Chapter 7

General discussion

The more I learn, the more I realize I don’t know.
The more I realize I don’t know, the more I want to learn.
- Albert Einstein
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The work presented in this thesis focused on the counterregulatory responses to hypoglycemia in rats, and the changes in those due to recurrent hypoglycemia. The first section of the thesis addressed the counterregulatory responses and the importance of insulin (Chapter 2) as well as the role of the nutritional state and other energy substrates (Chapters 2 and 3). The second section focused on the role of the central nervous system in the regulation of these counterregulatory responses, by studying the activation of specific brain areas (Chapter 4) or pharmacological blockade of these areas (Chapter 5). The third and final thesis section described the development of a rat model for recurrent hypoglycemia and the associated autonomic failure (Chapter 6).

Section I – Counterregulation, insulin, and the nutritional state

Insulin

In Chapter 2, the counterregulatory responses to insulin-induced hypoglycemia were investigated at different doses of insulin. The results describe in detail the glucagon, catecholamine, and corticosterone responses in rats subjected to hypoglycemia. One of the most important results is that, both in the fed and in the fasted state, higher insulin doses resulted in similar glucose levels but higher counterregulatory responses. From this we conclude that insulin plays a major role in the regulation of the counterregulatory responses to hypoglycemia, which has already been suggested in studies in humans (12, 21, 31). It is not known whether insulin acts directly on the counterregulatory responses, or whether it affects them indirectly through another mechanism (see below).

A direct action of insulin on the regulation of the counterregulatory responses would assume that insulin acts directly on the pancreas (glucagon), the adrenal medulla (catecholamines), and/or the brain centers that control these organs. Since insulin is a potent inhibitor of glucagon release, a direct activation of the glucagon response by exogenous insulin can be ruled out. Similarly there is no evidence that secretion of adrenaline from the adrenal medulla is stimulated by insulin. In contrast, a direct insulin effect on the nervous system seems much more likely. As one of the key centers of the autonomic nervous system, the brain is strongly involved in glucose homeostasis (41, 56), is responsive to insulin (36, 42, 57), and reacts differently to hyperinsulinemic euglycemia and hyperinsulinemic hypoglycemia (Chapter 4)). Furthermore it has been shown that insulin can modify noradrenergic neurotransmission by inhibiting noradrenaline reuptake (5), which could augment noradrenaline-mediated responses such as those in the hypothalamus (discussed later in this chapter). It is therefore very well possible that insulin directly modulates the counterregulatory responses to hypoglycemia by acting on the neural circuits controlling peripheral hormone secretion. This hypothesis presumes that exogenous insulin during
hypoglycemia enters the brain (50, 55) and affects these networks, an assumption now supported by the finding that mice lacking brain insulin receptors show defective counterregulation to hypoglycemia (15).

There are a couple of mechanisms that may underlie a possible indirect action of insulin on the regulation of the counterregulatory responses.

One consequence of exogenous administration of insulin is that endogenous insulin secretion is inhibited. Reduced endogenous insulin release removes the inhibition of glucagon secretion from the alpha cells and could therefore be an important determinant for this counterregulatory response (19). Indeed it is hypothesized that intra-islet insulin levels drop during insulin-induced hypoglycemia and thereby allow glucagon to be released (24, 59). It should be noted that estimatedly 75% of the glucagon response to hypoglycemia disappears when both branches of the autonomic nervous system are blocked (25). Hence the major part of the glucagon response seems to be due to a nervous stimulation of glucagon secretion, and/or a nervous inhibition of insulin secretion (26, 59), and perhaps not due to a direct effect of exogenous insulin on the pancreatic beta cells.

Insulin may also indirectly affect the counterregulatory responses through its effects on peripheral glucose homeostasis parameters (e.g. intracellular glucose levels, arterial/venous differences, or the speed of the decline in glucose levels). Some of these parameters may be recognized by the brain and thereby influence the counterregulatory responses to insulin-induced hypoglycemia. It is for example known that a drug that inhibits glucose utilization (the fructose analogue 2,5-anhydromannitoll) does not decrease blood glucose levels but leads to counterregulatory responses similar to those during insulin-induced hypoglycemia. This means that the brain receives information on decreased intracellular energy supply (due to the decreased glucose utilization), resulting in counterregulatory responses to restore energy balance (29, 43). It can be argued that the different insulin doses in studies like the one presented in this thesis may have led to local differences in peripheral glucose homeostasis (with hepatic intracellular energy availability as an example, or a factor such as speed of decline in glucose levels (35)), which may explain why different insulin doses but similar blood glucose levels were still accompanied by different counterregulatory responses.

Finally, higher insulin levels might have affected the body’s glucosensors, so that the same blood glucose level may result in different counterregulatory responses. Those glucosensors are located in the hepatic portal vein area and in the brain (18, 39), and insulin is known to augment the firing response of ventromedial hypothalamic neurons to glucose levels (for review see Ref. 64), which is in agreement with this hypothesis explaining insulin’s effect on the counterregulatory responses to hypoglycemia.

Our data confirm the observation that different glucose threshold levels exist for activation of glucagon, adrenaline, and other counterregulatory responses (37). This means that a hierarchy exists for the counterregulatory responses to hypoglycemia, where a moderate reduction of glucose levels already elicits a glucagon response, while greater reductions of glucose levels are needed to elicit an adrenaline response.

The current studies suggest this hierarchy not only exists for glucose levels, but also for insulin. At similar glucose levels, a low dose of insulin elicits a glucagon response only, while higher insulin doses are needed to elicit an adrenaline response. This suggests that
"insulin thresholds" may exist as well – or that the known glucose thresholds for counterregulatory responses are not fixed, but depend on the ambient insulin levels. The activation and magnitude of the counterregulatory responses to insulin-induced hypoglycemia therefore appear to be triggered by the decrease in glucose levels combined with the increase in insulin levels.

The nutritional state

Chapter 2 also addressed the effects of fasting on the counterregulation to hypoglycemia. Counterregulatory responses were greatly enhanced after fasting, which is in agreement with other studies showing increased responses to metabolic challenges in the fasting state (61, 62). The increased counterregulatory responses observed in our studies can first and foremost be linked to the lower glucose levels in the fasted condition, both at baseline and during administration of insulin. However, the reduced nutritional state by itself also plays a role, which is among others reflected by the very rapid corticosterone response, which already started at glucose and insulin levels that do not produce a corticosterone response in the fed condition.

The mechanism by which fasting enhances the counterregulatory responses to hypoglycemia in this way is not known. The enhanced insulin sensitivity in the fasted state might explain the increased stimulatory effect of insulin on the counterregulatory responses (as described in the previous paragraph). It may also be possible that periods of fasting alter the sensitivity of the glucosensors that respond to hypoglycemia, so that the magnitude of the counterregulatory responses at a given glucose level is increased. Furthermore, the liver might report a decreased combined energy status to the brain. As discussed before, the liver can give rise to afferent signals about energy content (existing as ATP) (43), and it can be hypothesized that the reduced glycogen availability negatively affects ATP availability and thereby further enhances the afferent signaling from the liver to the brain.

The latter hypothesized mechanism could also help explain the results described in Chapter 3. In that study, rats were subjected to hypoglycemia in a situation with reduced availability of energy from fatty acids (by inhibition of fatty acid oxidation). This lipoprivic condition also greatly enhanced the counterregulatory responses to hypoglycemia, at higher blood glucose levels than in the rats receiving hypoglycemia alone. While the fasting described in Chapter 2 started 48 hours before the hypoglycemic episode, the blockade of fatty acid oxidation in Chapter 3 was acute, and gradual adaptations to the reduction in energy availability can therefore be excluded. The fact that this acute effect on fatty acid oxidation still enhanced the counterregulatory responses to hypoglycemia, indicates a rapid and immediate integration of the information about decreasing blood glucose levels with the information about the changed nutritional state (in this case in the form of reduced fatty acid oxidation). This again could suggest the aforementioned involvement of the liver and brain. Both the brain (18, 32, 45) and the liver (28, 39) are glucose-sensitive, the liver is sensitive to lipoprivation (44, 49), and in addition the liver contains the glycogen reserves which become depleted during fasting.
The signals about glucoprivation and lipoprivation could then be integrated at two possible sites: the liver or the brain.

Firstly, it could be possible that the greatly potentiated responses to the combination of glucoprivation and lipoprivation may have been solely caused by the liver detecting a reduction in both fuel sources, resulting in a synergistically increased activity of the afferent hepatic vagal nerves. This is supported by data in the literature that have shown that a combination of glucoprivation and lipoprivation synergistically increased food intake (17) and decreased hepatic energy status (29). This means that the liver indeed may serve as an integration site for the signals about glucoprivation and lipoprivation, reporting a combined signal to the brain. It does not explain however, why the responses to glucoprivation are different than those to lipoprivation, which makes it less likely that the liver is the only energy substrate sensing organ.

The second possible place for the integration of the two signals is the brain. It is tenable that the brain is the key candidate for this, integrating the lipoprivic signal arising from afferent vagal liver nerves with a glucoprivic signal arising from the hypothalamus or the brain stem. This would imply that different neuronal networks exist for homeostatic control of the different energy substrates (49). This may also help explain why the body reacts differently to glucoprivation than to lipoprivation. The neuronal network involved in fatty acid homeostasis would then be interconnected to the neuronal networks involved in glucose homeostasis, bidirectionally modulating the responsiveness of each other. It has been indicated that indeed separate neuron populations in the hypothalamus are activated during glucoprivation versus lipoprivation (51), which supports this hypothesis.

Additionally or alternatively, the brain itself could be sensitive to both glucoprivation and lipoprivation. It is known that the brain can use other energy substrates than glucose, such as ketone bodies and lactate (27, 40), and there are indications that it can also metabolize fatty acids (53), so it is very well possible that some neurons can react to changes in fat oxidation, perhaps even the same neurons that sense hypoglycemia.

It has now become clear that the activation of the counterregulatory responses not only depends on glucose levels and on insulin levels, but also on the availability of other energy sources. The most important conclusion from this section is therefore that the activation and magnitude of the counterregulatory responses to insulin-induced hypoglycemia appear to be triggered by the decrease in glucose levels, the increase in insulin levels, and the decrease in other energy sources.

Section II – Central nervous mechanisms

Activation of noradrenergic pathways

In the second part of this thesis, the central nervous mechanisms involved in the counterregulatory responses to hypoglycemia were investigated. It is well-known that the brain – and especially the hypothalamus – is strongly involved in the control of energy balance (33, 54, 63). This certainly also includes glucose homeostasis, since both endogenous glucose production and the secretion of glucoregulatory hormones are coordinated by the
autonomic nervous system (48, 54, 58). As mentioned before, the hypothalamus seems to play a major role in the regulation of the counterregulatory responses to hypoglycemia (4). There are several lines of evidence for this. It is well known that the ventromedial hypothalamus (VMH) contains glucosensors, and local glucose administration into the VMH during hypoglycemia abolishes the counterregulatory responses (3). In addition, the hypothalamus receives signals from other glucose-sensitive areas, such as the hindbrain – an area also involved in the counterregulatory responses to hypoglycemia and glucoprivation (2). Noradrenaline is the main neurotransmitter in this neuronal pathway between the hindbrain and the hypothalamus, and we confirmed this in Chapter 4 by the elevation of noradrenaline levels in microdialysis samples from the PVN and the VMH during insulin-induced hypoglycemia.

As expected, the increase in noradrenaline (and GABA) levels in the VMH disappeared when the rats' blood glucose levels were kept normal throughout the experimental period by intravenous infusion of glucose. There was however still a noradrenaline response in the PVN, which therefore likely is due to the increased plasma insulin levels – confirming a possible role for insulin in the activation of the counterregulatory responses, as suggested in the previous paragraphs. It may therefore be hypothesized that the VMH is mainly involved in responses to low blood glucose levels (in line with its known glucosensory role (3)), while the PVN is mainly involved in the integration of information on glucose levels with information on insulin and perhaps other parameters, and the subsequent activation of the appropriate responses (in line with its known integratory and coordinating role (30, 34)).

**Blockade of noradrenergic pathways**

To substantiate the notion that the observed noradrenergic signal received by the PVN is indeed one of the steps in the activation of the counterregulatory responses to hypoglycemia, it was investigated whether inhibition of these signals would affect the counterregulatory responses. As described in Chapter 5, this was indeed the case. Rats received either an α₁- or an α₂-adrenergic antagonist into the PVN, whereafter they were subjected to insulin-induced hypoglycemia. Treatment with these antagonists inhibited the sympathoadrenal counterregulatory responses to hypoglycemia, and resulted in further reduced blood glucose levels – together indicating that the noradrenergic signal to the PVN described in Chapter 4 is indeed important in the counterregulation to hypoglycemia. This is in agreement with the previously published findings that anesthetizing the whole PVN impedes the counterregulatory responses (14), and refines those findings to α-noradrenergic pathways.

PVN administration of the α₂-adrenergic antagonist furthermore prevented the expected compensatory increased food intake response, in line with the known role of α₂-adrenergic signaling in food intake (6, 23, 38). This again suggests that the counterregulation to hypoglycemia is a complex and tightly-controlled system, and that the different responses (e.g. adrenaline secretion and food intake initiation) are controlled by different mechanisms. Current investigations are elucidating more details of these mechanisms, such as the origin of the noradrenergic signals observed in the hypothalamus during hypoglycemia (13, 20) and the different neurotransmitters involved in the different counterregulatory responses (7).
Together these data show that the low glucose levels and the high insulin levels associated with insulin-induced hypoglycemia lead to distinct noradrenergic signals to the paraventricular hypothalamus in rats. Blocking these noradrenergic signals impairs counterregulatory responses to insulin-induced hypoglycemia, showing that they are a functional part of the mechanisms activating the counterregulatory responses.

Section III – Recurrent hypoglycemia

Rat model for HAAF

The final section of this thesis addressed the issue of autonomic failure caused by recurrent hypoglycemia.

Recurrent hypoglycemia decreases the counterregulatory responses to hypoglycemia, a phenomenon called Hypoglycemia-Associated Autonomic Failure (HAAF) (1, 9, 11). HAAF impairs the counterregulatory responses to hypoglycemia, so that a further reduction of blood glucose levels is needed to trigger these important autonomic responses. At the same time, the awareness of hypoglycemia (by means of sensations such as sweating, trembling, increased pulse) turns into hypoglycemia unawareness (8, 22). This causes a vicious circle, ultimately leading to a situation where severe hypoglycemic episodes occur with almost no warning signals. As a result, patients fear the frequent hypoglycemic episodes, do not comply with the prescribed insulin therapy, and do not achieve optimal glucose control. It is therefore of great importance to find out why and how HAAF develops. This requires suitable animal models; and the aim of the studies described in Chapter 6 was thus to develop and characterize such a model.

As described in that chapter, protocols used to induce HAAF in humans – such as one or two antecedent hypoglycemic episodes on the day before the test episode – also induced HAAF in rats. HAAF was either evident as reduced counterregulatory responses at a given glucose level, or as unchanged counterregulatory responses but at a lower glucose level than in controls. The glucagon response seemed to be somewhat less susceptible to recurrent hypoglycemia than the adrenaline response, which has now also been shown in other studies (16, 52, 60). Finally it was remarkable that there were pronounced differences between individual animals in the development of HAAF.

In contrast to the hormonal counterregulatory responses, food intake – the behavioral counterregulatory response to hypoglycemia – was not much affected by recurrent hypoglycemia. Hypoglycemia by itself induces food intake, and can result in total 24h food intake being greater than on a control day, but recurrent hypoglycemia did not affect this response, which recently was confirmed by Sanders et al. (46). Interestingly, when animals could choose between carbohydrate- and fat-enriched chow, hypoglycemia shifted their nutrient preference towards carbohydrates. Again, this response to hypoglycemia did not change after multiple hypoglycemic episodes, confirming the differential and separate organization of the distinct counterregulatory responses to hypoglycemia described in the previous paragraph.
**HAAF is GAAF?**

Finally, the most interesting results described in Chapter 6 addressed the question whether HAAF is only related to insulin-induced hypoglycemia, or perhaps also to other forms of glucoprivation. Generally, the responses to other forms of glucoprivation (such as by the glucose antimetabolites 2-deoxyglucose or 2,5-anhydro mannitol) are very similar to the responses to hypoglycemia – i.e. secretion of glucagon, adrenaline, and corticosteroids (49). Furthermore, it is known that antecedent 2-DG administrations reduce the counterregulatory responses to subsequent 2-DG administration (47), suggesting that glucoprivation in general (rather than e.g. insulin-induced hypoglycemia only) can induce autonomic failure. In the last study described in Chapter 6, it was investigated whether antecedent recurrent 2-DG administrations reduced the counterregulatory responses to hypoglycemia, just like antecedent recurrent hypoglycemia does (as shown in Experiment 3 in Chapter 6). This was indeed the case – suggesting that glucoprivation might be the crucial factor in the development of Hypoglycemia-Associated Autonomic Failure. In other words, HAAF can probably be induced by any form of glucoprivation, and affects the counterregulatory responses to any form of glucoprivation. It might therefore not only be a Hypoglycemia-Associated Autonomic Failure, but also more generally a Glucoprivation-Associated Autonomic Failure.

This finding may also help pinpoint the mechanistic background of the development of this autonomic failure. Since 2-DG can induce this failure as well, exogenous insulin can likely be ruled out as an essential causal factor. The general glucoprivation-linked nature of the defect together with considerations about the likeliness of alternative hypothesis suggest the brain as the probable site for this defect, as that is the place where all glucose-related signals are integrated and lead to activation of counterregulatory responses (10). The precise reason why the brain changes counterregulatory responses after recurrent hypoglycemia remains to be resolved.

**Conclusions and perspective**

The studies presented in the three sections of this thesis have given several new insights into the counterregulation to insulin-induced hypoglycemia in rats. It was found that a complex but well-coordinated control of the individual counterregulatory responses exists, both in time and in required magnitude. It appeared that glucose levels may be defended above a certain level, independent of the exogenous insulin dose. It was shown that insulin by itself may be a significant contributor to the activation and magnitude of the counterregulatory responses. Also, the energetic state of the organism was of importance for these counterregulatory responses to hypoglycemia. These findings support the concept of "relative hypoglycemia", comprising that the effects and severity of hypoglycemia depend on many separate factors, including glucose levels, (exogenous) insulin levels, and the availability of other energy sources such as glycogen and fatty acids.

The brain mechanisms involved in these counterregulatory responses were also subject of investigation. It was shown that insulin-induced hypoglycemia activates noradrenergic signals into the ventromedial and paraventricular hypothalamus, and that pharmacological blockade of this signal in the paraventricular nucleus impairs the
counterregulatory responses to hypoglycemia, suggesting that the detection of hypoglycemia is relayed by noradrenergic signals through the paraventricular hypothalamus to the autonomic nervous system.

Finally, the issue of recurrent hypoglycemia and the associated autonomic failure was investigated. It was shown that this autonomic failure can be evoked in rats, and that the defect can also be induced by other glucoprivic agents. It is suggested therefore that the defect is a result of alterations in brain areas involved in the counterregulation to glucoprivation. However, a definitive mechanism hasn’t been identified yet, and more research will be needed to shed light onto the causes for developing Hypoglycemia-Associated Autonomic Failure.

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