Insulin-Stimulated Hydrogen Peroxide Increases Guanylate Cyclase Activity in Vascular Smooth Muscle

Ming Yang, Yu Yang, Sui Zhang, Andrew M. Kahn

Abstract—Insulin resistance is associated with vascular disease. Physiological concentrations of insulin inhibit cultured vascular smooth muscle cell (VSMC) contraction and migration by increasing nitric oxide (NO)-stimulated cGMP accumulation. The failure to do so in insulin-resistant states may aggravate vascular disease. We sought to determine the mechanism of insulin’s increase in cGMP accumulation. Isobutylmethylxanthine, an inhibitor of phosphodiesterase activity, inhibited the decline in cGMP levels measured by immunoassay in cGMP-loaded cultured rat aortic VSMCs, but 1 nmol insulin did not. Thus, insulin’s increase in cGMP accumulation is due to stimulated production, not inhibited hydrolysis and/or efflux. Insulin, which increases the NADH/NAD+ ratio in these cells, stimulated superoxide anion (O$_2^-$) accumulation measured by lucigenin luminescence to 256±25% (P<0.05) by a process that was blocked by the NADH oxidase inhibitor diphenyliodonium (DPI) and enhanced by the superoxide dismutase inhibitor diethyldithiocarbamate (DETCA). Insulin also stimulated hydrogen peroxide (H$_2$O$_2$) accumulation measured by horseradish peroxidase/luminol luminescence to 221±22% (P<0.05) by a DETCA-sensitive mechanism. H$_2$O$_2$ (100 µmol/L) in the absence of insulin increased NO-stimulated cGMP accumulation to 151±11% (P<0.05). Insulin alone increased NO-stimulated cGMP accumulation to 183±17% (P<0.05), and this was blocked by either DPI or DETCA. We conclude that insulin increases NADH oxidase-derived O$_2^-$ production in cultured rat VSMCs. This did not cause the expected scavenging of NO resulting in the reduction of NO-stimulated guanylate cyclase activity, but enough O$_2^-$ was metabolized to H$_2$O$_2$ to increase overall NO-stimulated cGMP production. (Hypertension. 2003;42:569-573.)

Key Words: muscle, smooth, vascular ■ insulin ■ cyclic GMP ■ nitric oxide

Atherosclerosis and restenosis after balloon catheter angioplasty are increased in insulin-resistant states.1–3 Some studies support the view that hyperinsulinemia, which is present in these conditions, stimulates vascular smooth muscle cell (VSMC) migration and proliferation, thereby accelerating the pathological process.4,5 Other data show that insulin can stimulate cGMP accumulation in normal cultured VSMCs6,7 and that cGMP inhibits migration and proliferation of those cells.8–10 The latter data are consistent with the notion that insulin normally inhibits atherosclerosis and restenosis in vivo and that the functional lack of insulin in insulin-resistant states contributes to vascular disease.

We have shown that insulin alone does not affect cGMP accumulation in cultured VSMCs, but when cGMP production is stimulated by a permissive amount of nitric oxide (NO), physiological concentrations of insulin increase the accumulation of cGMP.6 We found that insulin stimulates glucose uptake and aerobic glycolysis in these cells and that the resultant increase in lactate is responsible for insulin’s increase in NO-stimulated cGMP accumulation.6,11,12 Increased lactate in VSMCs has been reported to increase superoxide anion (O$_2^-$) production.13 O$_2^-$ is well known to scavenge NO, thereby decreasing VSMC guanylate cyclase activity.14 It was unclear how to reconcile these phenomenon with our previous finding that insulin-stimulated lactate increases NO-stimulated cGMP accumulation.6,12 The present study was designed to explain these discrepant findings and determine the mechanism of insulin’s increase in NO-stimulated cGMP accumulation. Since reactive oxygen species have been linked to decreased phosphodiesterase activity,15,16 the first goal of these studies was to determine whether insulin increases NO-stimulated cGMP generation or inhibits its hydrolysis and/or efflux.

Omar et al13 have shown that lactate increases cGMP-mediated relaxation of endothelium-denuded vascular smooth muscle tissue from calf pulmonary artery. They demonstrated that lactate’s increase in NADH, caused by the action of lactate dehydrogenase, stimulates the activity of NADH oxidase that yields O$_2^-$ in other experiments with endothelium-denuded bovine pulmonary artery, they showed that hydrogen peroxide (H$_2$O$_2$), the product of O$_2^-$ and superoxide dismutase (SOD), reacts with catalase and that the resultant intermediate form of the catalase enzyme (compound I) stimulates soluble guanylate cyclase activity.17 Others have reported that H$_2$O$_2$ stimulates guanylate cyclase activity in guinea pig aorta,18 rabbit urethral and cavernosal smooth...
muscle,\textsuperscript{19} and human platelets.\textsuperscript{20} Thus, it was possible that insulin’s increase in lactate in cultured VSMCs caused increased \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production and the latter increased guanylate cyclase activity. The second goal of this study was to test this hypothesis.

\textbf{Methods}

\textbf{Cell Culture}

Twelve male Sprague Dawley rats per cell culture preparation were killed with intraperitoneal pentobarbital sodium, and the thoracic aortas were disected free. Endothelia and adventitia were stripped away; the media of the arteries were minced, incubated at 37°C in a solution containing elastase (type V, Sigma) and collagenase (type I, Worthington Biochemical), and cultured VSMCs were prepared in 10% FBS as previously described.\textsuperscript{9} Confluent cultured cells of passages 3 to 10 in 35-mm plastic dishes after 14 hours without FBS were used in experiments. Since a permissive amount of NO is necessary for insulin to stimulate cGMP accumulation in cultured VSMCs, some experiments were performed with cells that had been induced to express iNOS with lipopolysaccharide and interleukin-1β as previously described.\textsuperscript{21}

\textbf{Cyclic GMP Assay}

Dishes of VSMCs were incubated for 30 minutes at 37°C with the desired agents in physiological salt solution (PSS) containing (in mmol/L) 136 NaCl, 4 KCl, 5 glucose, 1.8 CaCl\(_2\), 0.8 MgSO\(_4\), 10 HEPES-Tris, pH 7.4, plus 0.1% BSA. cGMP content of acetylated cell lysates was determined with a direct cGMP enzyme immunoassay kit (Assay Designs).

\textbf{Superoxide Anion Assay}

Dishes of VSMC were incubated for 30 minutes at 37°C with the desired agents in PSS; the cells were washed with Hanks balanced salt solution (HBSS) and released from the dishes by incubating them with collagenase (1 mg/mL), BSA (2 mg/mL), and soybean trypsin inhibitor (1 mg/mL) in HBSS at 37°C for 5 minutes. Cells were collected by centrifugation (200g for 5 minutes at 4°C) and the cell pellet was resuspended in HBSS; 20 μL of 2.5 mmol/L dark-adapted lucigenin in HBSS was added to 80 μL of cell suspension (≈40 μg protein) at room temperature, and photon emission was measured every 15 seconds for 10 minutes in a luminometer (model 20/20, Turner Designs) as previously described.\textsuperscript{22} Readings stabilized by 3 minutes, and that value minus a blank value obtained in the absence of cells was recorded. \( \text{O}_2^- \) content of samples was obtained from a standard curve generated with the use of xanthine/xanthine oxidase, as previously described.\textsuperscript{23}

\textbf{Hydrogen Peroxide Assay}

Dishes of cells were washed and incubated with the desired reagents in 2 mL of PSS at 37°C for 30 minutes. \( \text{H}_2\text{O}_2 \) content of 200 μL incubation solution was measured with the Peroxil Luminol Hydrogen Peroxide Determination Kit (World Precision Instruments). In this assay, horseradish peroxidase reacts with \( \text{H}_2\text{O}_2 \) to form reactive intermediates that oxidize luminol. The chemiluminescence emitted by luminol was measured in a luminometer, and \( \text{H}_2\text{O}_2 \) content of samples minus a blank was obtained from a standard curve with the use of authentic \( \text{H}_2\text{O}_2 \) in PSS.

Values for cGMP, \( \text{O}_2^- \), and \( \text{H}_2\text{O}_2 \) varied from experiment to experiment, but the relative effects of experimental perturbations were highly reproducible among different experiments. Thus, data are expressed as a percentage of values obtained under control conditions, and the mean absolute control values are stated in the figure legends. Statistical analysis was performed on paired data by use of Student \( t \) test and ANOVA, with multiple comparisons using the Newman-Keuls test. Statistical significance was taken as a value of \( P<0.05 \).

Bovine insulin, cGMP, IBMX, BSA, diphenylhydantoin (DP), diethylthiocarbamate (DETCA), and \( \text{H}_2\text{O}_2 \) were obtained from Sigma, and S-nitroso-N-acetylpenicillamine was from Alexis. Protein was measured by the method of Bradford.

\textbf{Insulin and cGMP Hydrolysis}

We have reported that insulin increases NO-stimulated cGMP accumulation in cultured VSMCs.\textsuperscript{6} A potential explanation for this finding is that insulin inhibits phosphodiesterase activity, thereby inhibiting the hydrolysis of NO-stimulated cGMP and/or inhibits cGMP efflux from the cell. To test this possibility, VSMCs were preloaded with cGMP, washed, incubated without NO in the presence and absence of 1 nmol insulin, and the decline in cell cGMP was measured over 1 hour. The permeable analogs of cGMP, 8-Br-cGMP and dibutyryl cGMP, are resistant to hydrolysis by phosphodiesterases, so cells were loaded with authentic cGMP, for which there is modest cell permeability.\textsuperscript{24} As shown in Figure 1, cell cGMP fell by 39% after 1 hour, and insulin did not inhibit its decline. To rule out the possibility that the observed fall in cGMP merely represented the release of extracellularly bound cGMP and to serve as a positive control, cGMP-loaded cells were also incubated with the phosphodiesterase inhibitor IBMX. As also shown in Figure 1, IBMX inhibited the fall of cGMP by 75%, indicating that most of the decline of cGMP under control conditions represented the hydrolysis of cGMP. Since cGMP levels in preloaded cells were approximately equal to insulin-stimulated values, insulin’s increase in NO-stimulated cGMP accumulation was not due to inhibition of cGMP hydrolysis and/or efflux.

\textbf{Effect of Insulin on \( \text{O}_2^- \)}

Insulin increases lactate content and the lactate-to-pyruvate ratio in these cells 3-fold, as we have previously reported.\textsuperscript{12} The concomitant increase in the NADH/NAD\(^+\) ratio would be expected to increase \( \text{O}_2^- \) production by NADH oxidase, as has been described in other vascular smooth muscle tissue.\textsuperscript{13} As shown in Figure 2, 30-minute exposure to 1 nmol insulin increased \( \text{O}_2^- \) levels in these cells. DPI, an inhibitor of NADH oxidase, did not affect basal \( \text{O}_2^- \) but completely blocked

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Effect of insulin and IBMX on cGMP hydrolysis. Concentrated dishes of VSMCs were preincubated in PSS plus 10 μmol/L cGMP for 1 hour at 37°C. Cells were washed 6 times with 1 mL PSS at 37°C and incubated for 1 hour in 1 mL PSS plus 0.1% BSA with or without 1 mmol/L IBMX or 1 nmol insulin, and cGMP was measured. Data are expressed as percentage ± SEM of cGMP content of cells after washing (time zero), which averaged 9.1 pmol/mg protein, and are from 4 separate experiments. \( P<0.05 \) vs control.}
\end{figure}
insulin stimulation of O$_2^-$ levels. DETCA, an inhibitor of SOD, increased both basal and insulin-stimulated O$_2^-$ levels. These data indicate that insulin stimulates O$_2^-$ production by NADH oxidase and that SOD is active in these cells.

O$_2^-$ Metabolism and cGMP

If insulin-stimulated O$_2^-$ production leads to an increase of insulin in NO-stimulated cGMP production, this effect of insulin should be blocked by DPI. As shown in Figure 3A, insulin alone had no effect on cGMP. SNAP, an NO donor, increased cGMP and insulin stimulated cGMP further, as we have previously described. As also shown in Figure 3A, DPI did not affect basal or SNAP-stimulated cGMP, but it blocked the further stimulation of cGMP by insulin. These data support the possibility that insulin-stimulated O$_2^-$ production leads to the increase of this hormone in cGMP production. Figure 3B shows that blocking SOD with DETCA did not affect basal cGMP levels, but it partially inhibited SNAP-stimulated cGMP levels and it completely blocked the additional stimulation of cGMP by insulin. These data indicate that the metabolism of insulin-stimulated O$_2^-$ by SOD to H$_2$O$_2$ mediates insulin's increase in NO-stimulated cGMP production.

H$_2$O$_2$ and cGMP

We then tested whether insulin increases H$_2$O$_2$ and whether the latter stimulates guanylate cyclase activity. As shown in Figure 4A, insulin increased H$_2$O$_2$ levels in VSMCs. As expected, inhibiting SOD activity with DETCA decreased basal and insulin-stimulated H$_2$O$_2$ levels. Figure 4B shows that H$_2$O$_2$ alone did not affect cGMP levels in these VSMCs, but when cGMP production was stimulated by NO from iNOS, H$_2$O$_2$ increased cGMP levels further. Since H$_2$O$_2$ was present in the incubation buffer, NO was provided by iNOS instead of SNAP in this experiment because oxidizing agents retard NO release from SNAP. H$_2$O$_2$ per se did not affect iNOS activity (data not shown). These data indicate that the insulin's increase in H$_2$O$_2$ increases NO-stimulated cGMP production.

Discussion

Cyclic GMP inhibits cultured VSMC migration and proliferation and we have shown that physiological concentrations of insulin increase cGMP accumulation in these cells, but only if NO is present. The present study indicates that insulin does not inhibit the hydrolysis and/or efflux of cGMP once its formation has been stimulated by NO, since insulin did not inhibit the decline of cGMP levels in preloaded cells. It is conceivable that insulin simultaneously stimulates or inhibits cGMP hydrolysis while inhibiting or stimulating cGMP efflux from the cell such that insulin has no net effect on the decline of cGMP. Nevertheless, the present data indicate that insulin increases NO-stimulated guanylate cyclase activity rather than retarding cGMP disappearance.

The mechanism of insulin-stimulated cGMP production is unknown, but we previously showed that insulin increases lactate accumulation in cultured VSMCs. If this effect of insulin was blocked, insulin failed to increase NO-stimulated cGMP levels. Raising lactate with specific metabolic sub-
showed in cultured VSMCs that insulin, which increases response decayed rapidly with time. In the present study, we demonstrated absence of NO. This occurred immediately, and the stimulation of guanylate cyclase activity in VSMCs in the apparent control experiments. We concluded that the increase in lactate increased NO-stimulated cGMP accumulation.12 We postulated that the increase of insulin in O2-derived H2O2, predominated over insulin increase in O2 scavenging of NO such that NO-stimulated cGMP production was overall increased by insulin. Even though the rate constant for the reaction of O2·− with NO (7×10^9 mol/s) exceeds that for O2·− with SOD (2×10^9 mol/s),29 under normal conditions, the relatively high abundance of SOD ensures that the latter reaction occurs preferentially.4 It is noteworthy that DETCA partially inhibited SNAP-stimulated cGMP production (Figure 3B). This may be explicable since DETCA increased basal O2·− levels (Figure 2), which would be expected to increase scavenging of NO, thereby lowering overall guanylate cyclase activity in the face of inhibited H2O2 production. Gutpe et al30 found that lactate decreased NO-induced relaxation in endothelium-removed bovine pulmonary artery in the presence of DETCA, presumably because of scavenging of NO by O2·−. In the present study, insulin, which increases lactate in cultured VSMCs, did not inhibit SNAP-stimulated cGMP production in the presence of DETCA (Figure 3B). The reason for this is unknown, but DETCA may not have completely blocked insulin-stimulated H2O2 production.

**Perspectives**

The present studies may have pathophysiological significance. Insulin-resistant states are associated with accelerated vascular disease.1–3 The failure of insulin to stimulate guanylate cyclase in the vascular smooth muscle of these individuals could contribute to their vascular pathology. Alternatively, it has been proposed that the hyperinsulinemia associated with those conditions can aggravate it.4,5 Although H2O2 is known to stimulate guanylate cyclase activity in several tissues, including VSMC,17–20 O2·−, H2O2, and other oxidants are associated with accelerated vascular disease.31 In the present study, insulin increased O2·− and H2O2, and the latter stimulated cGMP production. This should ameliorate vascular disease. On the basis of these studies, insulin might be expected to have beneficial or deleterious vascular effects, and it needs to be determined if the final effect of insulin on
vascular pathology depends on various factors that tip the net effect of insulin one way or the other.

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