺], to 204±10 nmol/L. In the presence of thapsigargin, insulin failed to significantly raise [Ca²⁺], (Table). When the cells were preexposed to thapsigargin, the calcium increases by Ang II, arginine vasopressin, and norepinephrine were 12±2, 14±5, and 8±3 nmol/L, respectively. These responses were significantly lower (P<.01) compared with responses in the absence of thapsigargin when Ang II increased calcium by 93±8 nmol/L, arginine vasopressin by 94±5 nmol/L, and norepinephrine by 46±7 nmol/L.

Discussion

This study demonstrates that insulin increases vascular smooth muscle calcium transients and attenuates...
FIG 2. Representative tracings show effect of angiotensin II (ANG) (1 nmol/L) on cytosolic free calcium concentration in a single vascular smooth muscle cell (A) and effect of Insulin (INS) (70 μU/mL) on unstimulated and angiotensin II (1 nmol/L)-stimulated cytosolic free calcium concentration in another vascular smooth muscle cell (B).

responses to Ang II, arginine vasopressin, and norepinephrine. In this study, unlike others, physiological doses of insulin were used, and cells from resistance arteries were examined. The blunted calcium response may explain in part how insulin decreases vasoconstrictor responses in isolated vessels and how it may play a role in blood pressure regulation.

Underlying mechanisms by which insulin induces changes in [Ca^{2+}], are unclear. This study shows that calcium antagonists inhibited insulin-evoked calcium increases, possibly by inhibition of voltage-dependent calcium channels. Voltage-dependent channels have been demonstrated in VSMCs derived from human and animal vessels. Staurosporine and calphostin C decreased the insulin-sensitive calcium response. Staurosporine is only relatively specific, whereas calphostin C is a highly selective protein kinase C inhibitor. Thus, the data suggest that calcium changes may be modulated via a protein kinase C-mediated mechanism. Protein kinase C is linked to Na^{+}-Ca^{2+} exchange via the Na^{+}-H^{+} transporter, and it is possible that insulin also exerts its effects on intracellular calcium through this system, as has previously been demonstrated in rat VSMCs and human mesangial cells.

Pretreatment of cells with thapsigargin, which inhibits endoplasmic reticular [Ca^{2+}]-ATPase and raises [Ca^{2+}], abolished the insulin-evoked calcium response. These data may imply that insulin increases [Ca^{2+}], by acting on intracellular calcium storage sites as well as by stimulating influx. However, thapsigargin resulted in [Ca^{2+}] similar to that attained with insulin, and insulin may not be able to further raise [Ca^{2+}]. Thus, the effect of insulin to increase intracellular calcium may occur mainly by stimulating calcium influx from the extracellular pool and possibly to a lesser extent by influencing calcium release from the endoplasmic reticulum. Baseline or plateau levels of [Ca^{2+}], occurred within a short time after insulin treatment. Normalization of [Ca^{2+}], was probably due to stimulation of Ca^{2+}-ATPase by insulin and by the increased [Ca^{2+}], itself.

The blunting effect of insulin on calcium transients by vasoactive agonists may be related to modulation of cellular calcium by insulin. Increased calcium influx may be counterbalanced by increased endoplasmic reticular calcium release and enhanced calcium efflux, which may deplete the cell of its intracellular calcium stores. Thus, further stimulation by agonists such as Ang II, vasopressin, and norepinephrine, which are known to increase [Ca^{2+}], by stimulating intracellular calcium mobilization and enhancing cellular influx, may result in an attenuated calcium response. Also, vasoactive agents such as vasopressin stimulate calcium inflow via nonspecific cation channels. These currents may be inhibited by insulin, suggesting another pathway by which insulin might blunt the agonist-induced calcium response in VSMCs.

In this study, insulin effects on agonist-stimulated calcium responses occurred within a short time period. Our results support other studies in which insulin-induced changes occurred within 5 minutes of insulin treatment. Previous studies demonstrated effects within 30 minutes of insulin introduction. Together with the results of our study, these data suggest that attenuation of agonist-induced calcium responses by insulin may result both from an early blunting of calcium influx and mobilization and from a late effect on calcium efflux.
If these in vitro data are extrapolated to in vivo situations, the blunting effect of insulin on agonist-stimulated calcium transients would manifest as decreased vasoconstriction. Thus, insulin may have a "protective" or inhibitory influence on the contractile effects of vasoactive agents. These phenomena may have important implications in blood pressure regulation and, if defective, may play a role in hypertension. Hypertension is associated with hyperinsulinemia and insulin resistance. It is proposed that in hypertension, insulin resistance is targeted at both glucose and calcium cellular responses. If this occurred in vascular smooth muscle, there would be a loss of the attenuation of agonist-induced calcium responses by insulin, and as a result, the vasoconstrictor response would be increased, producing an increased peripheral resistance and hypertension. In addition, hyperinsulinemic effects in some tissues (eg, sympathetic nervous system) may act synergistically with the effects of insulin resistance in other tissues (eg, vascular smooth muscle), contributing to hypertension.

Although insulin increases vascular smooth muscle calcium, studies have failed to demonstrate insulin-mediated vasoconstriction. The exact explanation for this remains unclear. Unlike vasoactive peptides, which increase intracellular calcium to very high concentrations, the insulin-stimulated increase is small. This elevation of calcium by insulin may not be high enough to induce vasoconstriction, particularly if it is not accompanied by a simultaneous increase in myofilament sensitivity to calcium or activation of other signal transduction mechanisms that act synergistically with calcium to induce vasoconstriction.

In conclusion, insulin modulates calcium transients in vascular smooth muscle. Possible mechanisms by which insulin increases [Ca\(^{2+}\)]\(_i\), are by stimulating calcium influx via calcium channels and protein kinase C-mediated mechanisms and also by influencing mobilization of intracellular calcium stores. These mechanisms remain to be demonstrated directly. By modulating cellular calcium metabolism, insulin attenuates vascular smooth muscle [Ca\(^{2+}\)]\(_i\) responses to Ang II, arginine vasopressin, and norepinephrine. Because the responses were similar for all three agents, the effects of insulin on agonist-induced calcium transients appear to be nonspecific. The influence of insulin on vascular smooth muscle calcium may be an important mechanism whereby agonist-induced responses are regulated. This regulatory function may be altered in hypertension, diabetes, and other insulin-resistant states.

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