Insulin attenuates agonist-mediated calcium mobilization in cultured rat vascular smooth muscle cells.

F Saito, M T Hori, M Fittingoff, T Hino, M L Tuck

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Research Article

Insulin has been shown to attenuate pressor-induced vascular contraction, but the mechanism for this vasodilatory action is unknown. This study examines the effect of insulin on angiotensin II (ANG II)-induced increments in cytosolic calcium in cultured rat vascular smooth muscle cells (VSMC). 20-min incubations with insulin (10 microU/ml to 100 mU/ml) did not alter basal intracellular calcium concentration ([Ca2+]i), but inhibited the response to 100 nM ANG II in a dose-dependent manner (ANG II alone, 721 +/- 54 vs. ANG II + 100 mU/ml insulin, 315 +/- 35 nM, P < 0.01). A similar effect of insulin on ANG II action was observed in calcium poor buffer. Moreover, insulin did not effect calcium influx. ANG II receptor density and affinity were not affected by 24-h incubation with insulin. To further clarify the mechanisms of these observations, we measured ANG II-induced production of inositol 1,4,5-triphosphate (IP3), and IP3-releasable 45Ca. Insulin treatment did not alter ANG II-stimulated IP3 production. However, IP3-stimulated release of 45Ca in digitonin permeabilized cells was significantly reduced after 5-min incubations with 100 mU/ml insulin. Thapsigargin induced release of calcium stores was also blocked by insulin. Thus, insulin attenuates ANG II-stimulated [Ca2+]i primarily by altering IP3-releasable calcium stores. Insulin effects on ANG II-induced [Ca2+]i were mimicked by preincubation of VSMC with either sodium nitroprusside or 8-bromo-cGMP. As elevations in […]

Insulin Attenuates Agonist-mediated Calcium Mobilization in Cultured Rat Vascular Smooth Muscle Cells

Fumio Saito, Mark T. Hori, Marianne Fittingoff, Toru Hino, and Michael L. Tuck
Division of Endocrinology, Veterans Affairs Medical Center, Sepulveda, California 91343; and UCLA School of Medicine, Los Angeles, California 90024

Abstract

Insulin has been shown to attenuate pressor-induced vascular contraction, but the mechanism for this vasodilatory action is unknown. This study examines the effect of insulin on angiotensin II (ANG II)-induced increments in cytosolic calcium in cultured rat vascular smooth muscle cells (VSMC). 20-min incubations with insulin (10 μU/ml to 100 mU/ml) did not alter basal intracellular calcium concentration ($[Ca^{2+}]_i$), but inhibited the response to 100 nM ANG II in a dose-dependent manner (ANG II alone, 721±54 vs. ANG II + 100 mU/ml insulin, 315±35 nM, $P < 0.01$). A similar effect of insulin on ANG II action was observed in calcium poor buffer. Moreover, insulin did not effect calcium influx. ANG II receptor density and affinity were not affected by 24-h incubation with insulin. To further clarify the mechanisms of these observations, we measured ANG II-induced production of inositol 1,4,5-triphosphate (IP_3), and IP_3-releasable ^{45}Ca. Insulin treatment did not alter ANG II-stimulated IP_3 production. However, IP_3-stimulated release of ^{45}Ca in digitonin permeabilized cells was significantly reduced after 5-min incubations with 100 mU/ml insulin. Thapsigargin induced release of calcium stores was also blocked by insulin. Thus, insulin attenuates ANG II-stimulated $[Ca^{2+}]_i$ primarily by altering IP_3-releasable calcium stores. Insulin effects on ANG II-induced $[Ca^{2+}]_i$ were mimicked by preincubation of VSMC with either sodium nitroprusside or 8-bromo-cGMP. As elevations in cGMP in vascular tissue lower $[Ca^{2+}]_i$, it is possible that insulin affects IP_3 release of calcium by a cGMP-dependent mechanism that would contribute to its vasodilatory effects. (*J. Clin. Invest.* 1993. 92:1161–1167.) Key words: cyclic nucleotides • hypotensive • inositol triphosphate • intracellular calcium stores • signal transduction

Introduction

Although a number of studies have emphasized the relationship between blood pressure and insulin in clinical and experimental hypertension (1–6), the direct effects of insulin on vascular tissue have yet to be clarified. While chronic hyperinsulinemia is thought to contribute to hypertension through several mechanisms including abnormal renal sodium handling (4, 7, 8), increased sympathetic nerve activity (8–10), and vascular reactivity (7), the acute action of insulin is vasodilatory as shown both in vivo (11–14), and in isolated vascular tissue (15, 16).

It is generally accepted that vasoactivity is, in part, modulated by alterations in cytosolic free calcium concentration (17). Recent studies have shown that insulin increases Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase) activity in the renal basolateral membrane (18) and that this effect is decreased in Zucker rats, an obese model of insulin resistance reported in some studies to be hypertensive (19). These observations might predict that the mechanism of insulin's acute vasodilatory actions occurs by alterations in Ca^{2+} pump activity and a decrease in intracellular calcium concentration. It follows then that chronic hyperinsulinemia and insulin resistance may contribute to the development of hypertension through impairment of this acute vasodilatory function.

This study examines the effect of insulin on angiotensin II (ANG II)[1]-mediated intracellular calcium $[Ca^{2+}]_i$ and calcium mobilization in cultured rat vascular smooth muscle cells (VSMC). To clarify mechanisms, ANG II-receptor responses, calcium influx, ANG II–stimulated inositol 1,4,5-triphosphate (IP_3), and IP_3 releasable calcium were measured in insulin-treated VSMC. In addition, the role of cGMP as the mediator of insulin action was assessed.

Methods

Cell culture. VSMC were isolated from rat thoracic aorta (250–300-g male Sprague-Dawley rats) by enzymatic dispersion as previously described (20). The resulting cells were grown in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (Intergen Co., Purchase, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma Chemical Co.). Confluent cultures were passed by treating with trypsin-EDTA and reseeding at a 1:4 ratio in fresh media. Cells (passages 4–12) were seeded onto 25-mm round glass coverslips and reached confluence in 3–5 d. Before experimentation, cell lines were randomly screened for smooth muscle actin expression as assayed by immunofluorescent staining with anti-rat smooth muscle actin (Enzo Diagnostics Inc., New York) and visualized with fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Organon Teknica Corp., West Chester, PA).

Measurement of $[Ca^{2+}]_i$. Confluent cells attached to the coverslips were deprived of serum for 24 h before study. Cells were loaded with 4 μM fura-2 acetoxymethylester (Molecular Probes, Inc., Eugene, OR) for 40 min at 37°C in physiologic salt solution (PSS; 143 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 6 mM glucose, and 10 mM Hepes, pH 7.4). Loaded cells were washed three times and then incubated with fresh PSS for an additional 15 min at

Address reprint requests to Dr. Michael Tuck, Division of Endocrinology (111E), Veterans Affairs Medical Center, UCLA School of Medicine, 16111 Plummer Street, Sepulveda, CA 91343.

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1. *Abbreviations used in this paper:* ANG II, angiotensin II; IGF, insulin-like growth factor; IP_3, inositol 1,4,5-triphosphate; L-NMMA, L-N^G-monomethyl-L-arginine; PSS, physiologic salt solution; TG, thapsigargin; VSMC, cultured vascular smooth muscle cells.

37°C to allow hydrolysis of the entrapped ester. Coverslips were then rinsed in fresh PSS, fixed to a specially designed chamber, and placed in a thermoregulated holder (maintained at 37°C) on the microscopic stage. Photon emission was monitored at 510 nm with excitation wavelength alternating between 340 (F340) and 380 nm (F380) using a Deltascan spectrofluorometer (Photon Technology International, South Brunswick, NJ). At the end of each experiment, the minimum (R_{min}) and maximum (R_{max}) ratio of F340 and F380 (R340/380) was determined in PSS containing no added Ca^{2+}, 4 mM EGTA and 1 μM ionomycin and in PSS containing 10 mM $CaCl_2$ and 1 μM ionomycin, respectively. Each coverslip was individually corrected for autofluorescence by Mn^{2+} quenching and $[Ca^{2+}]_i$ was calculated according to the formula (21):

$$[Ca^{2+}]_i = K_d \times \left[\frac{(R - R_{min})}{(R_{max} - R)}\right] \times \frac{Sf_2}{Sb_2},$$

where K_d represents the dissociation constant of fura-2 for calcium (224 nM), R represents the ratio of F340/F380, Sf_2 is the F380 intensity obtained from R_{min} (free fura-2), and Sb_2 is the F380 when the dye is fully saturated with calcium collected during the R_{max} determination. Intracellular calcium responses to 100 nM ANG II (Sigma Chemical Co.) were obtained in VSMC exposed to insulin (purified pork insulin, Regular ILETIN II, Eli Lilly & Co., Indianapolis, IN) 10 μU/ml to 100 mU/ml. For short-term studies, cells were incubated for 20 min with insulin diluted in PSS and ANG II was added without changing the buffer in the well. For long-term studies, insulin was added for 24 h in serum-free medium. Short-term studies were also done comparing ANG II-induced $[Ca^{2+}]_i$ response with insulin to insulin-like growth factors (IGF-I or IGF-II; 2.5 ng/ml, Intergen Co.).

ANG II receptors. ANG II receptors were estimated by displacement assay using a modification of standard techniques (22). Cells in six-well culture dishes were incubated in serum-free medium with or without added insulin at concentrations of 10 μU/ml to 1 mU/ml for 24 h. Cells were then washed in cold PBS and incubated with 0.1 μCi ^{125}I-ANG II (New England Nuclear, Boston, MA) and graded amounts of unlabeled ANG II from 1 nM to 1 μM for 4 h at 4°C in PBS containing 0.1% RIA grade BSA. Nonspecific background binding was determined by incubation with 50 μM ANG II. Unbound ANG II was then removed by three washes in fresh buffer, and then cells hydrolyzed in 1.0 N NaOH overnight. The resulting solution was then thoroughly mixed and a 0.5-ml aliquot was counted in a Gamma 4000 scintillation counter (Beckman Instruments Inc., Palo Alto, CA). The binding affinity and receptor density of all curves were analyzed by Scatchard plotting using the computer program LIGAND by Munson and Robard (23).

Calcium influx. Manganese (Mn^{2+}) was used as a surrogate for Ca^{2+} for the estimation of calcium influx rates. Mn^{2+} uses the same influx channels as Ca^{2+} and has a very high affinity for fura-2, and binding of Mn^{2+} to the fura-2 molecule quenches its fluorescence (24). To test the effect of insulin on calcium influx rates, 20 mM $MnCl_2$ was added to the incubation solution of fura-2–loaded VSMC. Total fluorescence was then monitored either by adding the 340- and 380-nm signals, or by monitoring the photon emission at 360 nm, the isosbestic wavelength for fura-2. The former method allows for simultaneous calculation of the calcium concentration. After a stable quench rate was established (usually 60–120 s) 100 mU insulin was added and fluorescence followed. ANG II-induced calcium influx (Mn^{2+} quench) was used as a positive control.

Measurement of ANG II-stimulated production of 1,4,5-IP$_3$. For instantaneous IP_3 measurements, confluent cells in six-well culture dishes were stimulated with 100 nM ANG II for 15, 30, 60, or 120 s. For measurement of total IP_3 production (phospholipase C activity), cells were incubated with ANG II for 5 min in the presence of 10 mM LiCl. In both experiments, reactions were stopped by addition of an equal volume of ice-cold 15% (wt/vol) TCA. The TCA was then removed by thrice washing with 10 vol of water-saturated diethyl ether. Samples were then titrated to neutral pH with saturated sodium bicarbonate, rapidly frozen, and lyophilized. Samples were subsequently reconstituted with distilled water, and IP_3 was measured using a radioligand binding assay system (Amersham Corp., Arlington Heights, IL).

Intracellular release of calcium. The IP_3-sensitive calcium pool was estimated using a modification of previously described methods (25). Briefly, confluent VSMC in 12-well culture dishes were loaded with 1 μCi/ml ^{45}Ca (New England Nuclear) for 30 min at 37°C. Loading, and all subsequent steps, were done in the presence of 5 mg/liter ruthenium red to prevent uptake of calcium by the mitocondria and to block membrane Ca-Mg ATPase activity and Ca-induced Ca mobilization. Cells were washed twice with HBSS and incubated with 100 mU/ml insulin or HBSS without insulin for 5 min at ambient temperature. After removal of the treatment buffer, cells were treated with 35 μM digitonin in Hepes buffer with or without 10 μM IP_3 (Calbiochem Corp., San Diego, CA) for 5 min, and then 1-ml aliquots were collected for counting. The IP_3-sensitive Ca^{2+} was calculated by subtracting digitonin released ^{45}Ca from digitonin plus IP_3-released ^{45}Ca. In order to confirm these observations, spectrofluorometric experiments were also conducted to investigate the effect of insulin on the release of calcium by 10 μM thapsigargin (TG) (26). Fura-2–loaded cells were preincubated with either 100 mU/ml insulin or heat-inactivated insulin for 20 min and then challenged with 10 μM TG and $[Ca^{2+}]_i$ calculated as described above. TG is known to release calcium from sarcoplasmic or endoplasmic reticulum and inhibit all isoforms of sarcoplasmic or endoplasmic reticulum Ca-ATPase (27).

Effect of cGMP on $[Ca^{2+}]_i$ and IP$_3$-releasable calcium. The effect of cGMP accumulation on $[Ca^{2+}]_i$ and IP_3-releasable calcium was also tested in VSMC. VSMC were treated with 10 μM 8-bromo-cGMP for 5 min before ANG II addition and determination of $[Ca^{2+}]_i$. Sodium nitroprusside, which stimulates nitrous oxide and cGMP production, was also tested for IP_3-sensitive calcium release in ^{45}Ca-loaded VSMC. To test the effect of nitrous oxide inhibition on insulin attenuation of ANG II-induced $[Ca^{2+}]_i$, fura-2–loaded VSMC were treated with 10 μM N^G-monomethyl-L-arginine (L-NMMA) (Calbiochem Corp.) for 5 min before insulin treatment and then stimulated with 100 nM ANG II and $[Ca^{2+}]_i$ was determined.

Statistics. Results are expressed as mean±SEM. Studies with two groups were compared by unpaired Student's t test. Studies with three or more groups were evaluated by analysis of variance (ANOVA) with subset analysis by Tukey contrast.

Results

Insulin dose effect on ANG II-stimulated $[Ca^{2+}]_i$. 20 min of incubation with insulin alone did not alter basal $[Ca^{2+}]_i$ level in VSMC (Fig. 1). However, mean ANG II–induced $[Ca^{2+}]_i$ levels were inhibited by 20-min insulin incubations in a dose-dependent manner (Fig. 1). Fig. 2 is a representative time course for ANG II–induced $[Ca^{2+}]_i$ responses showing that $[Ca^{2+}]_i$ responses were inhibited by 20-min addition of 1 and 100 mU/ml insulin. Cross-reactivity of insulin with IGF receptors in VSMC was also tested. The potency of insulin to displace ^{125}I-IGF is 1,000–2,000 times less than IGF itself as studied in rat cultured VSMC (28, 29). Therefore, control studies were performed using 2.5 ng/ml IGF-I and IGF-II, a concentration that does not significantly cross-react with other receptors (29, 30), but is approximately equivalent to the displacement of IGFs from their receptors by 100 mU/ml insulin. Fig. 3 shows mean basal and ANG II–stimulated peak $[Ca^{2+}]_i$ from control, 100 mU/ml insulin, 2.5 ng/ml of IGF-I– or IGF-II–treated VSMC. Insulin attenuated ANG II–stimulated $[Ca^{2+}]_i$, whereas IGF-I and IGF-II did not significantly alter $[Ca^{2+}]_i$ responses.

Extracellular calcium and calcium influx studies. To evaluate the contribution of extracellular calcium to insulin's effect

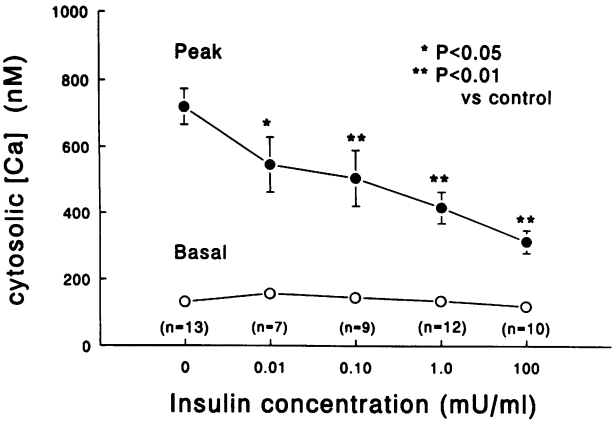

Figure 1. Effect of insulin on ANG II–induced calcium transients. Mean±SEM basal $[Ca^{2+}]_i$ (○), and peak responses (●) to 100 nM ANG II after 20-min of preincubation with control buffer or 10 μU/ml to 100 mU/ml insulin. No changes in basal $[Ca^{2+}]_i$ are noted but peak responses show a dose-dependent decrease in ANG II–induced $[Ca^{2+}]_i$ responses to insulin.

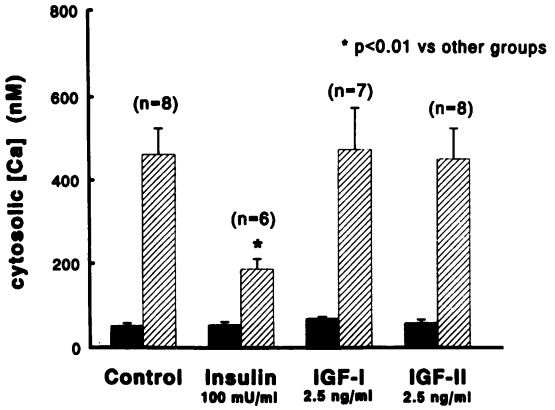

Figure 3. Comparison of insulin to IGF-I and IGF-II on $[Ca^{2+}]_i$. Mean±SEM basal $[Ca^{2+}]_i$ (*solid bars*) and peak responses (*hatched bars*) to 100 nM ANG II after 20 min of incubation with control buffer, 100 mU/ml insulin, and 2.5 ng/ml of IGF-I and IGF-II. No differences were observed in ANG II–induced $[Ca^{2+}]_i$ in responses to IGF-I or IGF-II.

on ANG II–induced $[Ca^{2+}]_i$ responses, 4 mM EGTA was added to the medium 19 min after insulin and 1 min before ANG II addition. There was no difference in basal $[Ca^{2+}]_i$ in control VSMC and VSMC exposed for 20 min to 100 mU/ml of insulin (Fig. 4). However, ANG II–stimulated $[Ca^{2+}]_i$ responses were significantly attenuated in insulin-treated VSMC (Fig. 4). Importantly, the percent attenuation of ANG II–induced $[Ca^{2+}]_i$ responses by 100 mU/ml insulin was similar in these experiments to studies using 2 mM extracellular $CaCl_2$.

Calcium influx was determined using Mn^{2+} quenching of fura-2 in VSMC in the presence of ANG II or insulin (Fig. 5). ANG II addition to Mn^{2+}-treated VSMC induced Ca^{2+} influx as demonstrated by an increase in the quench rate of the total fluorescence whereas insulin had no effect on Mn^{2+} quenching of fura-2 fluorescence.

Effect of 24 h of insulin treatment on ANG II–stimulated $[Ca^{2+}]_i$. Fig. 6 shows that basal $[Ca^{2+}]_i$ levels in VSMC preincubated for 24 h with 10 μU/ml to 10 mU/ml insulin did not change from control. However, 24-h insulin treatment attenuated ANG II–induced $[Ca^{2+}]_i$ responses at doses of 100 μU/ml or higher.

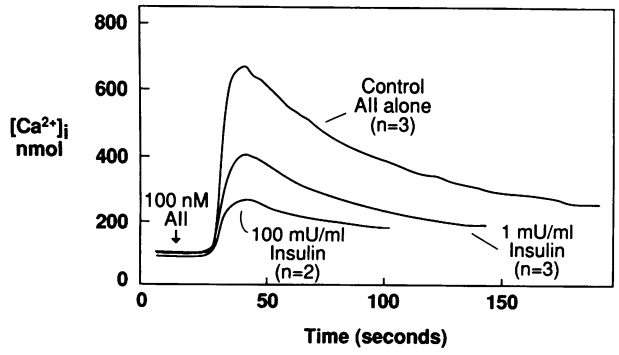

Figure 2. Time course of calcium transients. $[Ca^{2+}]_i$ responses to 100 nM ANG II after 20 min of preincubation with control buffer (n = 3), 1 mU/ml (n = 2), and 100 mU/ml (n = 3) insulin. Time 0 in the figure begins after 19 min of insulin treatment. Observed time course curves from 1 d were averaged at each concentration.

Effect of insulin on ^{125}*I-ANG II binding.* Competitive binding displacement of ^{125}I-ANG II in VSMC was not altered by incubation (24 h) in serum-free media with 0.05, 0.10, 0.5, and 1.00 mU/ml of insulin (Table I). Scatchard plot results show no significant changes in ANG II receptor density or affinity over the concentration range of insulin studied.

Effect of insulin on ANG II–stimulated 1,4,5-IP$_3$ production. VSMC incubated with four doses of insulin for 24 h showed a dose-dependent increase in mean values for peak ANG II–induced IP_3 production (Fig. 7). However, acute insulin incubation (20 min) had no effect on peak IP_3 production (30 s after ANG II stimulation). Phospholipase C activity (estimated by IP_3 production in the presence of 10 mM LiCl) was also not altered by acute insulin incubation.

Effect of insulin on IP$_3$-releasable ^{45}*Ca.* The effect of insulin on intracellular calcium stores was examined using IP_3-mediated release of ^{45}Ca from permeabilized VSMC preloaded with ^{45}Ca. Incubation of ^{45}Ca-loaded VSMC with 100 mU/ml

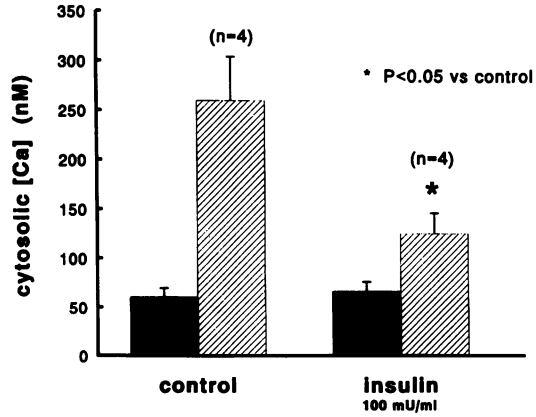

Figure 4. Effect of insulin on ANG II–induced $[Ca^{2+}]_i$ calcium-poor buffer. Mean±SEM basal $[Ca^{2+}]_i$ (*solid bars*) and peak responses (*hatched bars*) to 100 nM ANG II after 20 min of incubation with control buffer and 100 mU/ml insulin in calcium chelated medium. Insulin also attenuates ANG II–induced $[Ca^{2+}]_i$ responses at a reduced Ca^{2+} gradient.

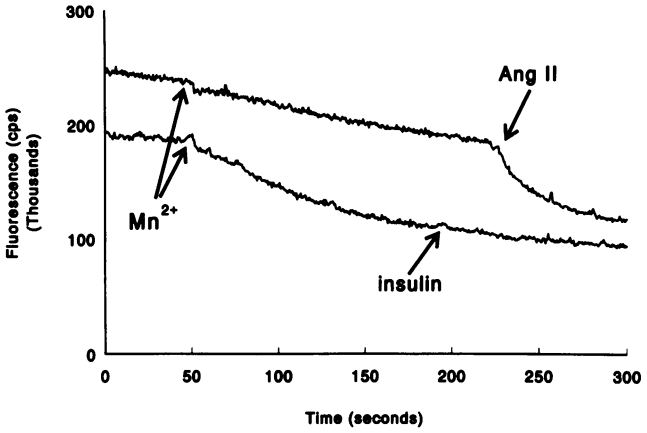

Figure 5. Effect of ANG II and insulin on Manganese influx. Fluorescence decay curves of fura 2–loaded VSMC over 300 s with addition of Mn^{2+} at 50 s and subsequent addition of ANG II or insulin between 200 and 240 s. Ang II reduces, whereas insulin has no effect on, Mn^{2+} quenching.

insulin caused a marked reduction ($P < 0.05$) in percentage from baseline of IP_3-releasable ^{45}Ca (Fig. 8). Ruthenium red was used to isolate IP_3-mediated calcium release from sarcoplasmic reticulum by blocking ^{45}Ca uptake in the mitochondria (25). Ruthenium red also blocks membrane Ca-Mg ATPase activity (31), and calcium-mediated Ca release from sarcoplasmic reticulum (32).

TG releases calcium from intracellular stores and prevents its reuptake, so it can be used as a means to estimate intracellular calcium pools (26). TG (10 μM) increased $[Ca^{2+}]_i$ from a basal level of 126.4±9.4 to 210.3 + 21.6 nM (Fig. 9). Preincubation with 100 mU/ml insulin for 20 min completely inhibited TG-induced $[Ca^{2+}]_i$ responses in VSMC indicating that its effect on reducing mobilization of calcium is from the same pool as TG effects.

Effect of cGMP on $[Ca^{2+}]_i$ and IP_3-releasable calcium. In fura 2–loaded VSMC, 5-min incubations with 10 μM of the cell-permeable, cGMP mimetic, 8-bromo-cGMP, attenuated mean ANG II–induced $[Ca^{2+}]_i$ levels (change in $[Ca^{2+}]_i$: 306.5±50.7 for control vs. 119.8±20.0 for 8-bromo-cGMP, $P < 0.01$). Fig. 10 shows a representative tracing from these experiments. In addition, incubation of VSMC with 8-bromo-cGMP blocked release of calcium by IP_3 as did addition of the nitrous oxide agonist sodium nitroprusside (50 μM) (data not shown).

Cultured VSMC have been shown to generate L-arginine-derived NO through pathways that are not sensitive to acetylcholine or bradykinin (33). Fig. 11 shows that pretreatment of VSMC with 10 μM of the NO inhibitor L-NMMA blocked the effect of insulin to attenuate ANG II–induced $[Ca^{2+}]_i$ responses.

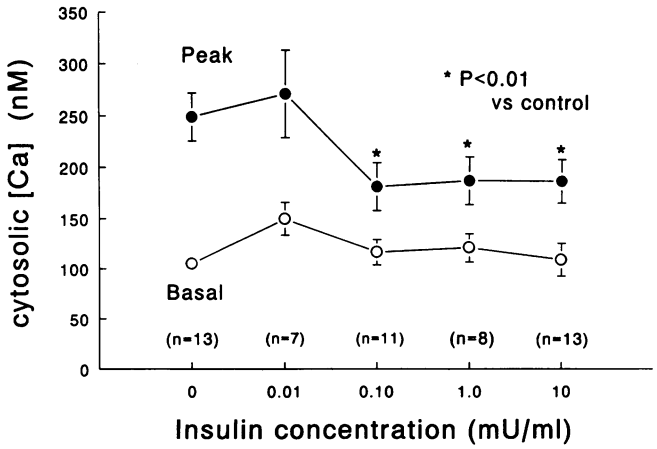

Figure 6. ANG II–mediated $[Ca^{2+}]_i$ responses after 24 h of incubation with insulin. Basal $[Ca^{2+}]_i$ (○), and peak responses (●) to 100 nM ANG II after 24 h of incubation with 0–10 mU/ml insulin. No significant differences are observed in basal $[Ca^{2+}]_i$, however insulin attenuates peak ANG II–induced $[Ca^{2+}]_i$ responses at doses of 100 μU/ml or higher.

Table I. Effect of Insulin on ANG II Receptor Number and Affinity

[Insulin]	B_{max}	K_d
mU/ml	$\times 10^{-11}$	$\times 10^{-9}$
Control	3.74±0.73	14.6±3.6
0.05	4.80±0.48	12.6±2.2
0.10	2.50±0.27	7.6±1.1
0.50	4.09±0.22	19.0±1.3
1.00	3.09±0.78	13.1±4.1

Scafit estimates of maximal binding (B_{max}) and dissociation constant (K_d)±residual variances as estimated from iterative curve fits of triplicate determinations of competative binding displacement of ^{125}I-ANG II by increasing concentrations of unlabeled ANG II. No differences were observed in ANG II receptor density or affinity after 24-h of incubation with insulin.

Discussion

One mechanism for insulin's vasodilatory action in vascular tissue may be through alterations in intracellular calcium metabolism. This action on calcium is not seen with insulin incubation alone, but appears to be an effect to interfere with pressor hormone mobilization of calcium in vascular cells. Insulin consistently produced a dose-dependent inhibition of ANG II–induced $[Ca^{2+}]_i$ responses. This effect on pressor hormone action may not be confined to ANG II in that arginine vasopressin-induced calcium transients in cultured VSMC are also in-

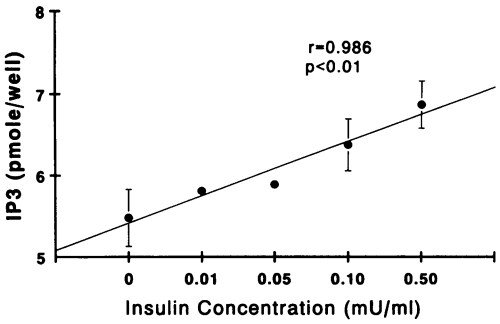

Figure 7. ANG II–mediated IP_3 responses after 24 h of incubation with insulin. Mean±SEM of triplicate determinations of the IP_3 response 30 s after ANG II stimulation. There are no significant changes in mean IP_3 responses with insulin.

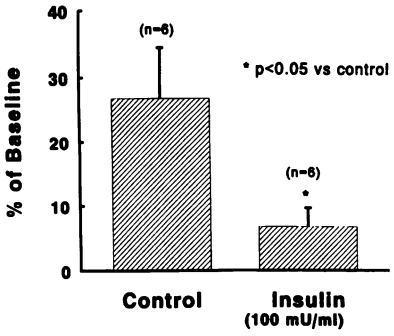

Figure 8. Effect of insulin on IP_3-releasable calcium. Mean±SEM of ^{45}Ca released (*hatched bars*) from preloaded VSMC by 10 μM IP_3 in control and after 5 min of exposure to 100 mU/ml insulin. Mean IP_3-releasable ^{45}Ca is reduced with addition of insulin.

hibited by insulin (34). Moreover, Kahn et al. (35) recently demonstrated reduced insulin attenuation of seratonin induced contraction and $[Ca^{2+}]_i$ in canine femoral artery VSMC. The dramatic effect of insulin to block IP_3-sensitive and TG sensitive calcium release indicates that its primary site of action is on intracellular calcium release from the sarcoplasmic reticulum. Although there may be effects of insulin on membrane calcium fluxes in vascular tissue, these appeared minimal in our studies because insulin's action was retained in calcium poor medium. There was also no influence of insulin on manganese influx, a newer method to measure real-time changes in calcium influx through calcium channels. In that stimulation of intracellular cGMP production with nitroprusside and 8-bromo-cGMP duplicated the effect of insulin on cytosolic calcium and IP_3-sensitive calcium release, we believe that insulin is acting through cyclic nucleotide pathways in altering calcium mobilization. It is thought that one of insulin's intracellular effects is to changes cyclic nucleotide metabolism (36) and elevation of cGMP in vascular tissue has been shown to reduce intracellular calcium (37). Insulin regulation of calcium in vascular cells may be via a cGMP-dependent pathway such as cGMP-dependent protein kinase or by regulation of phosphodiesterases, as has been demonstrated in hepatocytes and adipocytes (38, 39).

There are other mechanisms not tested in the present study to explain insulin's effect on calcium in blood vessels. The Na^+/Ca^{2+} exchanger and the Ca^{2+}-ATPase systems are present in VSMC to maintain a positive Ca^{2+} gradient (40–43). Insulin stimulates Ca^{2+}-ATPase activity in dog and rat kidney

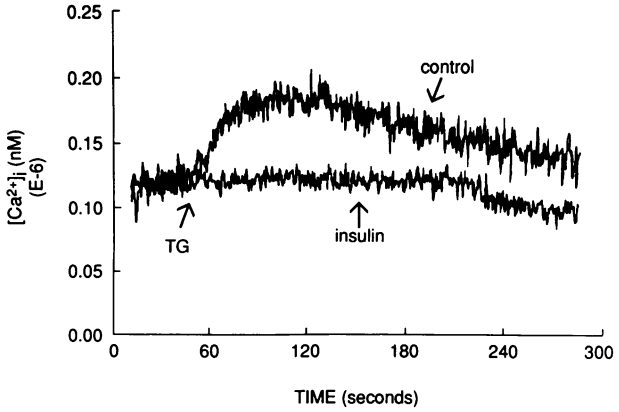

Figure 9. Effect of insulin on TG-induced $[Ca^{2+}]_i$ responses. $[Ca^{2+}]_i$ responses over 300 s to TG (10 μM) in control and 100 mU/ml insulin-treated VSMC. TG $[Ca^{2+}]_i$ responses are reduced in the presence of insulin.

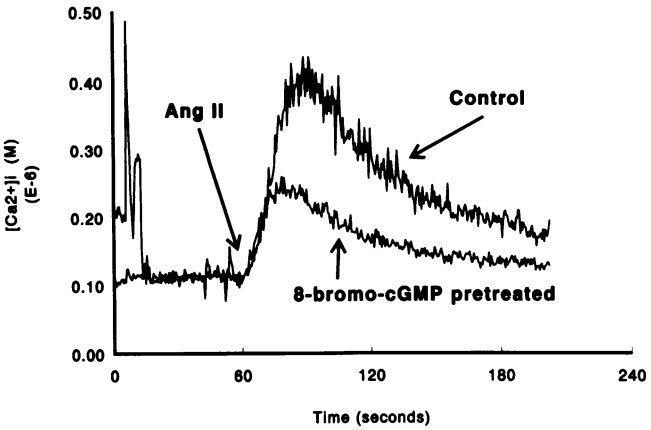

Figure 10. Effect of 8-bromo-cGMP on ANG II–induced $[Ca^{2+}]_i$ responses. $[Ca^{2+}]_i$ responses over 200 s to 100 nM ANG II in control and 8-bromo-cGMP–treated (10 μM) VSMC. $[Ca^{2+}]_i$ responses are reduced with 8-bromo-cGMP.

basolateral membranes (18, 44). Ca^{2+}-ATPase activity is reported decreased in kidney basolateral membranes in Zucker obese rats (19), an animal model of insulin resistance and hypertension (1). Decreased ^{45}Ca efflux is also seen in ^{45}Ca-loaded aortic strips from Zucker obese rats (45). However, in the present study we demonstrate effects of insulin that are probably independent of changes in the Ca^{2+}-ATPase pump. Since IP_3 release of ^{45}Ca was done in the presence of ruthenium red which blocks Ca^{2+}-ATPase, attenuation of calcium release from these stores by insulin must be from other mechanisms. Insulin has also been shown to stimulate the Na^+-K^+ pump (46), which leads to secondary increases in Na^+/Ca^{2+} exchange. This mechanism was not tested in our studies.

The affinity and density of ANG II receptors were not altered by 24-h incubation of VSMC with insulin and ANG II–stimulated phospholipase C activity was also unchanged. These findings indicate that insulin attenuation of Ang II–stimulated $[Ca^{2+}]_i$ responses is not by its receptor or immediate postreceptor signaling mechanisms. IP_3 formation is mediated by agonists such as ANG II, releasing calcium from intracellular stores in vascular smooth muscle cells (47). We observed no

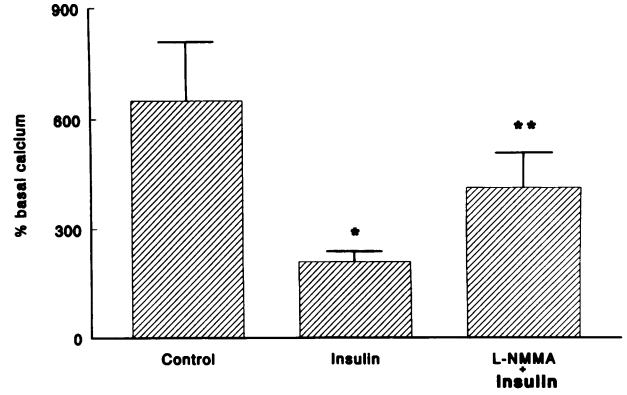

Figure 11. Effect of L-NMMA on $[Ca^{2+}]_i$ responses to ANG II. Mean±SEM $[Ca^{2+}]_i$ responses to ANG II (% over basal) during control, insulin, and insulin plus L-NMMA. Insulin reduces mean $[Ca^{2+}]_i$ responses to ANG II, whereas insulin + L-NMMA mean $[Ca^{2+}]_i$ is not different from control. *$P < 0.05$ vs. control; ** $P < 0.05$ vs. insulin alone.

change in IP_3 production in vascular cells after 20 min of incubation with insulin but noted a modest dose-dependent increase in IP_3 after 24-h exposure. However, in the acute studies, despite no alterations in IP_3 production, there was a decrease in IP_3-releasable ^{45}Ca with insulin treatment. Thus, insulin affects calcium not by altering IP_3 levels but by changing the sensitivity of IP_3 for calcium release or by depleting IP_3-releasable intracellular calcium stores.

When insulin is administered in vivo or added to in vitro vessel preparations, most studies find an acute vasodilatory response. For example, hypotension with insulin treatment has been reported in diabetic patients with the complications of diabetic neuropathy (11). Other studies in humans have also noted an acute dose-dependent vasodilatory effect of insulin (12–14). Infusion of increasing doses of insulin by euglycemic clamp in normal controls causes a substantial increase in leg blood flow suggesting a generalized vasodilator response (48). Interestingly, at the same doses of infused insulin, obese subjects have less increase in leg blood flow: results that could be interpreted to show resistance to insulin's vasodilatory action in the blood vessels of the obese. In vitro vascular preparation experiments also show that incubation with insulin for 30–120 min attenuates the vasoconstrictor response to norepinephrine in the resistance bed of the isolated perfused rat tail (15). Yagi et al. (16) performed extensive studies with rabbit femoral arteries and veins showing that 30-min incubations with insulin dose-dependently inhibits the vasoconstrictive responses to norepinephrine and Ang II.

Although the role of insulin as a regulator of cell calcium remains controversial, several effects on calcium mobilization and pump activity have been reported. Insulin has been found to alter calcium metabolism in skeletal tissue (49–51), cardiac muscle cells (52), adipocytes (51, 53), and kidney tissue (18, 19). Insulin-mediated increases in calcium efflux as studied in ^{45}Ca preloaded cells, have been reported in rat adipocytes and soleus muscle (51). It is not clear, however, what effect this insulin-mediated calcium efflux had on intracellular calcium. In another study insulin addition to adipocytes did increase cytosolic calcium (54). Direct effects of insulin on calcium channels have also been demonstrated in cultured skeletal muscle cells. In primary cultured rat embryo hindlimb muscle cells, 5–6 min of incubation with insulin inhibits depolarization-induced calcium inward current (50). However, in cultured human muscle cells, more prolonged (2 wk) treatment with insulin potentiates 40 mM K^+ induced $^{45}Ca^{2+}$ uptake (49). From these findings, it has been proposed that insulin acutely induces a vasodilator action by altering intracellular calcium metabolism. The present study supports these findings and offers an additional mechanism of action.

In contrast to insulin's acute vasodilating action, other reports have described potential hypertensive effects of insulin. Yanagisawa-Miwa et al. (55) observed an enhancing effect of insulin (incubation for 120 min) on contraction to a thromboxane A_2 analogue in porcine coronary artery. Potentiation by insulin might be selective for this agonist, since no enhancing effect was observed with high K^+, norepinephrine, histamine, or serotonin-induced contractions. In alloxan- or streptozotocin-induced diabetic animals, insulin reverses diabetes-induced inhibition of vascular smooth muscle contractility (56, 57) and long-term (8–12 wk) insulin therapy tended to increase vascular contractility to high potassium (57). Our laboratory has reported increased pressor responses to graded-dose ANG II infusions in normotensive and hypertensive non–insulin-dependent diabetic patients (7). These patients were obese with varying degrees of insulin resistance and insulinemia. The fact that acute studies demonstrate a vasodilatory effect of insulin whereas more chronic exposure results in a vasoconstrictor effect suggest a transition in insulin's vascular actions with exposure over time. One hypothesis suggests that chronic hyperinsulinemia may cause vascular hypereactivity by a desensitization of its acute vasodilatory effect as was shown in the study of obese subjects where leg blood flow responses to insulin were reduced (48).

In summary, our findings suggest that insulin attenuates ANG II–stimulated increases in $[Ca^{2+}]_i$ in VSMC by decreasing the pool of IP_3-releasable calcium. In addition, these studies demonstrate that insulin's effects can be mimicked by treatment of VSMC with a cGMP analogue (8-bromo-cGMP) or stimulation of a cGMP generating system (Na-nitroprusside). Moreover, we have demonstrated that inhibition of arginine-derived NO generation blocks insulin's effects on IP_3-sensitive Ca^{2+} release. This effect may explain the acute vasodilatory action of insulin, however, the chronic effects of insulin on calcium metabolism and vascular reactivity are yet to be clarified.

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