

# Cholinergic Involvement in Vascular and Glucoregulatory Actions of Insulin in Rats

Martin Lévesque,<sup>1</sup> Marta Santur ,<sup>1</sup> Maryse Pitre,<sup>1</sup> Andr  Nadeau,<sup>2</sup> and H l ne Bachelard<sup>1</sup>

**This study was designed to test the glucose metabolic and vasodilator actions of insulin in rats and its relation to cholinergic system-dependent mechanisms. The first group of rats had pulsed Doppler flow probes and intravascular catheters implanted to determine blood pressure, heart rate, and regional blood flows. Insulin sensitivity was assessed by the euglycemic-hyperinsulinemic clamp technique carried out in the absence or presence of atropine. The second group of rats was used to determine the cholinergic contribution to in vivo insulin-mediated glucose utilization in individual muscles. Glucose uptake was examined by using [<sup>3</sup>H]2-deoxy-D-glucose. Muscarinic cholinergic blockade was found to significantly ( $P = 0.002$ ) reduce insulin sensitivity and to completely abrogate the renal ( $P = 0.008$ ) and hindquarter ( $P = 0.02$ ) vasodilator responses to euglycemic infusion of insulin. A significant reduction in insulin-stimulated in vivo glucose uptake was also noted in soleus ( $P = 0.006$ ), quadriceps ( $P = 0.03$ ), gastrocnemius ( $P = 0.02$ ), and extensor digitorum longus (EDL) ( $P = 0.001$ ) muscles, when insulin was infused at a rate of  $4 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , whereas at the rate of  $16 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , a significant reduction in glucose uptake was only observed in EDL ( $P = 0.03$ ) and quadriceps ( $P = 0.01$ ) muscles. Together, these results demonstrate a potential role for cholinergic involvement with physiological insulin actions in glucose clearance and blood flow regulation in rats. *Diabetes* 55:398–404, 2006**

Insulin is well recognized for its ability to stimulate glucose uptake in skeletal muscles, heart, and adipose tissues. On a quantitative basis, skeletal muscle has been identified as the predominant site of insulin-stimulated glucose disposal and as a major tissue responsible for postprandial hyperglycemia in insulin-resistant states (1,2). In addition to its effects on glucose metabolism, insulin was shown to increase skeletal muscle blood flow and to decrease vascular resistance in humans (3). The hemodynamic responses to insulin have attracted a great deal of attention over the last few years, particularly in relation to its glucose lowering action, and

studies in both experimental animal models and humans have confirmed the vasodilating action of insulin over a range of physiological and pharmacological insulin concentrations and by using different techniques (4–7). The vascular effect of insulin has been proposed as an important physiological mechanism that amplifies the overall action of insulin to promote the disposal of glucose and other substrates (8). Particularly relevant to this is the demonstration that when the action of insulin on total blood flow (9) or capillary recruitment (10) is prevented in vivo, it concomitantly induces an acute state of insulin resistance. Among the most likely mechanism responsible for the insulin-hemodynamic effects, there is evidence indicating a role for endothelium-derived nitric oxide (NO). Abrogation of NO release by the NO synthase inhibitor *N*<sup>G</sup>-monomethyl-L-arginine was shown to prevent the vasodilating action of insulin in skeletal muscle (11,12), whereas a concomitant reduction in insulin sensitivity was also reported (8).

The mechanisms underlying insulin stimulation of NO release remain to be fully elucidated. Among the possible mechanisms, insulin could conceivably stimulate NO release by a direct, local effect at the vascular endothelium, as previously suggested (13), or indirectly through the activation of a cholinergically mediated stimulation of NO release, as proposed in liver (14). Evidence for cholinergic neurogenic vasodilation in skeletal muscle has been provided in many animal species (15–18), and interruption of nerve supply to skeletal muscle was shown to result in the development of insulin resistance in the affected muscle (19). Furthermore, applying atropine during the infusion of insulin in anesthetized cats and rats was reported to reduce the effect of insulin on glucose uptake in skeletal muscle (14,20). Therefore, in light of these previous findings, we hypothesized that part of the mechanism underlying the glucose metabolic and vasodilator actions of insulin involves a cholinergic component. Thus, in continuity with our previous work indicating a blood flow regulatory action of insulin in rats (5,21) and its paradoxical effect in insulin-resistant rats (5,22), we undertook the present study to examine the involvement of cholinergic system-dependent mechanisms in vascular and glucoregulatory actions of insulin in rats. In this study, rats were chronically instrumented with intravascular catheters and pulsed Doppler flow probes to assess the effect of an acute treatment with atropine on vascular responses to euglycemic infusion of insulin and to determine insulin sensitivity in conscious, unrestrained rats. Furthermore, in the present study, the influence of cholinergic mechanisms on insulin action on glucose uptake in muscles was examined in vivo in conscious and undisturbed rats.

From the <sup>1</sup>Lipid Research Unit, Centre Hospitalier de l'Universit  Laval (CHUL) Research Center, Centre Hospitalier Universitaire de Qu bec (CHUQ), Ste-Foy, Quebec, Canada; and the <sup>2</sup>Diabetes Research Unit, CHUL Research Center, CHUQ, Ste-Foy, Quebec, Canada.

Address correspondence and reprint requests to Dr. H l ne Bachelard, Lipid Research Unit, CHUL Research Center, CHUQ, 2705 Blvd. Laurier Ste-Foy, Quebec, Canada G1V 4G2. E-mail: helene.bachelard@crchul.ulaval.ca.

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EDL, extensor digitorum longus.

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## RESEARCH DESIGN AND METHODS

All experimental procedures were approved by the animal care and handling committee of Laval University and conformed to the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Male Sprague-Dawley rats (Charles River, St. Constant, Canada) weighing 250–300 g were housed in stainless steel cages. They were randomly divided into two groups. One group ( $n = 78$ ) was used for euglycemic-hyperinsulinemic clamp studies and hemodynamic measurements in conscious rats. In this group of rats, there were two surgical procedures undertaken in all animals. The first surgical procedure was to place the flow probes around specific arteries, and 7 days later, the second surgical procedure was to place the vascular catheters. Then, a euglycemic-hyperinsulinemic clamp study was conducted 72 h after the second surgical step. The second group of rats ( $n = 37$ ) was used to determine in vivo glucose utilization in individual muscles. In this group of rats, only one surgical procedure was undertaken to place the vascular catheters.

**Surgical preparation.** The rats were anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg i.p., respectively) and had pulsed Doppler flow probes implanted around the left renal and superior mesenteric arteries and the lower abdominal aorta to monitor renal, mesenteric, and hindquarter blood flows, according to Gardiner and Bennett (23) and as previously described (5). After surgery, the rats were given subcutaneous injections of buprenorphine (0.05 mg/kg), returned to their cages, and allowed to recover for at least 7 days. After the recovery period, the rats were reanesthetized (as above), and the leads of the implanted probes were soldered to a microconnector (Microtech). Three separate catheters were implanted in the right jugular vein (for intravenous infusions) and one catheter in left femoral artery (for blood pressure and heart rate measurements). The catheters were tunneled subcutaneously to emerge at the same point as the probe wires. The rats were given subcutaneous injections of buprenorphine (0.05 mg/kg) and returned to their cages. Recovery after surgery was deemed satisfactory by the resumption of growth and normalization of 24-h food intake the day after this second surgical procedure. Experiments began 72 h after this last surgical step.

**Euglycemic-hyperinsulinemic clamp studies.** The rats were deprived of food for 10–12 h before the glucose clamp study. Before each experiment, blood glucose and plasma insulin were determined, and the resting heart rate, blood pressure, and regional blood flows were recorded over 15 min in quiet, unrestrained rats. After that time, the first group of rats ( $n = 50$ ) received a continuous intravenous infusion of saline (0.9% NaCl), and the second group ( $n = 28$ ) received atropine (1 mg/kg, administered intravenously as a bolus, followed by a continuous intravenous infusion of  $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). The rate of infusion was 0.3 ml/h. The two groups of rats were further divided in three subgroups each. The euglycemic-hyperinsulinemic clamp was performed in two subgroups receiving saline only ( $n = 34$ ) and in two other subgroups receiving atropine ( $n = 18$ ). The other subgroups of saline-treated ( $n = 16$ ) or atropine-treated ( $n = 10$ ) rats were infused with saline/BSA 0.2%, instead of insulin and dextrose, to approximately match the saline load delivered during the clamp studies. Sixty minutes after the beginning of intravenous infusion of atropine or saline, a second blood sample (0.3 ml) was collected for blood glucose and plasma insulin determinations. The euglycemic-hyperinsulinemic clamp was then carried out over 120 min in the designated groups as previously described (5). Insulin was infused at a rate of 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in the four subgroups of rats receiving atropine or saline (only one dose of insulin was tested per subgroup). Heart rate, blood pressure, and regional blood flows were continuously measured.

**Effect of atropine on in vivo [ $^3\text{H}$ ]2-deoxy-D-glucose uptake in individual muscles.** The second group of rats ( $n = 37$ ) was used for measurement of steady-state insulin-stimulated glucose uptake in individual muscles, during a euglycemic infusion of insulin (at 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) carried out in the absence or presence of atropine, as described above. This was performed by measuring the incorporation of [ $^3\text{H}$ ]2-deoxy-D-glucose according to Kreamer et al. (24) and as previously described (21). Briefly, a bolus injection of 250  $\mu\text{Ci/kg}$  [ $^3\text{H}$ ]2-deoxy-D-glucose and 25  $\mu\text{Ci/kg}$  [ $^{14}\text{C}$ ]sucrose in a 0.5-ml saline solution were injected during the last 25 min of a euglycemic clamp performed at the doses of 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  insulin and in the absence or presence of atropine. Blood samples were taken at regular intervals (2.5, 5, 7.5, 10, 15, and 20 min) after the bolus injection for determination of plasma glucose, [ $^3\text{H}$ ]2-deoxy-D-glucose, and [ $^{14}\text{C}$ ]sucrose. Twenty-five minutes after the bolus, the animals were rapidly killed by decapitation, and hindlimb muscles (soleus, extensor digitorum longus [EDL], gastrocnemius, and quadriceps) were rapidly excised, frozen into liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Subsequently, tissue samples (30–50 mg) were dissolved in 1 ml 0.5 mol/l ammonium hydroxide at  $55^\circ\text{C}$  for 16 h. Thereafter, hydrogen peroxide (30%) was added for 60 min at  $55^\circ\text{C}$  to decrease quenching, followed by the addition of 10 ml scintillation fluid (Amersham, Mississauga, Ontario, Canada). The

TABLE 1

Effects of intravenous infusion of saline or atropine on plasma insulin and blood glucose concentrations and baseline values of heart rate, mean arterial blood pressure, and regional Doppler shift and vascular conductance in conscious rats

	Saline (0.9%)	Atropine (1 mg · $\text{kg}^{-1} \cdot \text{h}^{-1}$ )
<i>n</i>	50	28
Insulin (pmol/l)	71 ± 8	71 ± 5
Plasma glucose (mmol/l)	4.1 ± 0.1	4.2 ± 0.2
Heart rate (bpm)	335 ± 5	389 ± 6*
Mean blood pressure (mmHg)	89 ± 2	95 ± 2*
Renal Doppler shift (kHz)	7.5 ± 0.5	8.4 ± 1.0
Mesenteric Doppler shift (kHz)	12.9 ± 0.7	10.1 ± 1.0*
Hindquarter Doppler shift (kHz)	6.7 ± 0.4	7.6 ± 0.6
Renal vascular conductance ([kHz mmHg $^{-1}$ ] $10^3$ )	86 ± 6	92 ± 11
Mesenteric vascular conductance ([kHz mmHg $^{-1}$ ] $10^3$ )	148 ± 8	110 ± 11*
Hindquarter vascular conductance ([kHz mmHg $^{-1}$ ] $10^3$ )	76 ± 5	83 ± 8

Data are means ± SE. The groups represent those used to assess hemodynamic effects of insulin intravenously infused during the euglycemic-hyperinsulinemic clamp studies. \* $P < 0.05$  vs. vehicle group (Student's *t* test for unpaired data).

samples and plasma (20  $\mu\text{l}$ ) were then counted in a liquid scintillation counter (Wallach 1409). The accumulation of [ $^3\text{H}$ ]2-deoxy-D-glucose in muscle, corrected for the extracellular space with [ $^{14}\text{C}$ ]sucrose, was used as an index of glucose uptake rates (25).

**Drugs.** The drugs used were atropine (Sigma Aldrich, Oakville, Ontario, Canada), dextrose (Sigma Aldrich), BSA (Sigma Aldrich), insulin (Humulin R; Eli Lilly, Toronto, Ontario, Canada), [ $^3\text{H}$ ]2-deoxy-D-glucose (Perkin Elmer, Woodbridge, Ontario, Canada), [ $^{14}\text{C}$ ]sucrose (Mandel-NEN Lifesciences, Guelph, Ontario, Canada), buprenorphine (Schering-Plough, Welwyn Garden City, Hertfordshire, U.K.), ketamine (Rogar/STB, London, Ontario, Canada), and xylazine (Bayer, Toronto, Ontario, Canada).

**Analytic methods.** Blood for plasma glucose and insulin determinations in the basal state and during insulin infusion was drawn, put in untreated polypropylene tubes, and centrifuged with an Eppendorf microcentrifuge (Minimax; International Equipment). Plasma was stored at  $-20^\circ\text{C}$  until assay. The glucose concentration of the supernatant was measured by the glucose oxidase method (26) using a glucose analyzer (Technicon RA-XT), and plasma insulin level was measured by radioimmunoassay using porcine insulin standards and polyethylene glycol for separation (27).

**Data analysis.** Data are means ± SE; *n* is the number of observations. Data were analyzed for statistical significance by an ANOVA for repeated measurements. Post hoc comparisons were made using Fisher's test. A *P* value  $< 0.05$  was taken to indicate a significant difference.

## RESULTS

Table 1 illustrates the effects of continuous intravenous infusion of atropine on plasma levels of insulin and glucose and baseline values of heart rate, mean arterial blood pressure, regional blood flows, and vascular conductances in conscious, unrestrained rats. Thus, the continuous intravenous infusion of atropine had no effect on plasma glucose or insulin concentrations. However, the infusion of atropine was found to slightly but significantly increase basal heart rate and mean arterial blood pressure values and to decrease basal superior mesenteric blood flow and vascular conductance values, whereas no changes in resting values of renal or hindquarter blood flows or vascular conductances were noted.

**Hemodynamic responses to insulin during the euglycemic-hyperinsulinemic clamp.** Figure 1A and B shows that insulin infusion at a rate of 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$

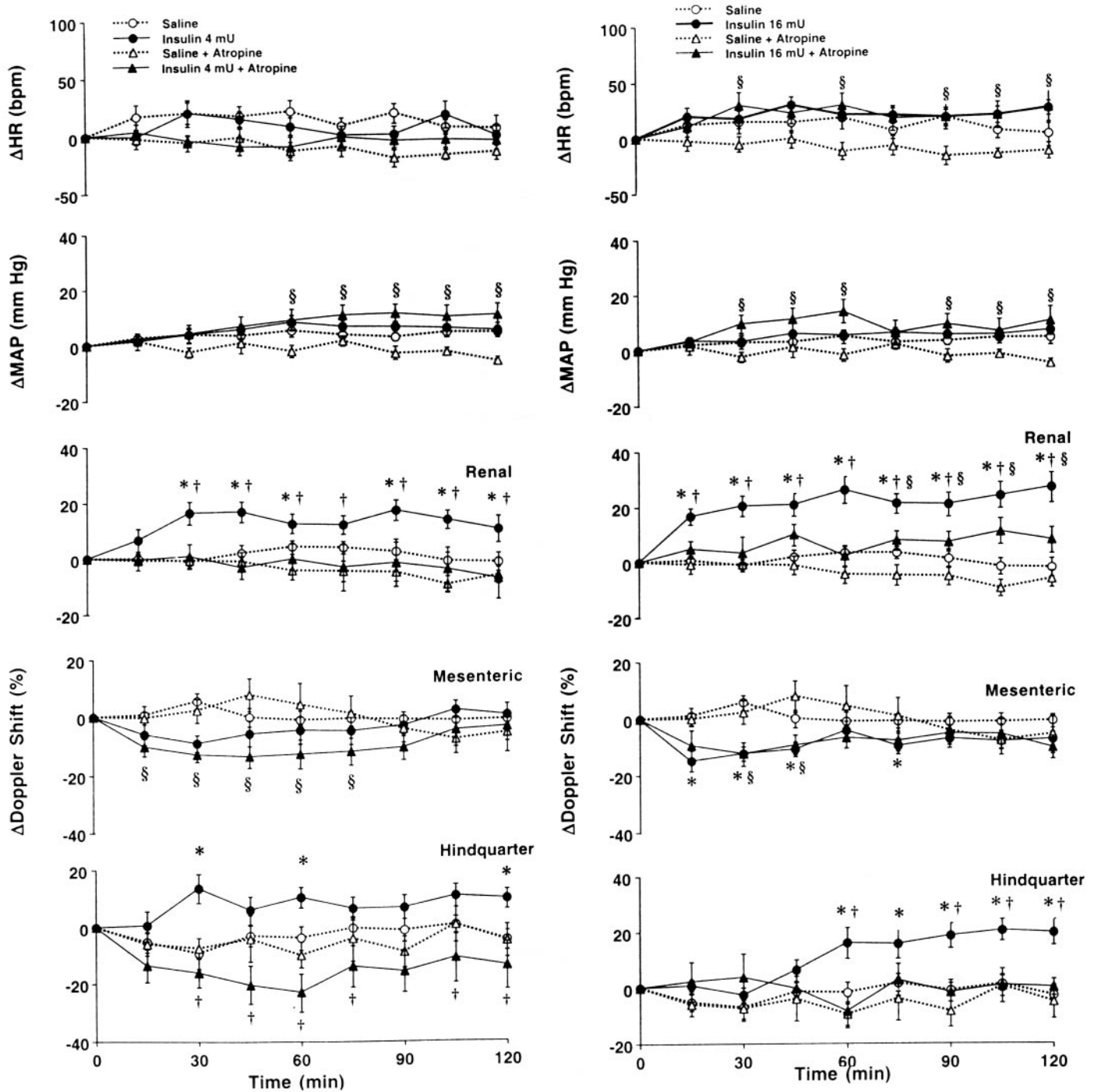


FIG. 1. Cardiovascular changes elicited by control intravenous infusion of saline/0.2% BSA ( $n = 16$ ) or euglycemic infusion of insulin at a rate of 4 ( $A$ ;  $n = 17$ ) or 16 ( $B$ ;  $n = 17$ )  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in conscious untreated rats. These changes were compared with those elicited by control intravenous infusion of saline/BSA ( $n = 10$ ) or euglycemic infusion of 4 ( $A$ ;  $n = 9$ ) or 16 ( $B$ ;  $n = 9$ )  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  insulin in rats acutely treated with atropine (1 mg/kg i.v. bolus, 1  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). Effects of saline/BSA or insulin were assessed relative to baseline values. Data are means  $\pm$  SE shown by vertical lines. \* $P < 0.05$  for the untreated insulin-infused group vs. its respective control saline/BSA group (ANOVA followed by Fisher's test). § $P < 0.05$  for the insulin-infused group treated with atropine vs. its respective atropine-treated control group (ANOVA followed by Fisher's test). † $P < 0.05$  for the untreated insulin-infused group vs. the insulin-infused group treated with atropine (ANOVA followed by Fisher's test). HR, heart rate; MAP, mean arterial pressure.

caused significant increases in renal and hindquarter blood flows but had no effect on heart rate or mean arterial pressure compared with measurements after a control infusion of vehicle (saline/0.2% BSA). Furthermore, a significant decrease in superior mesenteric flow was observed at the highest dose of insulin tested (Fig. 1B). These cardiovascular responses were associated with increases

in renal (Fig. 2A and B) and hindquarter vascular conductances (Fig. 2B) and decreases in superior mesenteric vascular conductance (Fig. 2B) when compared with the effects of control infusion of vehicle.

In rats receiving atropine, the same infusions of insulin were found to cause slight but significant increases in heart rate (Fig. 1B) and mean blood pressure (Fig. 1A and

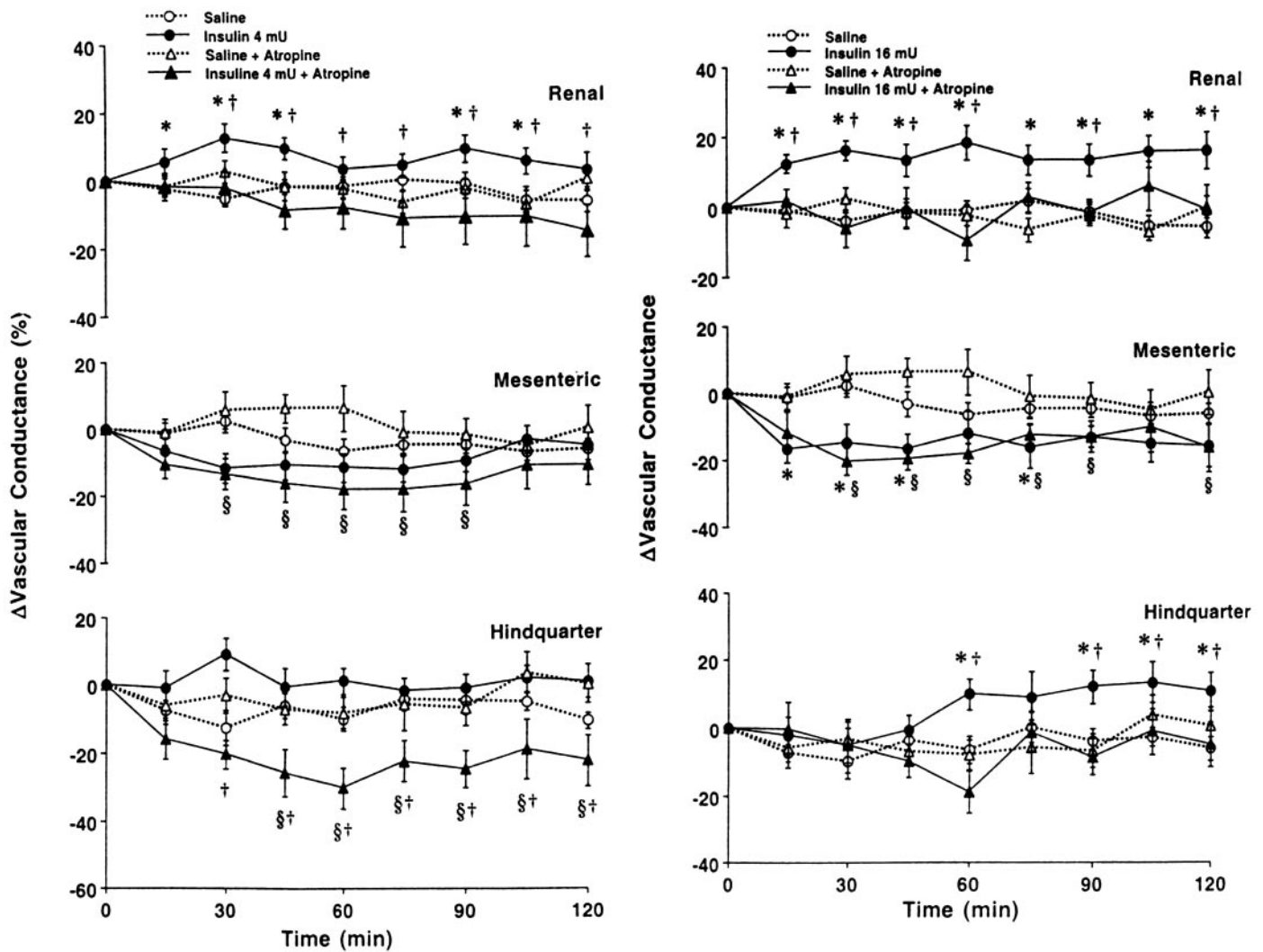


FIG. 2. Changes in regional vascular conductances elicited by control intravenous infusion of saline/BSA ( $n = 16$ ) or euglycemic infusion of insulin at a rate of 4 ( $A$ ;  $n = 17$ ) or 16 ( $B$ ;  $n = 17$ )  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in conscious untreated rats. These changes were compared with those elicited by control intravenous infusion of saline/BSA ( $n = 10$ ) or euglycemic infusion of 4 ( $A$ ;  $n = 9$ ) or 16 ( $B$ ;  $n = 9$ )  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  insulin in rats acutely treated with atropine (1 mg/kg i.v. bolus, 1 mg  $\cdot$  kg $^{-1} \cdot$  h $^{-1}$ ). Data were derived from data shown in Fig. 1. Effects of saline/0.2% BSA or insulin were assessed relative to the baseline values. Data are means  $\pm$  SE shown by vertical lines. \* $P < 0.05$  for the untreated insulin-infused group vs. its respective control saline/BSA group (ANOVA followed by Fisher's test). § $P < 0.05$  for the insulin-infused group treated with atropine vs. its respective atropine-treated control group (ANOVA followed by Fisher's test). † $P < 0.05$  for the untreated insulin-infused group vs. the insulin-infused group treated with atropine (ANOVA followed by Fisher's test).

$B$ ), when compared with the effects of control infusion of vehicle in atropine-treated rats. These responses did not differ from those observed in untreated rats, although in those animals, the heart rate and blood pressure responses to insulin did not reach the level of significance. However, the increases in renal and hindquarter flows and vascular conductances previously noted in untreated rats were completely abolished by the treatment with atropine (Figs. 1 and 2). A marked and long-lasting hindquarter vasoconstriction was observed at the dose of 4  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  insulin (Fig. 2A). Moreover, there were significant decreases in superior mesenteric blood flows and vascular conductances (Figs. 1 and 2), which did not differ from those observed in rats receiving insulin alone.

**Responses during euglycemic-hyperinsulinemic clamp.** During the euglycemic-hyperinsulinemic clamp, which was performed at an insulin infusion rate of 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  and in the presence or absence of atropine, we found that for each dose of insulin tested, fasting plasma insulin levels rose acutely and achieved similar

plateaus, according to the dose of insulin, whereas normal plasma glucose levels were maintained in every group of rats (Table 2). However, the average glucose infusion rate required to maintain euglycemia during the last hour of the clamp, the conditions of which closely approximated a steady-state insulin concentration and which represented the whole-body glucose utilization, was significantly smaller in the atropine-treated group than in the group receiving only insulin at the dose of 4  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  (Table 2). This difference was not observed when insulin was infused at a rate of 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in both experimental groups.

**Effect of atropine on in vivo [ $^3\text{H}$ ]2-deoxy-D-glucose uptake in individual muscles.** Figure 3 shows that in untreated rats, the euglycemic infusion of insulin at a rate of 4 or 16  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  elicited significant increases in glucose uptake in soleus, quadriceps, gastrocnemius, and EDL muscles when compared with that measured after a control infusion of vehicle. However, atropine treatment was found to markedly and significantly reduce

TABLE 2  
Euglycemic infusion of insulin in conscious untreated and atropine-treated rats

	n	Dose of insulin (mU · kg <sup>-1</sup> · min <sup>-1</sup> )	Plasma glucose		Plasma insulin		GIR <sub>60-120</sub> (mg · kg <sup>-1</sup> · min <sup>-1</sup> )
			Basal (mmol/l)	60-120 min (mmol/l)	Basal (pmol/l)	60-120 min (pmol/l)	
Untreated rats	17	4	4.2 ± 0.1	4.0 ± 0.1	67 ± 14	694 ± 70	31.1 ± 1.1
	17	16	4.2 ± 0.1	4.2 ± 0.1	62 ± 9	2,643 ± 84	40.6 ± 0.9
Atropine-treated rats	9	4	4.3 ± 0.2	4.0 ± 0.2	71 ± 7	706 ± 58	24.6 ± 1.9*
	9	16	4.1 ± 0.3	4.1 ± 0.2	71 ± 8	2,767 ± 169	40.7 ± 1.3

Data are means ± SE. The euglycemic-hyperinsulinemic clamp has been performed by infusing regular porcine insulin. GIR<sub>60-120</sub>, glucose infusion rate required to maintain euglycemia during steady-state (60-120 min) plasma insulin concentration. \*P < 0.05, sucrose-fed group vs. their respective chow-fed control group.

(~50%) in vivo insulin-stimulated glucose uptake in every studied muscle, when insulin was infused at a rate of 4 mU · kg<sup>-1</sup> · min<sup>-1</sup>. However, at the rate of 16 mU · kg<sup>-1</sup> · min<sup>-1</sup>, a slight but significant reduction in glucose uptake was only observed in EDL and quadriceps muscles. In the absence of insulin infusion, no effect of atropine was noted on basal glucose uptake in any muscles, when compared with that measured after a control infusion of vehicle only.

DISCUSSION

**Atropine and insulin sensitivity.** The present study indicates that at the whole-animal level acute treatment with atropine significantly reduces insulin sensitivity, as determined using a euglycemic-hyperinsulinemic clamp performed in conscious rats at a low dose of insulin. Thus, at the dose of 4 mU · kg<sup>-1</sup> · min<sup>-1</sup> insulin, we found that the glucose infusion rate required to maintain euglycemia in the face of physiological hyperinsulinemia was significantly lower (~25%) in the group of rats receiving atropine

than in the control group. However, atropine had no effect on basal plasma glucose or insulin concentrations, suggesting that muscarinic cholinergic antagonism has no net systemic glucoregulatory effect in terms of the plasma glucose concentration. Moreover, during the euglycemic infusion of insulin, atropine had no effect on steady-state plasma insulin concentrations and variability of plasma glucose concentrations. However, the reduction in insulin sensitivity was no longer observed at supra-physiological levels of insulin. Thus, it is likely that the apparent insulin resistance noted at a low dose of insulin was overcome at higher insulin levels (16 mU · kg<sup>-1</sup> · min<sup>-1</sup>) (5). These latter findings contrast with those of Xie and Lutt (28), who used a higher dose of insulin than us and showed a reduction in insulin sensitivity in fed anesthetized rats intravenously treated with atropine. Part of the discrepancy could be explained by differences in the prandial status of the animals, because fed rats would be anticipated to be more responsive to insulin action and to have a higher degree of basal parasympathetic tone than overnight-fasted rats (29).

Our results are compatible with the involvement of cholinergic input on the effect of insulin on whole-body clearance of glucose. This contention is supported by several pieces of evidence implicating the parasympathetic nerves on glucoregulation and indicating that parasympathetic neuropathy could be contributory to insulin resistance. Insulin resistance is commonly observed in clinical conditions that are associated with autonomic dysfunction, such as hypertension (30), diabetes (31), and obesity (32). Furthermore, electrical stimulation of the lateral hypothalamic nucleus (parasympathetic area) was shown to reduce blood glucose level in rabbits (33), and vagal nerve stimulation (34) and selective stimulation of hepatic efferent parasympathetic nerves (35,36) were found to decrease hepatic glucose output and increase hepatic glucose uptake and glycogen synthesis. In recently published studies, and using a modified euglycemic technique to quantify insulin sensitivity, Lutt (14) described a novel neurohormonal mechanism by which postprandial elevation in insulin levels activates a hepatic parasympathetic reflex release of a putative hepatic insulin-sensitizing substance, which sensitizes skeletal muscle to the effects of insulin. The reflex is mediated by acetylcholine, which binds to muscarinic receptors and stimulates the production of NO within the liver (14). Surgical denervation of the liver, cholinergic blockade by using atropine, or prevention of hepatic NO liberation by the use of NO synthase antagonists were all found to cause severe insulin resistance that was attributed to the blockade of hepatic insulin-sensitizing substance release (14). Al-

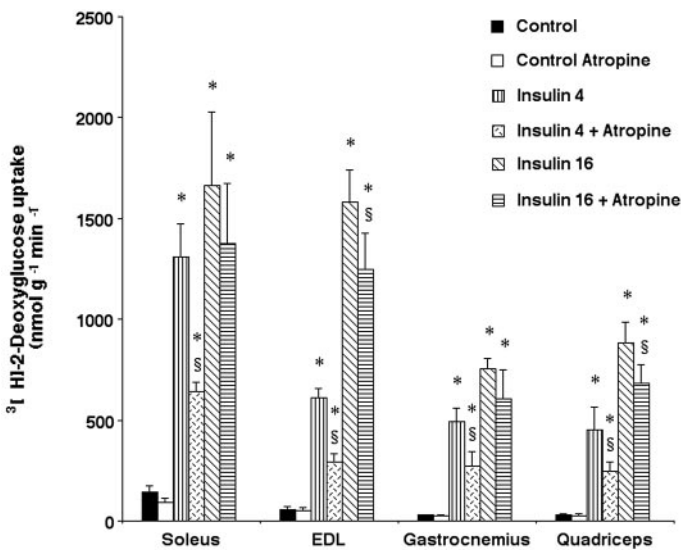


FIG. 3. Bar graphs showing in vivo [<sup>3</sup>H]2-deoxy-D-glucose uptake in individual skeletal muscles during a control intravenous infusion of saline/0.2% BSA in the absence (n = 15) or presence (1 mg/kg i.v. bolus, 1 mg · kg<sup>-1</sup> · h<sup>-1</sup>; n = 6) of atropine or during a euglycemic infusion of insulin at a rate of 4 (Insulin, n = 4; Insulin + Atropine, n = 4) or 16 (Insulin, n = 4; Insulin + Atropine, n = 4) mU · kg<sup>-1</sup> · min<sup>-1</sup> in conscious rats. Bars represent means ± SE shown by vertical lines. Comparisons were made between insulin-evoked responses in the presence or absence of atropine vs. those evoked by their respective control infusion of vehicle (ANOVA followed by Fisher's test). \*P < 0.05 for insulin-evoked responses in the presence or absence of atropine vs. those evoked in the absence of atropine (ANOVA followed by Fisher's test). §P < 0.05 for insulin-evoked responses in the presence of atropine vs. those evoked in the absence of atropine (ANOVA followed by Fisher's test).

though the present findings are unable to support or deny the hepatic autonomic reflex control of peripheral insulin action, as proposed by Lutt (14), our results agreed, however, with the presence of a cholinergic mediation of the action of insulin on glucose metabolism. Furthermore, in the experiments in which we have combined atropine treatment with *in vivo* administration of [<sup>3</sup>H]2-deoxy-D-glucose during insulin clamps, which permits maintenance of insulin at a steady-state level during investigation of its effect on glucose uptake in muscles, we found a marked reduction (~50%) in insulin-mediated glucose uptake by a physiological dose ( $4 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) in every tested muscle. At the dose of  $16 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  insulin, a significant reduction in glucose uptake in the presence of atropine was only noted in EDL and quadriceps muscles. These results highly suggest the existence of a cholinergic mediation of the actions of insulin on glucose uptake and blood flow regulation (see below) at the skeletal muscle level. Furthermore, the demonstration here that atropine treatment alone (i.e., in the absence of insulin) did not affect basal glucose uptake in any tested muscles excluded the possibility that glucose uptake may have already been substantially inhibited, before insulin addition (i.e., as a result from a redistribution of blood flow in the leg muscles to the detriment of glucose uptake).

**Atropine and insulin-mediated hemodynamic responses.** In untreated rats, we found that the euglycemic infusion of insulin elicited vasodilations in renal and hindquarter vascular beds and a transient vasoconstriction in the superior mesenteric vascular bed but no changes in mean arterial blood pressure or heart rate. These hemodynamic responses are consistent with those we previously reported in normal Wistar, Wistar-Kyoto, and Sprague-Dawley rats (5,21). In contrast, in the group of rats receiving atropine, we found that the physiological effects of insulin to vasodilate skeletal muscle and renal vasculatures were completely abolished, whereas the superior mesenteric vasoconstrictor response to insulin was not affected by the treatment. These observations highly suggest that cholinergic vasodilation could play an important role in the mediation of the vascular response to insulin, particularly at the skeletal muscle level. Our results are consistent with those of Mahajan et al. (37), showing that local methacholine enhances insulin-mediated muscle glucose uptake *in vivo* by augmenting capillary recruitment in anesthetized rats, but our results contrast with those of Randin et al. (38), demonstrating an absence of effect of atropine on the vascular response to euglycemic hyperinsulinemia in lean healthy humans. The reason for the conflicting results is not readily apparent. Differences in the experimental design, such as the dose of insulin, amount of atropine infused, and the site and method used to measure blood flow, may have contributed to these seemingly disparate results. Furthermore, we must consider that the extrapolation of the data in rats to humans may be difficult, because evidence for cholinergic vasodilator nerves in human skeletal muscle (in contrast to many animal species) has not been established (although the presence of local non-neuronal cholinergic mechanisms is not excluded) (39). It appears, therefore, that differences between rats and humans, in the effects of atropine on insulin stimulation of whole-body glucose uptake and muscle blood flow could be related to anatomical rather than to methodological differences.

Previous studies have clearly demonstrated that the insulin-mediated skeletal muscle vasodilation is mediated

by NO, because this effect is abolished by  $N^G$ -monomethyl-L-arginine (11,12), and by inhibition of tetrahydrobiopterin synthesis, a cofactor essential for NO synthesis (40). It was suggested that insulin stimulates NO release by a direct, local effect at the vascular endothelium (13). However, the present findings raise the possibility that a cholinergically mediated stimulation of NO-dependent vasodilatory mechanisms could contribute, at least in part, to the endothelium-dependent vasodilator response to insulin. Alternatively, our results could also indicate that part of the vasodilator response to insulin is related to inhibition of adrenergic neurotransmission by stimulation of inhibitory presynaptic cholinergic receptors located on vascular adrenergic nerve endings (41). Thus, in the presence of atropine, the vasodilator response to euglycemic infusion of insulin might have been limited by stimulation of sympathetic vasoconstrictor outflow. This contention is supported by previous studies carried out in humans and animals and demonstrating that insulin administration stimulates sympathetic nerve activity as determined by measurements of plasma catecholamine levels or by direct microneurographic recordings of sympathetic nerve action potentials targeted specifically at the skeletal musculature (42–45). Thus, in the presence of atropine, it is likely that the endothelium-dependent vasodilator response to insulin was overcome by unopposed sympathetic vasoconstrictor influences.

Impairment in the vascular responses to insulin is thought to contribute to deficient uptake of glucose by peripheral tissues (presumably because of less delivery of glucose) (3). Therefore, according to that concept, it is likely that cholinergic blockade during the infusion of insulin (at a rate of  $4 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) has contributed to reduce insulin sensitivity by altering the hemodynamic responses to insulin, thus restricting insulin and glucose distribution through insulin-sensitive tissues and then reducing insulin action on glucose extraction at the level of skeletal muscle. Furthermore, the present findings raise the possibility that interruption of the cholinergic contribution to the blood flow response to insulin may have contributed, at least in part, to the state of insulin resistance previously reported by Buse and Buse (19) in denervated skeletal muscles, although this is not supported by a recent study using a different approach and technique to measure insulin sensitivity and blood flow in anesthetized rats (46). At the highest dose of insulin tested ( $16 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), we found that the reduction in insulin sensitivity previously noted at the low dose of insulin was no more observed, despite a persistent defect in the vascular responses to insulin in the atropine-treated rats and a slight but significant reduction in glucose uptake in some but not all muscles *in vivo* (EDL and quadriceps only). The failure of atropine to achieve the same outcome at a higher dose of insulin might suggest that at a supra-physiological concentration of insulin (~2,700 pmol/l), the “atropine-sensitive” insulin blood flow effect is not rate-limiting on glucose uptake, as previously described for physiological insulin and that steps in insulin action beyond any effect on blood flow are important determinants of muscle glucose uptake.

In conclusion, the overall concept presented in this study is novel and original. Taken together, the results of our experiments carried out *in vivo* strongly suggest that in rats, a high percentage of insulin-mediated muscle glucose uptake by a physiological dose involves a cholin-

ergic mechanism. This atropine-sensitive effect is largely attributable to the vascular action of insulin in rats.

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