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Effects of insulin on vascular responses to spinal cord stimulation and vasoactive agents in pithed rats

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1 Effects of insulin (2–600 pmol kg^{-1} min^{-1}, i.v.) on vascular responses to spinal cord (lower thoracic vertebra, Th 9–12) stimulation (SCS) and to i.v. injection of noradrenaline (NA, 125–500 ng kg^{-1}), angiotensin II (Ang II, 40–200 pmol kg^{-1}), acetylcholine (ACh, 1 nmol kg^{-1}), calcitonin gene-related peptide (CGRP, 0.1 nmol kg^{-1}) and sodium nitroprusside (SNP, 5 μg kg^{-1}) were examined in pithed rats.

2 In euglycemic pithed rats, low and medium doses of insulin dose-dependently potentiated vasopressor responses to SCS (2–8 Hz), NA, while higher doses of insulin had little effect on SCS- and NA-induced pressor responses. All doses of insulin significantly augmented pressor responses to Ang II.

3 In pithed rats with artificially increased blood pressure, SCS (2 and 4 Hz) induced a frequency-dependent depressor response, which was blocked by infusion of CGRP(8–37) (CGRP receptor antagonist, 60 nmol kg^{-1} min^{-1}).

4 In euglycemic pithed rats, low-doses of insulin significantly attenuated depressor responses to SCS and CGRP, but medium and high doses of insulin remained unaffected.

5 All doses of insulin significantly inhibited depressor response to ACh, while SNP-induced depressor response was not significantly affected by any doses of insulin.

6 These results suggest that insulin at low and medium concentrations increases adrenergic vasoconstriction, which is partly associated with inhibition of CGRPergic nerve function and endothelium function. It is also suggested that lack of insulin effect at higher concentrations may result from acute desensitization of insulin action, possibly *via* insulin receptors.
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Abbreviations: ACh, acetylcholine; Ang II, angiotensin II; BP, blood pressure; CGRP, calcitonin gene-related peptide; HR, heart rate; NA, noradrenaline; NO, nitric oxide; SCS, spinal cord stimulation; SNP, sodium nitroprusside

Introduction

Accumulating evidence indicates that the effects of insulin are not limited to carbohydrate metabolism and that insulin plays a role in blood pressure control. Insulin has been reported to increase sympathetic activity (Lembo *et al*., 1992) and renal sodium reuptake (Defronzo, 1981), and promote the proliferation of vascular smooth muscle cells (Hsueh & Law, 1999), which could bring about an increase in blood pressure (BP). In fact, hyperinsulinemia has been shown to contribute to increased sympathetic activity (Reaven, 1988; Marigliano *et al*., 1990), and a close association between insulin resistance and hyperinsulinemia has been suggested in essential hypertension (Ferrannini *et al*., 1987; Pollare *et al*., 1990). This relationship implies that insulin resistance and hyperinsulinemia contribute to the pathogenesis of hypertension and, furthermore, that insulin might play an important role in the pathophysiological regulation of the cardiovascular system. On the other hand, recent investigations have shown that insulin acts as an endogenous vasodilator (Creager *et al*., 1985; Steinberg *et al*., 1994). Thus, insulin-mediated vascular responses have been found in various blood vessels in a number of different studies; however, the vascular effects of insulin have not been fully clarified.

Insulin has been shown to induce nonadrenergic, noncholinergic and endothelium-independent vasodilation of the rat mesenteric resistance artery, which is partially mediated by calcitonin gene-related peptide (CGRP) receptors (Mimaki *et al*., 1998). CGRP is a potent vasodilator (Brain *et al*., 1985) and acts as a vasodilator neurotransmitter of the perivascular nerves (CGRPergic nerves) (Kawasaki *et al*., 1988). In addition, CGRP has a partially similar amino-acid sequence to insulin, and has been reported to inhibit both insulin secretion and actions *in vitro* (Wimalawansa, 1996; 1997). These findings raise the possibility that insulin may interact with CGRP receptors *in vivo*.

Therefore, in the present study, to characterize further the vascular effects of insulin, we examined the effects of continuous infusion of low, medium and high doses of insulin on the cardiovascular responses to spinal cord stimulation

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(SCS) and to intravenous (i.v.) injection of vasoactive drugs. In the present study, we evaluated the effect of insulin on not only the sympathetic nerve-mediated pressor response, but also the CGRPergic nerve-mediated depressor response using pithed rats.

Methods

Animals

In all, 95 male Wistar rats weighing 350–400 g were used in this study. The animals were given food and water *ad libitum*. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of $22 \pm 10^{\circ}C$ with $50 \pm 10\%$ relative humidity and with a 12-h light/12-h dark cycle (lights on at 08:00 h).

Pithing and measurement

The animals were anesthetized with sodium pentobarbital ($50\,mg\,kg^{-1}$, i.p.). Polyethylene catheters (PE-10) were positioned in the right and left jugular veins for administration of drugs, and a bilateral vagotomy was performed at the midcervical level. A polyethylene catheter (PE-50) was inserted into the left carotid artery and connected to a pressure transducer (model DX-100, Nihon Kohden, Tokyo, Japan). The arterial BP and mean BP were recorded on a polygraph (model RM-6000, Nihon Kohden). The heart rate (HR) triggered by arterial pulses was measured using a cardiotachometer (model AT-600G, Nihon Kohden), and was recorded on the polygraph.

After the trachea was cannulated, the animals were pithed by inserting a stainless-steel rod (1.4 mm in diameter) through the right orbit and the foramen magnum and down into the spinal cord to the level of the sacral end, and then the tip of the rod was raised to the thoracolumbar vertebra (Th 9–12), according to the method described previously by Taguchi *et al*. (1992). Artificial respiration ($4.5\,ml\,beat^{-1}\,kg^{-1}$, $70\,beats\,min^{-1}$) with room air was immediately started using a respirator (model 683, Harvard Apparatus, South Natick, MA, U.S.A.). The pithing rod served as the stimulating electrode, which was insulated except for 5 mm at the tip. The level of SCS was determined by varying the depth of insertion of the rod. The position of the rod within the vertebral canal was determined from the length of the exposed rod. A stainless-steel needle was inserted subcutaneously in the dorsum, parallel to the vertebral column, to serve as an indifferent electrode. After the animals were pithed, *d*-tubocurarine ($1\,mg\,kg^{-1}$, i.v.) was injected to prevent skeletal muscle contraction during electrical stimulation of the spinal cord. The rectal temperature was maintained at approximately $37^{\circ}C$ using a heating mat (model KN-475, Natsume, Tokyo, Japan).

Spinal cord stimulation (SCS)

After allowing BP and HR to stabilize, electrical stimulation at 2, 4 and 8 Hz, which all induced a sharp increase in BP without changing HR, was applied to verify the position of the rod in the spinal column. Rectangular pulses (1 ms in duration and 20 V) were delivered for 30 s at 5–10-min intervals with an electronic stimulator (model SEN-3201, isolator 20865, Nihon Kohden).

In another series of experiments, mean BP was increased and maintained at a level of approximately 100 mmHg by continuous infusion of the α_1-adrenoceptor agonist methoxamine ($20\,\mu g\,kg^{-1}\,min^{-1}$, i.v.). The autonomic ganglionic blocker hexamethonium ($2\,mg\,kg^{-1}\,min^{-1}$, i.v.) was also infused to block autonomic outflow. The increased BP was allowed to stabilize for 30 min, and then the spinal cord was electrically stimulated. Rectangular pulses (1 ms in duration and 20 V) at 2 and 4 Hz were delivered for 30 s at 5–10-min intervals with an electronic stimulator (model SEN-3201, isolator 20865, Nihon Kohden).

Experimental protocols

To assess the underlying mechanisms involved in the vascular effect of insulin, the effects of various vasoactive agents were evaluated. After the animals had been pithed and both the BP and HR had stabilized, electrical stimulation (2, 4 and 8 Hz) and bolus injections of noradrenaline (NA; 125, 250 and $500\,ng\,kg^{-1}$, i.v.) and angiotensin II (Ang II; 40, 100 and $200\,pmol\,kg^{-1}$, i.v.) were applied. Thereafter, continuous infusions of insulin (2, 6, 20, 60, 200 and $600\,pmol\,kg^{-1}\,min^{-1}$, i.v.; $n = 4$–9 animals per group) and glucose (5–15%) were concomitantly carried out with an infusion pump (model 11, Harvard Apparatus) to maintain a normal glucose level. At 30 min after starting the insulin infusion, SCS and bolus injections of NA and Ang II were applied while continuing the insulin infusion. During the insulin infusion, the blood glucose levels were monitored using a glucose analyzer (ADVANTAGE, Boehringer Mannheim, Tokyo, Japan).

In another series of experiments, the mean BP of pithed rats was increased by continuous infusion of methoxamine ($25\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) concomitant with infusion of hexamethonium ($2\,mg\,kg^{-1}\,min^{-1}$, i.v.). After the elevated BP had stabilized, responses to SCS (2 and 4 Hz), bolus injections of acetylcholine (ACh; $1\,nmol\,kg^{-1}$, i.v.), rat CGRP ($0.1\,nmol\,kg^{-1}$, i.v.) and sodium nitroprusside (SNP; $5\,\mu g\,kg^{-1}$, i.v.) were measured as the control response. Thereafter, the infusion of insulin (2, 6, 20, 60, 200 and $600\,pmol\,kg^{-1}\,min^{-1}$, i.v.; $n = 4$–9 animals per group) was started and the vascular responses to SCS and to bolus injections of ACh, CGRP and SNP were examined during the insulin infusion. Blood glucose levels were monitored before and after 30 min of each insulin infusion.

As a control, saline (0.9%) at a volume of $0.2\,ml\,min^{-1}$ was infused and the vascular responses to SCS and to bolus injections of NA, Ang II, ACh, CGRP and SNP were examined during the saline infusion.

In the insulin infusion experiments, a combination of 2 and $6\,pmol\,kg^{-1}\,min^{-1}$ for low doses, 20 and $60\,pmol\,kg^{-1}\,min^{-1}$ for medium doses or a combination of a low dose (2 or $6\,pmol\,kg^{-1}\,min^{-1}$) and a medium dose (20 or 60 pmol $kg^{-1}\,min^{-1}$) was tested in the same animal. A separate group of animals was tested during infusion of 200 and $600\,pmol\,kg^{-1}\,min^{-1}$. The effect of insulin on pressor responses to SCS and to NA or Ang II injection was determined in different animals, but depressor responses to SCS and to CGRP, ACh and SNP injection were examined in the same animals.

In other experiments, to confirm the nature of the pressor response induced by SCS, NA and Ang II, tetrodotoxin (neurotoxin, 100 $\mu g\,kg^{-1}$, i.v.), guanethidine (adrenergic neuron blocker, 5 $mg\,kg^{-1}$, i.v.), prazosin (α_1-adrenoceptor antagonist, 0.1 $mg\,kg^{-1}$, i.v.) or CV-11974 (Ang II Type-1 receptor antagonist, 0.1 $mg\,kg^{-1}$, i.v.) were administered 10–20 min before SCA, NA or Ang II injection in different animals. The nature of the depressor response to SCS and CGRP injection was determined by continuous infusion of CGRP(8–37) at a dose of 60 $nmol\,kg^{-1}\,min^{-1}$, i.v.

Statistical analysis

The experimental results are presented as mean ± s.e.m. Statistical analyses were performed using one-way analysis of variance followed by the Tukey's test. A P-value less than 0.05 was considered significant.

Drugs

The following drugs were used: ACh chloride (Daiichi Pharmaceutical Co.,. Tokyo), Ang II (Peptide Institute, Osaka, Japan), CV-11974 (donated by Takeda Chemical Co., Osaka, Japan), D-glucose (Nakalai Tesque Inc., Kyoto, Japan), guanethidine sulfate (Tokyo Kasei, Tokyo, Japan), hexamethonium bromide (Sigma Chemical Co., St Louis, MO, U.S.A.), human CGRP(8–37) (Peptide Institute), insulin human (Eli Lilly Co., Osaka, Japan), methoxamine hydrochloride (Nihon Shinyaku Co., Kyoto, Japan), NA hydrochloride (Sankyo Co., Tokyo, Japan), prazosin hydrochloride (Sigma), rat α-CGRP (Peptide Institute), tetrodotoxin (Sigma), *d*-tubocurarine (Sigma) and SNP (Sigma). All drugs were dissolved in 0.9% saline and infused at a rate of 0.2 $ml\,min^{-1}$ using an infusion pump (model 11, Harvard Apparatus), or given as bolus doses (0.2 $ml\,kg^{-1}$).

Results

Cardiovascular responses to SCS and bolus injections of NA and Ang II in pithed rats

The basal mean BP and HR in pithed rats were 28.1 ± 1.3 mmHg and 272.0 ± 2.6 beats min^{-1} ($n = 95$), respectively. In pithed rats, electrical stimulations of the higher thoracic region (Th 1–4) evoked a sharp rise in BP and an increase in HR (Figure 1a). Stimulation (2–8 Hz) of the lower thoracic region (Th 9–12) evoked a frequency-dependent increase in BP without altering HR (Figure 1b). Therefore, in the following experiments, the lower thoracic region (Th 9–12) was stimulated to minimize the cardiac effect on vascular responses. The pressor responses to SCS were abolished by tetrodotoxin and by guanethidine (data not shown).

As shown in Figure 1c, bolus injections of NA (125–500 ng kg^{-1}, i.v.) caused a dose-dependent increase in BP with a slight increase in HR. The pressor response to NA was sharp and transient and mimicked the pressor response to SCS. The pressor responses to injection of NA were inhibited by prazosin (data not shown). Injections of Ang II (40–200 pmol kg^{-1}, i.v.) induced a dose-dependent pressor response without changing HR. The Ang II-induced pressor response was antagonized by CV-11974, but not by prazosin (data not shown).

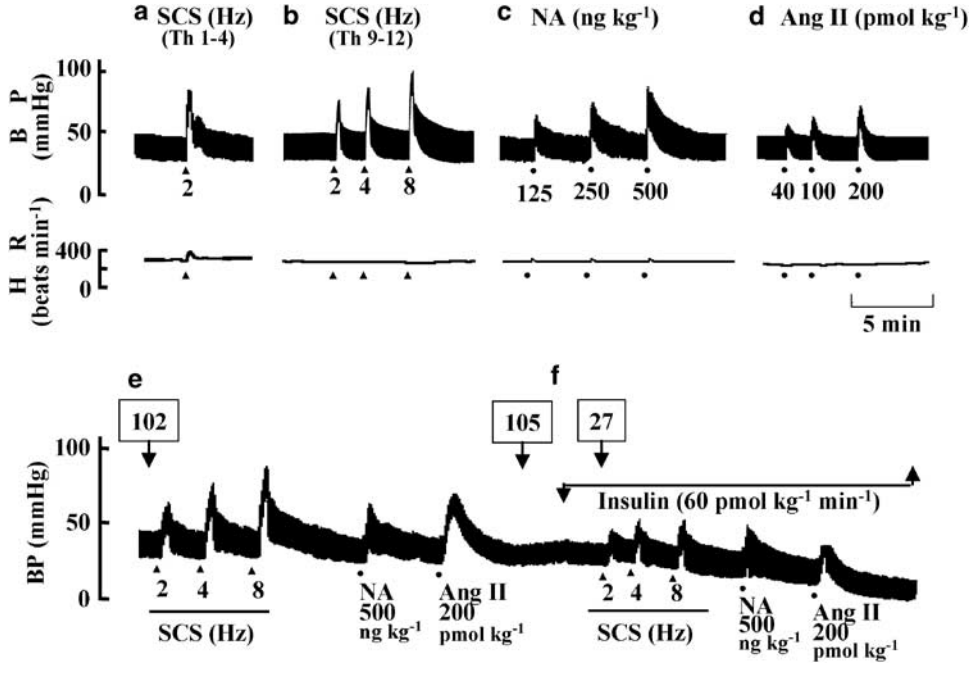

Figure 1 Typical records showing cardiovascular responses to SCS (2–8 Hz; triangles) and to bolus injections (circles) of NA (125–500 ng kg^{-1}, i.v.) and Ang II (40–200 pmol kg^{-1}, i.v.) in pithed rats. Tracing a: increase in BP and HR in response to upper thoracic (Th 1–4) SCS. Tracing b: pressor responses to lower thoracic (Th 9–12) SCS. Tracings c and d: dose-dependent pressor responses induced by NA and Ang II, respectively. Tracings e and f: the effect of insulin infusion (60 pmol $kg^{-1}\,min^{-1}$, i.v.) on cardiovascular responses to SCS, NA and Ang II in pithed rats under normal blood glucose level (e) and without euglycemia maintenance (f). The numbers in the box represent blood glucose level (mg dl^{-1}). BP, blood pressure; HR, heart rate.

Effect of insulin on pressor responses to SCS and bolus injections of NA and Ang II

As shown in Figure 1f, continuous infusion of insulin (60 pmol kg^{-1} min^{-1}, i.v.) markedly reduced the blood glucose level from 105 to 27 mg dl^{-1} and pressor responses both to SCS and to bolus injections of NA (500 ng kg^{-1}, i.v.) and Ang II (200 pmol kg^{-1}, i.v.) were markedly inhibited. Therefore, in the following experiments, the blood glucose level was maintained to minimize the hypoglycemic effect of insulin on vascular responses.

In pithed rats with a constant blood glucose level, continuous infusion of insulin did not affect the basal BP and HR. The mean BP and HR before SCS and each agonist injection after saline or infusion of insulin at doses of 2, 6, 20, 60, 200 and 600 pmol kg^{-1} min^{-1} were 28.9 ± 2.8 mmHg and 280.0 ± 4.6 beats min^{-1} ($n=8$) or 30.5 ± 1.0 mmHg and 270.5 ± 2.2 beats min^{-1} ($n=36$), 27.9 ± 0.8 mmHg and 272.6 ± 1.7 beats min^{-1} ($n=39$), 29.3 ± 1.8 mmHg and 268.0 ± 3.3 beats min^{-1} ($n=39$), 27.4 ± 0.9 mmHg and 271.2 ± 2.0 beats min^{-1} ($n=38$), 29.7 ± 3.1 mmHg and 278.1 ± 1.5 beats min^{-1} ($n=30$), 31.0 ± 2.2 mmHg and 285.0 ± 2.0 beats min^{-1} ($n=30$), respectively. Infusion of insulin at doses of 2–60 pmol kg^{-1} min^{-1} caused a dose-dependent potentiation of pressor responses to SCS (2–8 Hz) (Figures 2a and 3a). Significant differences ($P<0.05$ and 0.01) were found with doses of 2, 6, 20 and 60 pmol kg^{-1} min^{-1} at 2, 4 and 8 Hz SCS. Pressor responses to exogenous NA at all doses were significantly intensified by low (2 and 6 pmol kg^{-1} min^{-1}; $P<0.01$) and medium doses (20 and 60 pmol kg^{-1} min^{-1}; $P<0.01$) of insulin infusion (Figures 2a and 3b). However, neither SCS- nor NA-induced pressor responses were significantly affected by high doses of insulin (200 and 600 pmol kg^{-1} min^{-1}) when euglycemia was maintained (Figures 2b and 3a, b). As shown in Figure 3c, pressor responses to Ang II at all doses were significantly augmented by all doses of insulin infusion ($P<0.05$ and 0.01) except for the response to 100 pmol kg^{-1} Ang II during insulin infusion of 600 pmol kg^{-1} min^{-1}. However, the augmentation of the Ang II-induced pressor responses by high doses (200 and 600 pmol kg^{-1} min^{-1}) of insulin was smaller than those to low (6 pmol kg^{-1} min^{-1}) and medium doses (20 and 60 pmol kg^{-1} min^{-1}).

Depressor responses to SCS and bolus injection of ACh, CGRP and SNP in pithed rats with artificially increased BP

In pithed rats with resting BP, SCS (4 Hz) at the lower thoracic region (Th 9–12) induced a pressor response without changing HR (Figure 4a), and this was abolished by hexamethonium (2 mg kg^{-1} min^{-1}, i.v.) (Figure 4b). The mean BP of pithed rats was artificially increased and maintained at approximately 100 mmHg by continuous infusion of methoxamine (25 μg kg^{-1} min^{-1}, i.v.) in the presence of hexamethonium (2 mg kg^{-1} min^{-1}, i.v.). As shown in Figure 4c, SCS (2 and 4 Hz) of the lower thoracic region (Th 9–12) induced a frequency-dependent decrease in arterial BP. The depressor response appeared 10–20 s after the stimulation began and reached the maximum at 1–2 min after the stimulation ended, and the HR did not change during the depressor response. Figure 4d shows vascular responses to bolus i.v. injections of ACh, CGRP and SNP in pithed rats with artificially increased BP. Bolus injection of rat CGRP (0.1 nmol kg^{-1}) caused a long-lasting fall in BP without changing HR, which mimicked the depressor responses to SCS. However, bolus injections of ACh (1 nmol kg^{-1}) and SNP (5 μg kg^{-1}) induced a sharp and transient fall in BP without changing HR.

Continuous infusion of CGRP(8–37), a CGRP receptor antagonist, markedly inhibited the depressor responses both to SCS and to exogenously applied CGRP, while the antagonist

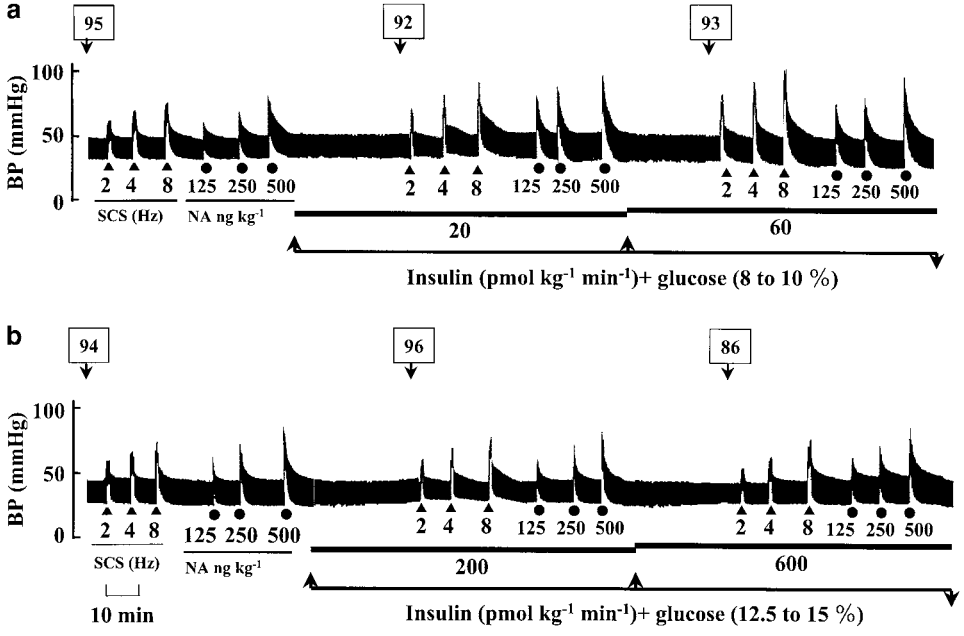

Figure 2 Typical records showing the effects of insulin on vasoconstrictor responses to SCS (2–8 Hz; triangles) and to bolus injection (circles) of NA (125–500 ng kg^{-1}, i.v.) in pithed rats under euglycemia maintenance. Tracing a: the effect of insulin at medium doses (20 and 60 pmol kg^{-1} min^{-1}, i.v.). Tracing b: the effect of insulin at high doses (200 and 600 pmol kg^{-1} min^{-1}, i.v.). The numbers in the box represent blood glucose level (mg dl^{-1}). BP, blood pressure.

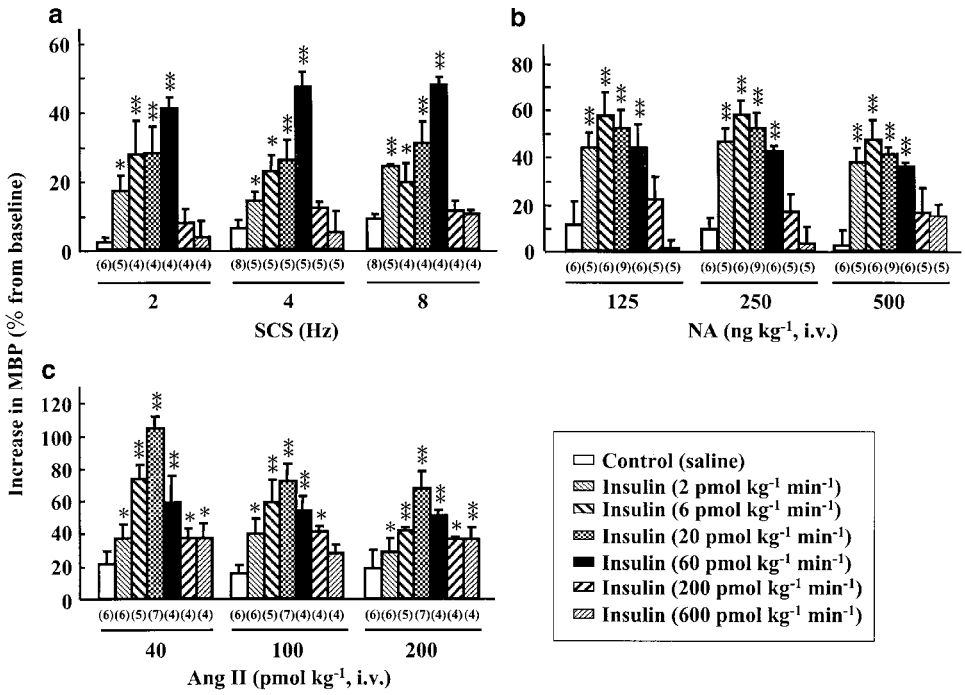

Figure 3 Bar graphs showing the effects of insulin on pressor responses to SCS (2–8 Hz) (panel (a)) and bolus injections of NA (125–500 ng kg^{-1}, i.v.) (panel (b)) and Ang II (40–200 pmol kg^{-1}, i.v.) (panel (c)) in pithed rats under euglycemia maintenance. Experimental conditions were as described in Figure 2. The ordinate indicates the percent increase in mean blood pressure (MBP), which is expressed as the percentage increase in MBP by SCS (2–8 Hz), NA (125–500 ng kg^{-1}, i.v.) and Ang II (40–200 pmol kg^{-1}, i.v.), respectively. Each bar indicates the mean ± s.e.m. Numbers in the parentheses below each bar indicate the number of animals. $*P<0.05$, $**P<0.01$ compared with saline control.

did not affect the depressor responses to ACh and SNP injection (Figure 4e).

As shown in Figure 4f, continuous infusion of insulin caused a hypoglycemic effect and a marked inhibition of depressor responses to SCS and to bolus injections of CGRP, ACh and SNP.

Effect of insulin on depressor responses to SCS and bolus injections of ACh, CGRP and SNP

As shown in Figures 5 and 6, in pithed rats with artificially increased BP, continuous infusion of low doses of insulin (2 and 6 pmol kg^{-1} min^{-1}) inhibited depressor responses induced by SCS (2 and 4 Hz) when maintained at a constant blood glucose level (Figures 5a and 6a). There was a significant difference ($P<0.01$) in the response to 2 and 4 Hz SCS between control and insulin infusion at 2 and 6 pmol kg^{-1} min^{-1}. Low dose of insulin at 2 but not 6 pmol kg^{-1} min^{-1} caused a significant inhibition ($P<0.05$) of the depressor response to exogenous CGRP (Figures 5a and 6b). On the other hand, medium (20 and 60 pmol kg^{-1} min^{-1}) and high (200 and 600 pmol kg^{-1} min^{-1}) doses of insulin did not affect the depressor responses either to SCS (4 Hz) (Figures 5b and 6a) or to bolus injections of CGRP (Figures 5b and 6b). The ACh-induced depressor responses were significantly inhibited ($P<0.05$ and 0.01) by all doses of insulin (Figures 5a, b and 6c), while depressor responses to SNP injections were not influenced by any doses of insulin (Figures 5 and 6d). There was no dose-responsiveness in the inhibitory effect of insulin on the ACh-induced depressor response (Figure 6c).

Discussion

The present study demonstrated that, in pithed rats, insulin at low doses significantly augmented vasoconstrictor responses induced by exogenously applied NA and Ang II, while no further augmentation in the pressor response to SCS and NA was observed with high doses of insulin. Since the pressor response to SCS is mediated by vasoconstriction of endogenous NA, which is released from sympathetic adrenergic nerves, it is likely that insulin augments adrenergic neurotransmission. In *in vivo* studies, insulin has been shown to increase sympathetic nerve activity, which suggests that insulin acts, in part, on the central nervous system such as the medial hypothalamus (Sauter *et al*., 1983; Anderson & Mark, 1993; Scherrer & Sartori, 1997). In addition, there is accumulating evidence that insulin stimulates peripheral sympathetic efferent outflow in humans through its action on the central nervous system (Scherrer & Sartori, 1997). However, an *in vivo* study with rats showed that insulin stimulates the lumbar but not the adrenal or renal sympathetic outflow (Morgan *et al*., 1993). In addition, in an *in vitro* study, insulin reduced NA release from the sympathetic nerves in rat mesenteric vascular preparations (Shimosawa *et al*., 1992). Furthermore, insulin has been reported to blunt sympathetic vasoconstriction through a direct peripheral effect on the reduced amount of NA reaching the receptor site (Lembo *et al*., 1994). The pithed rats used in the present study had no central outflow of sympathetic nerve activity and SCS was performed at thoracic regions. Furthermore, the augmenting effect of insulin on the pressor response was more pronounced for exogenously applied NA than for SCS. In addition, all doses of insulin potentiated the pressor

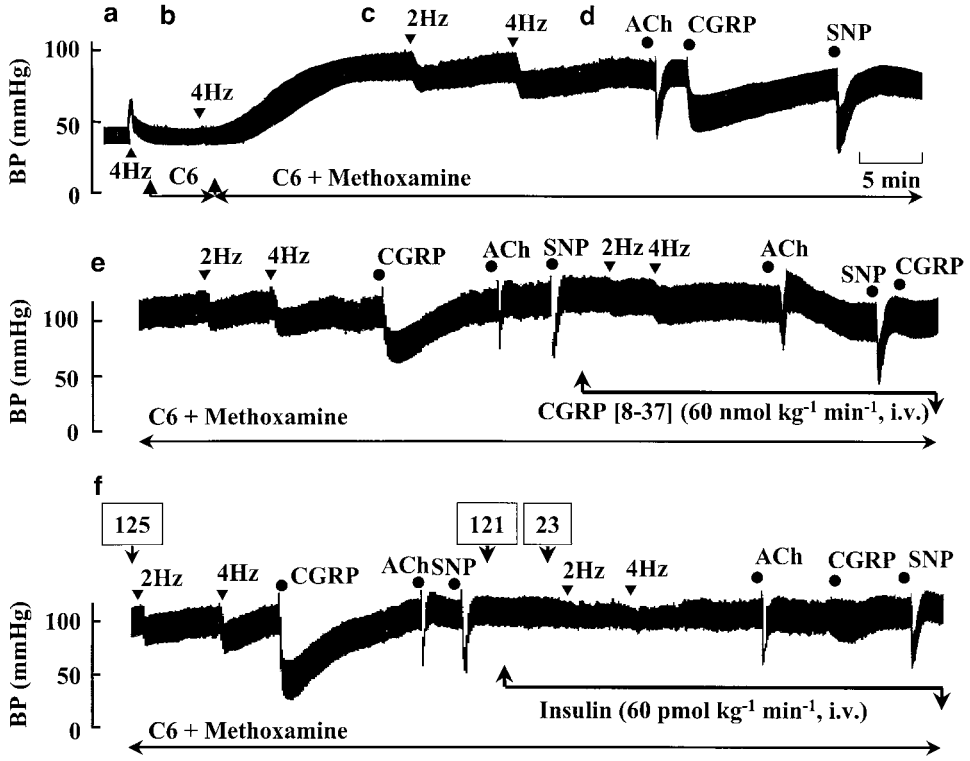

Figure 4 Typical records showing cardiovascular responses induced by SCS and to bolus injections (circles) of ACh (1 nmol kg^{-1}, i.v.), rat CGRP (0.1 nmol kg^{-1}, i.v.) and SNP (5 $\mu g\,kg^{-1}$, i.v.) in pithed rats with artificially increased BP. The BP was artificially increased by continuous infusion of methoxamine (20 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) in the presence of hexamethonium (C6, 2 mg $kg^{-1}\,min^{-1}$, i.v.). Tracing a: a pressor response to 4 Hz SCS. Tracing b: 4 Hz SCS in the presence of C6. Tracings c and d: depressor responses induced by SCS (2 and 4 Hz, inverted triangles) and injection (circles) of ACh, CGRP and SNP. Tracing e: depressor responses to SCS and bolus injection of ACh, SNP and CGRP during infusion of CGRP(8–37) (60 nmol $kg^{-1}\,min^{-1}$, i.v.). Trace f: effect of insulin (60 pmol $kg^{-1}\,min^{-1}$, i.v.) infusion on cardiovascular responses to SCS and bolus injection of ACh, CGRP and SNP in pithed rats under normal blood glucose level and without euglycemia maintenance. The numbers in the box represent blood glucose level (mg dl^{-1}).

response to injection of Ang II, which is mediated by Ang II receptors. Thus, it is more likely that the potentiating effect of low and medium doses of insulin on the pressor response mainly results from increased vasoreactivity of the blood vessels and may be, in part, mediated by other mechanisms. This is supported by the findings of Townsend *et al.* (1992), who reported that insulin augments the pressor responsiveness to exogenously administered NA without changing the amount of NA released. Furthermore, it has been reported that insulin potentiates the vasoconstrictor responses to NA *in vivo* (Gans *et al.*, 1991; Henrion & Laher, 1994), although insulin has been shown to attenuate the NA-induced vasoconstriction in rat arteries *in vitro* (Wambach & Liu, 1992). However, since insulin has been reported to increase sympathetic nerve activity, the potentiated pressor response to SCS induced by low and medium doses of insulin may result in part from the increased release of NA from the adrenergic nerves.

In contrast, high doses of insulin rapidly reduced the potentiating effect on pressor responses both to SCS and to exogenous NA and rather inhibited the responses to NA. It is possible that this rapid reduction resulted from a decrease in the blood glucose level caused by insulin, since decreased blood glucose resulted in a diminished responsiveness to SCS or exogenously applied NA and Ang II, as shown in Figure 1f. However, this possibility can be ruled out since constant blood glucose levels were maintained during the infusion of insulin. There is a large body of evidence indicating that insulin has a direct vasodilatory action. Moreover, it has been shown that insulin-induced vasodilation is not dependent on adrenaline release from the adrenal medulla (Randin *et al.*, 1994). Therefore, it is presumed that higher doses of insulin induce marked vasodilation and that this action may offset the augmenting effect of insulin on the pressor responses. However, infusions of high doses of insulin induced only a slight or almost no decrease in artificially increased mean BP of pithed rats under euglycemia maintenance. Therefore, it is unlikely that the vasodilatory properties of insulin are a major factor in the rapid reduction of the augmenting effect of insulin.

The previous and present studies demonstrated that SCS in pithed rats with an artificially increased mean BP caused a frequency-dependent depressor response (Taguchi *et al.*, 1992). Since the depressor response to SCS was blocked by CGRP(8–37), a CGRP receptor antagonist, the response is mediated by endogenous CGRP, which is released from CGRPergic vasodilator nerves. Therefore, it appears that the SCS-induced pressor response in pithed rats without an autonomic ganglion blocker, which resulted from both activation of sympathetic nerves and CGRPergic nerves, was attenuated by the simultaneous activation of CGRPergic nerves. Thus, it is

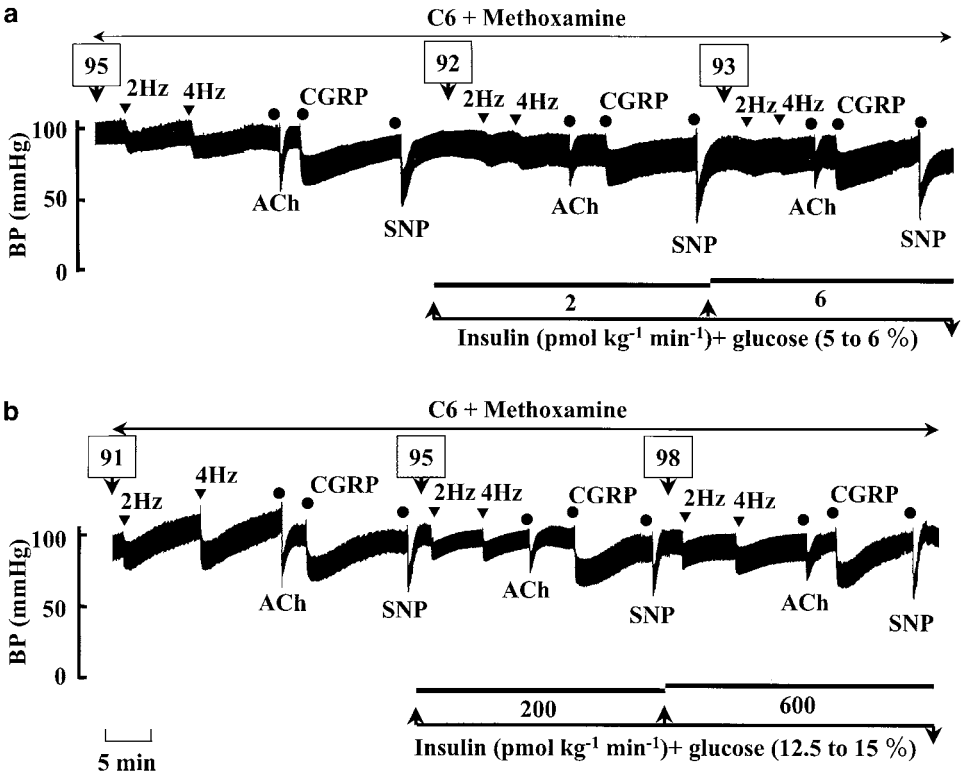

Figure 5 Typical records showing effects of insulin (tracing a, low doses at 2 and 6 pmol kg^{-1} min^{-1}, i.v.; tracing b, high doses at 200 and 600 pmol kg^{-1} min^{-1}, i.v.) on depressor responses to SCS (2 and 4 Hz; inverted triangles) and to bolus injections (circles) of ACh (1 nmol kg^{-1}, i.v.), rat CGRP (0.1 nmol kg^{-1}, i.v.) and SNP (5 μg kg^{-1}, i.v.) in pithed rats under euglycemia maintenance. BP, blood pressure; C6, hexamethonium. The BP was artificially increased by continuous infusion of methoxamine (20 μg kg^{-1} min^{-1}, i.v.) in the presence of C6 (2 mg kg^{-1} min^{-1}, i.v.). The numbers in the box represent blood glucose level (mg dl^{-1}).

assumed that the rapid reduction of the facilitatory effect observed with high doses of insulin may be due to an augmented depressor response to SCS induced by insulin. However, the present finding showed that the depressor response to SCS was inhibited by low doses of insulin and this inhibitory effect disappeared when high doses of insulin were applied, as observed in the augmenting effect of insulin. In addition, depressor responses induced by the bolus injection of CGRP were depressed by low doses of insulin and this effect also disappeared with high doses of insulin. It is, therefore, likely that the augmenting effect of low doses of insulin on the pressor response to SCS results in part from the inhibition of vasodilation mediated by CGRPergic nerves.

In a previous *in vitro* study, insulin was shown to act on CGRP receptors and cause vasodilation in mesenteric resistance blood vessels of rats (Mimaki *et al*., 1998). Therefore, it appears possible that the decreased vasodilatory response to CGRP in the presence of insulin may be due to the inhibition of CGRP receptor activity by blocking the CGRP receptor itself or by altering the signal transduction at the postreceptor level. However, whether or not insulin suppresses the CGRP-receptor-mediated vasodilation remains unknown.

Insulin at higher doses did not significantly affect the vasoconstrictor responses to SCS and NA or the vasodilatory responses to SCS and to bolus injection of CGRP. Furthermore, the augmenting effect of higher doses of insulin on the Ang II-induced pressor response was smaller than that of lower doses of insulin. The disappearance of the effect of insulin at higher doses was observed in not only the augmenting effect on vasoconstrictor responses to SCS and NA, but also in the inhibitory effect on vasodilatory responses to SCS and CGRP. If insulin increases the mechanisms of vasodilation to minimize the augmentation of the vasoconstrictor response observed at low doses, insulin at higher doses should facilitate vasodilation induced by SCS and CGRP. However, the present findings showed lesser or no effects of high doses of insulin. It is presumed that the higher doses of insulin used in the present experiments induced a higher plasma insulin level than the normal physiological level. Insulin has been shown to cause physiological action *via* the insulin receptor. Therefore, it is assumed that the disappearance of the effect of insulin at higher doses may result from the acute desensitization of insulin action, possibly *via* insulin receptors. There has been no report that high concentrations of insulin cause acute desensitization of vascular action. Therefore, further study is required to clarify the mechanisms underlying the lack of insulin action at higher concentrations.

On the other hand, low, medium and high doses of insulin infusion significantly inhibited the depressor response to bolus injection of ACh, which is well known to be mediated by endothelium-derived relaxing factors such as nitric oxide (NO) (Furchgott & Zawadzki, 1980). However, depressor responses to the administration of SNP, an NO donor, were not significantly affected by any doses of insulin. The present findings concurred with the findings of Kimura *et al*. (2002), who reported that insulin attenuated the ACh-induced

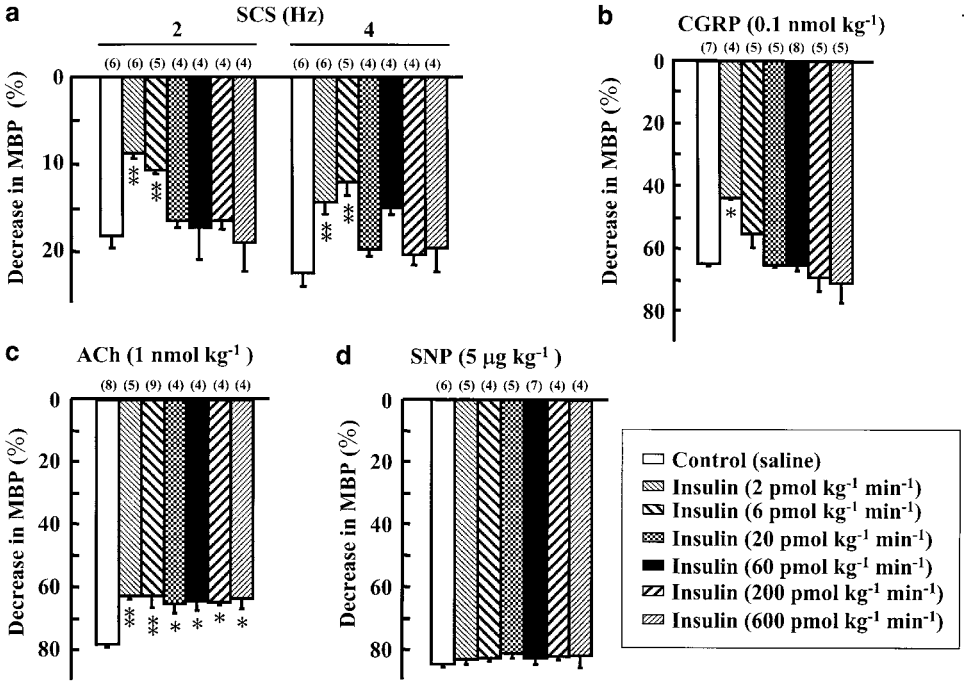

Figure 6 Bar graphs showing the effects of insulin (2–600 pmol kg^{-1} min^{-1}, i.v.) on depressor responses induced by SCS (2 and 4 Hz; panel (a)) and to bolus injections of rat CGRP (0.1 nmol kg^{-1}, i.v.; panel (b)), ACh (1 nmol kg^{-1}, i.v.; panel (c)) and SNP (5 $\mu g\,kg^{-1}$, i.v.; panel (d)) in pithed rats under euglycemia maintenance. The blood pressure was artificially increased by continuous infusion of methoxamine (20 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) in the presence of hexamethonium (2 mg kg^{-1} min^{-1}, i.v.). The responses are percentages compared to baseline values. Each bar indicates the mean ± s.e.m. Numbers in the parentheses above each bar indicate the number of animals. MBP, mean blood pressure. $^{*}P<0.05$, $^{**}P<0.01$, compared with saline control.

vasodilation in the rat mesenteric arteries and this attenuation was apparent only when NO production was blocked. In addition, under hyperinsulinemic conditions, the endothelium function and the vasodilation induced by ACh have been shown to be attenuated (Pieper *et al*., 1995; Katakam *et al*., 1999). Taken together, it is suggested that continuous infusion of insulin, in euglycemic pithed rats, mediates the decreased depressor response to ACh, a response that participates in the enhanced vasopressor responses to SCS, NA and Ang II injection. It appears that the insulin-induced decrease in vasodilatory function of the endothelium may contribute to the augmenting effect of insulin on vasopressor responses to SCS, Ang II and NA infusions.

In conclusion, the findings of the present study suggest that insulin at low and medium concentrations augments sympathetic vasoconstriction, which may be partly mediated by the inhibition of CGRPergic nerve function and endothelium function. It is also suggested that high concentrations of insulin may reduce its vascular effect due to the acute desensitization of insulin action, possibly *via* insulin receptors.

References

ANDERSON, E.A. & MARK, A.L. (1993). The vasodilator action of insulin. Implication for the insulin hypothesis of hypertension. *Hypertension,* **21,** 136–141.

BRAIN, S.D., WILLIAMS, T.J., TIPPINS, J.R., MORRIS, H.R. & MACINTYRE, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature,* **313,** 54–56.

CREAGER, M.A., LIANG, C.S. & COFFMAN, J.D. (1985). Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J. Pharmacol. Exp. Ther.,* **235,** 709–714.

DEFRONZO, R.A. (1981). The effect of insulin on renal sodium metabolism. A review with clinical implications. *Diabetologia,* **21,** 165–171.

FERRANNINI, E., BUZZIGOLI, G., BONADONNA, R., GIORICO, M.A., OLEGGINI, M., GRAZIADEI, L., PEDRINELLI, R., BRANDI, L. & BEVILACQUA, S. (1987). Insulin resistance in essential hypertension. *N. Engl. J. Med.,* **317,** 350–357.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373–376.

GANS, R.O., BILO, H.J., VON MAARSCHALKERWEERD, W.W., HEINE, R.J., NAUTA, J.J. & DONKER, A.J. (1991). Exogenous insulin augments in healthy volunteers the cardiovascular reactivity to noradrenaline but not to angiotensin II. *J. Clin. Invest.,* **88,** 512–518.

HENRION, D. & LAHER, I. (1994). Insulin potentiates norepinephrine-induced vascular tone by activation of protein kinase C and tyrosine kinase. *Can. J. Physiol. Pharmacol.,* **72,** 849–854.

HSUEH, W.A. & LAW, R.E. (1999). Insulin signaling in the arterial wall. *Am. J. Cardiol.,* **84,** 21J–24J.

KATAKAM, P.V., UJHELYI, M.R. & MILLER, A.W. (1999). EDHF-mediated relaxation is impaired in fructose-fed rats. *J. Cardiovasc. Pharmacol.,* **34,** 461–467.

KAWASAKI, H., TAKASAKI, K., SAITO, A. & GOTO, K. (1988). Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature,* **335,** 164–167.

KIMURA, M., JEFFERIS, A.M., WATANABE, H. & CHIN-DUSTING, J. (2002). Insulin inhibits acetylcholine responses in rat isolated mesenteric arteries *via* a non-nitric oxide nonprostanoid pathway. *Hypertension,* **39,** 35–40.

LEMBO, G., IACCARINO, G., RENDINA, V., VOLPE, M. & TRIMARCO, B. (1994). Insulin blunts sympathetic vasoconstriction through the alpha 2-adrenergic pathway in humans. *Hypertension,* **24,** 429–438.

LEMBO, G., NAPOLI, R., CAPALDO, B., RENDINA, V., IACCARINO, G., VOLPE, M., TRIMARCO, B. & SACCA, L. (1992). Abnormal sympathetic overactivity evoked by insulin in skeletal muscle of patients with essential hypertension. *J. Clin. Invest.,* **90,** 24–29.

MARIGLIANO, A., TEDDE, R., SECHI, L.A., PALA, A., PISANU, G. & PACIFICO, A. (1990). Insulinemia and blood pressure. Relationships in patients with primary and secondary hypertension, and with or without glucose metabolism impairment. *Am. J. Hypertens.,* **3,** 521–526.

MIMAKI, Y., KAWASAKI, H., OKAZAKI, M., NAKATSUMA, A., ARAKI, H. & GOMITA, Y. (1998). Involvement of calcitonin gene-related peptide (CGRP) receptors in insulin-induced vasodilatation in mesenteric resistance blood vessels of rats. *Br. J. Pharmacol.,* **123,** 1684–1690.

MORGAN, D.A., BALON, T.W., GINSBERG, B.H. & MARK, A.L. (1993). Nonuniform regional sympathetic nerve responses to hyperinsulinemia in rats. *Am. J. Physiol.,* **264,** R423–R427.

PIEPER, G.M., MEIER, D.A. & HAGER, S.R. (1995). Endothelium dysfunction in a model of hyperglycemia and hyperinsulinemia. *Am. J. Physiol.,* **269,** H845–H850.

POLLARE, T., LITHELL, H. & BERNE, C. (1990). Insulin resistance is a characteristic feature of primary hypertension independent of obesity. *Metabolism,* **39,** 167–174.

RANDIN, D., VOLLENWEIDER, P., TAPPY, L., JEQUIER, E., NICOD, P. & SCHERRER, U. (1994). Effect of adrenergic and cholinergic blockade on insulin-induced stimulation of calf blood flow in humans. *Am. J. Physiol.,* **266,** R809–R816.

REAVEN, G.M. (1988). Role of insulin resistance in human disease. *Diabetes,* **37,** 1595–1607.

SAUTER, A., GOLDSTEIN, M., ENGEL, J. & UETA, K. (1983). Effect of insulin on central catecholamines. *Brain Res.,* **260,** 330–333.

SCHERRER, U. & SARTORI, C. (1997). Insulin as a vascular and sympathoexcitatory hormone. Implication for blood pressure regulation, insulin sensitivity, and cardiovascular morbidity. *Circulation,* **96,** 4104–4113.

SHIMOSAWA, T., ANDO, K., ONO, A., TAKAHASHI, K., ISSHIKI, M., KANDA, M., OGATA, E. & FUJITA, T. (1992). Insulin inhibits norepinephrine overflow from peripheral sympathetic nerve ending. *Biochem. Biophys. Res. Commun.,* **188,** 330–335.

STEINBERG, H.O., BRECHTEL, G., JOHNSON, A., FINEBERG, N. & BARON, A.D. (1994). Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Invest.,* **94,** 1172–1179.

TAGUCHI, T., KAWASAKI, H., IMAMURA, T. & TAKASAKI, K. (1992). Endogenous calcitonin gene-related peptide mediates non-adrenergic noncholinergic depressor response to spinal cord stimulation in the pithed rat. *Circ. Res.,* **71,** 357–364.

TOWNSEND, R.R., YAMAMOTO, R., NICKOLS, M., DIPETTE, D.J. & NICKOLS, G.A. (1992). Insulin enhances pressor responses to norepinephrine in rat mesenteric vasculature. *Hypertension,* **19** (Suppl. II), II105–II110.

WAMBACH, G.K. & LIU, D. (1992). Insulin attenuates vasoconstriction by noradrenaline, serotonin and potassium chloride in rat mesenteric arterioles. *Clin. Exp. Hypertens. A,* **14,** 733–740.

WIMALAWANSA, S.J. (1996). Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. *Endocr. Rev.,* **17,** 533–585.

WIMALAWANSA, S.J. (1997). Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. *Crit. Rev. Neurobiol.,* **11,** 167–239.

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