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Primacy of liver glucosensors in the sympathetic response to progressive hypoglycemia

(counterregulation/hepatic glucoreceptors/catecholamines)

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ABSTRACT **The impact of hepatic glucose concentration on the sympathetic response to progressive hypoglycemia was examined in chronically cannulated conscious male dogs (n = 6). Graded hypoglycemia was induced via peripheral insulin infusion (30 pmol·kg^{-1}·min^{-1}) with either peripheral (PER) or portal (POR) glucose infusion. Over the 260-min experimental period, arterial glycemia was adjusted from 5.2 ± 0.1 to 2.5 ± 0.1 mM in decrements of ≈0.5 mM every 40 min. Arterial glycemias were not significantly different between PER and POR at any measured level. However, hepatic glycemia was significantly elevated at all times during POR (8.4 ± 0.8 to 3.4 ± 0.2 mM) when compared to PER (5.2 ± 0.2 to 2.5 ± 0.1 mM). Plasma epinephrine values were significantly greater during PER vs. POR at all arterial glycemias below 4.0 mM. At the lowest level of arterial glycemia studied (2.5 ± 0.2 mM) the epinephrine response above basal was 3-fold greater for PER (8.7 ± 1.7 nM) when compared to POR (2.6 ± 0.6 nM) ($P < 0.01$). Plasma norepinephrine results were similar for the two protocols, with PER demonstrating a 3-fold greater response above basal when compared to POR at 2.5 mM arterial glycemia ($P < 0.05$). While the sympathetic response was markedly different between protocols when expressed as a function of arterial glycemia, when expressed as a function of hepatic glycemia this discrepancy was largely eliminated. This latter observation supports the liver as the primary locus for glycemic detection relevant to the sympathoadrenal response when hypoglycemia develops slowly—i.e., over a period of 2–3 h. A comparison of the current findings with our previous observations suggests that the hepatic glucosensors may play a greater role in hypoglycemic counterregulation as the rate of fall in glycemia is less.**

In 1924 Walter B. Cannon and coworkers (1) provided the first convincing evidence that insulin-induced hypoglycemia resulted in increased sympathetic output. At that time, they proposed that the enhanced sympathetic activity was likely due to glucopenia "local" to the autonomic nervous system. This position was supported by the earlier work of Claude Bernard (2) and others (3, 4), demonstrating that lesions to specific aspects of the brain had a profound impact on glucose metabolism. The existence of specific "glucoreceptors" within the hypothalamus was later proposed by Mayer and Marshall (5). Subsequent studies in which direct microinjections were used have now clearly identified glucosensitive neurons within the ventromedial and lateral hypothalamus (6). In addition, substantial evidence has accumulated over the years delineating the efferent capacity of the central nervous system (CNS) to impact upon glucoregulation (6, 7). This has led to the prevailing concept that the brain "senses" ambient glycemia and effects the requisite glucoregulatory mechanisms.

Evidence for the role of the CNS in glycemic detection has relied largely on nonphysiological stimuli, such as brain lesions, electrical stimulation, glucose analogues, and direct (in the CNS) glucose administration (6, 7). These procedures, while elucidating glucoregulatory aspects of the brain, do not provide insight into the actual quantitative role of the brain in glycemic detection. To better quantify the contribution of the CNS toward hypoglycemic detection *in vivo*, we introduced the local glucose irrigation procedure—i.e., "brain clamp." This procedure involved induction of systemic hypoglycemia in the conscious animal via insulin infusion, while brain euglycemia was maintained via carotid or vertebral glucose irrigation (8–10). The counterregulatory responses to the brain clamp were then compared with those of a control experiment in which an equivalent level of systemic hypoglycemia was elicited but the brain was also hypoglycemic. After several studies in which CNS glycemia was clamped via either the carotid or vertebral arteries, we were unable to establish any quantitative role for the brain in hypoglycemic detection or counterregulation (8–10). However, a subsequent study by Biggers *et al.* (11), using similar methodology, reached a different conclusion; i.e., that the brain is essential for full counterregulation.

These equivocal findings from the various brain-clamp studies cast some doubt on the exclusivity of the brain in glycemic detection. This led us to examine the quantitative importance of an alternative purported site for hypoglycemic detection—the liver. Glucose-sensitive afferents, presumably components in several hepatoglucoregulatory reflexes, have been identified in the portohepatic region (12, 13). These afferents demonstrate activities inversely proportional to the portal glucose concentration and have been shown to be functionally linked to glucosensitive neurons within the hypothalamus (14, 15). Applying the local irrigation approach to the liver—i.e., liver clamp—we have demonstrated that hepatic hypoglycemia is essential to eliciting the full sympathoadrenal response to hypoglycemia (16).

The current study was undertaken to ascertain (*i*) whether the liver is a locus for glycemic detection (i.e., a site at which the glycemic threshold for sympathetic activation is determined) and (*ii*) the quantitative importance of the liver in mediation of the sympathetic response for progressive hypoglycemia. Germane to this second objective, all brain- and liver-clamp studies to date (8–11, 16, 17) have involved the rapid induction of hypoglycemia—i.e., within 30–60 min. The rapid induction of hypoglycemia is likely to involve a

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Abbreviations: CNS, central nervous system; POR, portal glucose infusion; PER, peripheral glucose infusion; ICG, indocyanine green.

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number of metabolic disturbances distinct from those occurring during a more gradual development of hypoglycemia. Therefore, it is not clear that the quantitative contribution of the various glucosensor loci as currently elucidated is applicable to all hypoglycemic scenarios. In the present studies, two trials were conducted in which identical graded stepwise decrements in arterial glycemias were established by systemic insulin infusion, coupled with either peripheral (PER) or portal (POR) glucose infusion. These two experimental protocols yielded progressive and identical decrements in central glycemia. However, liver was relatively normoglycemic during POR but hypoglycemic during systemic glucose infusion. This approach allowed us to ferret out the relative importance of liver sensing *per se* in the integrated counterregulatory response to hypoglycemia.

MATERIALS AND METHODS

Animals and Surgical Procedures. Experiments were conducted on conscious male mongrel dogs (27.3 ± 1.2 kg; n = 6) in the resting state. Dogs were housed under controlled conditions (12 h light/12 h dark) in the university vivarium and were fed once per day with standard chow (25% protein/9% fat/49% carbohydrate; Wayne Dog Chow, Alfred Mills, Chicago). Dogs were used for experiments only if judged to be in good health as determined by body temperature, hematocrit, regularity of food intake, and direct observation. All surgical and experimental procedures were preapproved by the University of Southern California Institutional Animal Care and Use Committee.

One week prior to initiating experiments, animals were chronically cannulated under anesthesia induced with sodium thiamylal (Biotal; Bio-Ceutic Labs, St. Joseph, MO) and maintained with 0.5–1.0% halothane and nitrous oxide. Cannulas (Tygon; i.d., 0.13 cm) were placed in the portal vein for glucose infusion, in the carotid artery for arterial sampling, and in the jugular vein for insulin infusion. In addition, the femoral vein was cannulated (Tygon; i.d., 0.13 cm) with the tip of the catheter advanced into the inferior vena cava, rostral to the hepatic vein. An inflatable cuff (model VO-4; Rhodes Medical) was surgically implanted around the inferior vena cava just caudal to the hepatic vein. Inflation of the cuff temporarily occludes flow caudal to the cuff, allowing mixed hepatic venous blood to be sampled from the femoral catheter (18). All cannulas and the actuating tubing for the inflatable cuff were tunneled subcutaneously and exteriorized at the back of the neck. Cannulas were filled with heparinized saline (100 units/ml), coiled, capped, and placed in a pouch protected by a heavy denim collar.

Experimental Procedures. Each animal was used for two experimental protocols, which differed only in the site of glucose infusion: the portal vein vs. a peripheral (cephalic) vein. For PER, intracatheters (19 gauge; Deseret, Sandy, UT) were acutely placed in the right and left cephalic veins for indocyanine green (ICG) dye and glucose infusion, respectively. Infusion of ICG (0.13 mg/min) was initiated at −120 min, followed by a 90-min equilibration period. A 30-min basal sampling period (−30 to 0 min) followed, during which serial blood samples, arterial (glucose, insulin, ICG, epinephrine, norepinephrine, and glucagon) and hepatic venous (ICG), were taken at 15-min intervals. At 0 min, insulin infusion (30 pmol·kg^{-1}·min^{-1}) was initiated and maintained for the remaining 260 min of the experimental period. Peripheral glucose infusion was initiated simultaneously so as to clamp arterial glycemia at ≈5.0 mM for the next 60 min. Thereafter, the glucose infusion rate was adjusted every 40 min so as to clamp arterial glycemia at 0.5 mM below the preceding arterial glycemia. This provided a stepwise decrease in arterial glycemia, reaching a nadir of 2.5 mM between 220 and 260 min. Between 0 and 260 min, serial blood samples were taken every 10 min for glucose and insulin assays and every 20 min for ICG measurements. Additional arterial blood samples were taken every 10 min during the final 20 min at each glycemic level (i.e., 20, 30, and 40 min at each glycemic level) for measurements of epinephrine, norepinephrine, and glucagon. The POR protocol was identical to that described above for PER except that glucose was infused via the portal vein. The order of treatments, POR vs. PER, was randomized among animals.

Assays. Glucose was assayed by the glucose oxidase method (YSI, Yellow Springs, OH). Plasma ICG concentrations were determined spectrophotometrically at 805 nm. Radioimmunoassays were used to determine insulin (19) and glucagon (kit no. 32; antisera K-5563; Novo-Nordisk, Copenhagen). Epinephrine and norepinephrine concentrations were assayed by a single-isotope radioenzymatic approach (20).

Calculations. Hepatic plasma flow (HPF; liters/min) was determined as follows:

$$HPF = ICG_{inf}/(ICG_a - ICG_{hv}),$$

where ICG_{inf} = infusion rate for ICG (mg/min), ICG_a = concentration of ICG in arterial plasma (mg/liter), and ICG_{hv} = concentration of ICG in hepatic venous plasma (mg/liter).

For PER, the hepatic glycemia—i.e., the average glucose concentration entering the liver—was assumed equal to the ambient arterial glucose concentration. For POR, the flow-weighted hepatic glycemia (G_h) was calculated as

$$G_h = G_a + (GINF_{por}/HPF),$$

where G_a = arterial glucose concentration (mM) and $GINF_{por}$ = portal glucose infusion rate (mmol/min).

Data Analysis. Comparisons between treatments over time were by repeated measures ANOVA utilizing Tukey's test for post hoc comparisons. For determination of the glycemic thresholds, hormonal responses as a function of glycemia were analyzed via ANOVA with profile analysis. This modification of the repeated measures ANOVA tests the differences in hormonal response between adjacent glycemic points. Thus, this analysis determined the glycemic value at which a significant elevation in hormone concentration above basal was first observed. Comparisons between treatments at a given glycemic plateau and between glycemic thresholds were made by using paired t tests.

RESULTS

Basal arterial glucose values were not significantly different between PER and POR protocols (5.5 ± 0.2 and 5.2 ± 0.1 mM, respectively) (Fig. 1). Insulin infusion, initiated at time 0, increased plasma insulin from basal (72 ± 8 pM) to plateaus of 1401 ± 198 and 1355 ± 178 pM for POR and PER, respectively (not significant; $P > 0.05$). The simultaneous initiation of glucose infusion at t = 0 (82 ± 8 μmol·kg^{-1}·min^{-1} for PER and POR) clamped arterial glucose concentration at 5.2 ± 0.1 mM between 40 and 60 min, a value not significantly different from basal (5.4 ± 0.1 mM). Between 60 and 260 min, the glucose infusion rate was adjusted at 40-min intervals to 84 ± 5, 72 ± 5, 60 ± 7, 46 ± 6, and 26 ± 6 μmol·kg^{-1}·min^{-1}, yielding mean arterial glucose concentrations of 4.9 ± 0.1, 4.3 ± 0.1, 3.6 ± 0.1, 3.1 ± 0.1, and 2.5 ± 0.1 mM, respectively. Glucose infusion rates and arterial glucose concentrations were not significantly different between protocols at any time during the clamp ($P > 0.05$). In contrast, hepatic glycemia was significantly elevated during POR (0.9–2.6 mM) when compared with PER at all times during the experimental period ($P \leq 0.05$). During POR, hepatic glycemia ranged from 8.4 ± 0.8 to 3.4 ± 0.2 mM, while PER values reached the deep hypoglycemic range, ranging from 5.2 ± 0.2 to 2.5 ± 0.1 mM.

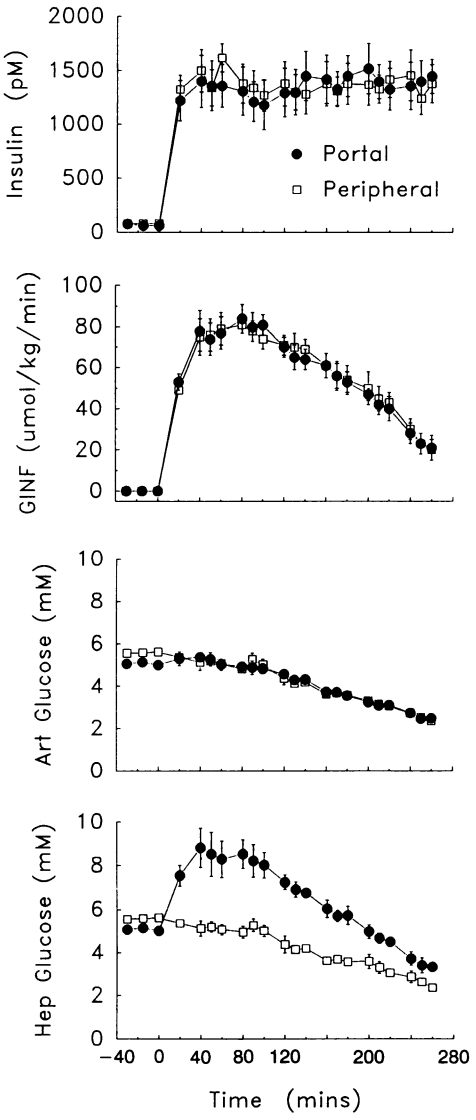

FIG. 1. Values (means ± SE) for insulin, glucose infusion rate (GINF), arterial (Art) glucose, and hepatic (Hep) glucose as a function of time. Solid symbols represent PORtal and open symbols represent PERipheral (cephalic vein) glucose infusion. No significant differences were observed between treatments for insulin, GINF, and arterial glucose. Hepatic glucose concentrations were significantly different ($P < 0.05$) between treatments at all times beyond 0 min.

In response to graded systemic hypoglycemia during PER, the catecholamine concentrations increased markedly above baseline. Epinephrine increased from a basal value of 0.3 ± 0.04 nM to a mean of 8.7 ± 1.7 nM during the final sampling period, a 25-fold increase (Fig. 2). However, when the equivalent systemic hypoglycemia was not present in the liver due to direct portal glucose infusion, the mean epinephrine value for the final sampling period was 70% lower, 2.6 ± 0.6 nM ($P < 0.05$); i.e., only a 7-fold increase above basal. A similar pattern was observed for norepinephrine, which increased 3.7-fold from a basal value of 0.9 ± 0.07 nM to a value of 3.5 ± 0.3 nM during PER and was markedly suppressed (1.8 ± 0.4 nM) during POR. The mean norepinephrine response above basal was suppressed by 67% during POR when compared to PER, despite matched arterial glycemia.

As noted above, when epinephrine and norepinephrine values were expressed as a function of arterial glycemia, PER diverged from POR, with PER demonstrating proportionally greater responses with declining arterial glycemia (Fig. 2). In contrast, when epinephrine and norepinephrine responses were expressed relative to hepatic glycemia no such differences were observed between protocols (Fig. 3). The difference in the catecholamine response as a function of arterial versus hepatic glycemia was also reflected in changes for the glycemic threshold. The glycemic threshold for any given counterregulatory hormone is ideally defined as the level of glycemia at which secretion is first activated above basal. For the current study, the glycemic threshold was determined as the glycemia at which the catecholamine concentration first departed from basal. During PER, the glycemic threshold was determined to occur at an arterial glycemia of 3.6 mM. For POR, this arterial glycemic threshold was significantly suppressed to a value of 3.1 mM ($P < 0.05$). However, when the glycemic thresholds were based on the hepatic glycemia no significant differences were observed between PER and POR (3.6 mM and 3.7 mM, respectively). Furthermore, the hepatic glycemic thresholds for both PER and POR were not significantly different from the arterial glycemic threshold during PER but were elevated above the arterial glycemic threshold for POR. Glycemic thresholds for norepinephrine, while lower than those for epinephrine, demonstrated similar relationships between PER and POR for both arterial and hepatic glycemias. The arterial glycemic threshold for norepinephrine was 3.2 mM during PER, but it was only 2.7 mM during POR. For POR, the corresponding hepatic glycemic threshold, 3.3 mM, was not significantly different from that for PER.

Glucagon values tended to be suppressed throughout the experimental period for both protocols (Table 1). Only during the final time period (240–260 min) for PER was the glucagon concentration elevated above the value from the preceding time period (200–220 min). This resulted in a significantly higher glucagon value during PER when compared to POR at an arterial glycemia of 2.5 mM ($P < 0.05$).

DISCUSSION

The current findings demonstrate that the magnitude of the sympathoadrenal response to progressive hypoglycemia is dictated primarily by the blood glucose concentration entering the liver, not the arterial glycemia. Clamping arterial glucose concentrations during the graded hypoglycemic protocols ensured that all tissues, excluding the liver, were exposed to virtually identical levels of glycemia during PER and POR (this includes the CNS). Yet, during POR, the magnitude of the epinephrine and norepinephrine responses (i.e., elevations above basal) was decreased by 73% and 67%, respectively, when arterial glycemia reached 2.5 mM (Fig. 2). Even this dramatic suppression of the sympathetic response does not reflect the full magnitude of control imposed by the hepatic glucosensors. During POR at an arterial glycemia of 2.5 mM, the liver was exposed to a modest level of hypoglycemia, 3.4 mM. Even if the sympathetic response were controlled completely by liver glycemia, some elevation in catecholamines during POR would be expected. Simply comparing the sympathetic responses between PER and POR at any given arterial glycemia will tend to underestimate the hepatic glucosensor contribution. Comparing the catecholamine responses for PER vs. POR as a function of hepatic glycemia better illustrates the true quantitative contribution of the hepatic glucosensors toward control of sympathetic output during hypoglycemia. While PER and POR generated different ranges for hepatic glycemia, there was adequate overlap to allow for such a comparison. When this was done (Fig. 3), the catecholamine response for any given level of glucose entering the liver (i.e., hepatic glycemia) was observed to be essentially identical for the two protocols. This was true despite the fact that at any given hepatic glycemia for PER and POR the corresponding arterial glycemias were markedly different. The extent to which hepatic glucosensors control the sympathetic response was further reflected in the

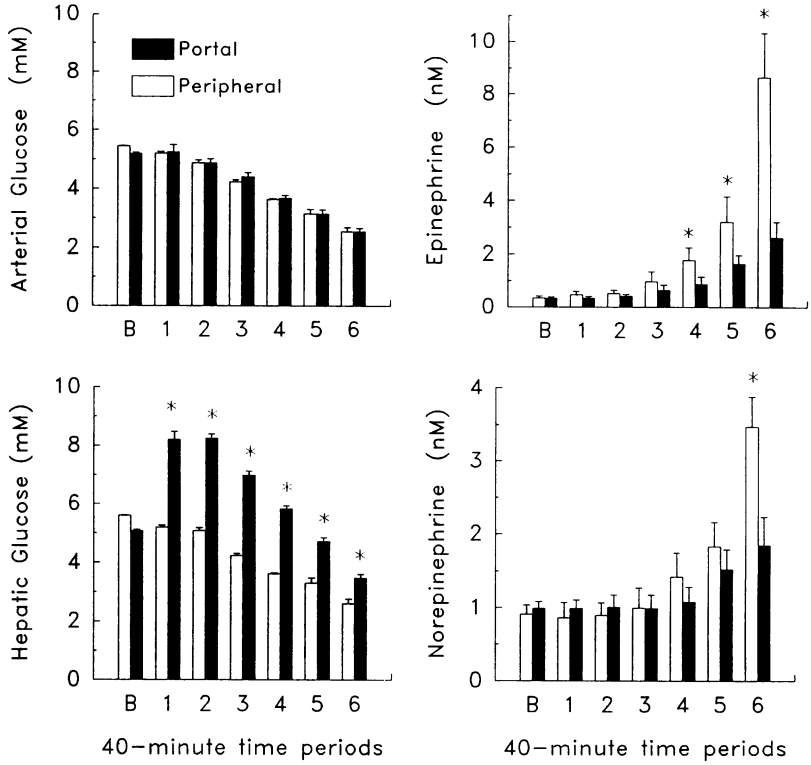

FIG. 2. Average values (means ± SE) for arterial glucose, hepatic glucose, epinephrine, and norepinephrine are presented for the basal (B) and experimental (40 min) sampling periods (bars 1–6). Average values for each animal were determined from three samples taken 10 min apart during each sampling period. Solid bars represent POR and open bars represent PER (cephalic vein). *, $P < 0.05$.

glycemic threshold—i.e., the glycemia at which sympathoadrenal output is stimulated beyond basal. When the glycemic threshold for epinephrine was based on arterial glycemia, it was observed to vary significantly between PER and POR (3.6 and 3.1 mM, respectively). Yet, when the glycemic threshold was determined from hepatic glycemia, it was essentially the same for PER and POR (3.6 and 3.7 mM, respectively). As with the magnitude of the sympathetic response, the glycemic thresholds for epinephrine and norepinephrine were observed to be a function of hepatic glycemia. Thus, under the current experimental conditions the hepatic glucosensors appear to be the primary mediators of the activation and magnitude of the sympathoadrenal response for hypoglycemia.

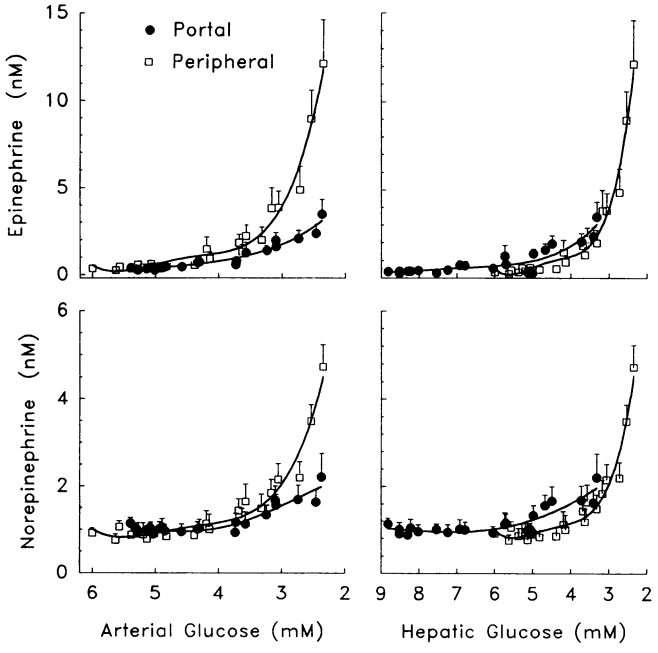

FIG. 3. Epinephrine and norepinephrine (means ± SE) as a function of arterial and hepatic glycemia. Solid symbols represent POR and open symbols represent PER (cephalic vein). Regressions are 4th order polynomials for POR and PER protocols. Polynomial order for each regression was optimized according to the Akaiki information criterion.

That hepatic glucosensors might mediate the sympathoadrenal response to hypoglycemia is not entirely unexpected. While the brain has long been perceived as the locus for hypoglycemic detection, a role for the liver has previously been proposed based on delineation of the requisite components for a hepatosympathoadrenal reflex (7, 15, 21). Glucose-sensitive afferents, which have been identified in the portohepatic region, demonstrate a firing rate inversely proportional to the portohepatic glucose concentration (12, 13). These afferents have been shown to be linked with glucose-sensitive neurons within the lateral hypothalamus and nuclear tractus solitarius (14, 15). Stimulation of the hypothalamus is in turn associated with increased sympathetic output, including output from the terminal ganglia comprising the adrenal medulla (21–23). The importance of hepatic glycemia for the hypoglycemic response *in vivo* was recently shown when we demonstrated that the epinephrine response to hypoglycemia was significantly suppressed by clamping the liver at euglycemia (16). Thus, a functional role for the hepatic glucosensors in modulating the sympathetic response to hypoglycemia had been previously suggested.

The quantitative contribution of the liver glucosensors to modulation of the sympathoadrenal response during progressive hypoglycemia proved much larger than expected. As indicated above, under conditions of the current study the epinephrine and norepinephrine responses were shown to be functions of hepatic glycemia and not arterial glycemia (Fig.

Table 1. Glucagon values (ng/liter) for basal and experimental (40 min) sampling periods during constant insulin infusion and decreasing PER and POR

	Sampling period						
	Basal	1	2	3	4	5	6
PER	141 ± 12	98 ± 10	106 ± 10	95 ± 8	101 ± 7	101 ± 12	141 ± 4
POR	121 ± 20	100 ± 17	101 ± 21	90 ± 21	81 ± 18	94 ± 14	101 ± 20*

Values are means ± SE.
*Significant difference between PER and POR ($P \leq 0.05$).

3). That is, the sympathoadrenal response for PER and POR at any comparable level of hepatic glycemia was the same, despite the fact that the concomitant arterial glycemias were different. Previously, we had noted only a 40% suppression in the epinephrine response to moderate systemic hypoglycemia (3.3 ± 0.2 mM) when the liver was clamped at euglycemia (16). More recently, we observed a 50% and 46% suppression of the epinephrine and norepinephrine response, respectively, to a systemic hypoglycemia of 2.6 ± 0.1 mM when hepatic glycemia was maintained at 4.0 ± 0.1 mM (17). While the absolute magnitude of the sympathoadrenal response to hypoglycemia during peripheral glucose infusion in this latter study was similar to current results for PER, the influence of hepatic glycemia (i.e., portal glucose infusion) was much more profound in the current study. The primary difference between these two studies appears to be the time required to achieve the hypoglycemic nadir, 230 min in the current study and only 30–40 min in our previous work (i.e., a 5- to 7-fold difference in the rate of fall for glycemia). Thus, in our studies as well as others (24–26), when glucose is infused peripherally the rate of fall has little impact on the magnitude of the sympathoadrenal response. However, it is possible that this may not be the case when glucose is infused portally. With a slow fall in glycemia, the sympathoadrenal response may primarily be the domain of the hepatic glucosensors. If the fall in glycemia is rapid, alternative glucosensors may well be recruited or the gain of the extant receptors may be modified (i.e., rate sensitivity). Further studies will be required to test directly the rate sensitivity of the putative portal hepatic glucoreceptors.

The lack of any significant response for glucagon in the current study may reflect the hyperinsulinemic, euglycemic (or near euglycemic) conditions established during the initial phase of each experiment. Several studies have reported declining glucagon values during such hyperinsulinemic euglycemic clamps (26, 27). This may be particularly important for the dog model in which even a modest infusion of glucose during insulin-induced hypoglycemia has been shown to result in very modest and transient glucagon responses (8, 9, 11). Despite the general suppression in plasma glucagon over most of the experiment, as arterial glycemia dropped to 2.5 mM glucagon values increased for PER (Table 1). This was not observed to occur during POR. As a result, glucagon values were significantly higher for PER when compared with POR at a glycemia of 2.5 mM. The lack of a significant glucagon response is of some concern in quantifying the contribution of the hepatic glucosensors toward counterregulation *in vivo*. Glucagon, a normal component of the counterregulatory response that impacts directly on hepatic glucose metabolism, may alter the sensitivity of the hepatic glucosensor to ambient glycemia and the subsequent sympathoadrenal response.

In conclusion, the liver appears to be the primary locus for glycemic detection during slowly developing hypoglycemia. The magnitude of the sympathoadrenal response during progressive hypoglycemia was observed to be a function of the hepatic glucose concentration and largely independent of the prevailing arterial glycemia. A comparison of the current findings with our earlier reports suggests that hepatic glucosensors are quantitatively more important for hypoglycemic detection as the rate of fall in glycemia decreases. Given the clinical prevalence of slowly developing (i.e., graded) hypoglycemia (28), hepatic glucosensors appear critical for hypoglycemic detection.

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