Acute noradrenergic activation induces insulin resistance in human skeletal muscle

GIUSEPPE LEMBO, BRUNELLA CAPALDO, VIRGILIO RENDINA, GUIDO IACCARINO, RAFFAELE NAPOLI, RAFFAELE GUIDA, BRUNO TRIMARCO, AND LUIGI SACCÁ.
Department of Internal Medicine, School of Medicine, Federico II University, 80131 Naples, Italy

Lembo, Giuseppe, Brunella Capaldo, Virgilio Rendina, Guido Iaccarino, Raffaele Napoli, Raffaele Guida, Bruno Trimarco, and Luigi Saccá. Acute noradrenergic activation induces insulin resistance in human skeletal muscle. *Am. J. Physiol.* 266 (*Endocrinol. Metab.* 29): E242–E247, 1994.—We assessed in normal subjects the effects of an acute increase in forearm norepinephrine (NE) release, evoked by −20 mmHg lower body negative pressure (LBNP), on insulin-mediated muscle glucose uptake. Seven normal subjects underwent the following two insulin euglycemic clamps in random sequence: one during application of LBNP and the other without LBNP (control study). In the control study, hyperinsulinemia (≈60 μU/ml) produced a significant increment in forearm NE release, measured by using the forearm perfusion technique combined with infusion of tritiated NE (from 4.91 ± 1 to 7.94 ± 1.33 $ng \cdot l^{-1} \cdot min^{-1}$; $P < 0.05$). Forearm glucose uptake rose from 0.97 ± 0.13 to 5.2 ± 0.2 $mg \cdot l^{-1} \cdot min^{-1}$ in response to insulin infusion. When the insulin clamp was performed during LBNP, forearm NE release rose to significantly higher values than those of the control study (from 4.33 ± 0.52 to 12.7 ± 1.46 $ng \cdot l^{-1} \cdot min^{-1}$; $P < 0.01$ vs. control). Under these conditions, the stimulatory effect of insulin on forearm glucose uptake was markedly reduced (from 0.78 ± 0.10 to 3.2 ± 0.7 $mg \cdot l^{-1} \cdot min^{-1}$; $P < 0.02$ vs. control). Forearm blood flow and plasma epinephrine and free fatty acid concentrations were comparable in the two study sessions. These data demonstrate that an acute activation of endogenous NE release antagonizes insulin-mediated glucose uptake in forearm skeletal muscle, probably accounted for by a direct metabolic effect of NE.

norepinephrine; insulin sensitivity; forearm norepinephrine release; glucose uptake; forearm blood flow

THE SYMPATHOADRENAL SYSTEM is known to exert important effects on glucoregulation. Particularly extensive is the information regarding epinephrine. This catecholamine possesses a powerful insulin antagonistic action resulting from multiple mechanisms (10, 28, 32, 33), among which induction of muscle insulin resistance is particularly striking (4).

The question whether norepinephrine (NE) also affects insulin sensitivity has been more difficult to explore adequately. This depends on the fact that NE is essentially a neurotransmitter and, consequently, the circulating NE levels are a poor reflection of the synaptic cleft concentrations. On the other hand, when the metabolic effects of NE are tested by means of exogenous infusion, an enormous reverse gradient is needed to simulate a state of noradrenergic activation. This is probably the reason why previous acute studies based on exogenous NE infusions have provided variable results ranging from barely perceptible effects to clear-cut alterations of glucose homeostasis and insulin sensitivity (7, 15, 23, 29, 35, 37). A further limitation of studies employing NE infusions resides in the uncertainty that the metabolic sites reached by exogenous NE coincide exactly with those stimulated by the neurotransmitter when physiologically released by the axon terminals. In theory, one cannot exclude that the metabolic effects of exogenous NE are in part mediated by activation of regions normally not influenced by neural NE.

The idea that endogenous NE may potentially alter insulin sensitivity is suggested by the observation that, in various models of complete sympathectomy (epinephrine + NE deficiency), the glucose recovery from insulin hypoglycemia is impaired (25, 30, 34). Because the same does not occur with selective lack of epinephrine (adrenalectomy; see Ref. 14), the data suggest a metabolic role for neural NE. Furthermore, when NE is administered in very large amounts to produce plasma levels projected to exist in the synaptic clefts, dramatic changes in glucose metabolism are observed (7).

Although attractive, the hypothesis of a noradrenergic modulation of insulin sensitivity has not been tested directly. Thus the question whether NE of neural origin does have an effect on insulin sensitivity remains unsettled. An answer to this question could be particularly relevant to a better understanding of the mechanisms operating in many conditions, e.g., during stress, in which insulin resistance and sympathetic overactivity coexist.

Thus we planned the present study in normal humans to determine whether a reflex activation of NE release, elicited by lower body negative pressure (LBNP), leads to an impairment of insulin sensitivity. The insulin-NE antagonistic effect on glucose uptake was tested in the skeletal muscle by using the forearm perfusion technique. This was combined with a tracer method ([^{3}H]NE) to get a simultaneous estimate of the degree of muscle noradrenergic activation.

METHODS

Subjects. Fourteen forearm studies were performed in seven normal male subjects (mean age 30 ± 3 yr; body mass index 24.9 ± 0.5), with each subject serving as his own control. A medical history and physical examination were performed to exclude any illness, hypertension, or use of medication. Renal, liver, and endocrine functions were normal. No subject had recent changes in body weight or dietary habits. All subjects had a normal tolerance to a 75-g oral glucose load (according to the criteria of the National Diabetes Data Group). No subject was engaged in competitive sports or did intense physical activity during the days preceding the study. Written informed consent was obtained from all participants. The experimental protocol was approved by the Ethical Committee of the University of Naples School of Medicine.

Procedures. The studies began at 8:00 A.M. in a quiet room with a constant temperature of 22 to 24°C. All subjects were

studied in the postabsorptive state in the supine position after a 12- to 15-h overnight fast. The forearm perfusion technique (1) was performed as previously described (4). A plastic cannula was introduced in a retrograde manner into a large antecubital vein and threaded as deeply as possible. Under these conditions, the effluent venous blood drained predominantly muscle tissue. A second cannula was inserted into the ipsilateral brachial artery. This was used for infusion of indocyanine green dye (Cardio-Green; Hynson, Westcott, and Dunning, Baltimore, MD) to measure forearm blood flow, to sample blood entering the forearm, and to measure systemic arterial pressure by means of a Statham P23Db pressure transducer (Cleveland, OH). Systolic and diastolic blood pressures were simultaneously recorded on a multichannel polygraph (Gould, Oxnard, CA). An electrocardiographic lead was monitored during the study to measure heart rate. A contralateral arm vein was also cannulated for the infusion of test substances. During blood collection, a sphygmomanometer cuff placed around the wrist was inflated 100 mmHg above the systolic arterial pressure to exclude the hand from the circulation. Soon after blood collection, indocyanine green dye was infused through the arterial catheter while keeping the cuff inflated around the wrist. After 4–5 min, two consecutive venous blood samples were taken to measure the plasma concentration of the dye.

Forearm NE release was measured with a tracer technique based on primed constant infusion of tritiated NE. In particular, 40 min before starting the study, the subjects received intravenously a priming dose (27 μCi) of L-[2,5,6-^{3}H]NE (New England Nuclear, Boston, MA; sp act 43.7 Ci/mmol) diluted in 0.9% saline containing ascorbic acid (2 mg/ml), followed by a constant infusion (0.63 μCi/min), which was continued throughout the study period.

An airtight chamber similar to that described by Mark and Kerber (24) was placed over the lower portion of each subject's body, from the iliac crest down. Pressure within the chamber was monitored with a pressure transducer. It has been previously shown that LBNP of approximately −15 mmHg produces a decrease in central venous pressure and unloading of cardiopulmonary baroreceptors without alteration in arterial blood pressure and consequent activation of arterial baroreflexes (2).

Protocol. The following two studies were performed on each subject in random order at an interval of 7–10 days: *1*) euglycemic insulin clamp (control study) and *2*) euglycemic insulin clamp plus LBNP.

After complete instrumentation, a minimum of 30 min of quiet rest preceded the two measurements of basal hemodynamics and venous and arterial blood samplings made at 15-min intervals. In both study sessions, the subjects received an infusion of human regular insulin in a contralateral vein at a rate of 1 $mU \cdot kg^{-1} \cdot min^{-1}$ for 75 min to raise peripheral insulin concentration to levels comparable to those normally achieved postprandially. To maintain plasma glucose (PG) uptake at its basal value, in spite of systemic hyperinsulinemia, a variable amount of glucose was also infused. The glucose infusion rate was adjusted by measuring arterial PG levels at 5-min intervals by means of a glucose analyzer (Beckman Instruments, Fullerton, CA). The hemodynamic and metabolic variables were measured after 60 and 75 min of insulin infusion in both study sessions.

The only difference between the two studies was whether or not LBNP was applied at −20 mmHg during the whole period of insulin infusion.

Analytical methods. PG was determined on a Beckman glucose analyzer and converted to blood glucose (BG) by using the formula BG = PG(1 − 0.3 × hematocrit). Plasma insulin was measured by radioimmunoassay (9). Plasma free fatty acids (FFA) were determined with an enzymatic procedure (27). The plasma concentration of indocyanine green dye was measured spectrophotometrically. Plasma catecholamines were partially purified by batch alumina extraction, separated using ion-pairing reverse-phase high-pressure liquid chromatography (μBondapak C_{18} column, Powerline 600A chromatography system and WISP 700 as autoinjector; Waters, Milford, MA), and quantified by a current produced upon exposure of the column effluent to oxidizing and then reducing potentials connected in series (Coulochem II, ESA; Bedford, MA; see Ref. 39). Recovery through the alumina extraction step, calculated using dihydroxybenzylamine as an internal standard, ranged 60–70%, and each sample was corrected for its recovery. Detection limits were 3 and 5 pg injected for NE and epinephrine, respectively. Intra-assay and interassay variation coefficients were 4.1 and 9.8% for NE and 6.2 and 12% for epinephrine, respectively.

Calculations and data analysis. Forearm plasma flow was estimated by dividing the amount of indocyanine green dye infused by its concentration in the venous plasma and converted to blood flow according to the hematocrit. The intrasubject coefficient of variation was 7%, as based on two consecutive measurements taken at 1-min intervals. Thus it includes any source of variability (laboratory and clinical). The forearm balance was calculated by multiplying the arterial-deep vein concentration difference by the forearm blood (glucose) or plasma (NE) flow and was normalized by the forearm volume in liters measured by water displacement.

Forearm NE kinetics were calculated by using the two available approaches, based on the arterial [venous to arterial (V-A) mode] or venous specific activity [arterial to venous (A-V) mode], respectively. The relative equations to quantify forearm NE uptake (FNU) are as follows (31)

$$FNU = f(A^* - V^*)/Sa_a \quad \text{(V-A mode)}$$

and

$$FNU = f(A^* - V^*)/SA_v \quad \text{(A-V mode)}$$

where A^* and V^* are the arterial and venous [^{3}H]NE concentrations, respectively; SA_a and SA_v are the specific activities of NE in the arterial and venous plasma, respectively; and f is the forearm plasma flow. Once FNU was calculated, forearm NE release was easily obtained by subtracting FNU from the net NE balance.

The question of which approach is more appropriate to quantify NE kinetics has not been faced so far. As recently discussed (31), the right choice between the two approaches must be based on knowledge of the specific activity at which substrate uptake takes place. Although such data are very difficult to be obtained in view of the fact that NE is a distributed metabolite with simultaneously ongoing processes of release, reuptake, and degradation, in all likelihood the venous specific activity reflects the NE specific activity at the synaptic clefts more closely than the arterial one. However, we calculated NE kinetics with both formulas to facilitate comparison with previous data. It should be stressed also that, for the purpose of the current study, one needs to document that forearm NE release was activated by LBNP to a consistently greater extent compared with insulin alone.

For each parameter the two observations made in the basal state (−15 and 0 min) or during insulin infusion (60 and 75 min) were averaged. This was possible since, within each condition (basal or insulin period), there was no statistical difference in the mean values of the various parameters. Thus subsequent statistical analysis to test the effects of noradrener-

gic activation was performed by the paired t test since only two means were involved.

Results are presented as means ± SE.

RESULTS

Table 1 shows the changes in arterial glucose, insulin, and FFA concentrations. Plasma insulin levels were identical in the basal state and rose to a similar extent both in the control and LBNP study. Arterial BG concentration was maintained at near-basal values during insulin infusion in both studies. Plasma FFA levels decreased markedly during insulin infusion in the two experiments.

Table 2 shows the effects of euglycemic hyperinsulinemia alone or combined with application of LBNP on the hemodynamic parameters. Baseline systolic and diastolic blood pressure as well as heart rate were comparable in the two studies and remained unchanged during insulin infusion. Also the values of forearm blood flow were similar in the two studies in the basal state. Application of LBNP failed to modify forearm blood flow that remained stable during insulin infusion whether combined or not with LBNP.

The basic data regarding forearm NE kinetics are summarized in Table 3. Isolated insulin infusion induced a significant increase in arterial NE levels ($P < 0.05$). When insulin was infused during application of LBNP, the increment in systemic NE concentration was greater than that seen in the control study ($P < 0.02$). As shown in Fig. 1, forearm NE release (based on the venous specific activity; A-V mode) increased significantly during infusion of insulin alone from the basal value of 4.91 ± 1.0 to 7.94 ± 1.33 $ng \cdot l^{-1} \cdot min^{-1}$ ($P < 0.05$; Fig. 1). This response was not apparent on examination of the balance data of unlabeled NE because a simultaneous increase in FNU occurred (Table 3). Application of LBNP during insulin infusion evoked a much more pronounced noradrenergic activation, as documented by the marked increase in net forearm NE balance (Table 3) as well as by the twofold greater elevation in forearm NE release compared with the control study (from 4.33 ± 0.52 $ng \cdot l^{-1} \cdot min^{-1}$ in the basal state to 12.7 ± 1.46 $ng \cdot l^{-1} \cdot min^{-1}$ in the insulin + LBNP study, $P < 0.01$; Fig. 1). When forearm NE release was calculated according to the V-A mode (arterial specific activity), the values were ≈60% lower than the corresponding values based on the A-V mode (from 1.77 ± 0.37 to 2.95 ± 0.37 $ng \cdot l^{-1} \cdot min^{-1}$ in the control study and from 1.75 ± 0.23 to 5.43 ± 0.71 $ng \cdot l^{-1} \cdot min^{-1}$ in the insulin + LBNP study).

Table 1. *Changes in arterial insulin, glucose, and FFA during infusion of insulin alone or combined with LBNP*

	Arterial Plasma Insulin, μU/ml	Arterial Blood Glucose, mg/dl	Arterial Plasma FFA, mmol
Base	6 ± 1	84 ± 3	0.75 ± 0.12
Insulin	60 ± 10	89 ± 3	0.15 ± 0.01
Base	6 ± 2	83 ± 4	0.78 ± 0.14
Insulin + LBNP	66 ± 6	82 ± 4	0.24 ± 0.04

Values are means ± SE. FFA, free fatty acid; LBNP, lower body negative pressure.

Table 2. *Changes in arterial systolic and diastolic pressure, heart rate, and forearm blood flow during infusion of insulin alone or combined with LBNP*

	SAP, mmHg	DAP, mmHg	HR, beats/min	FBF, $ml^{-1} \cdot min^{-1}$
Base	132 ± 6*	70 ± 5	68 ± 5	19.4 ± 2.5
Insulin	136 ± 5	73 ± 4	72 ± 4	22.3 ± 3.1
Base	133 ± 6	70 ± 3	68 ± 4	20.0 ± 3.2
Insulin + LBNP	134 ± 5	70 ± 4	74 ± 4	22.1 ± 3.2

Values are means ± SE. SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, heart rate; FBF, forearm blood flow.

The effects of insulin on muscle glucose uptake (FGU) are depicted in Fig. 1. In the basal state, FGU was similar in the two studies. Insulin infusion produced a consistent increase in FGU in both studies. However, the values attained during LBNP were ≈40% lower compared with those of the control study (3.2 ± 0.7 vs. 5.2 ± 0.2 $mg \cdot l^{-1} \cdot min^{-1}$ in the LBNP and control study, respectively; $P < 0.02$).

Venous plasma epinephrine levels (35 ± 10 pg/ml, basal) were not affected by insulin infusion in the control experiment (39 ± 10 pg/ml) and showed a marginal increase when insulin was infused during LBNP (40 ± 8 in the basal state and 57 ± 8 pg/ml during LBNP).

DISCUSSION

Our results indicate that, in normal subjects, an acute increase in skeletal muscle noradrenergic activity, as measured by NE outflow, is able to antagonize insulin-stimulated muscle glucose disposal.

All previous attempts to determine the effects of NE on glucose metabolism and insulin sensitivity are based on exogenous NE infusions (7, 15, 23, 29, 35, 37); therefore, the data generated are of limited physiological significance. In one of these studies the effect of infused NE to reduce insulin sensitivity in human subjects is reported (23). It should be stressed that, besides the limitations inherent in using exogenous NE, the minimal model approach used in that study (23) precludes accurate assessment of the potential contribution of hepatic glucose production and insulin secretion to the changes in glucose disposal. Further, the study does not provide precise information on NE-insulin competition on muscle tissue or any other specific site.

Clinical investigation of the potential metabolic role of neural NE has been limited by technical difficulties. In the current study, we tried to explore directly the role of the NE released physiologically through its neural route by using LBNP in conjunction with the forearm perfusion technique. This model offers several additional advantages.

First, LBNP is a simple and effective technique extensively used to investigate the function of baroreflexes both in physiology and pathological states (2, 16, 24). This maneuver reduces central venous pressure, and, if the applied pressure does not exceed −20 mmHg, it

Table 3. *Changes in arterial NE, arterial [^{3}H]NE, NE uptake, and NE balance during infusion of insulin alone or combined with LBNP*

	Arterial Plasma NE, pg/ml	Arterial Plasma [^{3}H]NE, dpm/ml	Forearm NE Uptake, ng·l^{-1}·min^{-1}	Forearm NE Balance, ng·l^{-1}·min^{-1}
Base	234 ± 16	794 ± 49	5.05 ± 0.76	−0.088 ± 0.335
Insulin	293 ± 26*	860 ± 53	7.89 ± 1.05	−0.048 ± 0.330
Base	219 ± 37	774 ± 69	4.94 ± 0.77	−0.087 ± 0.317
Insulin + LBNP	376 ± 70*	994 ± 132	10.83 ± 1.50*†	−1.427 ± 0.301*†

Values are means + SE. NE, norepinephrine; dpm, disintegrations/min. *$P < 0.05$ compared with baseline (paired t test). †$P < 0.05$ compared with insulin (paired t test).

elicits a reflex sympathetic discharge preferentially to the skeletal muscle that involves specifically the noradrenergic component. Consistent with this, the values of plasma epinephrine in the current experiments showed only marginal increments. Second, the magnitude of the NE response to LBNP may be regarded as being well within the physiological range. This is supported by the fact that the noradrenergic reflex elicited by LBNP produced increments in circulating NE levels (≈150 pg/ml) that are comparable to or even lower than those observed under physiological circumstances such as moderate stress, physical exercise, and mild hypoglycemia (8). Finally, another distinct feature of the model resides in the fact that both the response of glucose uptake to insulin and the degree of noradrenergic activation were measured in the same tissue (muscle). This, on the other hand, is a main target of insulin action and the most likely site of the anti-insulin effect of sympathetic overactivity (4).

Some limitations of the present approach should also be made clear. First, LBNP cannot be easily applied for a long period of time. In our experience, this maneuver is well tolerated without causing stress and epinephrine release when applied for no longer than 75 min. Thus the insulin infusion period in our experiments was reduced accordingly. This precluded evaluation of systemic metabolic responses (whole body glucose disposal) that require a longer period of time to be correctly measured. Second, our approach cannot provide any information on whether NE antagonizes insulin with respect to other metabolic functions, e.g., hepatic glucose production, because LBNP evokes a noradrenergic response preferentially in the skeletal muscle (2).

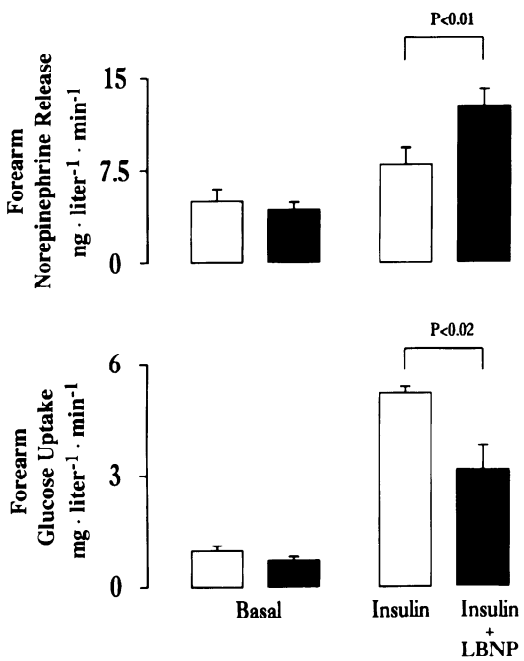

Fig. 1. Changes in forearm norepinephrine release and forearm glucose uptake induced by infusion of insulin alone (open bars) or combined with lower body negative pressure (LBNP) (solid bars).

The possible mechanisms whereby LBNP impairs insulin action on muscle glucose uptake are essentially as follows: *1*) a hemodynamic mechanism leading to a reduced muscle perfusion and *2*) a direct metabolic effect of NE at the cellular level.

The first possibility is suggested by recent data emphasizing the importance of the hemodynamic component as a contributing factor to the insulin resistance associated with obesity and type II diabetes (17, 18). Our results seem to rule out such a mechanism since forearm blood flow was very similar in the two study sessions. This finding deserves further considerations.

First, in the control study, forearm blood flow showed only a marginal and not significant increase. This finding is in line with the majority of previous studies showing no vasodilating effect of physiological insulin increments in the forearm vasculature (3, 13, 20, 26, 41). This does not imply that insulin is devoid of significant hemodynamic effects. What is likely to occur under physiological conditions is that the insulin effect to reduce vascular resistance is offset by the concomitant sympathetic activity. Furthermore, the reflex sympathetic activation elicited by LBNP usually results in an increase in forearm vascular resistance (2). Thus the lack of forearm vasoconstriction during LBNP in our study prima facie could be surprising. However, it has long been reported that insulin reduces NE-induced vascular reactivity in vitro (40). Further, we have recently demonstrated in healthy humans that an increase in plasma insulin concentration similar to that achieved in the present study markedly reduces the reflex forearm vasoconstriction induced by −20 mmHg LBNP (20). The current observation of stable levels of arterial blood pressure despite the inevitable reduction in cardiac output normally accompanying LBNP and the lack of forearm vasoconstriction strongly suggest a diversified effect of insulin on other vascular beds. In this regard, it has been reported that, in the dog, insulin increases renal and splanchnic vascular resistances while inducing vasodilatation in the skeletal muscle (21).

The second possibility is that the insulin resistance induced by reflex sympathetic overactivity was mediated by a direct metabolic effect of NE. Actually, the other known factors that are able to reduce insulin sensitivity in a very acute fashion, and that may be operative in the context of a sympathetic activation, are FFA and epinephrine. With regard to the former, previous studies using exogenous NE infusion have emphasized a potential role of FFA in mediating NE-induced insulin resistance (23). In our study, FFA levels were only slightly less suppressed when insulin was infused during LBNP, and the possibility that such small underbasal differences in FFA concentration may affect glucose disposal is very unlikely (3). Thus an essential role for FFA in the mediation of the anti-insulin effect of neural NE cannot be invoked.

With regard to a potential role for epinephrine, the plasma levels of this hormone showed only a marginal increment during LBNP and remained far below the threshold for changes in glucose metabolism (6). Thus a metabolic effect of NE probably mediated by the β-adrenoreceptors is the likely explanation for the insulin resistance induced by LBNP. In this regard, extensive research has documented the detrimental effect of acute β-adrenoceptor stimulation on glucose uptake and insulin sensitivity both in vitro and in vivo in human subjects (5, 10, 28).

A definite proof that NE was indeed the only mediator of LBNP through a β-adrenergic effect required reversal of the insulin resistance by β-adrenergic blockade. Actually, the hypothesis of a β-adrenergic mediation was not able to be tested due to intrinsic limitations of the current model. When we tried to use adrenergic blockers, marked changes in forearm blood perfusion occurred, making the interpretation of the data impossible. Furthermore, since infusion of insulin alone already induces muscle sympathetic activation, what should be abolished to verify the hypothesis of a β-receptor mediation is only the effect of the sympathetic extra activation evoked by LBNP, which would require a fine graduation of the β-blockade that is very difficult to achieve.

If the hypothesis of a β-receptor mediation holds true, one can also try to explain the discrepancy between acute and chronic effects of sympathetic stimulation on insulin sensitivity. Chronic NE infusion or chronic β-adrenoceptor stimulation has been reported to cause an increase rather than a decrease in insulin sensitivity (22, 36). Because it is well established that chronic stimulation of β-adrenoceptors leads to their downregulation (11), one can attribute the enhanced insulin sensitivity after chronic sympathetic stimulation to the removal of the negative influence of β-adrenoceptors.

This formulation would presuppose that normally the insulin action on glucose uptake is buffered by the concomitant noradrenergic activation. Transposed to the forearm model, the response of muscle glucose uptake to hyperinsulinemia would represent the balance between the stimulatory effect of insulin and the inhibitory indirect effect of the hormone mediated by the reflex sympathetic activation (19). If this interpretation is correct, one may speculate that an alteration of this balance represents a potential pathophysiological mechanism of insulin resistance. For example, we have recently demonstrated that, in patients with essential hypertension, a reduced insulin sensitivity during euglycemic hyperinsulinemia is associated with a threefold greater increase in muscle sympathetic activity than that observed in normal subjects (19). This is not to infer a causal role of the increased sympathetic nervous system activity in the genesis of insulin resistance in hypertension, as most of the recent data would rather argue in favor of the opposite sequence of events (12, 38). However, the current data make very real the possibility that a primary defect in insulin sensitivity in hypertension may be further aggravated by the greater sympathetic response evoked by episodic stimuli, such as postprandial hyperinsulinemia.

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