Inhibition of NO synthesis or endothelium removal reveals a vasoconstrictor effect of insulin on isolated arterioles

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Schroeder, C. Andrew, J r., Ya-Li Chen, and Edward J. Messina. Inhibition of NO synthesis or endothelium removal reveals a vasoconstrictor effect of insulin on isolated arterioles. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H815–H820, 1999.—In this study we tested the hypothesis that insulin may differentially affect isolated arterioles from red (RGM) and white gastrocnemius muscles (WGM) because of their differences in function and metabolic profile. We also determined whether the responses of these arterioles are endothelium dependent and mediated by either prostaglandins or nitric oxide (NO). Arterioles were isolated, pressurized to 85 mmHg, equilibrated in Krebs bicarbonate-buffered solution (pH 7.4) gassed with 10% O2 (5% CO2–85% N2), and studied in a no-flow state. Control diameters for first-order arterioles from RGM averaged 77 ± 8 µm and from WGM averaged 77 ± 5 µm. Cumulative dose-response curves to insulin (10 µU/ml, 100 µU/ml, 1 µU/ml, and 10 µU/ml) were obtained in arterioles before and after endothelium removal or administration of either indomethacin (Indo, 10−3 M) or Nω-nitro-L-arginine (L-NNA, 10−4 M). Insulin evoked concentration-dependent increases in control diameter of intact RGM and WGM arterioles of 6–26% and 9–28%, respectively. Indo was without any effect on insulin-induced dilation in RGM and WGM arterioles. Insulin-evoked dilation in both RGM and WGM arterioles was completely inhibited and converted to vasoconstriction by endothelium removal and administration of L-NNA. These results indicate that in endothelium-intact arterioles from RGM and WGM, insulin evokes an endothelium-dependent dilation that is equivalent and mediated by NO. In contrast, in the absence of a functional endothelium, insulin evokes arteriolar constriction. The finding that insulin can constrict arterioles, at physiological concentrations, suggests that insulin may play a more significant role in the regulation of vascular tone and total peripheral resistance than previously appreciated.

Key Words: rat; skeletal muscle; gastrocnemius muscle; dilation; constriction; nitric oxide; microcirculation; resistance; insulin resistance

Insulin exerts profound effects on the cardiovascular systems of both animals and humans (4). Skeletal muscle is one of the more important insulin-sensitive tissues, requiring insulin for stimulation of facilitative diffusion of glucose uptake and accounting for >80% of whole body glucose disposal (6). The effects of insulin are not limited to glucose uptake and metabolism. Recent evidence indicates that insulin can also increase skeletal muscle blood flow, which has the effect of enhancing glucose uptake (5, 7).

However, controversy surrounds insulin’s ability to affect total peripheral resistance and to lower blood pressure (2, 8). This is not surprising when one reviews the reported in vivo actions of insulin on the cardiovascular system and in particular the manner in which insulin is administered, the amounts of insulin administered, and the amounts of glucose given to maintain normal glucose concentrations. Normal plasma concentrations in humans range from basal (10 µU/ml) to postprandial (100 µU/ml) levels (13, 14), but many studies have exceeded these levels. Insulin administration has been shown to increase heart rate and cardiac output (4), stimulate the sympathetic nervous system (2), release epinephrine (11), alter membrane ion fluxes (26, 35), and alter vascular reactivity (1, 34). However, any attempt to clarify the effects of insulin on blood pressure or total peripheral resistance should include the direct study of the arteriolar vessels from insulin-sensitive tissue, such as skeletal muscle. An in vitro approach could determine insulin’s direct effect on arterioles by eliminating neural and reflex influences, tissue-derived metabolites, hormones, and flow-dependent phenomena.

On this basis we decided to test the hypothesis that insulin could differentially dilate isolated arterioles from red (RGM) and white gastrocnemius muscles (WGM) because of their differences in function and metabolic profile. Furthermore, we determined whether the responses of these arterioles are endothelium dependent and mediated by either prostaglandins or nitric oxide (NO). To test this hypothesis, four experimental aims were established. They were 1) to establish the vasomotor actions of insulin on RGM and WGM arterioles, 2) to determine whether any differences exist in reactivity to insulin between RGM and WGM arterioles, 3) to assess whether the effects of insulin are endothelium mediated, and 4) to determine whether the responses are mediated by prostaglandins or NO.

Materials and Methods

Male Wistar rats (average weight 363 ± 5 g) were anesthetized with pentobarbital sodium (50 mg/kg im), and the gastrocnemius muscles were exposed and excised. First-order arterioles were dissected free from adhering skeletal muscle and transferred to a special microvessel chamber (Living Systems Instrumentation, Burlington, VT) containing Krebs bicarbonate-buffered (pH 7.4) physiological salt solution (KPSS), equilibrated with 10% O2, 5% CO2, and the balance nitrogen, containing (in mM) 110 NaCl, 5 KCl, 2.5 CaCl2, 1 MgSO4, 1 K2HPO4, 10 glucose, 24 NaHCO3, and 0.02 EDTA. The arteriole was cannulated with glass inflow and outflow microcannulas and prepared for study as previously described (29). Temperature of the KPSS in the microvessel chamber was maintained at 37°C, and the Po2 was 75 ± 5 mmHg. All drugs were added to the reservoir, and final
concentrations are reported. Transmural pressure in the arteriole was set at 85 mmHg and maintained constant with a pressure-servo system (Living Systems Instrumentation).

Arterial luminal diameters were measured with an electronic image-shearing device (Video Caliper, Microcirculation Research Institute, Texas A & M University, College Station, TX). Internal arteriolar diameters were continuously recorded on a strip-chart recorder (Omega Engineering, Stamford, CT). When arterioles from RGM were initially mounted and pressurized to 85 mmHg, the passive diameters ranged from 95 to 131 µm and averaged 108 ± 5 µm; the passive diameters for WGM ranged from 75 to 144 µm and averaged 110 ± 9 µm. During the 60-min period of equilibration, arterioles developed myogenic tone and achieved resting control diameters averaging 77 ± 8 µm in RGM and 77 ± 5 µm in WGM arterioles. After the equilibration period, arterioles were routinely tested for their capacity to dilate to ACh µm in WGM arterioles. After the equilibration period, arterioles developed myogenic tone and achieved resting dilator responses to ACh (10 µM) as previously described (29). Physiological assessment of arteriolar dilation to arachidonic acid (20) and ACh (29), respectively, was conducted to evaluate smooth muscle function by the maintenance of responses to adenosine (10⁻⁷ M) and PE (10⁻⁷ M). We have previously reported that the concentrations of adenosine and PE employed in this study significantly inhibit arteriolar dilation to arachidonic acid (20) and ACh (29), respectively. The endothelium was removed from arterioles with air as previously described (29). Physiological assessment of endothelial removal was determined by the absence of dilator responses to ACh (10⁻⁷ M) and assessment of vascular smooth muscle function by the maintenance of responses to adenosine (10⁻⁷ M) and PE (10⁻⁷ M). We have previously reported that this procedure completely removes the endothelium, as determined by histology, while preserving vascular smooth muscle function (29).

It took insulin 5–11 min to produce a maximum response, which was sustained for several minutes. Therefore, maximum insulin responses were recorded after 15 min of exposure to each concentration of insulin. We have previously shown that concentration responses repeated over 3- to 4-h time periods were not statistically different, indicating reproducibility of responses (9).

Porcine insulin, ACh, and PE were purchased from Sigma Chemical (St. Louis, MO). ACh and PE were dissolved in KPSS. Insulin was first dissolved in normal saline, with further dilutions made in KPSS. All salts and chemicals were analytic grade and obtained from J. T. Baker Chemical (Phillipsburg, NJ). All concentrations of drugs refer to the base in molar amounts.

Statistical analyses were performed on paired samples with one variable using a Student-Newman-Keuls t-test. Paired samples with more than one variable were analyzed with a two-way analysis of variance test with repeated measures (Sigmastat; Jandel, San Rafael, CA). Data are presented as means ± SE, and n represents both the number of arterioles and animals studied. A P value of 0.05 or less was considered statistically significant.

**RESULTS**

Effects of insulin on RGM and WGM arterioles. In 33 experiments, cumulative concentration-response curves were generated to insulin (10 µU/ml, 100 µU/ml, 1 µU/ml, and 10 µU/ml). In this group, control diameters averaged 76.9 ± 8 µm in RGM arterioles (n = 15) and 77.3 ± 5 µm in WGM arterioles (n = 18). Increasing insulin concentrations evoked significant increases in arteriolar diameter (P < 0.05) (Fig. 1). Depending on the concentration administered, increases in arteriolar diameter in response to insulin were apparent within 3 min of administration but required 5–11 min to reach the maximum increase in diameter. The average increases in diameter, as percent change from control diameter, in RGM arterioles were 6, 12, 20, and 26%, and for WGM were 9, 15, 21, and 28% for insulin concentrations of 10 µU/ml, 100 µU/ml, 1 µU/ml, and 10 µU/ml. As shown in Fig. 1, responses to insulin in RGM and WGM arterioles were not significantly different (P > 0.05).

**Effects of endothelium removal on insulin-evoked dilation in RGM and WGM arterioles.** In 12 experiments, cumulative concentration-response curves were generated in response to insulin (10 µU/ml, 100 µU/ml, 1 µU/ml, and 10 µU/ml) before and after endothelium removal. In the RGM group of arterioles (n = 6), control diameters averaged 91.9 ± 11 µm before endothelium removal and 77.2 ± 13 µm after endothelium removal, for a decrease in diameter of 14.7 µm or 16%. Figure 2 shows the effects of insulin before and after endothelium removal in RGM arterioles, represented as percent change from control diameter. Before endothelium removal, arterioles increased their diameters by 5.50 ± 0.4, 11.0 ± 1, 17.0 ± 2, and 21.8 ± 1 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 µU/ml, and 10 µU/ml, respectively. After endothelium removal, the same arterioles changed their diameters by -0.60 ± 1, -1.10 ± 1, -1.10 ± 1, and -1.40 ± 1 µm (negative sign reflects vasoconstriction) to the same doses of insulin. Therefore, endothelium removal significantly inhibited insulin-evoked dilation in RGM and resulted in insulin-induced arteriolar constriction (P < 0.05).

![Fig. 1. Insulin dilates isolated arterioles from red (RGM, solid bars) and white gastrocnemius muscle (WGM, open bars). Administration of insulin (10 µU/ml–10 µU/ml) evoked similar concentration-dependent dilation. RGM, n = 15; WGM, n = 18.](http://ajpheart.physiology.org)
In the WGM group of arterioles (n = 6), control diameters averaged 73.2 ± 8 µm before endothelium removal and 57.2 ± 7 µm after endothelium removal for a decrease in diameter of 16.0 µm, or a 22% decrease. Figure 3 shows the effects of insulin before and after endothelium removal in WGM arterioles, represented as percent change from control diameter. Before endothelium removal, arterioles increased their diameters by 6.20 ± 1.20, 12.0 ± 1.69, and 23.3 ± 3 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml, respectively. After endothelium removal, the same arterioles changed their diameters by 0 ± 0, -1.20 ± 1, -1.00 ± 1, and -1.30 ± 2 µm (negative sign reflects vasoconstriction) to the same concentrations of insulin. Therefore, endothelium removal significantly inhibited insulin-evoked dilation in WGM arterioles and resulted in insulin-induced arteriolar constriction (P < 0.05).

Effects of Indo on insulin-evoked dilation in RGM and WGM arterioles. Cumulative concentration-response curves were generated in response to insulin (10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml) before and after Indo administration (10⁻⁵ M). Figure 4 shows the effects of insulin before and after Indo in RGM arterioles, represented as percent change from control diameter. In this group of RGM arterioles (n = 5), control diameters averaged 49.3 ± 3.4 µm before Indo administration and 58.0 ± 10 µm after Indo. Before Indo, arterioles increased their diameters by 1.83 ± 0.5, 4.83 ± 0.5, 6.67 ± 0.8, and 9.17 ± 0.8 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml, respectively. After Indo administration, the same arterioles increased their diameters by 3.50 ± 0.7, 5.67 ± 1, 10.17 ± 2, and 12.67 ± 2 µm to the same doses of insulin. Indo had no significant effect on the arteriolar dilation to insulin in RGM arterioles (P > 0.05).

Figure 5 shows the effects of insulin represented before and after Indo in WGM arterioles, as percent change from control diameter. In this group of WGM arterioles, endothelium removal (WGM - EC, open bars) inhibits arteriolar dilation to insulin in arterioles taken from WGM; n = 6. *P < 0.05 compared with WGM + EC (solid bars).
arterioles (n = 6), control diameters averaged 71.2 ± 1 µm before Indo administration and 80.0 ± 10 µm after Indo. Before Indo, arterioles increased their diameters by 5.67 ± 1.11, 14.3 ± 3, and 18.2 ± 3 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml, respectively. After Indo administration, the same arterioles increased their diameters by 8.33 ± 2, 14.8 ± 4, and 17.8 ± 4 µm to the same concentrations of insulin. Indo had no significant effect on arteriolar dilation to insulin in WGM arterioles (P > 0.05).

Effects of L-NNA on insulin-evoked dilation in RGM and WGM arterioles. In 12 experiments, cumulative concentration-response curves were generated in response to insulin (10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml) before and after L-NNA administration (10−4 M). Figure 6 shows the effects of insulin before and after L-NNA in RGM arterioles represented as percent change from control diameter. In this group of RGM arterioles (n = 6), control diameters averaged 49.3 ± 3 µm before L-NNA administration and 35.3 ± 2 µm after L-NNA. Before L-NNA, arterioles increased their diameters by 1.83 ± 0.5, 4.83 ± 0.5, 6.67 ± 0.8, and 9.17 ± 0.8 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml, respectively. After L-NNA administration, the same arterioles changed their diameters by −1.73 ± 0.4, −1.83 ± 0.5, −2.00 ± 1, and 0.17 ± 2 µm to the same concentrations of insulin (negative sign reflects vasoconstriction). Therefore, L-NNA administration significantly inhibited arteriolar dilation to insulin (Fig. 6, P < 0.05) and contributed to a significant insulin-induced constriction of RGM arterioles (Fig. 6, P < 0.05 compared with zero).

Figure 7 shows the effects of insulin before and after L-NNA in WGM arterioles represented as percent change from control diameter. In this group of WGM arterioles (n = 6), control diameters averaged 87.7 ± 8 µm before L-NNA administration and 67.3 ± 9 µm after L-NNA. Before L-NNA, these arterioles increased their diameters by 6.67 ± 1, 12.8 ± 2, 15.0 ± 2, and 18.8 ± 3 µm in response to insulin concentrations of 10 µU/ml, 100 µU/ml, 1 mU/ml, and 10 mU/ml, respectively. After L-NNA administration, the same arterioles changed their diameters by −4.00 ± 0.9, −2.17 ± 1, 0.67 ± 2, and 7.00 ± 4 µm to the same concentrations of insulin (negative sign reflects vasoconstriction). Therefore, L-NNA administration significantly inhibited insulin-evoked dilation in WGM arterioles (Fig. 7, P < 0.05) and contributed to an insulin-induced arteriolar constriction.

L-NNA was more effective in inhibiting insulin-induced arteriolar vasodilation and in converting the dilator effect of insulin into an arteriolar constrictor effect in RGM than in WGM vessels.

DISCUSSION

In this study we have shown that in endothelium-intact arterioles isolated from red and white rat gastrocnemius muscle, insulin evokes an equivalent concentration-dependent dilation. We have also shown that Indo had no effect on the insulin-induced arteriolar dilation and that endothelium removal or the administration of L-NNA inhibits the insulin-induced arteriolar dilation, revealing an insulin-induced vasoconstriction. On the basis of these findings we conclude that the insulin indirectly evokes an arteriolar dilation that is endothelium dependent and nitric oxide-mediated and, through a direct effect on vascular smooth muscle, evokes arteriolar constriction. To our knowledge this is the first study to utilize isolated arterioles from red and white gastrocnemius skeletal muscle and to report the effects of insulin on arterioles from these muscles.

The basic rationale for this study emerged from the facts that skeletal muscle is an insulin-sensitive tissue responsible for >80% of whole body glucose uptake (6) and is a large vascular compartment constituting 43% of body weight (21). As a result it is capable of influencing total peripheral resistance and blood pressure under resting and exercise conditions. We therefore
decided to investigate the effects of insulin on red and white skeletal muscle because insulin could potentially exert different vascular actions on arterioles from these muscles because of their differences in function and metabolic profile. This proved to be false even though NO synthase (NOS) has been reported to be differentially distributed in skeletal muscles (15) and insulin-stimulated glucose uptake in skeletal muscles is NO dependent (27).

The lowest concentrations of insulin we chose to study are equivalent to basal (10 µU/ml) and postprandial (100 µU/ml) plasma levels in humans (13, 14). Basal plasma insulin levels in rats are in the range of 40–50 µU/ml (32). The two higher concentrations of insulin that we employed (1 and 10 mU/ml) represent hyperinsulinemic doses. Insulin concentrations over 200 µU/ml are not likely to occur in vivo; therefore, the latter two concentrations tested should be considered pharmacological.

Therefore, we have been able to demonstrate that insulin, in physiological and clinically relevant concentrations, has the ability to affect vascular tone in red and white skeletal muscle arterioles by inducing an NO-mediated dilation in endothelium-intact vessels and vasoconstriction in arterioles in which the endothelium is completely (endothelium removed) or partially (L-NNA treated) functionally compromised. Thus we feel that through its vasodilator and vasoconstrictor actions insulin has the potential to play an important physiological or pathophysiological role in contributing to total peripheral resistance and arterial blood pressure, depending on the physiological state of the endothelium and the NOS system.

The results of the present study confirm our earlier report (9) of insulin-evoked, endothelium-dependent, NO-mediated dilation of arterioles isolated from the rat cremaster muscle. This skeletal muscle is classified as containing intermediate and mixed fiber types (3). It is small in size, with a specific function of thermoregulation of the testis. In in vivo studies of arterioles in the rat cremaster muscle, it was found that topical administration of hyperinsulinemic concentrations were also capable of evoking dilation of first-, second-, and third-order arterioles, whereas lower concentrations dilated only third-order vessels (25). Dilation in these arterioles was evident after 15 min of exposure to insulin (25), which is similar to our previous findings (9) and the present results. In contrast, intravenous euglycemic administration of insulin (6 mU/ml) required −60 min for cremaster arteriolar vasodilation to become evident, but only after treatment of the animals with a ganglionic blocker (25). This latter study demonstrates the complexity of insulin's actions in vivo and the difficulty of interpreting insulin's direct vascular actions. Further illustrating this point is a study in the hamster cremaster muscle in which topical suffusion of 200 µU/ml of insulin produced arteriolar dilation that was only partially blocked by inhibition of NO production but completely eliminated by administration of an adenosine-receptor antagonist (19). These findings implicate not only a role for NO in the mediation of insulin's action but also a role for the stimulation of metabolism with the consequent release of adenosine (19), a local metabolic vasodilator. None of the above studies evaluated the effects of insulin after endothelium removal and none reported any vasoconstrictor effects of insulin after NO synthesis inhibition. However, this may just reflect the complexity of in vivo studies versus in vitro studies. Vasoconstriction to insulin in vitro has been reported to occur in the isolated perfused mesenteric preparation of rats (33). This may reflect a difference in insulin reactivity in different vascular beds; however, it should be noted that this study required the induction of mesenteric tone by arginine vasopressin (33).

On the basis of our current finding that insulin-induced vasodilation of skeletal muscle arterioles is NO dependent and the work of others demonstrating that insulin-stimulated glucose uptake in skeletal muscles is also NO dependent (27), it can be hypothesized that insulin-resistant states may coincide with a failure to stimulate NOS, thus contributing to hypertension and disturbances in glucose metabolism. It is further reasonable to suggest that a compromised ability to increase blood flow in skeletal muscle in response to insulin release is an indirect mechanism that could contribute to a decreased glucose uptake in insulin-resistant diabetes (16). Our observation of arteriolar constriction to insulin after endothelium removal or NOS inhibition takes on additional importance when viewed against the established incidence of hypertension in insulin-resistant states both in animals and humans (16, 26). Chronic hypertension (17) and diabetes (18) cause profound reductions in endothelium-dependent relaxation to ACh, and exposing the endothelium to high circulating levels of glucose leads to endothelial cell dysfunction (23, 30) and the blunting of arteriolar responses to ACh (10, 31). Several lines of clinical evidence indicate an association between insulin resistance, hyperinsulinemia, and hypertension (2) and that insulin resistance is associated with essential hypertension and impaired insulin action on skeletal muscle metabolism (22).

In light of the above observations and our current findings, endothelial dysfunction may lead to insulin's failure to stimulate the NOS system, thus accounting for insulin resistance (4). As a result, NOS would be a valid target for therapy aimed at regulating glucose metabolism, skeletal muscle vascular responsiveness, and blood pressure in diabetics (24). In a similar manner, the beneficial effects of exercise in diabetic and hypertensive patients may be explained on the basis of exercise-induced increases in NOS gene expression (12, 28).

In summary, we propose that insulin may play a more important role than previously appreciated as a tonic stimulator of endothelial cell NOS under physiological conditions and, as such, plays an important role in the regulation of skeletal muscle blood flow, glucose metabolism, and total peripheral resistance. However, under conditions in which the endothelium is compromised, insulin in physiological concentrations can evoke arteriolar constriction and contribute to
increases in total peripheral resistance and arterial blood pressure.

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