Insulin Increases Acid Production and May Directly Stimulate Na^+/H^+ Exchange Activity in Cultured Vascular Smooth Muscle Cells

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Key Words

Insulin · Intracellular pH · Smooth muscle cells · Vascular physiology

Abstract

Insulin was reported to decrease Na^+/H^+ exchange activity in murine vascular smooth muscle (VSM) tissue. In most other cells, insulin increases activity. We tested the effects of insulin on Na^+/H^+ exchange activity in primary cultured canine VSM cells. Intracellular pH (pH_i) was measured with 2',7'-bis(2-carboxyethyl)-5-carboxyfluorescein fluorescence and Na^+ uptake by isotopic methods. Insulin alone did not significantly affect pH_i (7.13 ± 0.05 vs. 7.10 ± 0.03 for control and insulin, respectively; p = not significant), and EIPA alone lowered it to 6.98 ± 0.04 ($p < 0.05$), upon which insulin lowered it further to 6.91 ± 0.04 ($p < 0.05$). In the presence of a pH_i clamp, pH_i /extracellular pH (pH_o) 7.1/7.4, insulin increased amiloride-sensitive ^{22}Na uptake by $98 \pm 25\%$ ($p < 0.05$). At pH_i/pH_o 6.0/7.4 or 6.7/7.4, amiloride-sensitive ^{22}Na uptake was stimulated by 378 ± 59 and $105 \pm 27\%$, respectively, compared to pH_i/pH_o 7.1/7.4 ($p < 0.05$ for all 3 versus each other), but was insulin insensitive. In acid-loaded cells (pH_i 6.0), addition of extracellular Na^+ (pH_o 7.4) caused rapid intracellular alkalinization, and the initial rate was not affected by insulin. It is concluded that insulin stimulates acid accumulation in VSM cells that is normally effluxed by in-

creased Na^+/H^+ exchange activity, and insulin may directly stimulate Na^+/H^+ exchange activity when cells are clamped at resting pH_i but not at acidic pH_i .

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Introduction

Resistance to insulin-induced glucose disposal and resistance to insulin-induced vasodilation have been found in patients with essential hypertension, obesity and type II diabetes mellitus [1–3]. The latter two conditions are also associated with arterial hypertension [4]. It is an attractive hypothesis that the resistance to insulin-induced vasodilation in the above-mentioned conditions may contribute to the concomitant elevated blood pressure. Insulin is known to stimulate endothelial cell nitric oxide production which increases cyclic GMP formation in the underlying vascular smooth muscle cells (VSMCs), thereby inhibiting their contraction [5, 6]. Insulin also inhibits contraction of VSMCs in the absence of endothelial cells, but the mechanisms are not fully understood [7, 8].

The activity of VSMC sodium-proton exchange (NHE-1) [9] has been linked to intracellular pH (pH_i), intracellular Na^+ concentration, cell growth and proliferation [10, 11]. VSMC pH_i and intracellular Na^+ concentration have

been linked to VSM tone [12–18]. Although a number of growth factors are known to increase cultured VSMC Na^+/H^+ exchange activity [10, 11, 19], recently, insulin has been reported to decrease Na^+/H^+ exchange activity in intact VSM tissue [20]. We are not aware of other publications on the subject. However, insulin is known to increase Na^+/H^+ exchange activity in other cells such as red blood cells, skeletal muscle, fibroblasts and proximal tubular cells [21–24].

The present study was undertaken to determine whether insulin affected Na^+/H^+ exchange activity in primary cultured VSMCs from the canine femoral artery. The data indicate that insulin stimulates acid accumulation in these cells that is normally effluxed by increased Na^+/H^+ exchange activity, and insulin may directly stimulate Na^+/H^+ exchange activity by mechanism(s) independent of pH_i .

Materials and Methods

Cell Culture

Adult mongrel dogs of either sex were sacrificed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. Primary cultures of VSMCs were prepared as previously described [25]. In brief, the media of the arteries were minced and incubated at 37°C in a solution containing elastase (type V; Sigma Chemical Co.) and collagenase (type I; Worthington Biochemical Corp.). After 2 h, the enzyme solution was discarded and replaced with fresh solution, and the tissue was incubated for an additional 2 h. The dispersed cells were pelleted and suspended in Dulbecco's modified Eagle's medium (Gibco Laboratories) that contained 10% fetal calf serum (Cyclone), 1% glutamine and 1% PS (10,000 units/ml penicillin, 10 mg/ml streptomycin; Sigma). To grow cells on coverslips, the cell suspension was adjusted to 1.7×10^6 cells/ml, and 0.3 ml was placed on the surface of 15×22 -mm glass coverslips. To grow cells on the bottom of culture dishes, the suspension of dispersed cells was adjusted to 2×10^5 cells/ml, and 1 ml was placed in 35-mm plastic dishes (Falcon). The coverslips and dishes were placed in a humidified tissue culture incubator maintained at 37°C and equilibrated with 5% CO_2 -95% air. After 48 h and every 48 h thereafter, the media were replaced with 1 ml fresh media. The cells reached confluence between the 10th and 15th day.

Measurement of pH_i

pH_i was measured as previously described [26]. A coverslip with confluent cells was placed in a polystyrene cuvette inside a fluorescent spectrophotometer and superfused with solution at 37°C containing (in mM) 136 NaCl, 4 KCl, 1.8 CaCl_2 , 0.8 MgCl_2 , 5 glucose, 10 HEPES-Tris, pH 7.4, plus 0.1% bovine serum albumin (physiological salt solution, PSS). Before the cells were exposed to pH-sensitive dye, they were excited at 450 nm, the light at 530 nm was monitored and the intensity was blanked to zero units. The excitation beam was changed to 506 nm and the emission signal at 530 nm was arbitrarily set to 150 units. This value

was subtracted from all subsequent values obtained after cells had been loaded with dye. To load the cells with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5-carboxyfluorescein (BCECF), the perfusion was stopped, and 10 μM of the permeable acetoxymethyl derivative, BCECF AM (Molecular Probes), was added to the cuvette from a stock of 2 mM in dimethyl sulfoxide. The cells were preincubated for 30 min at 37°C without perfusion. Perfusion was restarted at 3 ml/min for 20 min and then for 10 min in the presence and absence of 50 μM EIPA. Fluorescence was continuously monitored by activating at 450 nm, alternating with 506 nm and recording the emission at 530 nm. pH_i was determined by the fluorescence ratio method (530-nm emission intensity at 506-nm excitation divided by that at 450-nm excitation). After 10 min, a stable baseline was obtained and 1 nM insulin was added to the superfusion solution. Preliminary data showed that baseline values remained constant for over 30 min. A fluorescence ratio/ pH_i calibration curve was obtained at the end of each experiment by determining the fluorescence ratios at pH_i values of 7.5, 7.25, 7.0 and 6.5. pH_i was set by incubating the coverslip at 37°C with high KCl PSS containing (in mM) KCl 140, CaCl_2 1.8, MgSO_4 0.8, glucose 5, and HEPES 10 (buffered to the desired pH with Tris), along with 7 μM nigericin (Sigma-Aldrich), a K^+-H^+ exchanger. Unknown pH_i values were determined from a linear regression line obtained from the calibration data.

In order to measure extracellular Na^+ (Na^+_o)-dependent pH_i recovery from an acid load, cells on coverslips were loaded with BCECF as above and placed on the stage of an epifluorescence microscope, as previously described [15]. Cells were superfused with PSS in the presence and absence of 1 nM insulin or 10 μM serotonin (5-HT) for 5 min and excited with alternating 100-ms pulses of light at 500 and 448 nm, and the emitted light at 530 nm was measured. Cells were acid loaded by superfusing them with modified PSS such that NaCl was replaced with KCl and buffered with 10 HEPES-Tris, pH 6.0, plus nigericin for 5 min with or without insulin or 5-HT. In order to scrub out nigericin but maintain pH_i 6.0, cells were perfused with the above solution without nigericin plus 1 g bovine serum albumin, with or without insulin or 5-HT, for 10 min. Cells were then superfused with original PSS [Na^+_o 135, extracellular pH (pH_o 7.4)] with or without insulin or 5-HT for 8 min. pH_i was calculated by the ratiometric method described above utilizing the same pH_i calibration method. The initial rate of pH_i recovery upon adding Na^+_o (dpH_i/dt) was measured by drawing a straight line along the pH_i recovery curve during the first 30 s on an expanded plot of the 506/450-nm excitation/530-nm emission ratio versus time.

²²Na Uptake

²²Na uptake was assayed as previously described [26]. pH_i was preset to 7.1, 6.7 or 6.0 by preincubating cells in 35-mm culture dishes for 30 min at 37°C in a modified PSS which was buffered to pH 7.1, 6.7 or 6.0, respectively, and contained zero Na^+ , 140 mM K^+ plus 7 μM nigericin. The uptake of Na^+ was assayed by incubating the cells at 37°C in another modified PSS, pH 7.4, containing (in mM) 70, 28 or 6 K^+ , respectively, plus enough N-methylglucamine such that K^+ plus N-methylglucamine equaled 130 mM, plus 10 mM ²²Na (1.0 μCi ; Perkin Elmer) with 7 μM nigericin. Taking intracellular K^+ to be 140 mM, the continued presence of nigericin in the uptake solution in the presence of 70, 28 or 6 mM K^+ should keep pH_i clamped to 7.1, 6.7 or 6.0, respectively. 1 mM ouabain was added to uptake solutions to inhibit Na^+ efflux via the

Fig. 1. Graphs showing effects of insulin and EIPA on pH_i in VSMCs. **a** Cells on a coverslip were loaded with BCECF and superfused with PSS with or without 50 μ M EIPA for 10 min. 1 nM insulin was added to the superfusion solution at the arrow. pH_i was continuously monitored by measuring BCECF fluorescence. Data are traces from representative experiments. **b** Experiments were performed as described in the legend of figure 1a. pH_i values were recorded before and 15 min after adding insulin to the superfusion solution. Data are means $\pm$ SE from 5 separate experiments each with or without EIPA. ^a $p < 0.05$ versus basal control. ^b $p < 0.05$ versus basal EIPA.

Na⁺/K⁺ pump. Uptake was terminated at 1 min, and the cells were washed by rapidly rinsing the dishes 6 times with 5.5 ml ice-cold 100 mM MgCl₂. The cells were dissolved in 1 ml of 1 M NaOH. The ²²Na content was measured by scintillation spectroscopy. In each experiment, the isotopic uptake solution was applied to cells and then removed 1–2 s later, and the cells were washed, dissolved in NaOH and counted to estimate binding and/or trapping of isotope by the cells and plastic dishes. This value was subtracted from all uptake values. ²²Na uptake studies were performed in triplicate. The absolute uptake of ²²Na per milligram protein varied from one preparation to another, but the relative effects of the various perturbations within each experiment were consistent from experiment to experiment. Thus, the data are expressed as a percent of ²²Na uptake under control conditions.

Statistical analysis was performed on paired and unpaired data by use of Student's t test and ANOVA with multiple comparisons using the Newman-Keuls test. Statistical significance was taken as a value of $p < 0.05$.

Results

Effects of Insulin on pH_i at Resting pH_i

If insulin increased or decreased the Na⁺/H⁺ exchange activity, it was expected that insulin would increase or decrease pH_i, respectively. As shown in a representative experiment in figure 1a, insulin did not affect pH_i. However, in the presence of EIPA, basal pH_i was lower and insulin lowered it further. As shown in figure 1b, basal pH_i was 7.13 ± 0.04 and 7.10 ± 0.03 in the presence and

absence of insulin for 15 min, respectively ($p =$ not significant). In the presence of EIPA, basal pH_i was 6.98 ± 0.04 ($p < 0.05$ vs. no EIPA) and decreased to 6.91 ± 0.04 after adding insulin ($p < 0.05$ vs. no insulin). These data indicate that insulin causes intracellular acidification when the Na⁺/H⁺ exchanger is inhibited and that insulin-induced acid production is largely effluxed by the Na⁺/H⁺ exchanger in the absence of EIPA, but do not reveal whether Na⁺/H⁺ exchange activity is affected directly by insulin.

Effects of Insulin on ²²Na Uptake

To see whether insulin could stimulate Na⁺/H⁺ exchange activity in the absence of the hormone's putative effect on pH_i by increasing intracellular acid production, the effects of insulin on ²²Na uptake, pH_o 7.4, was measured in the absence and presence of 1 mM amiloride in cells whose pH_i was clamped to the resting pH_i of 7.1 by the high potassium-nigericin technique [27]. Amiloride at this concentration was chosen to match our prior experiments to measure Na⁺/H⁺ exchange activity in these cells using isotopic techniques [27]. As shown in figure 2, insulin stimulated ²²Na uptake by $76 \pm 20\%$ ($p < 0.05$). Amiloride inhibited ²²Na uptake with or without insulin. The amiloride-sensitive component of ²²Na uptake was stimulated to $198 \pm 25\%$ of the control value by insulin ($p < 0.05$). These data show that insulin stimulated amiloride-sensitive ²²Na uptake independently of the

Fig. 2. Graph showing effects of insulin and amiloride on $^{22}\text{Na}^+$ uptake in VSMCs. Cells grown in dishes were preincubated at 37°C for 30 min with the appropriate solution to clamp pH_i 7.1 (see Materials and Methods), with or without 1 nM insulin. The 1-min uptake of 10 mM $^{22}\text{Na}^+$ was assayed in the presence of solution with the appropriate K^+ concentration, pH_o 7.4, to maintain pH_i clamped to 7.1 in the continued presence of nigericin, with or without 1 nM insulin and/or 1 mM amiloride. Amiloride-sensitive $^{22}\text{Na}^+$ uptake was the difference between uptake under a given experimental condition without and with amiloride and taken as Na^+/H^+ exchange activity. All data are expressed as a percent of Na^+ uptake under control conditions which averaged 3.2 nmol/mg protein. Data are the means $\pm$ SE from 5 experiments. ^a $p < 0.05$ versus control; ^b $p < 0.05$ versus Na^+/H^+ exchange without insulin; ^c $p < 0.05$ versus amiloride without insulin.

Fig. 3. Graph showing the effects of insulin on amiloride-sensitive $^{22}\text{Na}^+$ uptake in VSMCs clamped at pH_i 6.0, 6.7 or 7.1. Cells grown in dishes were preincubated in the presence and absence of 1 nM insulin at 37°C for 30 min with the appropriate solution to clamp pH_i to 6.0, 6.7 or 7.1 (see Materials and Methods). The 1-min uptake of 10 mM $^{22}\text{Na}^+$ was assayed in the presence of solution with the appropriate K^+ concentration, pH_o 7.4, to maintain pH_i clamped to 6.0, 6.7 or 7.1 in the continued presence of nigericin, with or without 1 nM insulin and/or 1 mM amiloride. Amiloride-sensitive $^{22}\text{Na}^+$ uptake in cells clamped to pH_i 7.1 without insulin averaged 1.5 nmol/mg protein. Data are the means $\pm$ SE from 5 experiments. ^a $p < 0.05$ versus the same conditions at pH_i 6.7; ^b $p < 0.05$ versus the same conditions at pH_i 7.1; ^c $p < 0.05$ versus control pH_i 7.1.

hormone's effect to increase intracellular acid production.

To see whether insulin stimulated amiloride-sensitive ^{22}Na uptake at pH_i values below the basal value of 7.1, the effects of insulin were measured in cells whose pH_i had been clamped to 6.0 or 6.7, as well as the basal pH_i 7.1. pH_o was 7.4 in all cases. As shown in figure 3, amiloride-sen-

sitive ^{22}Na uptake was stimulated at pH_i 6.0 and 6.7 by 378 ± 59 and $105 \pm 27\%$, respectively, of the value at pH_i 7.1 (both $p < 0.05$ vs. pH_i 7.1). Intracellular acid-stimulated amiloride-sensitive ^{22}Na uptake likely represents Na^+/H^+ exchange activity, and insulin did not significantly affect it. Insulin stimulated amiloride-sensitive ^{22}Na uptake when pH_i was clamped to 7.1 (by $88 \pm 17\%$; $p < 0.05$),

Fig. 4. Graphs showing the effects of insulin and 5-HT on Na^+_o -induced recovery of pH_i after an intracellular acid load. **a** Representative tracing of the ratio of 500- to 440-nm emission of BCECF fluorescence upon clamping pH_i to 6.0 in the absence of Na^+_o , followed by pH_i recovery (increased ratio of 500 to 400 nm) upon adding 135 mM Na^+_o , followed by a pH_i calibration curve. **b** The initial rate of Na^+_o -induced pH_i recovery after an acid load in cells preincubated with or without 1 nM insulin or 10 μ M 5-HT. Data represent the mean $\pm$ SE from 5 separate experiments. ^a $p < 0.05$ versus control and insulin.

similar to the stimulation in figure 2. At pH_i 6.0 or 6.7, insulin did not affect ^{22}Na uptake in the presence or absence of amiloride (data not shown).

Effects of Insulin on Recovery from Acidic pH_i

In order to check by another method whether insulin affects Na^+/H^+ exchange activity after lowering pH_i to 6.0, the initial recovery rate of pH_i (dpH_i/dt) upon adding extracellular 135 mM Na_o was measured via the BCECF

fluorescence technique. Figure 4a shows a representative tracing of pH_i recovery in control cells. As shown in figure 4b, initial dpH_i/dt was 0.31 ± 0.04 and 0.29 ± 0.03 pH units/min in the absence and presence of insulin, respectively ($p =$ not significant). As a positive control, dpH_i/dt was also measured in cells exposed to 10 μ M 5-HT. As shown in figure 4b, 5-HT increased initial dpH_i/dt to 0.53 ± 0.06 pH units/min ($p < 0.05$ vs. control or insulin), similar to previously described studies [28].

Discussion

Despite prior reports which demonstrated that a variety of growth factors stimulate Na^+/H^+ exchange activity in different cell types [21–24], we are aware of only one study which showed that insulin affects Na^+/H^+ exchange in VSMCs [20]. Boedtkjer and Aalkjaer [20] reported that insulin inhibited Na^+/H^+ exchange activity in VSMCs in isolated mouse mesenteric arteries. Others have shown that insulin inhibits the Na^+/H^+ exchanger in hepatocytes from diabetic animals [27]. In the present study using primary cultured VSMCs from canine femoral artery, we tested whether insulin affected pH_i to get insight into whether the hormone affected the Na^+/H^+ exchange activity. Insulin did not significantly affect resting pH_i but decreased it in the presence of EIPA. These data indicate that insulin stimulates the addition of intracellular acid in a Na^+/H^+ exchanger-independent mechanism, and that the Na^+/H^+ exchanger effluxes that extra acid in the absence of EIPA. This is in keeping with our prior data that 1 nM insulin increased aerobic glycolysis and lactate content by about 90% in primary cultured VSMCs from canine femoral artery [29] and rat aorta [30]. In order to test whether insulin directly affects the Na^+/H^+ exchange activity, we clamped pH_i . When pH_i was clamped to the resting value of pH_i 7.1, insulin stimulated amiloride-sensitive ^{22}Na uptake. It must be pointed out that amiloride is not a specific inhibitor of the Na^+/H^+ exchanger. It also inhibits the epithelial Na^+ channel (ENaC) in distal portions of the renal tubule [31]. Whether ENaC functions as a Na^+ channel in VSMCs is unknown. Previous data implicate members of the ENaC superfamily as mediating mechanotransduction in VSM from the brain and kidney [32]. Renal ENaC has an α , β and γ subunit [31]. It has been shown that β -ENaC and γ -ENaC message and protein are in VSMCs from rat cerebral arterioles, and amiloride blocks stretch-induced vasoconstriction of isolated perfused rat brain arteries and renal interlobular arteries and afferent arterioles [32–34]. Electrophysiological or other transport data demonstrating ENaC in VSMCs are not available. Van Renterghem and Lazdunski [35] demonstrated a non-voltage-dependent, epithelial-like Na^+ channel in VSMCs via patch clamp techniques, but this channel was insensitive to a high concentration of amiloride. Evidence for ENaC in VSMCs from sources other than the brain and kidney is not available. Thus, a limitation of the present study is that insulin-stimulated amiloride-sensitive ^{22}Na transport may be mediated, at least in part, by a putative ENaC and not by the Na^+/H^+ exchanger. The stimulatory effect of insulin

on amiloride-sensitive Na^{22} uptake was not seen when Na^+/H^+ exchange activities were already stimulated by clamping pH_i to 6.0 or 6.7. In accord with that finding, we showed that insulin did not affect Na^+_{o} -induced pH_i recovery after an acid load (pH_i 6.0) whereas 5-HT increased it, as previously described [28]. Thus, insulin did not affect intracellular acid-stimulated transport activity, which is likely mediated by the Na^+/H^+ exchanger. The present data are consistent with the possibility that insulin increases the affinity of H^+ to the intracellular modifier site which binds H^+ optimally near the resting pH_i [36, 37]. The latter has been reported as a mechanism by which other growth factors increase the Na^+/H^+ exchange activity in VSMCs [10, 11]. It is noteworthy that insulin increased amiloride-insensitive ^{22}Na uptake (fig. 2). This is explicable if amiloride did not completely inhibit the Na^+/H^+ exchange activity (or possibly ENaC) in these studies, or that insulin stimulates another transporter that mediates Na^+ uptake.

The present data do not support the conclusion of Boedtkjer and Aalkjaer [20] that insulin inhibits intracellular acid-stimulated Na^+/H^+ exchange activity in VSM. They showed that insulin inhibited Na^+_{o} -dependent pH_i recovery after an intracellular acid prepulse. Using a similar technique, we found no effect of insulin on Na^+_{o} -dependent pH_i recovery. Their data indicate that insulin inhibits the VSMC Na^+/H^+ exchange activity in isolated arteries. Thus, the opposite conclusions of the present studies and those by Boedtkjer and Aalkjaer [20] may be due to differences in concentrations of insulin employed (1 and 800 nM in the present study and that of Boedtkjer and Aalkjaer, respectively), sources of VSM, and/or a difference between the use of primary cultured cells versus intact isolated vascular tissue. It is important to note that primary cultured VSMCs from a canine femoral artery used in these studies had proliferated *in vitro*, were not contractile and were not from resistance vessels. In addition, these experiments were performed in the nominal absence of $\text{CO}_2/\text{HCO}_3^-$. Thus, the physiologic relevance of the present studies or their relevance to disorders of arterial hypertension are only speculative. We have not yet determined the isoform(s) of the Na^+/H^+ exchanger in these cells. To our knowledge, the only isoform reported in VSMCs is NHE-1 [9].

The mechanism by which insulin may have stimulated Na^+/H^+ exchange activity in the present study is unknown. We have shown that insulin stimulates aerobic glycolysis and lactate content in cultured VSMCs [29, 30]. Others have shown that plasma membrane-associated and compartmentalized glycolysis is responsible for pro-

ducing the ATP needed to fuel ATPase pumps in a variety of cells including VSM [38, 39]. It has also been reported that Na^+/H^+ exchange activity is inhibited when cellular ATP has been depleted [37, 39, 40]. It is possible that in the present study, insulin-stimulated ATP production in the region of the VSMC plasma membrane may have stimulated transporter activity by itself or by stimulating a number of kinases which are known to stimulate the Na^+/H^+ exchanger [10, 11, 39]. Ca-calmodulin-dependent protein kinase, protein kinase C and MAP kinase are known to stimulate Na^+/H^+ exchange activity in a variety of cell types including VSM [10, 11, 40]. In addition, locally produced ATP may be involved in other phosphory-

lation reactions such as the phosphatidylinositol pathway, thereby affecting Na^+/H^+ exchange activity by alterations in the level of PIP_2 [41]. Finally, insulin may stimulate any of the above kinases by mechanisms independent of raising ATP levels near the plasma membrane, with a resultant stimulation of the Na^+/H^+ exchange activity.

Future studies are needed to determine the isoform(s) of the Na^+/H^+ exchanger and whether a functional amiloride-sensitive ENaC is present in the cells used in the present study, and whether insulin stimulates Na^+/H^+ exchange activity in VSMCs from other sources and/or inhibits it. The relationship of insulin-affected Na^+ transport in VSMCs to vascular tone needs to be determined.

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