Regulation of Skeletal Muscle Blood Flow During Acute Insulin-Induced Hypoglycemia in the Rat

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The mechanism behind hyperemia in skeletal muscle during insulin-induced hypoglycemia was investigated in 42 anesthetized male Wistar rats using the microdialysis ethanol technique of monitoring nutritive blood flow. Microdialysis probes were inserted bilaterally into the gastrocnemius muscle and perfused with a modified Krebs-Henseleit buffer containing 20 mmol/l ethanol and one or more of the following compounds: propranolol (10^{-6} mol/l), phentolamine (10^{-6} mol/l), and calyculin A (1.0 μmol/l). Muscle blood flow increased, as indicated by a decrease in the ethanol outflow:inflow ratio ($P < 0.001$, $n = 6$), during hypoglycemia induced by a bolus intravenous infusion of insulin (680 mU/kg body wt). This increase was not present during normoglycemia or during hypoglycemia and local β-adrenergic blockade via propranolol. However, the hyperemic response was potentiated during hypoglycemia and local α-adrenergic blockade via phentolamine. A normal hyperemic response to hypoglycemia was detected during simultaneous α- and β-adrenergic blockade. This response was eliminated on further supplementation of the microdialysis perfusion medium with calyculin A. Therefore, although stimulation of the α- and β-adrenergic receptors does occur during insulin-induced hypoglycemia, it is not essential for the induction of hyperemia in this state. It may be concluded that hyperinsulinemia results in vasodilatation during hypoglycemia, although hyperinsulinemia does not have an effect on skeletal muscle blood flow under normoglycemic conditions. *Diabetes* 43:1340–1344, 1994

The increase in skeletal muscle blood flow during insulin-induced hypoglycemia is well documented (1–4). However, the regulation of the hyperemic response, as well as the relative roles of insulin and hypoglycemia per se in this response have not been fully elucidated. The hyperemia has been reported to be due to β-adrenergic stimulation (1,5), while α-receptor stimulation by norepinephrine, which is responsible for the maintenance of vasomotor tone during resting conditions (6), has been implicated in limiting this hyperemic response (5,7). However, some authors have suggested that the release of this α-adrenergic stimulation may allow for the increase in blood flow with hypoglycemia (1,8). Therefore, there may be many factors regulating skeletal muscle blood flow during insulin-induced hypoglycemia. The fact that the relative role of these factors has not been determined is partially because of the methods available for the study of local blood flow. The microdialysis ethanol technique (9,10) is a relatively new method that allows for the monitoring of tissue nutritive blood flow. The microdialysis probe can also be used as a vehicle for the local administration of pharmacological agents. This method has been used in the present study to investigate the local regulation of skeletal muscle blood flow during a 2-h period following an intravenous bolus infusion of insulin (680 mU/kg body wt). Propranolol and/or phentolamine were used to locally block the α- and β-adrenergic receptors during hypoglycemia. Glucose was also infused in one group in an attempt to maintain euglycemia following insulin infusion. Finally, calyculin A, a potent inhibitor of phosphoprotein phosphatases 1 and 2A, was perfused through the probe along with propranolol and phentolamine in an attempt to reveal possible effects due to insulin.

RESEARCH DESIGN AND METHODS

Male Wistar rats weighing 290–310 g (ALAB Laboratorietjänst AB, Sollentuna, Sweden) had free access to standard rat food (R3, ALAB) and water. The rats were housed in a room maintained at 22°C with a 12-h light/12-h dark cycle (light 7 A.M.–7 P.M.). Before the experiments, the rats were fasted overnight. This study was approved by the local ethical committee at the Karolinska Institute.

Microdialysis probes. The design of the microdialysis probes has been previously described (11). Briefly, the probe (CMA Microdialysis AB, Stockholm, Sweden) consists of a tubular membrane (polycarbonate, outer diameter 0.5 mm; length 10 mm; permeable to molecules with a molecular mass <5,000–20,000) glued to an outer steel shaft (length 30 mm; diameter 0.64 mm). Liquid enters an inner steel cannula, which extends inside the membrane and outer steel shaft, at the top of the probe. The liquid exits the inner steel cannula at the bottom of the probe through two small openings to flow upwards in the space between the inner steel cannula and the dialysis membrane (the site of dialysis). The liquid leaves the probe by way of a lateral cannula connected to the upper part of the outer stainless steel shaft. Before insertion into the tissue, the probes were connected to a CMA/100 microinfusion pump (CMA Microdialysis AB) and perfused with at least 150 μl buffer at 12 μl/min to remove the protective glycerol coating and air bubbles from the probe membrane.

Microdialysis probe insertion. The rats were anesthetized initially with sodium pentobarbital (1.2 ml/kg of 60 mg/ml pentobarbital intraperitoneally). The anesthesia was maintained by supplementary intraperitoneal injections of 0.12 ml/kg sodium pentobarbital (60 mg/ml) every 30 min. The rectal temperature was kept at 37.4 ± 0.2°C by adjusting the distance between a heating lamp and the rat. The left jugular vein was exposed, and a catheter was inserted and secured by silk thread. The contralateral jugular vein was also catheterized in experiments involving glucose infusion. Following the opening of the skin over both calves, the muscle fascia just above the Achilles tendon was punctured with a needle (20 gauge), and microdialysis probes were gently inserted superficially into the medial head of both gastrocnemius muscles. Probe position was visually verified during dissection at the end of each experiment.

Protocol. Probes, perfused at 0.5 μl/min with a modified Krebs-Henseleit bicarbonate buffer (KHB) containing 20 mmol/l ethanol at

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KHB, Krebs-Henseleit bicarbonate buffer; ANOVA, analysis of variance.

room temperature (10), were inserted bilaterally in the gastrocnemius muscle. In experiments with adrenergic receptor blockade, the perfusion medium of one of the probes was supplemented with propranolol (10^{-6} mol/l) and/or phentolamine (10^{-6} mol/l). In one group of rats, the perfusion medium of one of the probes contained propranolol and phentolamine, as well as calyculin A (1 μmol/l). The contralateral (control) probe (ctrl) was perfused with unsupplemented KHB that contained only 20 mmol/l ethanol. An initial 60-min equilibration period without sampling was allowed. Thereafter, 15-min samples were collected during 60 min, followed by intravenous infusion of insulin (Actrapid, Novo A/S, Gentofte, Denmark: 680 mU/kg body wt in a total volume of 300 μl 0.9% NaCl infused over 3 min). Dialysate samples were collected every 15 min for up to 2 h following insulin infusion. Sample collection times were adjusted to account for the presence of a dead space volume of 7.6 μl from the probe membrane to the collection site of the dialysate samples. In addition, 25-μl blood samples were collected in untreated tubes at these intervals from the jugular vein during experiments involving glucose infusion. Proteins were precipitated by addition of 50 μl of 3 N perchloric acid, and, following centrifugation, the resulting supernatant was stored at −20°C for subsequent analysis of glucose (12). Blood samples (100 μl) were also collected in untreated polyethylene tubes from the jugular vein every 30 min and allowed to clot. Following centrifugation, the supernatant was removed and stored at −70°C for subsequent analysis of serum insulin (12,13). Samples (50 μl) of the insulin infusates were also collected in untreated polyethylene tubes, stored at −70°C, and subsequently analyzed for insulin concentration (12,13).

Rats were divided into seven groups receiving the following treatments: C, intravenous saline infusion (control group); I, intravenous insulin infusion; PR, intravenous insulin infusion and local β-receptor blockade via propranolol (Inderal, ICI Pharmaceuticals, Macclesfield, U.K.); PH, intravenous insulin infusion and local α-receptor blockade via phentolamine (Regitine, Ciba-Geigy, Basle, Switzerland); PP, intravenous insulin infusion and local propranolol as well as phentolamine administration; G, intravenous insulin infusion with maintenance of relative euglycemia by continuous intravenous infusion of glucose (50%) following insulin administration (as a control for fluid volume, this intravenous glucose infusion protocol was replicated with 0.9% saline in all other groups); and CA, intravenous insulin infusion and local administration of propranolol, phentolamine, and calyculin A (Boehringer Mannheim, Biochemica, Mannheim, Germany). The contralateral (control) probe in groups PR, PH, PP, and CA were perfused with unsupplemented KHB and are denoted groups PRctrl, PHctrl, PPctrl, and CActrl.

Blood flow. Nutritive skeletal muscle blood flow was monitored using the microdialysis ethanol technique as described previously (9,10). For this, 20 mmol/l ethanol was included in the KHB perfusion medium. The concentration of ethanol in the perfusion medium, as well as that in the collected dialysate, was analyzed and expressed as the ethanol outflow:inflow ratio ($[\text{ethanol}]_{\text{collected dialysate}}/[\text{ethanol}]_{\text{infused perfusion medium}}$). The amount of ethanol remaining in the collected dialysate decreases with increasing blood flow. The ethanol outflow:inflow ratio will therefore be inversely related to blood flow in the area of the probe membrane (10). Samples were collected in capped 300-μl polyethylene vials, stored at 4°C, and assayed within 24 h for ethanol (10) and glucose (14).

Statistical analysis. Results are presented as means ± SE. A two-way analysis of variance (ANOVA) with repeated measures over time was performed to detect significant changes from basal and differences in the magnitude of change in dialysate ethanol concentration after insulin infusion between the various groups. The maximal increase in blood flow did not always occur at the same time point; therefore, the two points in time indicating the maximal treatment effect in each experiment were used for statistical analyses. A one-way ANOVA was used to analyze infusate-insulin data, and a one-way ANOVA with repeated measures over time was used to analyze serum insulin data. A significant difference between means was located by the Newman-Keul test when an overall significance was indicated by the F value. The level of significance was set at $P < 0.05$. The paired Student's t test was used to determine differences in the mean basal ethanol outflow:inflow ratios, as well as differences in the maximal effect between treatment and control probes within a given group. When multiple Student's t tests were performed, a Bonferroni correction was used to adjust the level of significance. Statistical analyses were performed with the Crunch statistical package (Crunch Software, Oakland, CA). The absence of a significant difference is denoted by NS.

RESULTS

Ethanol outflow:inflow ratio. Figure 1*A* displays results from experiments in which rats received a bolus infusion of either saline or insulin (680 mU/kg body wt). No significant change from basal was noted in the ethanol outflow:inflow ratio (a marker of skeletal muscle blood flow) in the control group (group C; $n = 6$, NS), while a decrease in outflow:inflow ratio was registered in the group receiving insulin (group I; $n = 6$, $P < 0.001$), indicating an increase in skeletal muscle blood flow during hypoglycemia. The supplementation of the perfusion medium with propranolol eliminated this decrease in ethanol outflow:inflow ratio (group PR, Fig. 1*B*). Control probes (group PRctrl) placed in these same animals displayed a decrease in outflow:inflow ratio ($n = 6$, $P < 0.001$). The decrease in ethanol outflow:inflow ratio found in group I was enhanced with the supplementation of the perfusion medium with phentolamine (group PH, Fig. 1*C*), the decrease in the phentolamine probes ($n = 6$, $P < 0.05$) being greater ($P < 0.01$) than the decrease in control probes (group PHctrl; $n = 6$, $P < 0.05$). Supplementation of the probe perfusion medium with both propranolol and phentolamine (group PP) resulted in a decrease in the ethanol outflow:inflow ratio ($n = 6$, $P < 0.001$) of similar magnitude to the decrease (group PPctrl; $n = 6$, $P < 0.001$) seen in unsupplemented control probes in the same group of rats (group PP vs. group PPctrl; NS, Fig. 1*D*). When relative euglycemia was maintained (group G), no significant change in the ethanol outflow:inflow ratio was detected ($n = 6$, Fig. 1*E*). Finally, when the perfusion medium was supplemented with propranolol, phentolamine, and calyculin A (group CA), no significant change in the ethanol outflow:inflow ratio was detected, but a decrease in outflow:inflow ratio was seen in the control probes in this group of rats (group CActrl; $n = 6$, $P < 0.001$). The observed difference between the supplemented and control probes was highly significant ($P < 0.001$).

Basal values for the ethanol outflow:inflow ratio were group C: 0.107 ± 0.007, group I: 0.140 ± 0.015, group PR: 0.126 ± 0.055, group PRctrl: 0.117 ± 0.016, group PH: 0.086 ± 0.018, group PHctrl: 0.108 ± 0.019, group PP: 0.104 ± 0.009, group PPctrl: 0.111 ± 0.0121, group G: 0.095 ± 0.010, group CA: 0.104 ± 0.006, and group CActrl: 0.083 ± 0.006 ($n = 6$, group PH and group PHctrl are significantly different from one another, $P < 0.01$).

Dialysate and blood glucose. Blood glucose concentration was not determined in this study during the hypoglycemia experiments. However, we have previously determined the relationship between changes in dialysate and blood glucose concentrations during this protocol of insulin-induced hypoglycemia (15). In that study, it was found that, although changes in dialysate glucose concentration in large part followed changes in blood glucose concentration, changes in dialysate glucose concentration were dependent on changes in blood flow observed in skeletal muscle during hypoglycemia. Most of the dialysate glucose results are, therefore, not shown. However, a similar decrease in blood glucose was expected in all groups. An example of the decrease in dialysate glucose in experiments in which no change in blood flow occurred is shown in Fig. 2. As can be seen, dialysate glucose decreased to ~30% of basal in both probe groups CA and PR in which no change in blood flow occurred.

Blood and dialysate glucose were determined in trials in

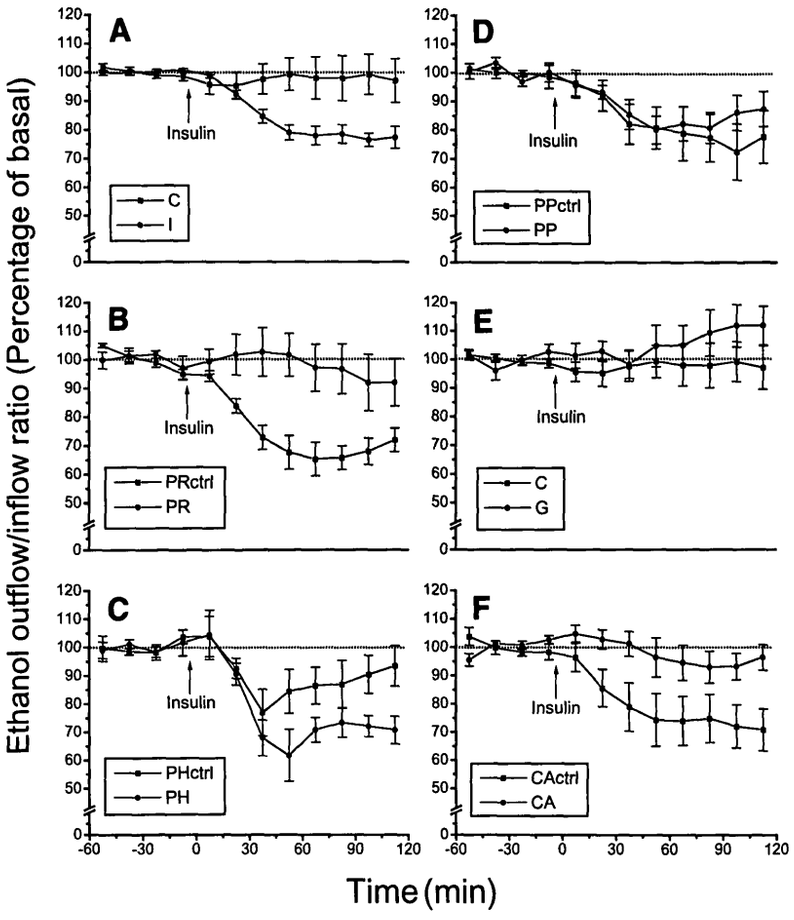

FIG. 1. Changes in the ethanol outflow:inflow ratio in response to insulin-induced hypoglycemia during adrenergic blockade (*A–D*) and calyculin A administration (*F*), or in response to hyperinsulinemia during relative euglycemia (*E*). Microdialysis probes were perfused at 0.5 μl/min with a modified KHB containing 20 mmol/l ethanol and inserted bilaterally in the m. gastrocnemius. Dialysate samples were collected every 15 min during a 60-min basal period, followed by (groups I, PR, PRctrl, PH, PHctrl, PP, PPctrl, G, CA, and CActrl) a pulse intravenous infusion of insulin (680 mU/kg body wt) or (group C) infusion of 0.9% saline. Dialysate samples were collected every 15 min for 2 h following insulin infusion. The perfusion medium in the microdialysis probes was supplemented with 10^{-6} mol/l propranolol (group PR); 10^{-6} mol/l phentolamine (group PH); 10^{-6} mol/l propranolol and 10^{-6} mol/l phentolamine (group PP); 10^{-6} mol/l propranolol, 10^{-6} mol/l phentolamine, and 1.0 μmol/l calyculin A (group CA); or was not supplemented (groups I, C, G, PRctrl, PHctrl, PPctrl, and CActrl). In group G, euglycemia (±10% of basal) was maintained by constant intravenous infusion of a 50% glucose solution. Values from group C in *E* are taken from *A*. At the maximal effect, groups I, PRctrl, PP, PPctrl, and CActrl are reduced from basal ($P < 0.001$), as are groups PH and PHctrl ($P < 0.05$). At the maximal effect, group I is lower than group C ($P < 0.001$), group PRctrl is lower than group PR ($P < 0.001$), and group PH is lower than group PHctrl ($P < 0.01$). Group CActrl is lower than group CA ($P < 0.001$). Basal values for the ethanol outflow:inflow ratio were group C: 0.107 ± 0.007, group I: 0.140 ± 0.015, group PR: 0.126 ± 0.055, group PRctrl: 0.117 ± 0.016, group PH: 0.086 ± 0.018, group PHctrl: 0.108 ± 0.019, group PP: 0.104 ± 0.009, group PPctrl: 0.111 ± 0.0121, group G: 0.095 ± 0.010, group CA: 0.104 ± 0.006, group CActrl: 0.083 ± 0.006. Values are presented as means ± SE (n = 6).

which relative euglycemia was maintained (group G). Blood glucose concentration was 4.09 ± 0.17 mmol/l at basal and was maintained ±10% of basal following insulin infusion. Basal dialysate glucose concentration was 1.66 ± 0.14 mmol/l. Dialysate glucose concentration mirrored blood glucose concentration. However, following a slight decrease in blood and dialysate glucose concentrations, the return of dialysate glucose to euglycemic levels lagged slightly behind that of blood glucose.

Insulin. The insulin infusate was collected at the outlet of the infusion catheter and determined to be 4,084,910 ± 159,395 pmol/l (n = 36). The insulin infusate was prepared to be ~7,000,000 pmol/l, delivering 1,200 mU/kg body wt to the rat. This result indicates that only 57% (680 mU/kg body wt) of the insulin actually reached the rat. Infusate-insulin concentration did not vary significantly between the six insulin-infusion groups. In group G in which both infusate and plasma insulin concentrations were determined, the infusate-insulin concentration of 4,263,900 ± 304,808 pmol/l (n = 6) resulted in a serum insulin concentration of 60,587 ± 5,882 pmol/l at 15 min, 59,369 ± 7,760 pmol/l at 45 min, 60,206 ± 6,522 pmol/l at 75 min, and 67,205 ± 3,343 pmol/l at 105 min following insulin infusion (n = 6, NS).

DISCUSSION

The circulatory response to insulin-induced hypoglycemia has been investigated previously (1–4), but results obtained from measurement of skeletal muscle blood flow, as opposed to limb blood flow, are few. Receptor blockade has also been performed, albeit with agents that have often been administered systemically. No results have been obtained from experiments in which the blocking agent has been administered at a local site in skeletal muscle at which blood flow has been determined. The microdialysis and ethanol techniques, which allow for the study of nutritive blood flow in tissue as well as the simultaneous local administration of pharmacological agents, have provided a unique means of gaining insight into the local regulation of skeletal muscle blood flow.

While the ethanol outflow:inflow ratio (a marker of skeletal muscle blood flow) was unaffected by a bolus infusion of 300 μl of saline, a significant decrease in the outflow:inflow ratio was registered in the group receiving a bolus infusion of insulin, indicating an increase in skeletal muscle blood flow during insulin-induced hypoglycemia. Because this decrease in the ethanol outflow:inflow ratio was eliminated with supplementation of the perfusion medium with propranolol (Fig. 1*B*), it is concluded that vasodilatation during hypoglycemia is largely due to β-receptor stimulation, most likely by adrenaline as reported previously (4,5). The decrease in ethanol outflow:inflow ratio found in group I was enhanced with the supplementation of the perfusion medium with phentolamine (group PH; Fig. 1*C*), thus demonstrating that α-adrenergic stimulation results in a concomitant vasoconstriction during hypoglycemia. This supports statements by Hilsted (7) and Liang et al. (5) that α-adrenergic vasoconstriction may compete with the β-adrenergic vasodilatation in skeletal muscle during insulin-induced hypoglycemia. Interestingly, supplementation of the perfusion medium with both propranolol and phentolamine (group PP) resulted in a decrease in the ethanol outflow:inflow ratio of equal magnitude to that seen with no supplementation (Fig. 1*D*). This

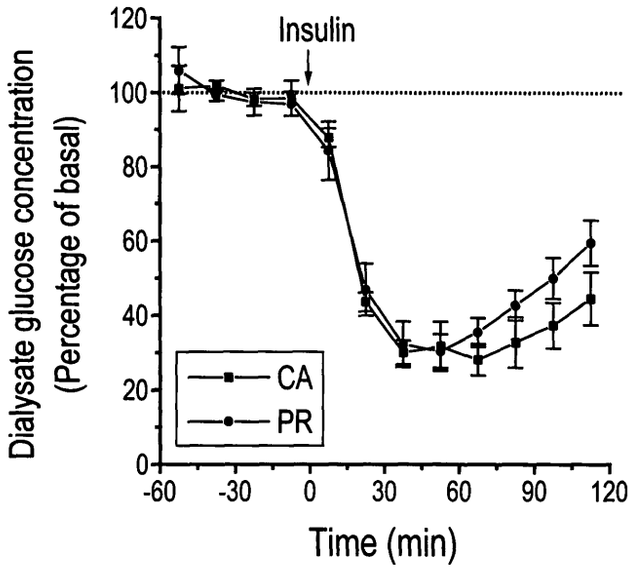

FIG. 2. Changes in the dialysate glucose concentration in response to insulin-induced hypoglycemia under local β-adrenergic blockade or local α- and β-adrenergic blockade and calyculin A administration. Microdialysis probes were perfused at 0.5 μl/min with modified KHB containing 20 mmol/l ethanol and inserted in the m. gastrocnemius. The perfusion medium of the probes in one group was supplemented with 10^{-6} mol/l propranolol, 10^{-6} mol/l phentolamine, and 1 μmol/l calyculin A (group CA), while the perfusion medium of the probes in the other group were supplemented with 10^{-6} mol/l propranolol (group PR). Dialysate samples were collected every 15 min during a 60-min basal period, followed by a pulse intravenous infusion of insulin (680 mU/kg body wt). Dialysate samples were collected every 15 min for 2 h following insulin infusion. Basal dialysate glucose concentrations were: group CA: 1.26 ± 0.11 mmol/l, group PR: 1.34 ± 0.11 mmol/l. Values are presented as means ± SE, n = 6.

finding indicates that, although α- and β-receptor stimulation have been shown in this study to occur in skeletal muscle during hypoglycemia, adrenergic stimulation is not necessary for the blood-flow response in the rat. Liang et al. (5) have also found this to be true in the dog. This may explain studies of adrenalectomized and sympathectomized humans, in whom an increase in blood flow was seen during hypoglycemia. These findings also support contentions by Liang et al. (5) that insulin alone is responsible for a portion of the vasodilatation during hypoglycemia.

When relative euglycemia was maintained (group G), no significant change in the ethanol outflow:inflow ratio was detected. Euglycemia was not strictly maintained, as blood glucose was ±10% of basal, indicating that these slight changes in glycemia are not sufficient to significantly affect skeletal muscle blood flow, even during extreme hyperinsulinemia. It is widely believed that vasodilatation in skeletal muscle does occur during a euglycemic hyperinsulinemic clamp (16–19), mainly because of a β-adrenergic mechanism (20). However, many authors have found no change in skeletal muscle blood flow under these conditions (21–24). Allwood et al. (4) also reported no change in forearm blood flow in subjects who failed to exhibit clinical evidence of hypoglycemia during hyperinsulinemia, finding an increased forearm blood flow only when frank hypoglycemia was attained. It was concluded that it is hypoglycemia, not insulin, that is the signal for the increase in forearm blood flow. Aside from differences in insulin dosages and methods used to measure blood flow, one possible explanation for discrepant results is that the α-mediated vasoconstriction may be greater in some subjects than in others, wholly or partially overriding the vasodilatation and resulting in a wide range of responses to insulin during euglycemia or near euglycemia. A similar mechanism has been proposed to account for the variable response to hypoglycemia in hand (skin) blood flow (4). That vasodilatation in the forearm in response to insulin infusion has a high degree of interindividual variation and is not found in all subjects was recently reported in a study of 65 normo- and hypertensive individuals (25). Further support for this hypothesis comes from the finding that norepinephrine release is greater with euglycemia than with hypoglycemia during hyperinsulinemia (5). Rowe et al. (26) have also reported increased norepinephrine concentrations in humans during insulin infusion and euglycemia.

As stated above, Liang et al. (5) have suggested that part of the vasodilator response during hypoglycemia is due to insulin. The authors stated that this vasodilator response is likely because of "the metabolic effects of insulin on tissue metabolism and ion fluxes," although direct and other indirect effects of insulin on the vascular endothelium cannot be ruled out. Results obtained during supplementation of the perfusion medium with both propranolol and phentolamine (group PP, Fig. 1*E*) and during supplementation with propranolol, phentolamine, and calyculin A (group CA, Fig. 1*F*) support these contentions. Alpha-mediated vasoconstriction must have masked the effect of insulin during propranolol perfusion, since the addition of propranolol to the perfusion medium eliminated the vasodilatation during hypoglycemia. It was found that the vasodilator response that occurs in the absence of adrenergic stimulation (group PP, Fig. 1*E*) can be eliminated by the inhibition of phosphoprotein phosphatases 1 and 2a with calyculin A (group CA, Fig. 1*F*), indicating that protein phosphorylation-dephosphorylation reactions are involved in this vasodilatation. These data should be taken with caution because there was a tendency for a vasoconstrictor effect of calyculin A under basal conditions. Baron, in his review (27), provides evidence for both direct and indirect mechanisms for insulin's vasodilator action. These data collectively indicate that insulin is involved in the hyperemic response during insulin-induced hypoglycemia. However, more specific and less toxic substances than calyculin A are needed to fully determine the role of insulin in this context.

In summary, while α- and β-adrenergic stimulation govern the blood-flow changes occurring in skeletal muscle during insulin-induced hypoglycemia, a sympathetic influence is not essential for the induction of hyperemia in this state. It can be concluded that hyperinsulinemia results in vasodilatation during hypoglycemia, although hyperinsulinemia does not have an effect on skeletal muscle blood flow under normoglycemic conditions. Alpha-mediated vasoconstriction, or some undescribed variable, may be the regulating factor responsible for the differential action of insulin during normoglycemia and hypoglycemia. Because the microdialysis ethanol technique is a safe method that can also be used to study nutritive skeletal muscle blood flow in humans, the possibility now exists to thoroughly investigate skeletal muscle vasodilatation during insulin-induced hypoglycemia in humans.

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