Insulin Inhibits Acetylcholine Responses in Rat Isolated Mesenteric Arteries via a Non–Nitric Oxide Nonprostanoid Pathway

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Abstract—Hyperinsulinemia is a risk factor for hypertension and arteriosclerosis. The mechanism by which it contributes to disease progression is not known. The present study examines the effects of insulin on endothelium-derived relaxing factors. Segments of rat mesenteric arterioles and aorta were set up for isometric recordings. The effect of insulin (1 mU/mL) on acetylcholine responses was examined with and without nitro-L-arginine, indomethacin, KCl (40 mmol/L), and apamin/charybdotoxin. Incubation with insulin (maximum response to acetylcholine 90.9 ± 8.7% versus 90.7 ± 4.5% for before versus after insulin, respectively), nitro-L-arginine, indomethacin, or high K⁺ alone had no effect on these responses in mesenteric arterioles. Apamin/charybdotoxin significantly blunted responses to acetylcholine. When coincubated with nitro-L-arginine but not with indomethacin or high K⁺ alone, insulin blunted the maximum response to acetylcholine (from 84.8 ± 8.2% to 40.7 ± 10.2% for before versus after insulin, respectively; P<0.01). When coincubated with apamin/charybdotoxin, insulin had no further effect. Coadministration of indomethacin with nitro-L-arginine had no greater effect than did nitro-L-arginine alone. The addition of insulin, together with nitro-L-arginine and indomethacin, significantly decreased the maximal response to acetylcholine from 96.6 ± 5.3% to 52.9 ± 10.8% (P<0.01). In the aorta, nitro-L-arginine abolished acetylcholine responses. Coadministration with insulin had no further effect. We conclude that insulin attenuates acetylcholine responses mediated by endothelium-derived hyperpolarizing factor in small but not large arteries. This effect of insulin is apparent only when NO is blocked and may be important in the development of hypertension or arteriosclerosis when reduced NO function has been reported. (Hypertension. 2002;39:35-40.)

Key Words: insulin ▪ endothelium-derived factor ▪ nitric oxide ▪ mesenteric arteries ▪ aorta ▪ rats

Hyperinsulinemia is common in patients with insulin resistance syndrome and results from a diminished metabolic effect of this hormone.¹,² The syndrome is often associated with the development of hypertension and arteriosclerosis,³,⁴ and previous reports have suggested that this may be due to the facilitatory effects of high levels of insulin on sodium reabsorption at the nephron,⁵ an activated sympathetic nervous system,⁶ and/or vascular smooth muscle cell proliferation.⁷

The roles of the endothelium and the endothelium-derived relaxing factors, NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), in the regulation of vascular function are well documented.⁸,⁹ Also well studied are the associations between these endothelium products and hypertension and/or arteriosclerosis.⁹,¹⁰ The effect of insulin on these endothelium-derived relaxing factors is, on the other hand, less well investigated. To date, most reports of insulin demonstrate a dilatory role or an augmenting effect on acetylcholine-induced vasodilatation. Both of these effects have been hypothesized to act via increased NO production.¹¹⁻¹³ Most of these studies have involved in vivo systemic hyperinsulinemia produced by euglycemic clamping. We have previously shown that insulin blunts bradykinin-induced venodilatation in human dorsal hand veins and also blocks the bradykinin-induced elevation of cytoplasmic calcium in porcine aortic endothelial cells.¹⁴ Responses to bradykinin, as to acetylcholine, are endothelium dependent and may be due to the release of several endothelial factors, such as NO,¹⁵,¹⁶ prostanoids,¹⁷,¹⁸ and/or EDHF, depending on the vascular bed.¹⁹

In the present study, we explore the pathway by which insulin inhibits endothelium-dependent vasodilatation in vessels isolated from rats.

Methods

This study was approved by the Animal Ethics Committee of the Baker Medical Research Institute of Melbourne, Australia.
Preparation of Vessels
Thoracic aortas and mesenteric arterioles were quickly removed from male rats. From the mesenteric arterioles, short segments (2 mm in length) of the second-order branch were isolated and mounted in isometric myographs. After a 30-minute equilibration period, each vessel was subjected to a length-tension stretch enabling calculation of stretch at an internal circumference equivalent to 90% of the transmural pressure of 100 mm Hg.20 A further equilibration period of 30 minutes was observed.

The thoracic aorta was cut into rings of 3-mm length and mounted in standard 20-mL organ baths. Each ring was set to a passive tension of 2 g and allowed to equilibrate for 60 minutes.

Assessment of Endothelium-Dependent Vasodilatation
A full cumulative concentration-response curve to norepinephrine (NE, 1 mmol/L to 10 mmol/L) was obtained in each preparation to determine the submaximal dose of NE. This was generally either 10 or 30 mmol/L in aortic preparations and either 0.1 or 0.3 mmol/L in mesenteric arteries.

Full concentration-dilatation curves to acetylcholine (1 mmol/L to 100 mmol/L) were then obtained on vessels preconstricted with NE. In mesenteric arterioles, responses to acetylcholine were obtained in the absence and presence of (1) insulin (1 mU/mL, 30-minute incubation), (2) nitro-L-arginine (100 mmol/L), (3) nitro-L-arginine (100 mmol/L)+insulin, (4) indomethacin (100 mmol/L), (5) indomethacin+insulin, (6) KCl (40 mmol/L), (7) KCl+insulin, (8) nitro-L-arginine+indomethacin, (9) nitro-L-arginine+indomethacin+KCl, (10) nitro-L-arginine+KCl, (11) apanin (100 mmol/L)+charybdotoxin (100 mmol/L), (12) apanin+charybdotoxin+insulin, and (13) apanin+charybdotoxin+nitro-L-arginine.

In aortic rings, responses to acetylcholine were obtained in the absence and presence of (1) insulin, (2) nitro-L-arginine, and (3) nitro-L-arginine+insulin.

Responses to acetylcholine were also obtained before and after 30 minutes, with no intervention (time control) in either vessel type. Only 1 protocol was tested on any 1 mesenteric artery or aortic ring from any 1 rat.

Assessment of Endothelium-Independent Vasodilatation in Mesenteric Arteries
Full concentration-dilatation curves to sodium nitroprusside (1 mmol/L to 100 mmol/L) were obtained on mesenteric arterioles preconstricted with NE. Responses to sodium nitroprusside were obtained in the absence and presence of (1) nitro-L-arginine, (2) insulin, and (3) insulin+nitro-L-arginine.

Data Analysis
The tension of each vessel at rest was defined as 100% relaxation. The tension augmented by NE at EC70 was defined as 0% dilatation. The tension of each vessel at rest was defined as 100% relaxation.

All data were analyzed as means±SEM. ANOVA was used to compare the maximal response (Emax) to acetylcholine. Statistical significance was set at P<0.05. Emax to acetylcholine in individual experiments was determined from each curve fitted to a sigmoidal curve by using the 4-parameter logistic equation with SigmaPlot (SigmaPlot 5.0, Jandel Scientific).

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results
Mesenteric Arteries
The mean±SEM diameter of mesenteric arterioles used in each protocol is listed in Table 1. These were not different between the groups. Similarly, the vasodilatation produced by acetylcholine before the administration of inhibitors or insulin was identical in every group (Table 2).

In the time-control experiments, there was no significant difference between the first and second concentration-response curve to acetylcholine (Emax 74.8±6.1% versus 82.6±4.8%, respectively; P>0.05; Table 2).

Incubation with insulin had no effect on responses to acetylcholine (Emax 90.9±8.7% versus 90.7±4.5% for absence versus presence of insulin, respectively; Table 2). Nitro-L-arginine trended toward attenuated responses to acetylcholine (Figure 1A), but this did not reach statistical significance (Emax 91.4%±6.3% versus 81.2±9.7% for absence versus presence of nitro-L-arginine, respectively; Table 2). When administered together with insulin, however, nitro-L-arginine attenuated the vasodilatation to acetylcholine significantly. Emax was reduced from 84.8±8.2% to 40.7±10.2% (P<0.01, Table 2 and Figure 1A). The Emax of acetylcholine after coadministration of nitro-L-arginine and insulin was also statistically less than that obtained after nitro-L-arginine administration by itself (P<0.01, Table 2 and Figure 1A). Neither indomethacin nor high K’ affected the maximal responses to acetylcholine. Additional administration of in-

| TABLE 1. Characteristics of Mesenteric Arterioles |
|------------------------------|----------------------|----------------|
| Antagonist | n | Diameter, μm |
| Control | 9 | 353.4±32.5 |
| Insulin | 8 | 386.6±29.2 |
| NOLA | 9 | 366.5±18.3 |
| NOLA+insulin | 9 | 357.1±16.7 |
| IND | 7 | 348.2±31.9 |
| IND+insulin | 7 | 331.8±23.6 |
| High K’ | 5 | 340.6±23.8 |
| High K’+insulin | 5 | 332.6±32.2 |
| NOLA+IND | 6 | 374.5±9.0 |
| NOLA+IND+insulin | 6 | 370.9±15.3 |
| NOLA+high K’ | 6 | 395.2±14.2 |

NOLA indicates Nω-nitro-L-arginine; IND, indomethacin; and high K’, Krebs’ solution containing high K’ concentration. Values are mean±SEM.

| TABLE 2. Maximum Effects of Acetylcholine on Mesenteric Arterioles |
|-----------------------|------------------|------------------|
| Antagonist | Before, % | After, % |
| Time control | 75.4±6.1 | 82.6±8.4 |
| Insulin | 90.9±8.7 | 90.7±4.5 |
| NOLA | 91.4±6.3 | 81.2±9.7 |
| NOLA+insulin | 84.8±8.2 | 40.7±10.2† |
| IND | 89.7±9.3 | 87.1±7.3 |
| IND+insulin | 81.7±10.2 | 79.2±8.2 |
| High K’ | 93.6±5.4 | 91.1±20.0 |
| High K’+insulin | 81.1±5.9 | 112.5±36.9 |
| NOLA+IND | 99.2±2.2 | 83.0±7.5 |
| NOLA+IND+insulin | 96.6±5.3 | 52.9±10.8† |

Values are mean±SEM.

*P<0.01 for before vs after antagonist; †P<0.01 for presence vs absence of insulin.
sulin had no further effect on responses to acetylcholine (Table 2 and Figure 1B and 1C). Coadministration of N\textsubscript{N}H\textsubscript{9275}-nitro- L -arginine and high K\textsubscript{11001} almost totally abolished the responses to acetylcholine (Figure 2B), whereas the coadministration of nitro-L-arginine and indomethacin did not significantly affect the vasodilating response (Emax 99.15\pm2.2% versus 83.0\pm10.8% for the absence versus presence of both inhibitors, respectively; Table 2 and Figure 2A). However, insulin administered with nitro-L-arginine plus indomethacin further blunted responses to acetylcholine such that E\textsubscript{max} was reduced significantly from 96.60\pm5.3% to 52.9\pm10.8% (P<0.01, Table 2 and Figure 2B). The maximal response to acetylcholine in the presence of nitro-L-arginine, indomethacin, and insulin was again significantly different from that obtained after the coadministration of just the 2 inhibitors in the absence of insulin (P<0.01).

Maximal responses to acetylcholine were significantly blunted by coincubation with apamin and charybdotoxin (percent dilatation [y\textsubscript{max}] 99.38\pm6.06 versus 83.12\pm9.9 for before versus after incubation, P=0.04). Insulin, together with apamin and charybdotoxin, also dampened the maximal response to acetylcholine (y\textsubscript{max} 97.82\pm1.53 versus 87.78\pm4.69 for before versus after incubation, P=0.019). However, the added incubation of insulin did not further add to the blunted effect already seen with apamin and charybdotoxin (y\textsubscript{max} 83.12\pm9.9 versus 87.78\pm4.69, respectively; P>0.05). However, the effects of apamin and charybdotoxin were further blunted with the added presence of nitro-L-arginine (y\textsubscript{max} 83.12\pm9.9 [n=7] versus 52.87\pm14.67 [n=4], respectively; P=0.015).

Responses to sodium nitroprusside were not affected by incubation with insulin (Figure 3A) or nitro-L-arginine (Figure 3B) or insulin coincubated with nitro-L-arginine (Figure 3C).

**Aorta**

Acetylcholine produced concentration-dependent vasodilatation in aortic rings isolated from rats. The responses were reproducible. As with mesenteric arteries, incubation with insulin had no effect on the responses to acetylcholine (Figure 4A). Nitro-L-arginine almost totally abolished the response to acetylcholine. Further administration with insulin did not reveal further inhibitory effects (Figure 4B).
Discussion

Our major finding from the present study is that insulin inhibits responses to acetylcholine in rat mesenteric arterioles and that this inhibition is probably accomplished through blocking EDHF-mediated relaxation. Similar to the effect of high K⁺, the inhibitory effect of insulin was seen only after inhibition of NO synthase. This is the first report of such a role for insulin and may suggest a means by which high levels of insulin in syndromes of hyperinsulinemia can contribute to the progression of disease, such as hypertension and arteriosclerosis.

In contrast to the present study, most reports of insulin demonstrate a dilatory role or an augmenting effect on acetylcholine-induced vasodilatation. Both of these effects have been hypothesized to act via increased NO production. Most of these studies have been on in vivo systemic hyperinsulinemia produced by euglycemic clamping. In isolated vessels, concentrations significantly higher (10 to 100 mU/mL) than those used in the present study are required to produce vasodilatation. It may be that additional systemic or metabolic effects of insulin, not observed in the in vitro situation, are required for the vasorelaxant effect of equal concentrations of hyperinsulinemia in vivo. Alternatively, the attenuation of responses observed to acetylcholine could reflect a change in the balance of vasoconstrictor versus vasodilator pathways of insulin response.

The inhibitory effect of insulin was not apparent on blockade of the cyclooxygenase system or on blockade of EDHF with a high K⁺ solution or with the combined use of apamin and charybdotoxin in the rat mesenteric arterioles. Neither insulin nor nitro-l-arginine nor indomethacin nor high K⁺, when administered alone, had any significant inhibitory effect on responses to acetylcholine. Apamin and charybdotoxin did significantly blunt the maximal response to acetylcholine. When coadministered with nitro-l-arginine, indomethacin had no further effect, suggesting that prostanooids play a negligible role in the responses of acetylcholine in this preparation. On the other hand, when coadministered with nitro-l-arginine, high K⁺ completely abolished the
responses to acetylcholine, suggesting that EDHF plays a major role in its dilatory capacity. Similarly, in the presence of nitro-L-arginine, maximal responses to acetylcholine were further blunted by apamin and charybdotoxin. Therefore, we suggest that the 2 relaxing factors, NO and EDHF, play a compensatory role in this preparation, such that one factor compensates for the diminished action of the other factor. Thus, when the effect of EDHF is abolished, as with the administration of high K\(^+\), a full relaxation response to acetylcholine that is due to the release of NO is still observed. In the presence of apamin and charybdotoxin, the maximal response to acetylcholine while blunted still achieves >80% dilatation. Similarly, when the effects of NO are blocked, as with the administration of nitro-L-arginine, again a full relaxation response to acetylcholine that is due, this time, to the release of EDHF is observed. However, when both systems are blocked, the relaxation response to acetylcholine is abolished. This synergistic effect of NO and EDHF has been previously alluded to by others.25-26 This synergism is not observed with indomethacin, suggesting that prostanooids play no role in the dilatory response to acetylcholine in this preparation. Because insulin inhibited the responses to acetylcholine only after the blockade of NO synthase, we surmise that insulin, like high K\(^+\), inhibits EDHF. This finding is further supported by the added observation that responses to acetylcholine were further diminished by insulin in vessels incubated with both nitro-L-arginine and indomethacin when the only other relaxing factor available is EDHF. The dilatory responses to acetylcholine were reproducible in this preparation, showing little change in the response over time.

Notably, in mesenteric arteries isolated from rats with insulin resistance and hyperinsulinemia, Miller, Katakam, and colleagues27,28 have shown that EDHF-mediated dilatation is attenuated and that the primary relaxant factor in these animals is NO. The evidence from the present study would suggest that in these insulin-resistant animals, insulin directly inhibits EDHF-mediated relaxation, allowing NO to compensate for the diminished EDHF role.

The production of NO, prostacyclin, and EDHF is associated with an elevation of cytoplasmic calcium ([Ca\(^{2+}\)]\(_i\)), which is triggered by agonist-receptor stimulation. [Ca\(^{2+}\)]\(_i\) elevation activates NO synthase and the production of NO, phospholipase A\(_2\), and prostacyclin.29,30 Although the chemical nature of EDHF is still unknown, it has been reported that production of this relaxing factor also requires [Ca\(^{2+}\)]\(_i\), elevation.\(^{31}\) In a previous study, we demonstrated that insulin attenuates the bradykinin-induced elevation of [Ca\(^{2+}\)]\(_i\) in cultured endothelial cells.\(^{14}\) Because the attenuation of [Ca\(^{2+}\)]\(_i\), was relatively modest in that report and because the production of prostacyclin and EDHF, but not of NO, is regulated by enzymes sensitive to small changes in [Ca\(^{2+}\)]\(_i\), levels,26,32 our prior hypothesis was that insulin inhibited either prostacyclin or EDHF production and that the attenuation was unlikely to be via suppression of NO synthesis. Indeed, from the present study, the evidence supports the notion that EDHF-mediated dilatation is diminished by insulin.

In the absence of nitro-L-arginine, insulin, like high K\(^+\), had no effect on responses to acetylcholine in the rat mesenteric artery. As previously discussed, this is likely to be because NO plays an augmented role when EDHF is blocked such that the inhibitory effect of insulin is not observable. However, it is worth noting that NO-mediated dilatation is diminished in patients with hypertension\(^{32}\) and arteriosclerosis,\(^{34}\) even in the early or preclinical stages.\(^{35}\) It is in these patients that the inhibitory effect of insulin on EDHF-induced vasodilatation is likely to play a key role in the progression of disease.

Although nitro-L-arginine only marginally inhibited responses to acetylcholine in rat isolated mesenteric arteries, it totally abolished the vasodilatation to this agonist in isolated aortic rings. This concurs with reports that NO is the dominant relaxing factor in large arteries and that the involvement of EDHF is minimal.\(^{8}\) Therefore, in this preparation, insulin has little effect, even in the presence of nitro-L-arginine.

We conclude that insulin attenuates EDHF-induced vasodilatation in small arteries but not in large arteries. This effect of insulin is especially efficacious in situations in which the role of NO is diminished; therefore, insulin may play a key role in the development of hypertension or arteriosclerosis in patients with hyperinsulinemia.

References


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