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Effects of fatty acids and over-stimulation on insulin secretion in man.

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Dr. med. thesis, 2003

ISBN: 82-471-5153-7

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1. ACKNOWLEDGEMENTS

This research was carried out at the Department of Endocrinology, the Medical Faculty at the Norwegian University of Science and Technology in Trondheim. The project was financially supported by the Medical Faculty at the Norwegian University of Science and Technology and the Norwegian Research Council, the Norwegian Diabetes Association, the Norwegian Society for Endocrinology and Torstein Erbo's Foundation.

I would especially like to thank:

The study participants for their positive attitude and endurance.

Valdemar Grill, my supervisor, who so bravely recruited me for this job. Working with him has been inspiring and I greatly appreciate his vast knowledge, but maybe more so his supportiveness and being readily available for discussions.

Ingrid Løvold Mostad for collaborating patiently and conscientiously with me in the Acipimox project, helping with statistical analyses, sharing despair over writing and laughs during tests.

Magnus Kollind, Magret Wæren, Eva Tingstad and colleagues, all at Levanger Hospital, for participating with such enthusiasm in the Diazoxide project. Henning Borgos for devoting a summer to the elaboration of protocol forms, screening and recruitment of participants in the Diazoxide study.

Kristian Bjerve and the staff at the Department of Clinical Chemistry for assistance in analysis of blood samples, especially so to Kristian for data analysis of fatty acids in the phospholipid fraction and valuable comments during manuscript preparation.

Ingrid H. Jørgensen, Veslemøy Malm and Kristin Rian, for instructions and assistance during my laboratory work.

Li Xin Li, Tie-Feng Shi and Hiroyasu Yoshikawa for contribution to the groups'discussions and progress in whole.

The nurses and secretarial staff at the Department of Endocrinology, St.Olavs Hospital, for patience and assistance during the clinical projects, and to the endocrinologists there, for their interest and support.

Professor Hans Krokan, leader of the former Institute of Cancer Research and Molecular Biology for providing a friendly and stimulating working atmosphere and good laboratory facilities. Dagmar Moholdt, Tove Oppdahl and Gerd Bromseth at the same Institute for their secretarial efforts.

In addition I would like to thank:

Sture Fallkmer and Eli Johanessen at the Institute of Morphology and the staff at the Animal Department for time and effort spent during a pilot study with db/db mice.

Rigmor Austgulen, Ann-Mari Brubakk, Pauline Verloove-Vanhorick and Jostein Halgunset for supporting my first interest in research.

Svanhild Schønberg, Carina Seidel, Trude Flo and Brit Dybdahl for friendship, laughs and discussions, whether professionally fruitful or not, during our common research progress.

Tone Løvik, Ekkehart Geiger, Anita S. Dalsbø, neighbors and colleagues for their friendship and support.

My parents and my brother for believing in me and helping out with the children.

And last, but not the least, Sunniva and Frithjof for being here, without them no work seems worth doing.

2. LIST OF PUBLICATIONS

1. **Effects of long term fasting on insulin responses in man.** *Scand J Clin Lab Invest 62: 271-8, 2002*

2. **Acute lowering of circulating fatty acids improves insulin secretion in a subset of type 2 diabetes subjects.** *Am J Physiol Endocrinol Metab 284: E129-E137, 2003.*

3. **Effects of a 3 day low-fat diet on lipids, metabolic control, insulin sensitivity and adipocyte hormones in Norwegian subjects with hypertriglyceridemia and type II diabetes.** *Submitted Eur J Clin Nutr Nov 11th 2002.*

4. **Nine weeks of bedtime diazoxide is well tolerated and beta cell beneficial in subjects with type 2 diabetes.** *Submitted Diabetic Medicine Sept 18th 2002.*

3. ABBREVIATIONS

BTI	bedtime insulin
Ca^{2+}	cytoplasmic free calcium
FA	fatty acids
GLUT-2 / GLUT-4	members of the glucose transporter family
GSIS	glucose-stimulated insulin secretion
K^{+}ATP channel	ATP-sensitive potassium channel
MODY	maturity onset of diabetes of the young
n-3 / n-6 FA	omega-3 / omega-6 fatty acids
PDH	pyruvate dehydrogenase
PPAR-γ	peroxisome proliferator activated receptor gamma
RIA	radioimmunoassay
SUR	sulphonylurea receptor
TG	triglycerides

4. GENERAL INTRODUCTION

4.1 DEFINITIONS, CLASSIFICATION AND ETIOLOGY OF DIABETES MELLITUS

Diabetes mellitus is a state of chronic hyperglycemia[1]. It encompasses two major diseases: Type 1 diabetes (previously termed juvenile onset, insulin dependent diabetes) and type 2 diabetes (previously termed maturity onset, "non-insulin dependent" diabetes).

In type 1 diabetes, autoimmune processes damage the pancreatic beta cells and this leads to a total failure of insulin secretion[2]. Type 2 diabetes is, phenotypically and genotypically heterogeneous. It accounts for 85% of all cases of diabetes with a prevalence of 3-6% in Scandinavia[3-4]. In certain populations such as the Pima Indians and the Nauruans this figure approaches 40%[5]. The incidence of type 2 diabetes has in recent years increased alarmingly in the Western hemisphere, also in adolescents and young adults[4].

Insulin resistance and insulin deficiency characterize type 2 diabetes. Insulin resistance signifies tissue resistance to insulin action, mainly in skeletal muscle and liver. Insulin resistance is in part determined by multiple genetic factors [6-8]. Physical inactivity and obesity, especially of abdominal distribution, impart insulin resistance and increase the risk of developing type 2 diabetes[9-10]. Other acquired factors causing insulin resistance are intrauterine growth retardation, smoking, persistent hyperglycemia ("glucotoxicity") and dyslipidemia ("lipotoxicity")[7].

Insulin deficiency is defined as an inappropriately low insulin response to glucose and other secretagogues. Aspects of insulin deficiency will be further described and discussed in relation to the normal physiology of beta cells (p.13).

The relative impact of insulin resistance vs. low insulin secretion in the pathogenesis of type 2 diabetes is still debated. A combination of relative beta cell deficiency and insulin resistance with a variable impact of each of the two factors in individual patients seems most likely. A

common clinical situation is that an acquired increase in insulin resistance unmasks a primary beta cell defect[11].

4.2 THE PHYSIOLOGICAL EFFECTS OF INSULIN.

In the liver, insulin increases the build-up of glycogen by stimulating the glycogen synthesis pathway and inhibits gluconeogenesis. Furthermore, protein and triglyceride (TG) synthesis are increased, as well as the formation of very low density lipoprotein cholesterol.

The most important peripheral effect of insulin is on muscle, where protein synthesis is promoted by increased uptake of amino acids and increased ribosomal protein synthesis. In addition, insulin promotes glycogen synthesis in the muscle.

In adipose tissue, insulin promotes TG storage by inducing the enzymatic intravascular hydrolysis of lipoproteins. By increasing glucose uptake, more alpha-glycerol-phosphate is made available for TG synthesis. Insulin also inhibits intracellular lipase, thus preserving TG stores. (Overview in [12].)

4.3 INSULIN SECRETION AND OTHER BETA CELL FUNCTIONS

Insulin secretion responds to minute changes in blood glucose levels. The level of blood glucose is the main regulator of insulin secretion. Acute regulation of insulin secretion relies on interplay between glucose related secretory signals and potentiating or inhibiting influences of nutrients, hormones or neurotransmitters[6;13-14]. Time-dependent effects supplement the acute effects. The time-dependent effects include stimulation of insulin biosynthesis[15] and beta cell replication and neogenesis[16].

4.3.1 Glucose stimulation of insulin secretion

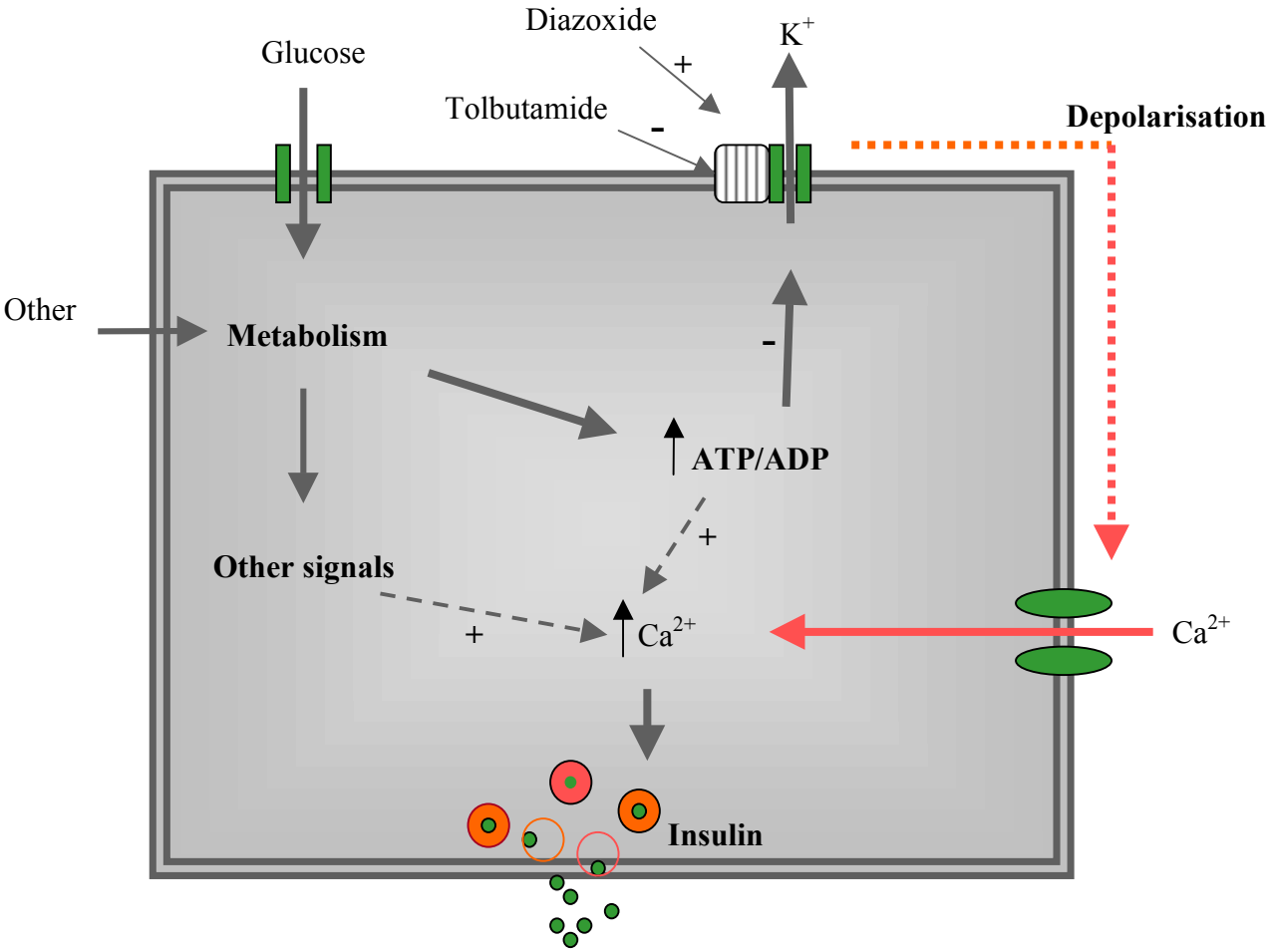

Figure 1. Schematic representation of glucose stimulated insulin secretion, + (stimulation); - (inhibition); dashed arrows indicate the amplifying pathways. Adapted from [14].

GLUT-2 is the main transporter of glucose into the beta cell. The inward transport of glucose is not a rate-limiting step. Instead it is the phosphorylating step (glucokinase enzyme activity) that has this function. An increase in extracellular glucose is coupled to increases in glucose phosphorylation. Glycolysis and in particular glucose oxidation follow with a dose-response relationship similar to that of insulin secretion, which leads to production of ATP from ADP[14]. The elevated concentration of ATP that results in an increased ATP/ADP ratio[14-17], triggers the closure of ATP sensitive potassium (K^+ATP) channels which are located in the plasma membrane. This results in a decrease of K^+ conductance through the plasma membrane and generates a wave of depolarization. Then, voltage dependent Ca^{2+} channels open with subsequent Ca^{2+} inflow. The resulting rise in cytoplasmic Ca^{2+} concentration is a signal for insulin secretion[17]. This stimulus-secretion pathway is amplified by another pathway that is K^+ATP independent[18]. The amplifying pathway is only functional when the intracellular Ca^{2+} concentration is at a stimulatory level, which occurs when glucose is above threshold level[18]. The amplifying pathway is activated by all metabolized nutrients that are oxidized similar to glucose metabolism. The second messenger(s) generated from the amplifying pathway are so far unknown.

Insulin release is phasic with a 1st phase that lasts less than 10 minutes, followed by an extended 2nd phase. A morphological basis for these phases may, in part, reside in the different pools of granules in the beta cell[13;19].

4.3.1.1 The K^+ATP channel

The K^+ATP channel is a complex between to intimately connected proteins: a tetramer high affinity sulphonylurea-receptor (SUR1) and a tetramer inwardly rectifying K^+channel (Kir 6.2)[17]. SUR1 is required for sulphonylurea- and Diazoxide-sensitivity towards the pore-forming Kir 6.2, and enabling these agents to close and open the channel, respectively[20].

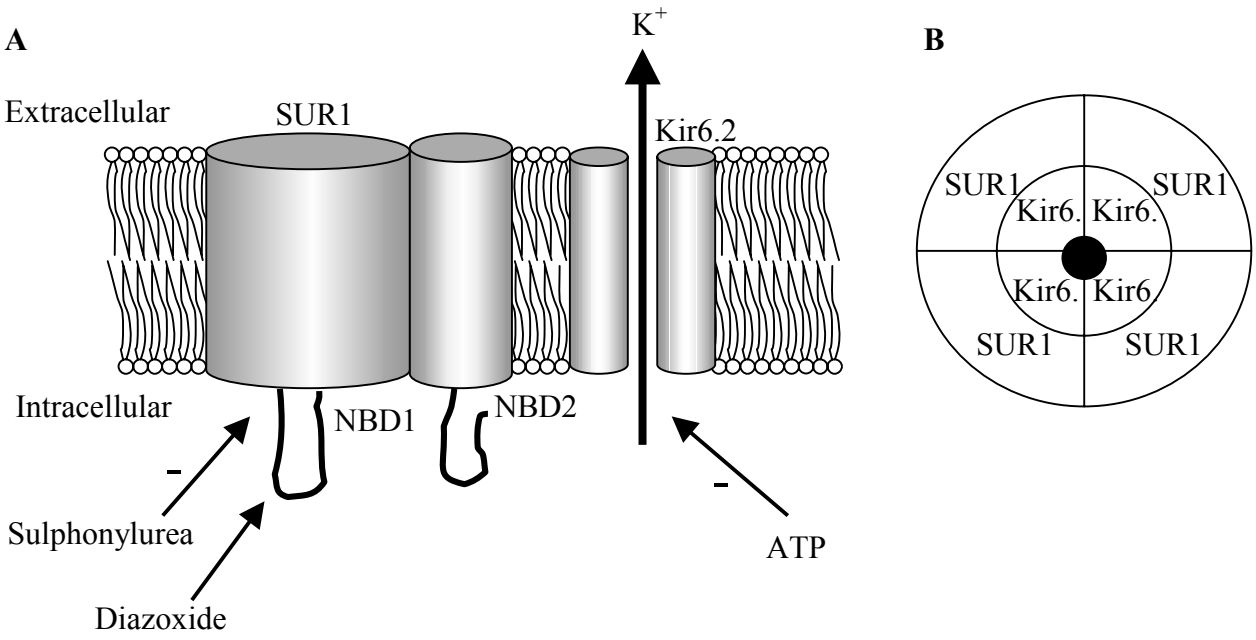

Figure 3. Regulation (A) and stochiometry (B) of the K^+ATP channels in the pancreatic beta cell (Nucleotide binding domains: NBD1, NBD2), adapted from [20].

4.3.2 Non-glucose influences on insulin secretion

Other nutrients, non-nutrient amino acids, neurotransmitters and hormones also influence insulin secretion[6]. Other nutrient secretagogues than glucose include fatty acids (FA, see below) and some amino acids, such as leucine, that are oxidized like glucose.

Other amino acids, mainly arginine, lysine and histidine, exert their effects as potentiators of glucose induced insulin secretion. They stimulate insulin secretion probably by adding positive intracellular charges[21].

Neurotransmitters such as acetylcholine enhance insulin release in response to vagal stimulation at mealtime. Glucagon-like-peptide-1 and gastrointestinal peptide are so-called incretin hormones. These hormones are released from the gastrointestinal tract during meals and enhance insulin responses postprandially. These and other hormones exert their effects mainly through the adenylate-cyclase pathway[22].

Inhibitors of insulin secretion are somatostatin, galanin and activation of alpha-adrenoreceptors. Inhibitors act by several mechanisms including interaction with G-membrane proteins[23].

4.3.3 Regulation of insulin biosynthesis and beta cell mass

Glucose is the main regulator of insulin biosynthesis. However, cAMP-raising agents such as the hormones and neurotransmitters mentioned above also participate. Fetal development of the beta cell population is regulated by transcription factors. Growth hormone and prolactin stimulate mitogenesis. Ex utero, the demands for insulin secretion are instrumental in regulating the beta cell mass[16]. Increased demands in pregnancy or obesity due to insulin resistance in these states will normally enhance the beta cell mass. The mechanisms behind such an enhancement are probably related to minute increases in blood glucose levels, which in turn induces hyperplasia, hypertrophy and neogenesis of beta cells.

5. INSULIN DEFICIENCY AND DYSREGULATION IN TYPE 2 DIABETES

5.1 GENERAL

Several aspects of insulin secretion are deficient or dysfunctional in type 2 diabetes[24] (Table 1). Insulin secretion in response to glucose is most profoundly affected[6;25]. First phase glucose induced insulin secretion is lost already in the prediabetic state[26-27]. The loss of 1st phase insulin secretion may be physiologically important, since it attenuates the important inhibiting effect of insulin on hepatic glucose production postprandially[28]. Type 2 diabetes of some duration displays further abnormalities of insulin secretion. Then, the glucose response can be totally obliterated, although insulin responses are evoked with other secretagogues[6]. Accompanying the insensitivity for glucose per se, potentiators fail to enhance secretion at elevated glucose levels[6;29].

Disordered pulsatility of insulin secretion is also frequently found in type 2 diabetes. This abnormality probably leads to a less efficient insulin secretion, which also is more energy demanding for the beta cell[30].

Increased proinsulin levels are also seen (absolute as well as relative to insulin levels)[31]. This abnormality could render the beta cell less efficient in lowering of blood glucose in target tissues. This is because proinsulin is less effective than insulin in lowering blood glucose. Proinsulin per se may also have negative effects, since proinsulin stimulates growth of endothelium, which possibly promotes atherogenesis[29].

Table 1. Abnormalities of insulin secretion in type 2 diabetes

1	*Loss of 1st phase insulin secretion after glucose stimulation*
2	*Reduced or blunted 2nd phase insulin secretion after correction for glycemia and insulin resistance.*
3	*Disturbed secretory oscillations*
4	*Increased release of partially processed precursors*
5	*Reduced potentiation by glucose of insulin responses to other secretagogues.*

Insulin secretion deteriorates with diabetes duration[32]. This deterioration is clinically very important, since it leads to worsening of metabolic control[32]. The causes for beta cell deterioration with time are so far not elucidated. Table 2 outlines possible causes of progressive beta cell dysfunction.

Table 2. Possible causes of progressive pancreatic beta cell dysfunction in type 2 diabetes.

	Causes	*Ref.*
1	*Morphological changes in islets* 1. Decreased beta cell mass 2. Accumulation of amyloid-like material	33 34
2	*Gene-related defects in stimulus secretion coupling* 1. Less efficient insulin synthesis and trafficking 2. Mutations in mitochondrial DNA	35 36
3	*Environmental alterations* 1. Insulin resistance	37
4	*Age*	38
5	*Diabetic state* 1. Hyperglycemia: glucose desensitization and/or toxicity 2. Hypertriglyceridemia with elevated FA 3. Overstimulation	11;39 40 41

5.2 GENETIC AND INTRAUTERINE CAUSES OF TYPE 2 DIABETES

Epidemiological and family studies demonstrate the importance of genetic factors for insulin secretion and beta cell function. Monogenic type 2 diabetes (maturity onset of diabetes of the young, MODY) may account for less than 5% of phenotypical type 2 diabetes. But the causes of MODY give insight in the importance of genetic effects on insulin secretion[42]. The remaining 95% of type 2 diabetes subjects probably have a polygenic and varied genetic predisposition for their disease.

Low birthweight entails a risk of type 2 diabetes as well as of cardiovascular disease [43]. This risk seems independent of genetic causes[44-45]. Low birth weight relates to insulin resistance in

man[46-47]. Also hyperglycemia during pregnancy may have negative effects on glucose homeostasis in the offspring[48-49].

5.3 EXTRAUTERINE CAUSES

Of relevance to type 2 diabetes are the effects of metabolic abnormalities (hyperglycemia and elevated FA).

5.3.1 Glucotoxicity

Chronic hyperglycemia is the principal cause of the late complications of diabetes such as retinopathy, neuropathy and nephropathy[32;50]. The mechanisms behind the effects of chronic hyperglycemia are probably multi-factorial, involving the formation of advanced glycosylation end products and other toxic compounds[51]. Effects of advanced glycosylation end products could be part of a more generalized negative effect of oxidative stress on beta cell. There is evidence that hyperglycemia could damage the beta cell by similar mechanisms[52-53]. However, hyperglycemia could also affect beta cell function through over-stimulation, i.e. through an indirect effect (see below).

5.3.2 Lipotoxicity

5.3.2.1 General

An acute elevation of FA results in a moderate increase in insulin secretion[54-55]. Longer-term effects of elevated FA levels are less well elucidated. Longer-term effects are clinically more important, since in type 2 diabetes, high levels of FA may persist for years and decades. Such levels are, in part at least, linked to lumbar obesity, especially in type 2 diabetes subjects[56].

5.3.2.2 In vitro and ex vivo

Time dependency and mechanisms

In pancreatic islets subjected to hyperglycemia, the long term elevation of FA levels exerts an additional negative influence on insulin secretion and glucose oxidation[57-58]. A time dependency for effects of FA on insulin secretion was demonstrated in rats. Hence, a 3-6h infusion of Intralipid stimulated glucose stimulated insulin secretion (GSIS), whereas a 48h infusion inhibited GSIS from perfused pancreas by 50%[57]. The negative effects of prolonged elevation of FA seem linked to FA oxidation. Thus, when Etomoxir (an inhibitor of FA oxidation[59]) was added in vitro to islets from rats formerly infused with Intralipid, GSIS and islet glucose oxidation improved.

In liver and muscle, pyruvate dehydrogenase (PDH) activity is a target for inhibitory FA effects[60]. In islets, a 48h FA exposure resulted in a reduction of the active form of PDH, and a concomitant increase of PDH kinase[61]. Some controversy exists as to the effects of FA on the PDH enzyme activity[62]. In any case effects on PDH do not completely explain negative effects on FA on insulin secretion[62-63] (Table 3). Other causes for negative effects of FA could include uncoupling of mitochondrial metabolism in the islets[64]. Such uncoupling would result in a less efficient glucose metabolism and possibly also an increase in reactive oxygen species[65]. Indeed, FA have been shown to induce the formation of uncoupling protein-2 in beta cells[66].

Table 3. Proposed mechanisms of long term effects of FA on beta cells.

Changes in islet glucose metabolism	Decreased PDH Increased PDH kinase activity Decreased pyruvate carboxylase activity Decreased glukokinase activity	[61] [61] [67] [68]
Alternative FA metabolism activated	FA esterification: TG formation	[69]
Excess acyl-CoA levels	Increased ceramide production resulting in nitric oxide production and apoptosis	[69]
Uncoupling	Partial uncoupling of oxidative phosphorylation	[65]

Triglyceride accumulation

Chronic elevation of FA results in an increased TG accumulation in islets[70-71]. Because of a leptin receptor mutation diabetic fa/fa rats accumulate 50-100 fold more TG than in non-diabetic rats[72]. In these animals accumulated TG have toxic effects on beta cells and cause cellular depletion and fibrosis. However, toxicity of TG stores in the beta cells of normal rats has not been verified[73].

The influence of FA on insulin biosynthesis, beta cell replication and beta cell mass

FA have negative effects on total protein biosynthesis and on glucose induced insulin biosynthesis, both in rat islets[74] and in human islets[58]. This could explain the lower insulin contents found in FA exposed beta cells, in which no crinophagy could be detected[75]. The negative effect on insulin biosynthesis seems maladapted and possibly deleterious in type 2 diabetes in light of the increased demands for insulin during hyperglycemia and insulin resistance.

FA have recently been shown to inhibit a glucose-dependent beta cell proliferation induced by insulin like growth factor-1[76], and can thus have the potential to reduce beta cell mass in vivo.

5.3.2.3 In vivo

Non-diabetes studies

In vivo studies are discrepant as to the documented inhibitory effects of FA on insulin secretion seen in vitro or ex vivo studies. Some of the divergence could result from a hyper-responsiveness to glucose in vivo, which may be secondary to FA-related decreases in sympathetic nerve activity as tested in the rat and in man[77-78].

Table 4. Effects of FA on insulin secretion in healthy individuals.

Conditions	*BMI (kg/m^2)**	*Change in insulin response*	*Ref.*
Intravenous glucose tolerance tests during 24h lipid infusion	25	Decrease	[79]
8,6mM glucose clamp with concomitant 48h lipid infusion	23	Increase	[80]
10/20mM glucose clamp following a 48h lipid infusion	*24*	Unaltered in spite of insulin resistance	[81]
Graded glucose infusion 1-8 mg/kg/min (~10mM) following a 48h lipid infusion	*31*	*Decrease*	[82]

*Mean of study group.

Divergent results were obtained also in non-diabetic man (Table 4). Paolisso et al. found decreased insulin secretion in response to an acute glucose challenge following a 24h Intralipid infusion[79]. The test procedure did not allow stabilization of blood glucose levels. Boden et al. corrected for glucose levels by running 8,6mM glucose clamps during a 48h Intralipid infusion[80] and found that the lipid infusions enhanced insulin levels.

Carpentier et al. performed high-glucose clamp studies after 48h lipid infusions in healthy individuals[81]. Insulin secretion was unchanged; this was judged as dysfunctional because of attendant FA-induced insulin resistance. Later the same group demonstrated that the lipid infusion a positive rather than negative effect on insulin secretion in type 2 diabetes patients[82]. However, the results varied much between the diabetic patients. Altogether, the discrepant results in man indicate that multiple factors influence the interaction of FA with beta cells. Genetic factors could be important, since a negative effect of Intralipid on insulin secretion was associated with a specific haplotype of the peroxisome proliferator activated receptor-γ (PPAR-γ) transcription factor[83].

The previous studies tested the effects of elevating FA (mostly for 24 to 48h). The effects of lowering FA have been studied only in one study[84]. Insulin secretion was improved after one week of the FA-lowering agent Acipimox. So far the effects of an acute lowering of FA levels on insulin secretion in subjects with chronically elevated FA levels, such as type II diabetic subjects, have not been investigated.

Effects of elevated FA during prolonged fasting

Stein and co-workers reported that acute lowering of FA by nicotinic acid inhibits glucose-induced insulin secretion in the rat in the fasted, but not in the fed state, and that the addition of FA reversed this inhibition[85]. The authors concluded that fasting made FA essential for

insulin secretion. They reproduced these findings to some extent in humans, using a similar protocol [86]. On the other hand, there is evidence in the rat that fasting induces an increase in fatty acid oxidation in pancreatic islets[87] and that increased fatty acid oxidation inhibits insulin secretion in islets from 48h fasted rats[88]. These findings, as well as others[57-58] are compatible with the operation of a glucose-fatty acid cycle also in pancreatic beta cells. Further studies are needed to resolve these discrepancies.

5.3.2.4 Treatment with FA lowering drugs

Acipimox is a nicotinic acid derivative. In man Acipimox acutely reduces levels of TG and VLDL by 30-40% and FA levels tenfold[89]. Acipimox reduces fasting blood glucose and hepatic glucose output and improves insulin sensitivity[90]. The drug has been used in clinical trials in type 2 diabetes[90-92]. However long term treatment with Acipimox did not ameliorate glucose control, probably due to rebound effects on FA levels[93]. Acipimox influences the levels of glycogen synthase, leptin, gastrointestinal peptide and glucagon-like peptide-1[94-96]. Apart from the rebound effects, the clinical use of Acipimox is somewhat limited by side effects such as flushing[97].

As mentioned above, one week of Acipimox reportedly enhanced GSIS[84], however, the acute effects of Acipimox on insulin secretion have not been tested in detail.

5.3.2.5 Effects of dietary fat on insulin secretion and sensitivity

A high fat diet inhibits beta cell function in diabetic mice[98-99]. These and other animal data suggest that dietary fat could be detrimental to beta cell function in a situation of fuel abundance with a preferential intake of fat (reviewed in [100]). Low fat diets increase insulin sensitivity in most dietary studies (in animals[101-102], and some, but not all human studies:[103-104]). In several of the diet intervention studies in diabetes, glucose control improved

markedly[105-106]. Such amelioration makes it impossible to distinguish effects of the diet per se on one hand and the well-documented and beneficial effect of glucose control[106-107] on the other. Another confounder is the weight reduction that is often seen and encouraged during low-fat dieting[106]. A negative energy balance is known to increase insulin sensitivity in type 2 diabetes subjects[105-106;108] and thus precludes the evaluation of any effect of fat reduction per se. There is thus a need for studies that examine the effects of low-fat dieting with minimal interference from the confounding factor mentioned. Such studies would also shed light on the influence of fat intake on the regulation of adipocyte-secreted hormones.

5.3.3 Effects of adipocyte-secreted hormones on insulin sensitivity

The discovery of leptin established the endocrine function of adipose tissue, with effects on satiety, but also metabolic pathways and control of starvation response[109-110]. Leptin regulates food intake, body weight, energy expenditure and neuroendocrine function[111-112]. Leptin levels are high in most models of obesity associated type 2 diabetes[113]. Leptin seems to exert a hypoglycemic effect independent of its weightreducing effects[114]. It also regulates peripheral glucose uptake in muscle and adipose tissue[115].

Adiponectin is a newly discovered adipocyte-derived hormone with potential importance for insulin sensitivity. Adiponectin levels are low in obese humans and low levels have been associated with insulin resistance[116-117]. So far, the receptor and downstream signalling pathways of adiponectin are unknown. However, studies in rodents[118] suggest that adiponectin equivalents might function in multiple tissues to ameliorate insulin resistance.

5.3.4 Overstimulation by hyperglycemia

Leahy[119] demonstrated that a period of hyperglycemia (48h) in normal rats, with insulin release tested in perfused pancreas, resulted in insensitivity that was glucose-specific. The

desensitization was reversible within 24h. Sako and Grill[41] blocked glucose-stimulated insulin secretion by a simultaneous Diazoxide infusion - in this setting, no desensitization occurred and insulin responses were even enhanced. Diazoxide reversibly blocks glucose-induced insulin secretion without any other major effects (see below). Therefore the desensitization by glucose described by Leahy could be attributed to overstimulation.

Using Diazoxide as a probe it was later shown that overstimulation also affects other aspects of beta cell function. Thus, tissue culture of human islets in high glucose media results in alterations in Ca^{2+} fluxes[120], some of which were protected against by Diazoxide. There is also evidence in a rat transplantation model that overstimulation can permanently damage beta cells[121].

5.3.4.1 Diazoxide

Diazoxide is non-diuretic thiazide that reversibly inhibits GSIS by opening K^+ ATP channels in the cell membrane of beta cells, thereby preventing beta cell depolarization[122]. An interesting peripheral effect is the recruitment and upregulation of insulin receptors[123].

The therapeutic use of Diazoxide is limited by side effects. The most common and noticeable side effects of Diazoxide are edema and lanugo hair growth. Other less common side effects are nausea and hypotension. Edema is treatable by diuretics and subsides, as do other side effects when Diazoxide treatment is discontinued[124]. Therefore the side effects are less serious, but still disturbing enough to create a major obstacle to treatment.

Clinical experience from treatment of insulinoma patients has shown that 100 mg Diazoxide three times daily is usually required to suppress glucose-induced insulin secretion[124]. Such or similar doses were given in the previous studies with diabetic patients[125-127]. Lower doses given three times daily could potentially diminish side effects, but would at the same time diminish the inhibitory effects on insulin secretion while not eliminating, in type 2 diabetes, a

potential need for 24h insulin treatment. Intermittent treatment with Diazoxide could give less side effects than a three times daily regimen.

Diazoxide inhibits endogenous insulin secretion. Therefore, to avoid deterioration in glycemic control, endogenous insulin should logically be replaced with exogenous insulin. A bedtime insulin regimen in combination with Diazoxide at bedtime could be added to daytime peroral anti-diabetic treatment without any need for major adjustments of treatment and less discomfort/adjustment for the patient. Sleep is a phase with low basal metabolic rate, most organs utilize this period for repair and regeneration. Intuitively, beta cell rest should take place at nightime.

To our knowledge, no studies have been performed to test this concept.

5.3.4.2 Clinical studies reducing overstimulation

Insulin treatment of type 2 diabetes patients improves insulin secretion[128-130]. Treatment of type 1 diabetes subjects treated with an intensive insulin regimen gave similar results[50]. These data do not distinguish between beneficial effects of "glucotoxicity" and over stimulation. Diazoxide also improved residual insulin secretion in type 1 diabetes[125-127]. Treatment with Diazoxide in type 2 diabetes patients for 7 days in an open trial resulted in an improved insulin secretion[125]. Diazoxide could possibly be more advantageous than insulin in reducing overstimulation, since the drug depresses insulin secretion more than insulin alone[131-132]. Also somatostatin[133] inhibits insulin secretion, but by mechanisms different from those of Diazoxide, i.e. by interaction with G-proteins in the beta cell membrane[23]. A somatotropin analogue exerted beneficial effects on beta cell function in humans[125].

6. AIMS

Overall objective:

To test for effects of alterations of fatty acid levels as well as beta cell stimulation on insulin secretion, insulin sensitivity and glycemic control in man.

Specific objectives:

- fasting protocol:

To test for an altered influence of FA on insulin secretion and insulin sensitivity after long-term fasting in healthy volunteers.

- Acipimox protocol:

To test in type 2 diabetes and in non-diabetic subjects for effects of acute lowering of FA and of re-introducing elevated levels of FA by lipid infusion on glucose-induced insulin secretion and insulin sensitivity.

- low-fat diet protocol:

To test whether a shift in nutrient utilization away from fat would affect insulin secretion, insulin sensitivity and adipocyte hormones in type 2 diabetes.

- Diazoxide protocol:

To test for improvement in endogenous insulin secretion and glycemic control by achieving beta cell rest through intermittent Diazoxide treatment in type 2 diabetes.
To monitor side effects of intermittent Diazoxide treatment.

7. SUBJECTS AND METHODS

7.1 SUBJECTS

All subjects were Caucasians. The subjects in paper 1 were healthy students recruited at the Medical Faculty at St. Olavs University Hospital.

The diabetic subjects in paper 2-3 were recruited from the outpatient clinic of the Department of Endocrinology, St. Olavs University Hospital. The non-diabetic subjects in paper 2 were blood donors from the blood donor unit at St. Olavs University Hospital.

Recruitment of diabetic subjects in paper 4 was from the Departments of Endocrinology at Levanger Hospital and St. Olavs University Hospital.

Table 6. Characteristics of the study participants and protocols in paper 1-4.

Paper	*No. of subjects*	*Age (years)*	*Body mass index (kg/m^2)*	*Duration of diabetes (years)*	*Type of clinical study*
1	14 (ND)	22 (18-28)	23,2 ± 0,8	-	Crossover, randomized
2	21 (D) 10 (ND)	56 (40-70) 55 (40-68)	31,2 ± 1,0 25,3 ± 0,6	6.9 -	Crossover, randomized
3	19 (D)	56 (40-69)	30,5 ± 1,1	6.6	Prospective intervention
4	27 (D)	59 (39-79)	28,6 ± 0,8	6.8	Double blinded, randomized

D=diabetes, ND=non-diabetes; results are given as mean ± SEM or mean (range).

7.2 HYPERGLYCEMIC CLAMP PROCEDURE

The (hyperglycemic clamp) procedure (Figure 4) is regarded as a gold standard to assess glucose stimulated insulin secretion. The procedure can also be used to assess insulin sensitivity [134-135].

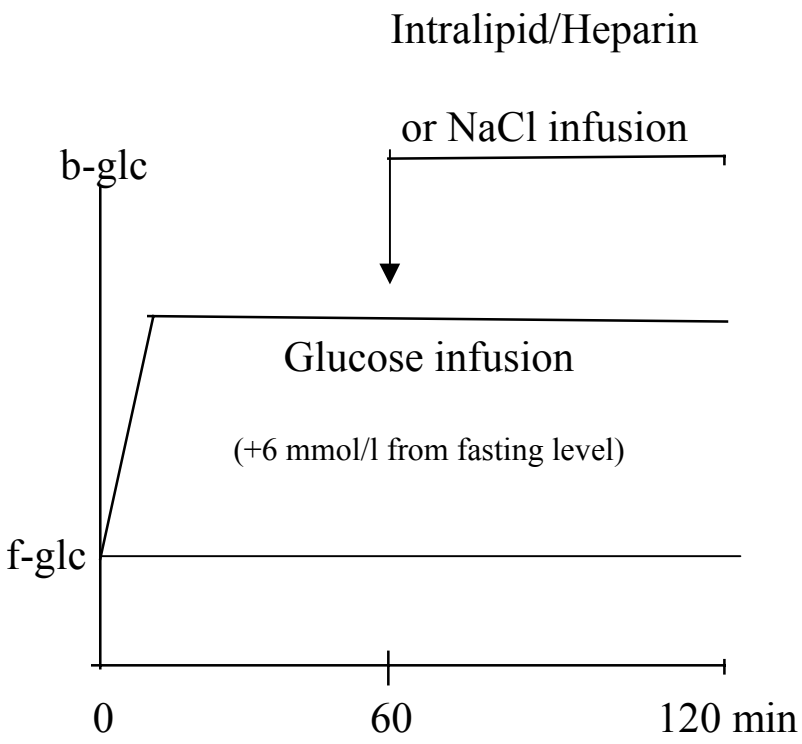

Figure 4. Schematic representation of the hyperglycemic clamp protocol used in papers 1-3.

In paper 1 (healthy subjects), we started a hyperglycemic clamp[136], aiming at a blood glucose level of 11mM. In paper 2 and 3 (diabetic subjects) the aim was to raise blood glucose 6mM above fasting levels. We measured blood glucose every 2,5 min in paper 1 and every 5 min in papers 2 and 3. The results of blood glucose measurements served to adjust the infusion rate of glucose. At min 60 of the on-going hyperglycemic clamp, an infusion of Intralipid (20%, 1 ml/min, Pharmacia, Uppsala, Sweden) and Heparin (0,24 U/kg/min, Leo, Ballerup, Denmark) was started. In paper 1, 6 subjects additionally received saline infusion during min 60-120.

7.3 C-PEPTIDE GLUCAGON TEST

Faber et al. introduced this test several decades ago[137]. It is a standard method of evaluating insulin secretion. There is extensive documentation of the test (reviewed in [138]). Studies of the test reproducibility are satisfactory[139].

The test was used in duplicate before and during interventions (paper 4). At each occasion we first collected fasting blood samples. Then, 1mg glucagon (Novo Nordisk) was injected i.v. Six min after the end of injection blood samples (C-peptide and insulin) were again drawn.

7.4 STANDARD MEAL TEST

In paper 4, patients ingested a standardized breakfast meal containing 47 energy % (E%) carbohydrates, 17 E% protein and 36 E% fat and a total of 470kcal. Repaglinide 0.5mg, (Novonorm™) was given together with the meal. We collected blood samples before the test and then every 15min for 2 hours.

7.5 NON-REGISTERED TEST SUBSTANCES

7.5.1 Acipimox

Acipimox (Olbetam™, Pharmacia Inc.) was requisitioned in the form of capsules from the hospital pharmacy.

7.5.2 Diazoxide

The Hospital Pharmacy, St.Olavs University hospital, Trondheim produced capsules of Diazoxide (Proglycem™, Schering Plough Inc.) and placebo. The Norwegian Drugs Control Authority approved the quality control standards of the production.

7.6 REGISTRATION OF DIET AND PHYSICAL ACTIVITY

In paper 2, we assessed diet and physical activity of diabetic subjects and non-diabetic subjects at inclusion, by a validated questionnaire[140]. The data were processed at the Institute of Nutrition Research, University of Oslo, in collaboration with one of the authors (ILM, clinical nutritionist).

The diabetic subjects registered food intake also by weighing records during 3 days of usual diet (6 days in paper 3) as well as during 3 days of dietary intervention (papers 2 and 3). To be comfortable with the method of weighing records, subjects individually practiced the method during the supervision by one of the authors (ILM). The intake of energy and nutrients was computed by using a food database (AKF96) and software systems (BEREGN) developed at the Institute of Nutrition Research, University of Oslo. The food database was mainly based on the official Norwegian Food Table[141].

7.7 BLOOD GLUCOSE MONITORING

Instructions in blood glucose monitoring were given individually by diabetes nurses when necessary. The subject monitored blood glucose at home (paper 3) with their own blood glucose measuring device. In paper 4 all subjects used the same type of device (Glucometer Elite XL, Medisense LTD).

7.8 LABORATORY ANALYSES

7.8.1 Blood glucose and glycosylated hemoglobin measurements

During hyperglycemic clamp procedures (paper 1-3), levels of blood and urinary glucose were determined by a glucose oxidase method (Yellow Springs Instrument, Yellow Springs, OH). In paper 4, we determined blood glucose levels using a reflectrometric device (Hemocue). Glycosylated hemoglobin was determined by DCA (Bayer).

7.8.2 Hormone analyses

In paper 1 immunoreactive insulin was measured by radioimmunoassay (RIA), using antibodies raised against porcine insulin (from the Department of Endocrinology, Karolinska Hospital, Stockholm, Sweden) and charcoal addition to separate bound and free insulin. This assay co-measures proinsulin. In papers 2-4 we used a RIA specific to human insulin (Linco Research Inc., Missouri, USA.).

We assayed C-peptide levels in paper 1 by RIA: in the case of fasting levels, by a kit from DPC, Los Angeles, USA and during clamps in 6 subjects by a kit from Linco Research Inc. C-peptide in papers 2-4 was measured by RIA (Linco Research Inc.).

Leptin and adiponectin levels (paper 3) were assayed with RIA kits from Linco Research Inc. Interassay coefficient of variation (CV) for leptin was 4,6% and the intrassay CV was 5,0%. Interassay coefficient of variation (CV) for adiponectin was 6,9% and the intrassay CV was 6,2%. Proinsulin was determined by ELISA (Dako Inc., Oslo, Norway). In glucagon samples a proteolytic inhibitor (Trasylol) was added to the tubes. Glucagon was measured by RIA (Linco Research Inc., paper 4). The other intra- and inter-assay coefficients of variance are given in the respective papers.

7.8.3 Lipid, fatty acid and ketone analyses

Samples for assay of FA were routinely stored at -20°C. Duplicate samples stored at -80°C and -20°C, did not differ with regard to the concentrations of FA measured in the assay, hence making it improbable that on-going lipolysis in the samples should affect the results as has been proposed[142]. The tubes for blood sampling for FA measurements contained EDTA. We did not routinely add other preservatives to samples for FA measurements. In two non-diabetic subjects we checked for the effect of adding Paraoxon (Sigma Chemical Co.) to the

samples. There was no effect of adding Paraoxon to samples obtained before the start of the Intralipid plus heparin infusion. However, in the samples obtained during the Intralipid + heparin infusion the FA levels were 40 % lower with than without Paroxon. An artefactual elevation of measured FA was thus restricted to the Intralipid infusion. We determined levels of FA by an enzymatic colorimetric method (NEFA-C-kit, Wako Pure Chemical Industries Ltd., Osaka, Japan). TG, cholesterol, and high-density lipoprotein cholesterol (HDL) were determined by standard laboratory techniques. Plasma phospholipid FA (PL-FA) were analyzed and quantified by gas chromatography[143]. Beta-hydroxybutyrate was determined by a Precision Xtra device (Medisense Products, Bedford, MA, USA[144].

7.8.4 Antibody analyses

The presence of antibodies against glutamic acid decarboxylase and islet cell antigen-2 was determined by RIA (Dianova GmbH, Hamburg, Germany). Insulin antibodies were determined by enzyme linked immunosorbent assay (Milenia-Biotec GmbH, Bad Nauheim, Germany).

7.9 RANDOMISATION PROCEDURES

We utilized drawing procedures in paper 1-2, i.e. for every chronological pair of subjects The first subject scheduled for testing would draw the sequence of tests for himself and the following subject.

Randomization in paper 4 was done by a computerized minimization procedure[145]. The randomization parameters were glycosylated hemoglobin (2), body mass index (1,5), age (1), C-peptide (1), sex (0,5) and diabetes duration (0,5) (weights in parentheses, based on [146]).

7.10 INSULIN SECRETION RATE

In papers 1-3, we computed insulin secretion rates (ISR) from the C-peptide data by a regression program (ISEC, version 3.4a) that derives parameters of C-peptide kinetics from the subject's sex, age, type (normal, obese, type 2 diabetes), weight and height[147]. Previous studies have shown that valid results can be obtained without adjusting for individual differences in C-peptide elimination[148].

7.11 ESTIMATES OF INSULIN SENSITIVITY AND INSULIN SECRETION

In paper 3 we calculated indices of beta cell function and insulin sensitivity based on fasting values before and after the low-fat diet intervention. Albareda et al. reviewed the existing methods of estimation and concluded that fasting indices give a fair representation of results obtained from more complex tests such as clamps. For beta cell function, the HOMA index gave the best estimate, whereas for insulin sensitivity, the fasting glucose to insulin ratio was the best alternative[149].

7.12 STATISTICAL METHODS

Statistical analysis was done using Statistical Package for the Social Sciences, version 10.0, Chicago, 2000. Significance testing was done by Student's paired *t* test, by independent samples *t*-test and, for non-normally distributed variables, by the Wilcoxon matched pairs signed-rank sum test or Mann-Whitney test. We evaluated bivariate correlations by Spearman's correlation coefficients. We performed linear regression by using Enter, Stepwise and Backward models. For repeated analyses ANOVA testing was done.

7.13 ETHICAL CONSIDERATIONS

The local ethical committee (paper 1-4) and the Norwegian Drug Control Authority (paper 2-4) approved the protocols that were used in the study. We conducted the studies in accordance with the Declaration of Helsinki. All subjects gave informed written consent.

8. SUMMARY OF THE RESULTS

Paper 1, fasting protocol:

We tested non-diabetic subjects after an overnight as well as after a 58h fast. They underwent hyperglycemic clamps during which either Intralipid + Heparin or saline was administered during the last 60 min. After the overnight fast insulin levels increased during Intralipid infusion, reaching at min 120 an increment of 33,0 ± 8,5 μU/ml vs. 9,5 ± 4,4 μU/ml during saline, $p<0,05$ for difference. Conversely, after the 58 h fast, the Intralipid infusion was not associated with any successive increase of insulin levels (increment during Intralipid at min 120: 0,5 ± 5,8 μU/ml versus -4,3 ± 2,5 μU/ml during saline, NS). Insulin sensitivity as assessed by the amount of infused glucose, M and ratios of M to insulin increased during Intralipid after an overnight fast but decreased after a 58 h fast. We conclude that long term elevation of FA during fasting associates with diminished beta cell responsiveness to an acute elevation of FA in conjunction with negative effects on insulin sensitivity.

Paper 2, Acipimox protocol:

Twenty-one subjects with type 2 diabetes and hypertriglyceridemia and ten non-diabetic subjects participated. Acipimox lowered FA levels and enhanced insulin sensitivity (i.e. amount of glucose infused) in diabetic and non-diabetic subjects alike. Acipimox administration failed to affect insulin secretion rates in non-diabetic subjects. In the diabetic subjects there was a significant effect of Acipimox in the dichotomized group having the lowest levels of glycosylated hemoglobin (HbA1c) on integrated insulin secretion rates during min 60-120 min 379 ± 34 with vs. 326 ± 30 $pmol \cdot kg^{-1} \cdot min^{-1}$ without Acipimox, $P<0.05$. In the diabetic subjects, three days of a low-fat diet diminished energy from fat by from 39 to 23% without improving an insulin response to glucose.

Paper 3, low-fat diet protocol:

Nineteen type 2 diabetes subjects with hypertriglyceridemia reduced energy % (E%) from fat from 39 to 23 without weight loss. Total cholesterol decreased to 95% of baseline (P<0.011), HDL to 97% (P<0.083), triglycerides to 91% (P< 0.055) and the ratio of n-6 to n-3 FA to 76% (P<0.001). Levels of blood glucose and fasting glucose to insulin ratios were unaltered, but fasting insulin concentrations tended to increase at moderate and decrease at pronounced fat reduction, as achieved in individual patients. Fasting levels of leptin decreased to 90% of baseline (p<0.013) and of adiponectin to 92% (P<0,055). The leptin decrease was unrelated to fat reduction and confined to women. A successful short-term fat reduction alters lipid variables in type 2 diabetes without affecting glycemic control. The effects of the intervention vary with gender and degree of fat reduction.

Paper 4, Diazoxide protocol:

Twenty-seven type 2 diabetic subjects (17M, 10F) using BTI and metformin participated. Half of the subjects received added treatment with Diazoxide, 100mg at bedtime, and half with placebo for 9 weeks. We registered no side effects of Diazoxide. Treatment with Diazoxide did not incur any increase in BTI. Plasma C-peptide responses to glucagon increased (0,15 ± 0,06nmol/l vs. -0,01 ± 0,04nmol/l for placebo, p<0,06 for difference). Plasma insulin levels were 66,2 ± 41,7pmol/l for Diazoxide vs. -84,2 ± 51,5, for placebo p<0,03. Bedtime Diazoxide decreased fasting glucagon levels by 41% vs. placebo, p<0,03. HbA1c levels did not change, whereas levels of blood glucose post breakfast were higher during Diazoxide (1,34 ± 0,43mmol/l, p<0,01 vs. placebo), but not at other time points. A breakfast test in the presence of repaglinide elicited a robust insulin response in Diazoxide treated subjects. Diazoxide taken at bedtime with BTI was well tolerated and produced beneficial effects on insulin and glucagon secretion, but failed to improve metabolic control.

9. DISCUSSION

9.1 CHARACTERISTICS OF THE STUDY POPULATIONS

This thesis included two different populations with type 2 diabetes. For the studies on FA we selected subjects who had abnormal FA metabolism as judged from the elevated levels of TG. Such selection is not particularly exclusive, since elevated levels of TG are commonly seen in type 2 diabetes. Therefore the subjects selected are representative for a large segment of type 2 diabetes patients in Norway[4].

In the Diazoxide study the subjects recruited were considered in need of insulin treatment by the physicians responsible for their treatment. Also this group constitutes an important part of all type 2 diabetes patients. Regarding representativity, the majority of type 2 diabetes patients in Norway are referred to the hospital outpatient clinics for commencement of insulin treatment. Thus, although we mainly recruited patients at two diabetes clinics, the patients may be less sub-selected than suspected.

For both study populations, we found considerable variability in several diabetes-related characteristics between individuals. It is becoming increasingly clear that such variability is typical for type 2 diabetes[150].

9.2 COMPLIANCE

In the fasting protocol, the biochemical parameters (pre-test blood glucose, ketones, C-peptide, insulin and FA levels) all showed the changes expected after long term fasting in healthy subjects[151]. These observations confirm a good compliance.

With regard to dietary interventions in papers 2-3, it should be noted that such dietary interventions often have problems with compliance. Conditions favorable for compliance in our dietary intervention, were the short-term nature of the dietary manipulation and the daily contacts with the subjects before, during and after the intervention. Also indicative of good

compliance with the prescribed diet were the observations that lipid levels including PL-FA changed in a manner commensurate with that of the dietary prescriptions[152-154].

In the Diazoxide trial, blood glucose measurements in the clinic corresponded well with the reported home monitoring levels. This observation confirms good compliance also in this study.

9.3 INFLUENCES BY FASTING, ACUTE PHARMACOLOGICAL REDUCTION OF FA LEVELS AND DIET INTERVENTION ON INSULIN SECRETION

9.3.1 The influence of fasting on FA interactions with insulin secretion.

The results in healthy volunteers indicate that long term fasting blunts a stimulatory effect of FA on glucose-induced insulin secretion. The effect did not seem transient, since the attenuation of FA-induced insulin secretion became successively more marked during the Intralipid infusion. At first glance, this effect of fasting is difficult to reconcile with the "essentiality" for FA in the fasted state as reported by Stein et al.[85]. However it cannot be excluded that a dose-response for FA effects on insulin secretion during long term fasting would include essentiality for low concentrations and blunted stimulation for high concentrations of FA.

Long term fasting exerted only minor effects on glucose-induced insulin secretion whereas other studies have shown somewhat larger effects[155]. It seems possible that the light restriction in physical activity that we imposed on the subjects during fasting would, by decreasing insulin sensitivity, counteract to some extent a decrease in insulin secretion. Indeed, long term fasting was associated with a marked decrease in insulin sensitivity.

9.3.2 The influence of Acipimox on FA interactions with insulin secretion.

In paper 2 we find differences in FA interactions between non-diabetic and diabetic subjects. Thus, Intralipid infusion on a hyperglycemic background was not accompanied by enhanced insulin secretion in diabetes whereas in non-diabetic subjects we found a stimulatory effect. Moreover we found distinct differences within the diabetic subjects that we studied. Thus, we demonstrate for the first time that an insulin response during hyperglycemia and Intralipid was detectable after Acipimox in the subset of the diabetic subjects that had the best metabolic control. Acipimox was on the other hand without effect in non-diabetic subjects. It seems probable – but not proven - that the Acipimox effect in the subset of diabetic subjects is related to the chronic hypertriglyceridemia and the chronically elevated FA in these patients rather than to the fact that the patients had diabetes. Such a conclusion is in line with the studies of Carpentier et al. Those investigations found no effect by an increase of FA in non-diabetic lean subjects[81], a negative one in obese subjects with hypertriglyceridemia[82], and a positive (enhancing) one in diabetic subjects[82] who did not demonstrate the same degree of hypertriglyceridemia as in the present study.

9.3.3 The influence of low-fat diet on FA interactions with insulin secretion and insulin sensitivity.

The Norwegian diabetic subjects in the low-fat diet study had, previous to the intervention, a diet with low carbohydrate content and a high fat and high normal protein intake compared to current recommendations[156]. Diet is reported to have a major influence on glucose metabolism and can, when combined with physical activity, prevent type 2 diabetes[157]. These measures seem to have as great an impact on prevention of type 2 diabetes as anti diabetic

medications[158]. However, although excessive caloric intake induces insulin resistance, it is not clear whether specific dietary constituents are important or not.

The dietary intervention was sucessful assessed both from weighing and from biochemical data.

Initially we were surprised to find reduced insulin sensitivity during low-fat diet intervention as evidenced from the tests of paper 2 and from the data presented in paper 3. However, closer perusal of the litterature revealed that beneficial effects on insulin sensitivity were usually coupled to attendant weight reduction (reviewed in [108]). There exists no clear discrepancy with other studies employing isoenergetic diets. In fact, our study could be seen to complement these earlier long term studies by demonstrating that a short term intervention produces similar results.

When our study group was dichotomized on basis of degree of fat reduction, a more complex response was seen. A moderate fat reduction decreased insulin sensitivity, whereas a more pronounced fat reduction tended to improve insulin sensitivity. Although the strength of these findings is compromised by their post-hoc nature, there was consistency between the clamp data and fasting parameters as to the diverse effect of diffents degrees of fat reduction.

The adipocyte-derived hormones leptin and adiponectin both decreased after the low fat diet intervention. With regard to adiponectin, we found a strong tendency for a decrease as a result of the dietary intervention. Low levels of adiponectin associate with insulin resistance[116]. Therefore, it seems possible that the decrease in adiponectin – for which we have no ready explanation – could be important for the insulin resistance effect by the low fat diet.

9.4 INFLUENCES ON OVERSTIMULATION BY GLUCOSE BY BETA CELL REST INDUCED BY DIAZOXIDE AND BTI TREATMENT

We found an improved insulin secretion during Diazoxide treatment in terms of responses to glucagon in the C-peptide glucagon tests. This improved insulin secretion was obtained in spite of other evidence that an inhibitory effect of Diazoxide on insulin secretion was still present, i.e. somewhat higher glucose levels in the morning. This possible discrepancy can be explained by the selectivity of Diazoxide´s inhibitory effect on glucose induced insulin secretion. Thus secretion by insulin secretagogues that operate through other signal-secretion pathways (e.g. glucagon and glucagon like peptide-1) are not inhibited by Diazoxide [55;159-161]. The requirement for BTI did not increase during Diazoxide relative to placebo treatment (using the same algorithm for adjusting doses[162]). This indicates an increase in insulin sensitivity. A precedent for such an effect exists in animal models[163-5]. In our study, the reduction of glucagon levels that we find could, in part at least, explain an insulin-sensitizing effect by Diazoxide.

We designed the standardized breakfast test to investigate potential problems in combining repaglinide and Diazoxide treatment in further clinical studies. Also, we wished to see whether subjects on Diazoxide could indeed respond with insulin release to a breakfast together with repaglinide, despite a lingering inhibition of glucose-induced insulin secretion. The meal test with a low dose of peroral repaglinide demonstrated a robust insulin response in the Diazoxide-treated group. Altogether these findings suggest that Diazoxide-treated subjects would enhance insulin secretion during meals more than subjects not treated with this drug, provided that a) higher doses of repaglinide and similar agents acting on the K^+ATP-channels were used, b) these agents were to be used also at later meals when the counteracting effect of Diazoxide is gone. This notion is supported by a study in dogs demonstrating that previous Diazoxide actually enhances the subsequent insulin response to tolbutamide[165].

Beta cell capacity correlates to the beneficial effect that normalization of blood glucose exerts on insulin secretion[166]. Our patients, however, had a rather long duration of known diabetes and were clinically in the stage of the disease in which some form of insulin treatment was considered in order to improve a deteriorating metabolic control. Specifically, one should modify the selection of patients in future studies.

9.5 CONSEQUENCES FOR RESEARCH AND TREATMENT STRATEGIES IN TYPE 2 DIABETES

Based on our findings with interventions on FA levels (papers 2) and on beta cell rest (paper 4), one may envisage a "window" for therapeutic intervention on beta cell function (Figure 5). Such a "window" could be present during the progression of metabolic dysfunction from pre-diabetic obesity to manifest type 2 diabetes, but could be lost at later stages of the disease.

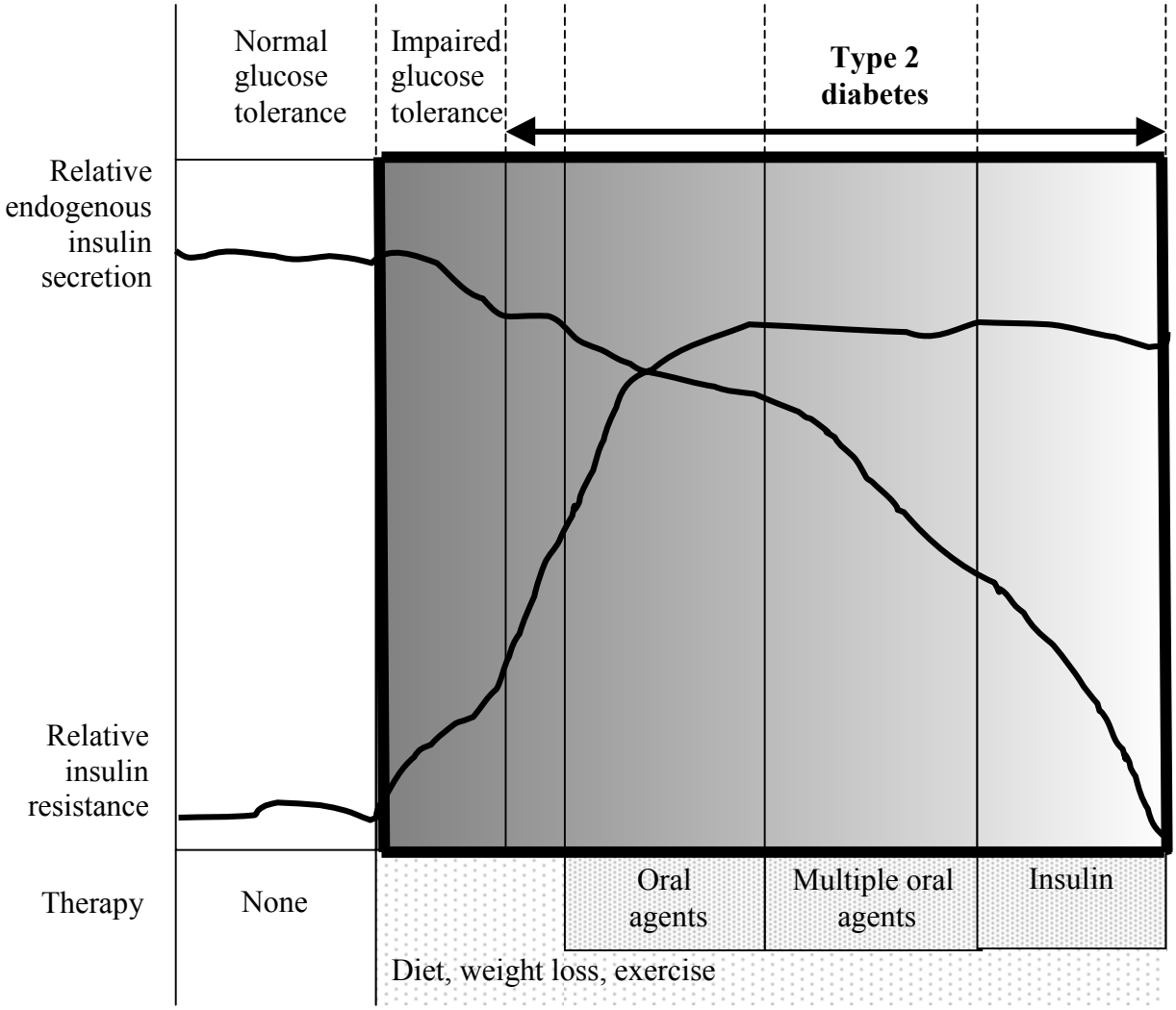

Figure 5. Hypothetical window for effect of intervention on beta cell function. The intensity of the "framed" area indicates the time segment where intervention seems to be most efficient (adapted from [167]).

10. SUMMARY AND CONCLUSIONS

Main findings:

- Long term elevated FA during fasting associates with diminished beta cell responsiveness to an acute elevation of FA in conjunction with negative effects on insulin sensitivity.
- An acute reduction of FA improves stimulated insulin secretion in a subset of type 2 diabetes subjects.
- A successful short-term reduction of fat intake in hypertriglyceridemic type 2 diabetic subjects fails to affect insulin secretion and metabolic control, whereas insulin sensitivity is unaltered or diminished.
- A low fat diet decreases levels of leptin and adiponectin without a direct relation to fat intake.
- A 9-week intervention with 100mg Diazoxide taken at bedtime with BTI is well tolerated, also when 100mg Diazoxide taken 8-10h previous to 0.5mg repaglinide. Treatment is associated with improvement in insulin secretion parameters.

11. REFERENCES

1. Alberti KG, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet.Med.* 15:539-553, 1998

2. Lernmark Å, Möller C, Kockum I, Sanjeevi C: Autoimmune endocrinopathies 3, islet cell autoimmunity. *J.Intern.Med.* 234:361-369, 1993

3. Lundman B, Engström L: Diabetes and it's complications in a Swedish county. *Diabetes Res.Clin.Pract.* 39:157-164, 1998

4. Midthjell K, Krüger O, Holmen J et al.: Rapid changes in the prevalence of obesity and known diabetes in an adult Norwegian population. The Nord-Trøndelag Health Surveys: 1984- 1986 and 1995-1997. *Diabetes Care* 22:1813-1820, 1999

5. McCarty, D. and Zimmet, P. Z. Diabetes 1994 to 2010: Global estimates and projections. 1994. Melbourne, Australia, International Diabetes Institute.

6. Kahn SE: Regulation of beta cell function in vivo: From health to disease. *Diabetes Rev* 4:372-389, 1996

7. Karam JH: Reversible insulin resistance in non-insulin-dependent diabetes mellitus. *Horm.Metab Res.* 28:440-444, 1996

8. Le Roith D, Zick Y: Recent advances in our understanding of insulin action and insulin resistance. *Diabetes Care* 24:588-597, 2001

9. Carlsson S, Persson PG, Alvarsson M et al.: Weight history, glucose intolerance, and insulin levels in middle-aged Swedish men. *Am.J.Epidemiol.* 148:539-545, 1998

10. Helmrich SP, Ragland DR, Leung RW, Paffenbarger RS, Jr.: Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus. *N.Engl.J.Med.* 325:147-152, 1991

11. Yki-Järvinen H: Pathogenesis of non-insulin-dependent diabetes mellitus. *Lancet* 343:91-95, 1994

12. Masharani U, Karam JH: Pancreatic hormones and diabetes mellitus. In Basic and clinical endocrinology. 6th ed. ed. Greenspan FS, Gardner DG, Eds. New York, McGrawHill, 2001, p. 623-698

13. Lang J: Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. *Eur.J.Biochem.* 259:3-17, 1999

14. Matschinsky F, Sweet I: Annotated questions and answers about glucose metabolism and insulin secretion of beta cells. *Diabetes Rev* 4:130-144, 1996

15. Dumonteil E, Philippe J: Insulin gene: organisation, expression and regulation. *Diabetes Metab* 22:164-173, 1996

16. Bonner-Weir S: Islet growth and development in the adult. *J Mol.Endocrinol.* 24:297-302, 2000

17. Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P: Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem.* 55 Suppl:54-65, 1994

18. Henquin JC: Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751-1760, 2000

19. Eliasson L, Renström E, Ding WG, Proks P, Rorsman P: Rapid ATP-dependent priming of secretory granules precedes Ca(2+)- induced exocytosis in mouse pancreatic B-cells. *J.Physiol* 503 (Pt 2):399-412, 1997

20. Ashcroft FM, Gribble FM: ATP-sensitive K+ channels and insulin secretion: their role in health and disease. *Diabetologia* 42:903-919, 1999

21. Strandgaard C, Curry DL: Differential insulin secretory responses to cationic and branched-chain amino acids. *Pancreas* 17:65-71, 1998

22. Cell signaling. In Molecular biology of the cell. 3rd ed. Alberts B, Ed. New York, Garland Publishing Inc., 1994, p. 721-785

23. Sharp GW: Mechanisms of inhibition of insulin release. *Am.J.Physiol* 271:C1781-C1799, 1996

24. Grill V, Björklund A: Dysfunctional insulin secretion in type 2 diabetes: role of metabolic abnormalities. *Cell Mol.Life Sci.* 57:429-440, 2000

25. Taylor SI, Accili D, Imai Y: Insulin resistance or insulin deficiency. Which is the primary cause of NIDDM?. *Diabetes* 43:735-740, 1994

26. Cerasi E, Luft R: "What is inherited--what is added" hypothesis for the pathogenesis of diabetes mellitus. *Diabetes* 16:615-627, 1967

27. Matsuda A, Kuzuya T: The prevalence of low insulin responders to oral glucose load among groups with various patterns of family history of diabetes. *Diabet.Med.* 13:S59-S62, 1996

28. Luzi L, DeFronzo RA: Effect of loss of first-phase insulin secretion on hepatic glucose production and tissue glucose disposal in humans. *Am.J Physiol* 257:E241-E246, 1989

29. Porte D, Jr.: Banting lecture 1990. Beta cells in type II diabetes mellitus. *Diabetes* 40:166-180, 1991

30. Porksen N: The in vivo regulation of pulsatile insulin secretion. *Diabetologia* 45:3-20, 2002

31. Hamman RF: Genetic and environmental determinants of non-insulin-dependent diabetes mellitus (NIDDM). *Diabetes Metab Rev.* 8:287-338, 1992

32. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes

(UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 352:837-853, 1998

33. Rahier J, Goebbels RM, Henquin JC: Cellular composition of the human diabetic pancreas. *Diabetologia* 24:366-371, 1983

34. Bennet WM, Smith DM, Bloom SR: Islet amyloid polypeptide: does it play a pathophysiological role in the development of diabetes?. *Diabet.Med.* 11:825-829, 1994

35. Steiner DF, Tager HS, Chan SJ, Nanjo K, Sanke T, Rubenstein AH: Lessons learned from molecular biology of insulin-gene mutations. *Diabetes Care* 13:600-609, 1990

36. van den Ouweland JM, Lemkes HH, Ruitenbeek W et al.: Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nat.Genet.* 1:368-371, 1992

37. Kahn SE, Prigeon RL, Schwartz RS et al.: Obesity, body fat distribution, insulin sensitivity and Islet beta cell function as explanations for metabolic diversity. *J Nutr.* 131:354S-360S, 2001

38. Coordt MC, Ruhe RC, McDonald RB: Aging and insulin secretion. *Proc.Soc.Exp.Biol.Med.* 209:213-222, 1995

39. Leahy JL, Bonner-Weir S, Weir GC: Beta cell dysfunction induced by chronic hyperglycemia. Current ideas on mechanism of impaired glucose-induced insulin secretion. *Diabetes Care* 15:442-455, 1992

40. Unger RH, Orci L: Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J.* 15:312-321, 2001

41. Sako Y, Grill VE: Coupling of beta cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats. *Diabetes* 39:1580-1583, 1990

42. Hattersley AT: Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity. *Diabet.Med.* 15:15-24, 1998

43. Hales CN, Barker DJ: Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 35:595-601, 1992

44. Poulsen P, Vaag AA, Kyvik KO, Moller JD, Beck-Nielsen H: Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs. *Diabetologia* 40:439-446, 1997

45. Carlsson S, Persson PG, Alvarsson M et al.: Low birth weight, family history of diabetes, and glucose intolerance in Swedish middle-aged men. *Diabetes Care* 22:1043-1047, 1999

46. Phillips DI, Barker DJ, Hales CN, Hirst S, Osmond C: Thinness at birth and insulin resistance in adult life. *Diabetologia* 37:150-154, 1994

47. Clausen JO, Borch-Johnsen K, Pedersen O: Relation between birth weight and the insulin sensitivity index in a population sample of 331 young, healthy Caucasians. *Am.J Epidemiol.* 146:23-31, 1997

48. Martin AO, Simpson JL, Ober C, Freinkel N: Frequency of diabetes mellitus in mothers of probands with gestational diabetes: possible maternal influence on the predisposition to gestational diabetes. *Am.J.Obstet.Gynecol.* 151:471-475, 1985

49. Pettitt DJ, Aleck KA, Baird HR, Carraher MJ, Bennett PH, Knowler WC: Congenital susceptibility to NIDDM. Role of intrauterine environment. *Diabetes* 37:622-628, 1988

50. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N.Engl.J.Med.* 329:977-986, 1993

51. Bertuzzi F, Saccomanno K, Socci C et al.: Long-term in vitro exposure to high glucose increases proinsulin-like- molecules release by isolated human islets. *J.Endocrinol.* 158:205-211, 1998

52. Kaneto H, Kajimoto Y, Miyagawa J et al.: Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta cells against glucose toxicity. *Diabetes* 48:2398-2406, 1999

53. Tanaka Y, Gleason CE, Tran PO, Harmon JS, Robertson RP: Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc.Natl.Acad.Sci.U.S.A* 96:10857-10862, 1999

54. Crespin SR, Greenough WB, III, Steinberg D: Stimulation of insulin secretion by infusion of free fatty acids. *J.Clin.Invest* 48:1934-1943, 1969

55. Malaisse WJ, Malaisse-Lagae F: Stimulation of insulin secretion by noncarbohydrate metabolites. *J.Lab Clin.Med.* 72:438-448, 1968

56. Lönnqvist F, Thome A, Nilsell K, Hoffstedt J, Arner P: A pathogenic role of visceral fat beta 3-adrenoceptors in obesity. *J Clin Invest* 95:1109-1116, 1995

57. Sako Y, Grill VE: A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580-1589, 1990

58. Zhou YP, Grill V: Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J.Clin.Endocrinol.Metab* 80:1584-1590, 1995

59. Wolf HP: Possible new therapeutic approach in diabetes mellitus by inhibition of carnitine palmitoyltransferase 1 (CPT1). *Horm.Metab Res.Suppl* 26:62-67, 1992

60. Randle PJ, Kerbey AL, Espinal J: Mechanisms decreasing glucose oxidation in diabetes and starvation: role of lipid fuels and hormones. *Diabetes Metab Rev* 4:623-638, 1988

61. Zhou YP, Grill VE: Palmitate-induced beta cell insensitivity to glucose is coupled to decreased pyruvate dehydrogenase activity and enhanced kinase activity in rat pancreatic islets. *Diabetes* 44:394-399, 1995

62. Liu YQ, Tornheim K, Leahy JL: Glucose-fatty acid cycle to inhibit glucose utilization and oxidation is not operative in fatty acid-cultured islets. *Diabetes* 48:1747-1753, 1999

63. Segall L, Lameloise N, Assimacopoulos-Jeannet F et al.: Lipid rather than glucose metabolism is implicated in altered insulin secretion caused by oleate in INS-1 cells. *Am.J.Physiol* 277:E521-E528, 1999

64. Dalgaard LT, Pedersen O: Uncoupling proteins: functional characteristics and role in the pathogenesis of obesity and Type II diabetes. *Diabetologia* 44:946-965, 2001

65. Carlsson C, Borg LA, Welsh N: Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140:3422-3428, 1999

66. Li LX, Skorpen F, Egeberg K, Jørgensen IH, Grill V: Induction of uncoupling protein 2 mRNA in beta cells is stimulated by oxidation of fatty acids but not by nutrient oversupply. *Endocrinology* 143:1371-1377, 2002

67. Iizuka K, Nakajima H, Namba M et al.: Metabolic consequence of long-term exposure of pancreatic beta cells to free fatty acid with special reference to glucose insensitivity. *Biochim.Biophys.Acta* 1586:23-31, 2002

68. Gremlich S, Bonny C, Waeber G, Thorens B: Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. *J Biol.Chem.* 272:30261-30269, 1997

69. Unger RH, Zhou YT: Lipotoxicity of beta cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 1:S118-S121, 2001

70. Zhou YP, Ling ZC, Grill VE: Inhibitory effects of fatty acids on glucose-regulated B-cell function: association with increased islet triglyceride stores and altered effect of fatty acid oxidation on glucose metabolism. *Metabolism* 45:981-986, 1996

71. Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH: Beta cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta cell relationships. *Proc.Natl.Acad.Sci.U.S.A* 91:10878-10882, 1994

72. Unger RH, Orci L: Lipotoxic diseases of nonadipose tissues in obesity. *Int.J.Obes.Relat Metab Disord.* 24 Suppl 4:S28-S32, 2000

73. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 50:1771-1777, 2001

74. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J.Clin.Invest* 93:870-876, 1994

75. Bollheimer LC, Skelly RH, Chester MW, McGarry JD, Rhodes CJ: Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J.Clin.Invest* 101:1094-1101, 1998

76. Rhodes CJ: IGF-I and GH post-receptor signaling mechanisms for pancreatic beta- cell replication. *J Mol.Endocrinol.* 24:303-311, 2000

77. Magnan C, Collins S, Berthault MF et al.: Lipid infusion lowers sympathetic nervous activity and leads to increased beta cell responsiveness to glucose. *J.Clin.Invest* 103:413-419, 1999

78. Magnan C, Cruciani C, Clement L et al.: Glucose-induced insulin hypersecretion in lipid-infused healthy subjects is associated with a decrease in plasma norepinephrine concentration and urinary excretion. *J Clin Endocrinol.Metab* 86:4901-4907, 2001

79. Paolisso G, Gambardella A, Amato L, Tortoriello R, D'Amore A, Varricchio M, D'Onofrio F: Opposite effects of short- and long-term fatty acid infusion on insulin secretion in healthy subjects. *Diabetologia* 38:1295-1299, 1995

80. Boden G, Chen X, Rosner J, Barton M: Effects of a 48-h fat infusion on insulin secretion and glucose utilization. *Diabetes* 44:1239-1242, 1995

81. Carpentier A, Mittelman SD, Lamarche B, Bergman RN, Giacca A, Lewis GF: Acute enhancement of insulin secretion by FFA in humans is lost with prolonged FFA elevation. *Am.J.Physiol* 276:E1055-E1066, 1999

82. Carpentier A, Mittelman SD, Bergman RN, Giacca A, Lewis GF: Prolonged elevation of plasma free fatty acids impairs pancreatic beta- cell function in obese nondiabetic humans but not in individuals with type 2 diabetes. *Diabetes* 49:399-408, 2000

83. Stefan N, Fritsche A, Haring H, Stumvoll M: Effect of experimental elevation of free fatty acids on insulin secretion and insulin sensitivity in healthy carriers of the Pro12Ala polymorphism of the peroxisome proliferator--activated receptor-gamma2 gene. *Diabetes* 50:1143-1148, 2001

84. Paolisso G, Tagliamonte MR, Rizzo MR et al.: Lowering fatty acids potentiates acute insulin response in first degree relatives of people with type II diabetes. *Diabetologia* 41:1127-1132, 1998

85. Stein DT, Esser V, Stevenson BE et al.: Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J.Clin.Invest* 97:2728-2735, 1996

86. Dobbins RL, Chester MW, Daniels MB, McGarry JD, Stein DT: Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. *Diabetes* 47:1613-1618, 1998

87. Tamarit-Rodriguez J, Vara E, Tamarit J: Starvation-induced changes of palmitate metabolism and insulin secretion in isolated rat islets stimulated by glucose. *Biochem.J.* 221:317-324, 1984

88. Zhou YP, Priestman DA, Randle PJ, Grill VE: Fasting and decreased B cell sensitivity: important role for fatty acid- induced inhibition of PDH activity. *Am.J.Physiol* 270:E988-E994, 1996

89. Shepherd, J. The action of nicotinic acid and its analogues on lipoprotein metabolism. Gotto, A. M. and Paoletti, R. (22), 207-212. 1991. New York, Raven Press Ltd. Atherosclerosis Reviews.

90. Fulcher GR, Catalano C, Walker M et al.: A double blind study of the effect of acipimox on serum lipids, blood glucose control and insulin action in non-obese patients with type 2 diabetes mellitus. *Diabet.Med.* 9:908-914, 1992

91. Worm D, Henriksen JE, Vaag A, Thye-Ronn P, Melander A, Beck-Nielsen H: Pronounced blood glucose-lowering effect of the antilipolytic drug acipimox in noninsulin-dependent diabetes mellitus patients during a 3- day intensified treatment period. *J.Clin.Endocrinol.Metab* 78:717-721, 1994

92. Saloranta C, Groop L, Ekstrand A, Franssila-Kallunki A, Eriksson J, Taskinen MR: Different acute and chronic effects of acipimox treatment on glucose and lipid metabolism in patients with type 2 diabetes. *Diabet.Med.* 10:950-957, 1993

93. Vaag AA, Beck-Nielsen H: Effects of prolonged Acipimox treatment on glucose and lipid metabolism and on in vivo insulin sensitivity in patients with non-insulin dependent diabetes mellitus. *Acta Endocrinol.(Copenh)* 127:344-350, 1992

94. Ranganath L, Norris F, Morgan L, Wright J, Marks V: The effect of circulating non-esterified fatty acids on the entero- insular axis. *Eur.J.Clin.Invest* 29:27-32, 1999

95. Worm D, Vinten J, Vaag A, Henriksen JE, Beck-Nielsen H: The nicotinic acid analogue acipimox increases plasma leptin and decreases free fatty acids in type 2 diabetic patients. *Eur.J.Endocrinol.* 143:389-395, 2000

96. Vaag A, Skott P, Damsbo P, Gall MA, Richter EA, Beck-Nielsen H: Effect of the antilipolytic nicotinic acid analogue acipimox on whole- body and skeletal muscle glucose metabolism in patients with non- insulin-dependent diabetes mellitus. *J.Clin.Invest* 88:1282-1290, 1991

97. Buckley, B. M. Long-term safety profile of acipimox. Pharmacological basis and clinical evidence. Gotto, A. M. and Paoletti, R. (22), 217-224. 1991. New York, Raven Press Ltd. Atherosclerosis Reviews.

98. Capito K, Hansen SE, Hedeskov CJ, Islin H, Thams P: Fat-induced changes in mouse pancreatic islet insulin secretion, insulin biosynthesis and glucose metabolism. *Acta Diabetol.* 28:193-198, 1992

99. Briaud I, Kelpe CL, Johnson LM, Tran PO, Poitout V: Differential effects of hyperlipidemia on insulin secretion in islets of langerhans from hyperglycemic versus normoglycemic rats. *Diabetes* 51:662-668, 2002

100. Shafrir E, Ziv E, Mosthaf L: Nutritionally induced insulin resistance and receptor defect leading to beta cell failure in animal models. *Ann.N.Y.Acad.Sci.* 892:223-246, 1999

101. Kraegen EW, James DE, Storlien LH, Burleigh KM, Chisholm DJ: In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. *Diabetologia* 29:192-198, 1986

102. Storlien LH, James DE, Burleigh KM, Chisholm DJ, Kraegen EW: Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. *Am.J.Physiol* 251:E576-E583, 1986

103. Swinburn BA: Effect of dietary lipid on insulin action. Clinical studies. *Ann.N.Y.Acad.Sci.* 683:102-109, 1993

104. Riccardi G, Parillo M: Comparison of the metabolic effects of fat-modified vs low fat diets. *Ann.N.Y.Acad.Sci.* 683:192-198, 1993

105. Heilbronn LK, Noakes M, Clifton PM: Effect of energy restriction, weight loss, and diet composition on plasma lipids and glucose in patients with type 2 diabetes. *Diabetes Care* 22:889-895, 1999

106. Markovic TP, Jenkins AB, Campbell LV, Furler SM, Kraegen EW, Chisholm DJ: The determinants of glycemic responses to diet restriction and weight loss in obesity and NIDDM. *Diabetes Care* 21:687-694, 1998

107. Byrne CD, Wareham NJ, Day NE, McLeish R, Williams DR, Hales CN: Decreased non-esterified fatty acid suppression and features of the insulin resistance syndrome occur in a sub-group of individuals with normal glucose tolerance. *Diabetologia* 38:1358-1366, 1995

108. Hu FB, van Dam RM, Liu S: Diet and risk of Type II diabetes: the role of types of fat and carbohydrate. *Diabetologia* 44:805-817, 2001

109. Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM: Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc.Natl.Acad.Sci.U.S.A* 94:8878-8883, 1997

110. Pelleymounter MA, Cullen MJ, Baker MB et al.: Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269:540-543, 1995

111. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763-770, 1998

112. Ahima RS, Prabakaran D, Mantzoros C et al.: Role of leptin in the neuroendocrine response to fasting. *Nature* 382:250-252, 1996

113. Maffei M, Halaas J, Ravussin E et al: Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat.Med.* 1:1155-1161, 1995

114. Sivitz WI, Walsh SA, Morgan DA, Thomas MJ, Haynes WG: Effects of leptin on insulin sensitivity in normal rats. *Endocrinology* 138:3395-3401, 1997

115. Kraus D, Fasshauer M, Ott V et al.: Leptin secretion and negative autocrine crosstalk with insulin in brown adipocytes. *J Endocrinol.* 175:185-191, 2002

116. Arita Y, Kihara S, Ouchi N et al.: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem.Biophys.Res.Commun.* 257:79-83, 1999

117. Weyer C, Funahashi T, Tanaka S et al.: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol.Metab* 86:1930-1935, 2001

118. Yamauchi T, Kamon J, Waki H et al.: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat.Med.* 7:941-946, 2001

119. Leahy JL, Cooper HE, Deal DA, Weir GC: Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic in vivo glucose infusions. *J.Clin.Invest* 77:908-915, 1986

120. Björklund A, Lansner A, Grill VE: Glucose-induced [Ca2+]i abnormalities in human pancreatic islets: important role of overstimulation. *Diabetes* 49:1840-1848, 2000

121. Hiramatsu S, Grill V: Influence of a high-fat diet during chronic hyperglycemia on beta cell function in pancreatic islet transplants to streptozotocin-diabetic rats. *Eur.J.Endocrinol.* 144:521-527, 2001

122. Trube G, Rorsman P, Ohno-Shosaku T: Opposite effects of tolbutamide and diazoxide on the ATP-dependent K+ channel in mouse pancreatic beta cells. *Pflugers Arch.* 407:493-499, 1986

123. Wigand JP, Blackard WG: Downregulation of insulin receptors in obese man. *Diabetes* 28:287-291, 1979

124. *Therapeutic Drugs*
46. Edinburgh, Churchill Livingstone, 1999

125. Greenwood RH, Mahler RF, Hales CN: Improvement in insulin secretion in diabetes after diazoxide. *Lancet* 1:444-447, 1976

126. Björk E, Berne C, Karlsson FA: Induction of beta cell rest in type 1 diabetes. Studies on the effects of octreotide and diazoxide. *Diabetes Care* 21:427-430, 1998

127. Björk E, Berne C, Kampe O, Wibell L, Oskarsson P, Karlsson FA: Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. *Diabetes* 45:1427-1430, 1996

128. Kosaka K, Kuzuya T, Akanuma Y, Hagura R: Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. *Diabetologia* 18:23-28, 1980

129. Garvey WT, Olefsky JM, Griffin J, Hamman RF, Kolterman OG: The effect of insulin treatment on insulin secretion and insulin action in type II diabetes mellitus. *Diabetes* 34:222-234, 1985

130. Ilkova H, Glaser B, Tunckale A, Bagriacik N, Cerasi E: Induction of long-term glycemic control in newly diagnosed type 2 diabetic patients by transient intensive insulin treatment. *Diabetes Care* 20:1353-1356, 1997

131. Puhakainen I, Taskinen MR, Yki-Järvinen H: Comparison of acute daytime and nocturnal insulinization on diurnal glucose homeostasis in NIDDM. *Diabetes Care* 17:805-809, 1994

132. Guldstrand M, Grill V, Björklund A, Lins P-E, Adamson U: Improved beta cell function after short-term treatment with diazoxide in obese subjects with type 2 diabetes. *Diabetes Metab* In press.: 2002

133. Laedtke T, Kjems L, Porksen N et al.: Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. *Am.J.Physiol Endocrinol.Metab* 279:E520-E528, 2000

134. Mitrakou A, Vuorinen-Markkola H, Raptis G et al.: Simultaneous assessment of insulin secretion and insulin sensitivity using a hyperglycemia clamp. *J.Clin.Endocrinol.Metab* 75:379-382, 1992

135. Bonadonna RC, Zych K, Boni C, Ferrannini E, DeFronzo RA: Time dependence of the interaction between lipid and glucose in humans. *Am.J.Physiol* 257:E49-E56, 1989

136. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am.J.Physiol* 237:E214-E223, 1979

137. Faber OK, Binder C: C-peptide response to glucagon. A test for the residual beta cell function in diabetes mellitus. *Diabetes* 26:605-610, 1977

138. Scheen AJ, Castillo MJ, Lefebvre PJ: Assessment of residual insulin secretion in diabetic patients using the intravenous glucagon stimulatory test: methodological aspects and clinical applications. *Diabetes Metab* 22:397-406, 1996

139. Göttsater A, Landin-Olsson M, Fernlund P, Gullberg B, Lernmark A, Sundkvist G: Pancreatic beta cell function evaluated by intravenous glucose and glucagon stimulation. A comparison between insulin and C-peptide to measure insulin secretion. *Scand.J.Clin.Lab Invest* 52:631-639, 1992

140. Nes M, Frost AL, Solvoll K et al.: Accuracy of a quantitative food frequency questionnaire applied in elderly Norwegian women. *Eur.J.Clin.Nutr.* 46:809-821, 1992

141. Rimestad AH, Blaker B, Flåten A-M, Nordbotten A: *Den store matvaretabellen (The Norwegian Food Table)*. Oslo, Universitetsforlaget, 1995

142. Zambon A, Hashimoto SI, Brunzell JD: Analysis of techniques to obtain plasma for measurement of levels of free fatty acids. *J.Lipid Res.* 34:1021-1028, 1993

143. Bønaa KH, Bjerve KS, Straume B, Gram IT, Thelle D: Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the Tromso study. *N.Engl.J.Med.* 322:795-801, 1990

144. Byrne HA, Tieszen KL, Hollis S, Dornan TL, New JP: Evaluation of an electrochemical sensor for measuring blood ketones. *Diabetes Care* 23:500-503, 2000

145. Jensen CV: A computer program for randomizing patients with near-even distribution of important parameters. *Comput.Biomed.Res.* 24:429-434, 1991

146. Yki-Järvinen H, Ryysy L, Nikkila K, Tulokas T, Vanamo R, Heikkila M: Comparison of bedtime insulin regimens in patients with type 2 diabetes mellitus. A randomized, controlled trial. *Ann.Intern.Med.* 130:389-396, 1999

147. Hovorka R, Soons PA, Young MA: ISEC: a program to calculate insulin secretion. *Comput.Methods Programs Biomed.* 50:253-264, 1996

148. Wiesenthal SR, Sandhu H, McCall RH et al.: Free fatty acids impair hepatic insulin extraction in vivo. *Diabetes* 48:766-774, 1999

149. Albareda M, Rodriguez-Espinosa J, Murugo M, de Leiva A, Corcoy R: Assessment of insulin sensitivity and beta cell function from measurements in the fasting state and during an oral glucose tolerance test. *Diabetologia* 43:1507-1511, 2000

150. Busch CP, Hegele RA: Genetic determinants of type 2 diabetes mellitus. *Clin Genet.* 60:243-254, 2001

151. Højlund K, Wildner-Christensen M, Eshøj O et al.: Reference intervals for glucose, beta cell polypeptides, and counterregulatory factors during prolonged fasting. *Am.J.Physiol Endocrinol.Metab* 280:E50-E58, 2001

152. Bjerve KS, Brubakk AM, Fougner KJ, Johnsen H, Midthjell K, Vik T: Omega-3 fatty acids: essential fatty acids with important biological effects, and serum phospholipid fatty acids as markers of dietary omega 3-fatty acid intake. *Am.J.Clin.Nutr.* 57:801S-805S, 1993

153. Andersen LF, Solvoll K, Drevon CA: Very-long-chain n-3 fatty acids as biomarkers for intake of fish and n- 3 fatty acid concentrates. *Am.J.Clin.Nutr.* 64:305-311, 1996

154. Block G, Woods M, Potosky A, Clifford C: Validation of a self-administered diet history questionnaire using multiple diet records. *J.Clin.Epidemiol.* 43:1327-1335, 1990

155. Björkman O, Eriksson LS: Influence of a 60-hour fast on insulin-mediated splanchnic and peripheral glucose metabolism in humans. *J.Clin.Invest* 76:87-92, 1985

156. Evidence-based nutrition principles and recommendations for the treatment and prevention of diabetes and related complications. *Diabetes Care* 25:202-212, 2002

157. Tuomilehto J, Lindström J, Eriksson JG et al.: Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance 6. *N.Engl.J Med.* 344:1343-1350, 2001

158. Knowler WC, Barrett-Connor E, Fowler SE et al.: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N.Engl.J Med.* 346:393-403, 2002

159. Gromada J, Holst JJ, Rorsman P: Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. *Pflugers Arch.* 435:583-594, 1998

160. Zunkler BJ, Lenzen S, Manner K, Panten U, Trube G: Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K+ currents in pancreatic B-cells. *Naunyn Schmiedebergs Arch.Pharmacol.* 337:225-230, 1988

161. Mariot P, Gilon P, Nenquin M, Henquin JC: Tolbutamide and diazoxide influence insulin secretion by changing the concentration but not the action of cytoplasmic Ca2+ in beta cells. *Diabetes* 47:365-373, 1998

162. Makimättila S, Nikkila K, Yki-Järvinen H: Causes of weight gain during insulin therapy with and without metformin in patients with Type II diabetes mellitus. *Diabetologia* 42:406-412, 1999

163. Alemzadeh R, Slonim AE, Zdanowicz MM, Maturo J: Modification of insulin resistance by diazoxide in obese Zucker rats. *Endocrinology* 133:705-712, 1993

164. Anderson JH, Jr., Byrd GW, Blackard WG: Hyperresponsiveness to tolbutamide of dogs pretreated with diazoxide. *Metabolism* 20:1023-1030, 1971

165. Urdanivia E, Pek S, Santiago JC: Inhibition of glucagon secretion by diazoxide in vitro. *Diabetes* 28:26-31, 1979

166. Clauson P, Alvarsson M, Grill V: Enhancement of B-cell secretion by blood glucose normalization in type 2 diabetes is associated with fasting C-peptide levels. *J.Intern.Med.* 241:493-500, 1997

167. DeFronzo RA: Lilly lecture 1987. The triumvirate: beta cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 37:667-687, 1988.

12. PAPERS AND MANUSCRIPTS

Paper I and II are not included due to copyright.

Effects of a 3 day low-fat diet on lipids, metabolic control, insulin sensitivity and adipocyte hormones in Norwegian subjects with hypertriglyceridemia and type II diabetes.

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This study was supported by funds from the Norwegian Research Council (grant no 11290/320), The Norwegian Diabetes Association, Bayer Inc./ The Norwegian Society for Endocrinology and Torstein Erbo's Foundation.

Short running title: Low-fat diet and type II diabetes

ABSTRACT

Objective: To investigate in type II diabetes with hypertriglyceridemia the effects of a short term low-fat diet on lipids, metabolic control, insulin sensitivity and adipocyte hormones**.**

Design: Baseline dietary intake assessed by weighing during two separate 3 day periods followed by a 3 day low-fat dietary intervention.

Setting: Out-patient.

Subjects: Nineteen subjects, 10 males, 9 females with type II diabetes and triglycerides >2.2 mmol/l at screening**.**

Interventions: Patients were advised to reduce isoenergetically 10 – 15 energy % (E%) from fat. They were to reduce visible fat, use low-fat products, and increase intake of fish and fibre-rich foods.

Results: E% from fat was reduced from 39 to 22 ($P<0.0001$). Total cholesterol decreased to 95 % of baseline ($P<0.011$), HDL cholesterol to 97 % ($P<0.083$), triglycerides to 91 % ($P<0.055$) and the ratio of n-6 to n-3 fatty acids to 76 % ($P<0.001$). Blood glucose, fasting insulin and fasting glucose to insulin ratios were unaffected in the whole group. However, glucose/insulin was lower at moderate than at pronounced degree of fat reduction ($P<0.009$ for difference between dichotomised groups). Concentrations of leptin decreased to 90 % of baseline ($P<0.013$) and of adiponectin to 92 % ($P<0.055$). The leptin decrease was unrelated to fat reduction and confined to women.

Conclusions: 1) The effects of a clinically relevant reduction in total and saturated fat in type II diabetes vary with gender and degree of fat reduction. 2) Effects on blood lipids and adipocyte hormones are not accompanied by beneficial effects on insulin sensitivity and metabolic control.

Sponsorship: University.

Descriptors: low-fat diet, type II diabetes, insulin sensitivity, leptin, adiponectin, hypertriglyceridemia.

INTRODUCTION

The influence of low-fat dieting on insulin sensitivity in type II diabetes is controversial (Riccardi & Parillo, 1993; Storlien et al, 1991; Berry, 1997). Evidence in animals indicates that reduced fat intake increases insulin sensitivity (Kraegen et al, 1986; Storlien et al, 1986). Documentation for a similar effect in humans is less clear, particularly in type II diabetes (Swinburn, 1993). When a beneficial effect on insulin sensitivity was reported it was often influenced by concomitant weight reduction (Markovic et al, 1998; de Man, van der et al, 1999). Differences in patient characteristics in different studies could be important for divergent results. A genetic heterogeneity in type II diabetes is by now obvious (McCarthy & Froguel, 2002). Such heterogeneity likely involves different aspects of intermediary metabolism in different subjects, to which gender-related differences in fatty acid metabolism (Blaak, 2001) could contribute. Furthermore, the clinical relevance can be questioned in studies using artificial diet regimens (for example liquid diet (Kolterman et al, 1979; Markovic et al, 1998) or mixing liquid and usual diet (Chen et al, 1988). Clearly then, the common recommendation for type II diabetes subjects to reduce total fat intake in order to improve insulin sensitivity rests on imperfect evidence.

The aim of this study was to intervene in type II diabetic patients with a short term but a clinically relevant, low-fat diet with an emphasis on restriction of saturated fat. The dietary effects to be tested were on lipids, metabolic control, insulin sensitivity and adipocyte hormones in a setting that minimised confounding. In order to ensure a clinically relevant diet it was a) based on normal food products, b) based on dietary principles in concrete terms but participant-selected food choices. Furthermore, the intervention c) attempted to reduce saturated fat and to promote unsaturated fat, especially n-3 fatty acids, in line with current dietary recommendations in Norway (SEF, 1997). In order to reduce heterogeneity in the study population of type II diabetes we recruited only hypertriglyceridemic subjects. We

furthermore reasoned that such individuals were the most likely to profit from a low-fat diet. To assess heterogeneity due to gender we tried to include an equal number of men and women into the study. We chose a short intervention time (3 days) since compliance decreases with length of intervention. A short intervention time also allowed us to collect detailed information on the diet consumed, by weighing all intake of food, and also on metabolic control through home glucose monitoring. Our results indicate that a low-fat diet that is intended to be isoenergetic, rapidly affects lipid variables but does not ameliorate metabolic control or insulin sensitivity. Furthermore, we find that leptin and adiponectin concentrations are reduced despite no weight reduction and without relationship to metabolic control or fat intake.

SUBJECTS AND METHODS

Subjects. Nineteen subjects (10 M, 9 F) participated. These subjects were recruited from the outpatient clinic of our Department of Endocrinology. Inclusion criteria were: type II diabetes as defined by clinical criteria, age 40 to 75 years, and hypertriglyceridemia with fasting triglyceride concentrations ≥ 2.2 mmol/l. Exclusion criteria were: insulin treatment, proliferative retinopathy, pregnancy or lactation, heart failure grade III or IV, allergy to fish or other ailment prohibiting diet intervention, and alcoholism or other serious diseases affecting the possibility of the subject to participate. The majority of subjects were treated with more than one antidiabetic medication. Eighteen subjects were treated with metformin in doses varying from 500 mg to 3000 mg/d and thirteen of these subjects were on combination therapy with glibenclamide or glipizide. Four of the subjects received antihyperlipemic treatment in the form of statins. Seven subjects received antihypertensive treatment. Five subjects were habitual smokers (3 M, 2 F).

Physical activity. The physical activity of the subjects at baseline was assessed by a questionnaire (Nes et al, 1992). The intensity of exercise was limited, since only 32 % of the participants exercised more than three times a week with a minimum duration of 20 min.

Experimental design. A physical examination was performed at screening. Food intake was registered by weighing records during 3 day periods. During two periods they consumed their usual diet (baseline) and during the third period they consumed a low-fat intervention diet. The interval between the periods of diet registration varied between 2 and 6 weeks. Most subjects underwent a sequential glucose and lipid infusion test at the end of each 3 day period. The results of these tests are reported separately (Qvigstad et al, *In press*). The local ethical committee and the Norwegian Drug Control Authority approved the protocols that were used in the study. The principles of the Helsinki Declaration were followed. All participants gave written informed consent.

Details on diet registration, home glucose monitoring and intervention. Food and beverages were weighed on an electronic kitchen scale (Philips HR 2385). To be comfortable with the method of weighing records, subjects individually practised the method during the supervision by one of the authors (ILM, clinical nutritionist). Subjects were to measure their blood glucose levels at home five times during the day: fasting, pre lunch, pre dinner, 2 h post dinner and at bedtime. All information about the low-fat diet (see below), weighing method and blood glucose monitoring was given both orally and in writing. The subjects were supported by telephone calls on the day before and then daily during each registration period.

The intervention diet was low-fat and fibre-rich. It was intended to be isoenergetic with the usual diet. At the start of the intervention subjects were told to reduce all visible fats, but to increase the intake of cereals (especially whole meal bread), potatoes, rice, pasta, fish, vegetables and fruits. Subjects were told to eat approximately the same amount of dairy and meat products as usual, but to prefer low-fat variants.

The weighing records were analysed for energy, carbohydrates without fibre, protein, fat, alcohol, sugar and total fibre. The intake of energy and nutrients was computed by using a food database (AKF96) and software systems (BEREGN) developed at the Institute of Nutrition Research, University of Oslo. The food database was mainly based on the official Norwegian Food Table (Rimestad et al, 1995).

Sampling procedures. All fasting variables were measured in the morning on the day that followed the 3x24 h dietary intervention. The subjects reported to the clinic between 8 and 9 a.m. Body weight and blood pressure were measured. Then a cannula (Venflon, Viggo, Helsingborg, Sweden) was inserted retrogradely into an antecubital vein of the contralateral arm. Fasting blood samples were collected. Blood samples were collected at standardised intervals. After centrifugation (3G, 15min, 20°C), the plasma was frozen and kept at –80ºC for later analysis. For glucagon measurements, 0,55 ml of aprotinin (Trasylol, Bayer AG,

Leverkusen, Germany) was added to chilled, pre-heparinized tubes and samples centrifugated (3G, 20min, 4°C) before freezing at –80 °C.

Assays. Leptin was measured by a human leptin RIA kit (Linco Res. Co., St. Charles, MO, USA). According to the manufacturer, there is no detectable cross-reactivity with insulin, proinsulin, C-peptid or glucagon. The inter-assay coefficient of variation (CV) of the leptin RIA is 4.6 % and the intra-assay CV 5.0 %, according to the manufacturer. Adiponectin was measured by a human adiponectin RIA kit (Linco Res. Co., St. Charles, MO, USA). The inter- and intra-assay CV are 6.9 and 6.2 % respectively, according to the manufacturer. Insulin and glucagon were also measured by RIA kit. The insulin assay was specific for human insulin (Linco Res. Co., St. Charles, MO, USA). According to the manufacturer, the inter-assay coefficient of variation (CV) of the insulin RIA is 9.7 % and the intra-assay CV 5.0 %. Cross-reactivity with proinsulin is approximately 0.2 %. C-peptide was assayed by a RIA kit from DPC, Los Angeles, CA, USA. Proinsulin was determined by ELISA (Dako Inc., Oslo, Norway). Cortisol was determined by competitive immunoassay using a commercial kit (DPC, Los Angeles, CA, USA). Concentrations of FA (free fatty acids) were determined by an enzymatic colorometric method (NEFA-C-kit, Wako Pure Chemical Industries Ltd, Osaka, Japan). Plasma phospholipid fatty acids (PL-FA) were determined by gas chromatography and glucose, triglycerides, cholesterol, HDL cholesterol (high density lipoprotein cholesterol) and glycosylated haemoglobin (HbA1c) by standard laboratory techniques.

Presentation of results. Results are generally given as median values and range unless otherwise stated. Concentrations of glucose and insulin are always given as mmol/l and mU/l respectively, with one exception: In the glucose/insulin ratio glucose was entered as mg/dl to obtain comparability with previous studies. A measure of insulin sensitivity was obtained from a ratio of fasting glucose- to insulin-concentrations (glu/ins, mg/10^{-4}U) (Legro et al, 1998; Albareda et al, 2000). The homeostasis model assessment index (HOMA, 20 x (fasting

insulin mU/l/(fasting glucose-3.5mmol/l)) calculated as per cent of normal was used as a measure of beta cell function (Matthews et al, 1985; Albareda et al, 2000). Statistical analysis was done using Statistical Package for the Social Sciences, version 10.0, Chicago, 2000. The results of variables measured after each of the two baseline periods on the patient's usual diet were averaged before comparisons were done with variables measured after the low-fat diet intervention. The distribution of nutrient intake and the delta values between baseline and intervention results was skewed. Therefore, non-parametric statistical methods were chosen. The differences between medians were tested with the unpaired Mann-Whitney Test, and the analysis after dichotomization was performed by the same test. Significance testing included the paired Wilcoxon Signed Ranks Test. Spearman's correlation coefficients were used to evaluate bivariate correlation. Most delta values were correlated to baseline values ($P<0.05$ or less). Therefore, all variables were expressed as percentages of the baseline values before being subjected to further analysis.

RESULTS

Baseline characteristics.

Clinical data. The subjects were obese, although to a varying extent (Table 1). The metabolic control as assessed by HbA1c was non-optimal with large variations between subjects (Table 1). Concentrations of triglycerides were, by design, markedly elevated (Table 1). Concentrations of cholesterol were above normal according to current guidelines (NCEP, 2001), as were cholesterol/HDL cholesterol ratios.

Insulin secretion and sensitivity. The HOMA estimate of beta cell function indicated reduced secretion (median 63 % of non-diabetic subjects (Matthews et al, 1985), range 11-186 %), table 1. There was a hyperbolic relation between this variable and the variable of insulin sensitivity (glucose/insulin ratio), data not shown.

Hormone concentrations. The fasting concentrations of different hormones are given in table 1. The leptin concentration tended to correlate with BMI ($r = 0{,}433$, $P<0{,}064$), as expected (Widjaja et al, 1997).

Baseline diet. Energy intake at baseline calculated from weighed records of 6 days was 7,9 MJ/d, with a median of 18 energy % (E%) from protein, 39 E% from fat and 41 E% from carbohydrates (Table 2).

There were no significant differences between total energy intake or the E% from the macronutrients or from the intake of different food constituents between the two periods of 3-days registrations when subjects consumed their usual diet at baseline (data not shown).

Gender differences. As expected (Widjaja et al, 1997) the concentrations of leptin were higher in women than in men (17,3 ng/ml compared to 8,1 ng/ml), $p< 0{,}003$ (Table 1). Other significant differences related to gender were age, total energy intake, E% from sugar, intake of fat and carbohydrate (g/d), and concentrations of C-peptide, glucagon, cholesterol, HDL

cholesterol as well as FA (Tables 1 and 2). The concentrations of adiponectin (Table 1) did not differ between men and women. The intake of food items, nutrients and distribution of macronutrients did not differ at baseline, except for the intake of beta-carotene and the E% from sugar (Table 2).

Effects of intervention.

Consumption of food items (Table 2). The subjects consumed more bread, potatoes, vegetables, fruit and fish during the low-fat diet and less of edible fats (margarine, butter and oils). Total consumption (g/d) of meat, milk, cheese and eggs was not altered. However, the intake of fat from these items was reduced because the subjects chose low-fat variants thereby reducing their intake of saturated fats (data not shown). The reduction of fat was primarily due to reduced intake of edible fats and to a lesser extent to reduced intake of fat from meat and dairy products. Consumption of refined sugar or of alcohol did not change. There was no significant difference between gender in fat reduction (g/d), but men increased their intake of potatoes more than women ($P<0.027$).

Effects on energy distribution (Table 2). The subjects decreased fat intake to 22 E% and increased carbohydrate and protein intake to 50 and 23 E%, respectively. The median effect on total energy intake for all subjects was – 0.8 MJ/d (range from –3.5 to + 0.9 MJ/d). The energy intake at low-fat diet was 89 % of the intake at baseline ($P<0.027$). The reduction did not differ between gender (88 % M, 89 % F). The median intake of fibre increased from 20 to 27 g/d in all subjects, with some difference between genders (men increased their fibre intake more than women, $p<0.050$). Gender did not affect the change in distribution of macronutrients.

Body Mass Index (BMI), blood pressure and blood glucose. The dietary intervention did not affect BMI or blood pressure (Table 1). Fasting blood glucose concentrations when monitored at home were marginally but significantly lower during the low-fat period, median 8.5, range 4.6-11.9 compared to 8.6, 4.8-13.0 mmol/l at baseline, P<0.030 (Figure 1). Intervention did not affect fasting concentrations of blood glucose as measured in the clinic on test days. Furthermore, intervention did not affect blood glucose concentrations measured pre lunch, pre dinner, 2 h post dinner or at bedtime (Figure 1). Neither were daytime averages affected (median 8.6 (range 5.2-13.7) during the low-fat diet, compared to 8.5 (5.3-12.9) mmol/l during the baseline diet).

Hormones (Table 1). The diet intervention did not significantly affect fasting concentrations of C-peptide, insulin, proinsulin, glucagon or cortisol. There was no difference between gender. Leptin decreased after 3 day low-fat diet to 90 % of baseline (P<0.013), and adiponectin to 92 % (P< 0.055). Women reduced leptin to 87 % (61-105) whereas the median effect was unchanged in men (101 % of baseline, range 64-113), P<0.072 for the difference between gender. There was no gender difference with regard to adiponectin.

Lipids (Table 1). Total cholesterol decreased to 95 % of baseline values (P<0.011) and HDL cholesterol tended to decrease, to 97 % (P<0.083). Also triglycerides (TG) tended to decrease, P<0.055. The results of LDL cholesterol were not available for all subjects[1]. Total FA and total plasma PL-FA were unchanged. However, the fractions SFA and n-6 fatty acids were significantly reduced, whereas the PL-FA content of MUFA and n-3 fatty acids increased. None of the lipid effects differed between gender.

[1] Only results from 10 subjects could be used to calculate LDL cholesterol and LDL cholesterol/HDL cholesterol ratio (because of TG > 4.1 mmol/l in the other subjects). In these 10 subjects LDL cholesterol decreased from 3.9 (2.0-5.2) to 3.3 (1.7-4.8) mmol/l (NS), and LDL cholesterol/HDL cholesterol ratio from 3.4 (2.2-4.8) to 3.0 (1.9-4.0)mmol/l (NS).

Insulin sensitivity and beta cell function (Table 1). In the whole group (n = 19) there were no significant changes in fasting glucose/insulin ratios (a measure of insulin sensitivity), nor on insulin secretion as assessed by HOMA. There was no significant difference between gender with regard to glucose/insulin ratio or HOMA.

Associations with effects on leptin and adiponectin. The reduction of leptin did not correlate with the reduction in total energy ($P<0.482$), changes in energy distribution (E% fat: $P<0.705$; E% carbohydrates: $P<0.567$; E% protein: $P<0.154$) or the extent of fat reduction in individuals ($P<0.955$). The same lack of correlation was seen with adiponectin, except that the change in adiponectin correlated with the change in intake of protein (g/d), $r = - 0.483$, $P<0.050$.

Effects of variation in fat reduction. In a post hoc analysis we dichotomised subjects into a moderate fat reduction group which reduced E% from fat with 4-15 E%, (n = 9, 4 M, 5 F) and a group with more pronounced reduction which reduced E% from fat with 16-30 E% (n = 10, 6 M, 4 F). None of the baseline characteristics of Tables 1 or 2 differed between the dichotomised fat reduction groups (data not shown), with the exception of edible fats. Thus, the moderate fat reduction group had a lower intake (26 g/d, (15-65)) than the other group (47 g/d (24-78)), $P<0.050$ for difference at baseline. During intervention the intake of fruit was 108 % of baseline in the moderate group, and 247 % in the pronounced group ($P<0.028$ for difference), whereas changes in the intake of all other food items was similar. Changes in energy intake, fibre intake or results of home glucose monitoring did not differ between groups. Further, there were no differences in effects on lipids or measured hormones, except for insulin. The moderate fat reduction group increased fasting insulin to 112 % compared to baseline, whereas the pronounced group decreased fasting insulin to 76 % of baseline, $p<0.050$ for the difference between groups. The group with moderate fat reduction reduced glucose/insulin ratio to 91 % compared to baseline whereas the group with pronounced

reduction increased the ratio to 114 %, P<0.009 for the difference between groups. Thus a moderate fat reduction seemed to worsen insulin sensitivity, whereas a pronounced fat reduction seemed to improve it.

DISCUSSION

This low-fat dietary intervention fails to improve insulin sensitivity in hypertriglyceridemic subjects with type II diabetes. Thus, the glucose to insulin ratios in the overnight fasted state did not change in a manner commensurate with improvement of sensitivity. Also the sequential glucose and lipid infusion tests performed in 17 of the 19 subjects at the end of intervention indicated decreased rather than increased sensitivity (Qvigstad et al, *In press*).

Our results on insulin sensitivity differ from some early reports that show improved sensitivity as a result of a low-fat diet (Swinburn, 1993). The short duration of our intervention could be a factor behind divergent results. However, the 3 days of intervention was sufficient to affect important lipid variables. Also, a recent study demonstrated that three days of changing the percentage of fat in the diet was sufficient to affect the concentration of intramuscular triglycerides (Bachmann et al, 2001).

As previously mentioned, our goal was to uphold energy balance, but this goal was not completely achieved. A negative energy balance has been shown to increase, not decrease insulin sensitivity in type II diabetic subjects (Markovic et al, 1998; Heilbronn et al, 1999). Hence, our study may overestimate insulin sensitivity in relation to effects of low-fat dieting per se.

The question arises to which extent the degree of fat reduction could influence insulin sensitivity. A post-hoc analysis indicated that a moderate reduction of fat intake was associated with decreased insulin sensitivity whereas a more pronounced degree of reduction was associated with better sensitivity or at least a reduction in fasting insulin. These findings are in line with those of the glucose and lipid infusion tests that were previously reported for the majority of the whole group (Qvigstad et al, *In press*). In these tests glucose was first infused for 60 min and clamped at 6 mmol/l above the fasting glucose concentrations.

Subgroup analysis showed a median reduction to 45 % (25-70) of the glucose infused/insulin ratio compared to baseline in the group with moderate fat reduction, but not in the group with pronounced fat reduction (106 % (41-631)), $P<0.004$ for the difference between groups. Further studies in which subjects are randomised to different degrees of fat reduction will be necessary to confirm the presently observed differences.

The type of fat of the prescribed diet could be important for our results. During the dietary intervention subjects consumed much less total fat, however their consumption of fat fish and consequent intake of n-3 fatty acids increased and the n-6/n-3 ratio in PL-FA decreased. The extra intake of n-3 fatty acids was calculated to be about 1 g/d (data not shown). This particular feature of the diet is one recommended in Norway and may be different from other studies where effects of fat reduction have been tested. However, in contrast to possible negative effects of n-3 fatty acids on insulin secretion (Dunstan et al, 1997), there exists to our knowledge no evidence for a negative effect of n-3 fatty acids on insulin sensitivity. Some studies report positive effects in animals (Luo et al, 1996; Chicco et al, 1996; Behme, 1996), as well as in humans (Kasim, 1993).

"Lipotoxicity" towards beta cells has been proposed to induce and aggravate type II diabetes (Grill & Qvigstad, 2000). In this context, it is interesting that the low-fat diet failed to affect a fasting estimate of beta cell function (HOMA). Also the glucose and lipid infusion tests failed to record any effect on by the low-fat diet on glucose-induced insulin secretion (Qvigstad et al, *In press*). The simultaneous absence of effects on insulin secretion and insulin sensitivity would agree with our finding of unaltered metabolic control during the intervention.

Could alterations in adipocyte hormones be of importance for insulin secretion and/or sensitivity during the low-fat diet? Concentrations of adiponectin are positively correlated with insulin sensitivity (Weyer et al, 2001). There was a strong tendency for a decrease in adiponectin levels as a result of the low-fat diet. This effect is the opposite of that seen after

weight reduction (Hotta et al, 2000). The tendency for a decrease that we see here would therefore indicate a negative effect on insulin sensitivity, an effect that appears compatible with our results.

As to regulation of adiponectin we note that the increase in protein that occurred as a consequence of the diet intervention was negatively correlated with the effect on adiponectin levels. Further studies are however needed to decide which food constituents that exert a negative effect on circulating adiponectin.

Leptin could potentially influence both insulin secretion and insulin sensitivity (Kieffer & Habener, 2000; Ceddia et al, 2002). We find that leptin concentrations were moderately but significantly decreased by the dietary intervention. However, we failed to find associations with estimates of insulin secretion and sensitivity.

There were no associations with changes in leptin concentrations and individual effects on energy balance or fat reduction per se. Studies in animals do indicate an effect of fat intake on leptin concentrations (Surwit et al, 1997). However, our results are in line with other studies in man which do not assign a major role to fat intake in the regulation of circulating levels of leptin (Jenkins et al, 1997; Coleman & Herrmann, 1999). As to a possible effect of specific types of fatty acids it is of interest that supplementation with 5 g daily of n-3 fatty acids correlated with a decrease in circulating leptin (Reseland et al, 2001). However, this supplementation was five times greater than the increased dietary intake of n-3 fatty acids in our study.

With regard to gender, we find, as others (Widjaja et al, 1997), that leptin concentrations are considerably higher in women than in, men. It is interesting that the diet-induced reduction in leptin concentrations tended to be more marked in women than in men. Another study examining the effect of short-time energy restriction on leptin concentrations found that a

decrease in leptin was more marked in women than in men despite similar weight loss (Dubuc et al, 1998). The gender difference in the previous and in the present study is unexplained.

The alterations in total cholesterol and HDL cholesterol that we observe are qualitatively the same as those observed in dietary studies of longer duration in non-diabetic individuals (Yu-Poth et al, 1999). Also the decrease in n-6/n-3 ratio is compatible with that found in studies in which n-3 fatty acids were increased in diets (Kasim, 1993). That we did not observe increased triglycerides could depend on increased intake of n-3 fatty acids through fat fish, since these fatty acids are known to decrease triglycerides (Connor et al, 1993a; Connor et al, 1993b; Dunstan et al, 1997). Increased fibre intake could also be of importance as indicated in other studies (Chandalia et al, 2000; Parks & Hellerstein, 2000).

To our mind, some conditions of our dietary intervention add strength and importance to the present findings. First, as detailed below, the compliance of participating subjects seemed satisfactory. Second, double recordings of subject's usual diet at baseline and other variables at different time-points documented that the intervention results were not affected by factors such as repeated measurements or a successive change in lifestyle as a result of inclusion into the study. Third, the intervention results were not confounded by weight reduction. Fourth, the group of type II diabetic subjects, although being heterogeneous in many respects did, by design, have hypertriglyceridemia as a common feature, therefore being the group of diabetic subjects in whom intervention by low-fat diet would potentially be the most interesting one to test.

Conditions favourable for compliance were the short-term nature of the dietary manipulation and the daily contacts with the subjects before, during and after the intervention. Also the food weighing data assembled by the subjects were carefully scrutinised by the clinical nutritionist. Furthermore, in our study effects on lipids, including PL-FA, changed in a manner commensurate with that of the dietary prescriptions. The effects on PL-FA seem

particularly important, since they have been shown to reflect dietary intake and tissue levels of fatty acids, especially long chain fatty acids after short time intervention (Bjerve et al, 1993; Andersen et al, 1996; Ma et al, 1995).

The present findings appear clinically significant despite the short duration of the low-fat intervention. The subject group studied is typical for many type II diabetic subjects. (In this context it is note-worthy that the patient's usual diet was much higher in fat content than recommended. Also the non-optimal glucose control is - unfortunately – found in a large segment of the type II diabetic population). Furthermore, the type of dietary intervention prescribed here seems clinically more relevant than diets employed in some earlier studies.

In summary, our results fail to find beneficial effects of a 3 day low-fat diet intervention on insulin sensitivity and metabolic control in type II diabetes. Some studies of longer duration also failed to show a beneficial effect of total fat reduction per se on insulin sensitivity (Swinburn, 1993; Riccardi & Parillo, 1993; Garg et al, 1992). Our study complements these findings by demonstrating that also a short-term intervention fails to produce beneficial effects despite similar alterations in cholesterol and HDL as in long-term studies. However, a low-fat diet with reduction of saturated fat may still be beneficial with regard to development of cardiovascular disease (Hooper et al, 2001; Hu et al, 2001). Also the variability of response to the intervention that we observe makes it possible that a low-fat diet could under certain conditions and in certain diabetic subjects be beneficial outside the effects on cardiovascular disease.

ACKNOWLEDGMENTS

We are grateful to Ms Ellen G Lystad and Ms Harriet Selle for assistance during the clamp procedures. The helpful advice of Dr Trine Ranheim and M Sc Elin B Løken at Johan Throne Holst's Institute of Nutrition Research, University of Oslo, is greatly appreciated. This study was supported by the Norwegian Research Council (grant no 11290/320), The Norwegian Diabetes Association, the Norwegian Endocrine Society and by Torstein Erbo's Foundation. Ingrid L Mostad was a recipient of a stipend from the Norwegian foundation Health and Rehabilitation during part of the scientific work.

REFERENCES

Albareda M, Rodriguez-Espinosa J, Murugo M, de Leiva A, and Corcoy R (2000): Assessment of insulin sensitivity and beta-cell function from measurements in the fasting state and during an oral glucose tolerance test. *Diabetologia* **43**, 1507-1511.

Andersen LF, Solvoll K, and Drevon CA (1996): Very-long-chain n-3 fatty acids as biomarkers for intake of fish and n- 3 fatty acid concentrates. *Am.J.Clin.Nutr.* **64,** 305-311.

Bachmann OP, Dahl DB, Brechtel K, Machann J, Haap M, Maier T, Loviscach M, Stumvoll M, Claussen CD, Schick F, Haring HU, and Jacob S (2001): Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes* **50,** 2579-2584.

Behme MT (1996): Dietary fish oil enhances insulin sensitivity in miniature pigs. *J.Nutr.* **126,** 1549-1553.

Berry EM (1997): Dietary fatty acids in the management of diabetes mellitus. *Am.J.Clin.Nutr.* **66,** 991S-997S.

Bjerve KS, Brubakk AM, Fougner KJ, Johnsen H, Midthjell K, and Vik T (1993): Omega-3 fatty acids: essential fatty acids with important biological effects, and serum phospholipid fatty acids as markers of dietary omega 3-fatty acid intake. *Am.J.Clin.Nutr.* **57,** 801S-805S.

Blaak E (2001): Gender differences in fat metabolism. *Curr.Opin.Clin.Nutr.Metab Care* **4,** 499-502.

Ceddia RB, Koistinen HA, Zierath JR, and Sweeney G (2002): Analysis of paradoxical observations on the association between leptin and insulin resistance. *FASEB J.* **16,** 1163-1176.

Chandalia M, Garg A, Lutjohann D, von Bergmann K, Grundy SM, and Brinkley LJ (2000): Beneficial effects of high dietary fiber intake in patients with type 2 diabetes mellitus. *N.Engl.J.Med.* **342,** 1392-1398.

Chen M, Bergman RN, and Porte D, Jr. (1988): Insulin resistance and beta-cell dysfunction in aging: the importance of dietary carbohydrate. *J.Clin.Endocrinol.Metab* **67,** 951-957.

Chicco A, D'Alessandro ME, Karabatas L, Gutman R, and Lombardo YB (1996): Effect of moderate levels of dietary fish oil on insulin secretion and sensitivity, and pancreas insulin content in normal rats. *Ann.Nutr.Metab* **40,** 61-70.

Coleman RA and Herrmann TS (1999): Nutritional regulation of leptin in humans. *Diabetologia* **42,** 639-646.

Connor WE, DeFrancesco CA, and Connor SL (1993b): N-3 fatty acids from fish oil. Effects on plasma lipoproteins and hypertriglyceridemic patients. *Ann.N.Y.Acad.Sci.* **683,** 16-34.

Connor WE, Prince MJ, Ullmann D, Riddle M, Hatcher L, Smith FE, and Wilson D (1993a): The hypotriglyceridemic effect of fish oil in adult-onset diabetes without adverse glucose control. *Ann.N.Y.Acad.Sci.* **683,** 337-340.

de Man FH, van der LA, Hopman EG, Gevers Leuven JA, Onkenhout W, Dallinga-Thie GM, and Smelt AH (1999): Dietary counselling effectively improves lipid levels in patients with endogenous hypertriglyceridemia: emphasis on weight reduction and alcohol limitation. *Eur.J.Clin.Nutr.* **53,** 413-418.

Dubuc GR, Phinney SD, Stern JS, and Havel PJ (1998): Changes of serum leptin and endocrine and metabolic parameters after 7 days of energy restriction in men and women. *Metabolism* **47,** 429-434.

Dunstan DW, Mori TA, Puddey IB, Beilin LJ, Burke V, Morton AR, and Stanton KG (1997): The independent and combined effects of aerobic exercise and dietary fish intake on serum lipids and glycemic control in NIDDM. A randomized controlled study. *Diabetes Care* **20,** 913-921.

Garg A, Grundy SM, and Unger RH (1992): Comparison of effects of high and low carbohydrate diets on plasma lipoproteins and insulin sensitivity in patients with mild NIDDM. *Diabetes* **41,** 1278-1285.

Grill V and Qvigstad E (2000): Fatty acids and insulin secretion. *Br.J.Nutr.* **83 Suppl 1,** S79-S84.

Heilbronn LK, Noakes M, and Clifton PM (1999): Effect of energy restriction, weight loss, and diet composition on plasma lipids and glucose in patients with type 2 diabetes. *Diabetes Care* **22,** 889-895.

Hooper L, Summerbell CD, Higgins JP, Thompson RL, Capps NE, Smith GD, Riemersma RA, and Ebrahim S (2001): Dietary fat intake and prevention of cardiovascular disease: systematic review. *BMJ* **322,** 757-763.

Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, and Matsuzawa Y (2000): Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler.Thromb.Vasc.Biol.* **20,** 1595-1599.

Hu FB, Manson JE, and Willett WC (2001): Types of dietary fat and risk of coronary heart disease: a critical review. *J.Am.Coll.Nutr.* **20,** 5-19.

Jenkins AB, Markovic TP, Fleury A, and Campbell LV (1997): Carbohydrate intake and short-term regulation of leptin in humans. *Diabetologia* **40,** 348-351.

Kasim SE (1993): Dietary marine fish oils and insulin action in type 2 diabetes. *Ann.N.Y.Acad.Sci.* **683,** 250-257.

Kieffer TJ and Habener JF (2000): The adipoinsular axis: effects of leptin on pancreatic beta-cells. *Am.J.Physiol Endocrinol.Metab* **278,** E1-E14.

Kolterman OG, Greenfield M, Reaven GM, Saekow M, and Olefsky JM (1979): Effect of a high carbohydrate diet on insulin binding to adipocytes and on insulin action in vivo in man. *Diabetes* **28,** 731-736.

Kraegen EW, James DE, Storlien LH, Burleigh KM, and Chisholm DJ (1986): In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. *Diabetologia* **29,** 192-198.

Legro RS, Finegood D, and Dunaif A (1998): A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. *J.Clin.Endocrinol.Metab* **83,** 2694-2698.

Luo J, Rizkalla SW, Boillot J, Alamowitch C, Chaib H, Bruzzo F, Desplanque N, Dalix AM, Durand G, and Slama G (1996): Dietary (n-3) polyunsaturated fatty acids improve adipocyte insulin action and glucose metabolism in insulin-resistant rats: relation to membrane fatty acids. *J.Nutr.* **126,** 1951-1958.

Ma J, Folsom AR, Shahar E, and Eckfeldt JH (1995): Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Am.J.Clin.Nutr.* **62,** 564-571.

Markovic TP, Jenkins AB, Campbell LV, Furler SM, Kraegen EW, and Chisholm DJ (1998): The determinants of glycemic responses to diet restriction and weight loss in obesity and NIDDM. *Diabetes Care* **21,** 687-694.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, and Turner RC (1985): Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28,** 412-419.

McCarthy MI and Froguel P (2002): Genetic approaches to the molecular understanding of type 2 diabetes. *Am.J.Physiol Endocrinol.Metab* **283,** E217-E225.

NCEP (2001): Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* **285,** 2486-2497.

Nes M, Frost AL, Solvoll K, Sandstad B, Hustvedt BE, Lovo A, and Drevon CA (1992): Accuracy of a quantitative food frequency questionnaire applied in elderly Norwegian women. *Eur.J.Clin.Nutr.* **46,** 809-821.

Parks EJ and Hellerstein MK (2000): Carbohydrate-induced hypertriacylglycerolemia: historical perspective and review of biological mechanisms. *Am.J.Clin.Nutr.* **71,** 412-433.

Qvigstad E, Mostad IL, Bjerve KS, and Grill V (*In press*): Acute lowering of circulating fatty acids improves insulin secretion in a subset of type II diabetes subjects. *Am.J.Physiol*.

Reseland JE, Haugen F, Hollung K, Solvoll K, Halvorsen B, Brude IR, Nenseter MS, Christiansen EN, and Drevon CA (2001): Reduction of leptin gene expression by dietary polyunsaturated fatty acids. *J.Lipid.Res.* **42,** 743-750.

Riccardi G and Parillo M (1993): Comparison of the metabolic effects of fat-modified vs low fat diets. *Ann.N.Y.Acad.Sci.* **683,** 192-198.

Rimestad AH, Blaker B, Flåten A-M, and Nordbotten A (1995): *Den store matvaretabellen (The Norwegian Food Table). In norwegian.* Universitetsforlaget, Oslo.

SEF (1997): *Norske næringsstoffanbefalinger (The Norwegian Recommendations of Food and Nutrient Intake). In norwegian.*

Storlien LH, Borkman M, Jenkins AB, and Campbell LV (1991): Diet and *in vivo* insulin action: of rats and man. *Diabetes.Nutr.Metab.* **4,** 227-240.

Storlien LH, James DE, Burleigh KM, Chisholm DJ, and Kraegen EW (1986): Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. *Am.J.Physiol* **251,** E576-E583.

Surwit RS, Petro AE, Parekh P, and Collins S (1997): Low plasma leptin in response to dietary fat in diabetes- and obesity- prone mice. *Diabetes* **46,** 1516-1520.

Swinburn BA (1993): Effect of dietary lipid on insulin action. Clinical studies. *Ann.N.Y.Acad.Sci.* **683,** 102-109.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, and Tataranni PA (2001): Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J.Clin.Endocrinol.Metab* **86,** 1930-1935.

Widjaja A, Stratton IM, Horn R, Holman RR, Turner R, and Brabant G (1997): UKPDS 20: plasma leptin, obesity, and plasma insulin in type 2 diabetic subjects. *J.Clin.Endocrinol.Metab* **82,** 654-657.

Yu-Poth S, Zhao G, Etherton T, Naglak M, Jonnalagadda S, and Kris-Etherton PM (1999): Effects of the National Cholesterol Education Program's Step I and Step II dietary intervention programs on cardiovascular disease risk factors: a meta-analysis. *Am.J.Clin.Nutr.* **69,** 632-646.

Table 1. Baseline and intervention characteristics of anthropometric and metabolic variables.

	Baseline all subjects n=19 **median** ***(min-max)***	**Baseline men n=10** **median** ***(min-max)***	**Baseline women n=9** **median** ***(min-max)***	**P-value[1]**	**Intervention All subjects n=19** **median** ***(min-max)***	**Intervention as % change of baseline n=19** **median** ***(min-max)***	**P-value[2]**
Age (y)	61 *(40-69)*	49 *(40-69)*	63 *(45-69)*	0.045			
Duration diabetes (y)	6 *(1-15)*	5 *(1-11)*	7 *(4-15)*	0.065			
BMI (kg/m^2)	30.3 *(24.7-41.9)*	30.4 *(25.3-39.0)*	30.3 *(24.7-41.9)*	0.683	30.0 *(24.5-41.6)*	99 % *(97-102)*	0.170
Systolic BP[3] (mm Hg)	146 *(103-168)*	143 *(123-165)*	160 *(103-168)*	0.069	140 *(115-180)*	94 % *(84-176)*	0.147
Diastolic BP (mm Hg)	88 *(70-103)*	90 *(70-100)*	88 *(70-103)*	0.533	85 *(75-100)*	101 % *(78-108)*	0.863
HbA1c (%)	7.8 *(5.4-10.3)*	7.2 *(5.4-9.6)*	7.8 *(7.2-10.3)*	0.221	7.9 *(5.2-10.4)*	100 % *(93-108)*	0.632
Glucose (mmol/l)	9.1 *(5.3-14.5)*	8.2 *(5.3-14.5)*	9.3 *(8.3-13.7)*	0.253	9.2 *(5.2-15.5)*	99 % *(74-132)*	0.507
Insulin (mU/l)	16.2 *(5.7-28.8)*	17.0 *(7.6-28.8)*	13.9 *(5.7-25.1)*	0.540	14.9 *(3.9-26.6)*	100 % *(35-164)*	0.520

[1] Mann-Whitney U-test was used to analyse differences between gender.
[2] Wilcoxon Signed Ranks Test was used to analyse the difference between baseline and intervention variables, n=19.
[3] BP = blood pressure

Glu/ins[4] ratio (mg/10^{-4}U)	11 *(4-43)*	11 *(4-34)*	11 *(7-43)*	0.514	11 *(4-60)*	95 % *(60-249)*	0.601
HOMA[5] (%)	63 *(11-186)*	64 *(14-186)*	58 *(11-87)*	0.327	48 *(8-313)*	105 % *(43-169)*	0.334
C-peptide (nmol/l)	1.1 *(0.7-2.2)*	1,2 *(0.9-2.2)*	0,9 *(0.7-1.3)*	0,033	0.8 *(0.6-2.3)*	95 % *(52-136)*	0.197
Glucagon (pmol/l)	35 *(20-70)*	42 *(31-74)*	32 *(20-74)*	0.041	36 *(17-77)*	92 % *(50-151)*	0.420
Proinsulin (pmol/l)	15 *(4-35)*	19 *(4-34)*	11 *(4-35)*	0,072	17 *(5-37)*	106 % *(70-150)*	0.965
Cortisol (nmol/l)	501 *(254-1045)*	454 *(254-852)*	559 *(266-1045)*	0.424	479 *(278-1182)*	99 % *(53-248)*	0.948
Leptin (ng/ml)	11.4 *(5.2-24.0)*	8,1 *(5.2-17.5)*	17.3 *(11.3-24.0)*	0.003	9.9 *(4.9-21.6)*	90 % *(61-113)*	0.013
Adiponectin (μg/ml)	1.1 *(0.3-1.8)*	1.1 *(0.3-1.8)*	1.1 *(0.8-1.4)*	0.627	1.0 *(0.3-1.8)*	92 % *(54-158)*	0.055
Cholesterol (mmol/l)	6.3 *(4.3-8.5)*	5.8 *(4.3-8.5)*	6.6 *(6.1-8.0)*	0.045	6.2 *(4.1-8.2)*	95 % *(76-110)*	0.011
HDL[6] (mmol/l)	1.11 *(0.84-1.52)*	1.00 *(0.84-1.45)*	1.21 *(0.97-1.52)*	0.025	1.10 *(0.86-1.50)*	97 % *(88-110)*	0.083
Cholesterol/HDL ratio	5.6 *(3.7-10.1)*	5.8 *(3.7-10.1)*	5.5 *(4.9-7.6)*	1.000	5.6 *(3.3-9.2)*	97 % *(75-111)*	0.133
Triglycerides (mmol/l)	2.7 *(1.4-11.1)*	3.1 *(1.4-11.1)*	2.7 *(2.2-6.4)*	0.713	2.7 *(1.1-10.3)*	91 % *(61-123)*	0.055

[4] glu/ins = fasting glucose (mg/dl)/fasting insulin (mU/l)
[5] HOMA = Homeostasis Model Assessment index
[6] HDL = High Density Lipoprotein cholesterol

FA[7] (mmol/l)	0.78 *(0.31-1.36)*	0.61 *(0.31-0.84)*	0.90 *(0.54-1.36)*	0.024	0.77 *(0.34-1.34)*	103 % *(71-158)*	0.372
PL-FA[8], total (mg/l)	1571 *(1198-2345)*	1480 *(1198-2345)*	1572 *(1474-1969)*	0.253	1552 *(1274-2342)*	98 % *(88-111)*	0.334
SFA[9] (g/100 g PL-FA)	41 *(39-43)*	41 *(39-42)*	41 *(40-43)*	0.191	41 *(38-42)*	97 % *(88-111)*	0.001
MUFA[10] (g/100 g PL-FA)	12 *(11-15)*	12 *(11-15)*	12 *(11-15)*	0.902	13 *(11-15)*	105 % *(94-127)*	0.002
n-6 FA (g/100 g PL-FA)	34 *(28-40)*	35 *(30-40)*	32 *(29-38)*	0.205	31 *(24-37)*	93 % *(78-107)*	0.001
n-3 FA (g/100 g PL-FA)	14 *(7-18)*	12 *(7-18)*	14 *(10-18)*	0.307	16 *(9-21)*	113 % *(72-192)*	0.003
n-6/n-3 ratio	2.4 *(1.6-5.7)*	2.9 *(1.6-5.7)*	2.3 *(1.7-3.9)*	0.253	1.9 *(1.2-4.2)*	76 % *(45-128)*	0.001

[7] FA = free fatty acids
[8] PL-FA = plasma phospholipid fatty acids
[9] SFA = saturated fatty acids
[10] MUFA = monounsaturated fatty acids

Table 2. Baseline and intervention characteristics of dietary intake of energy, nutrients and food items.

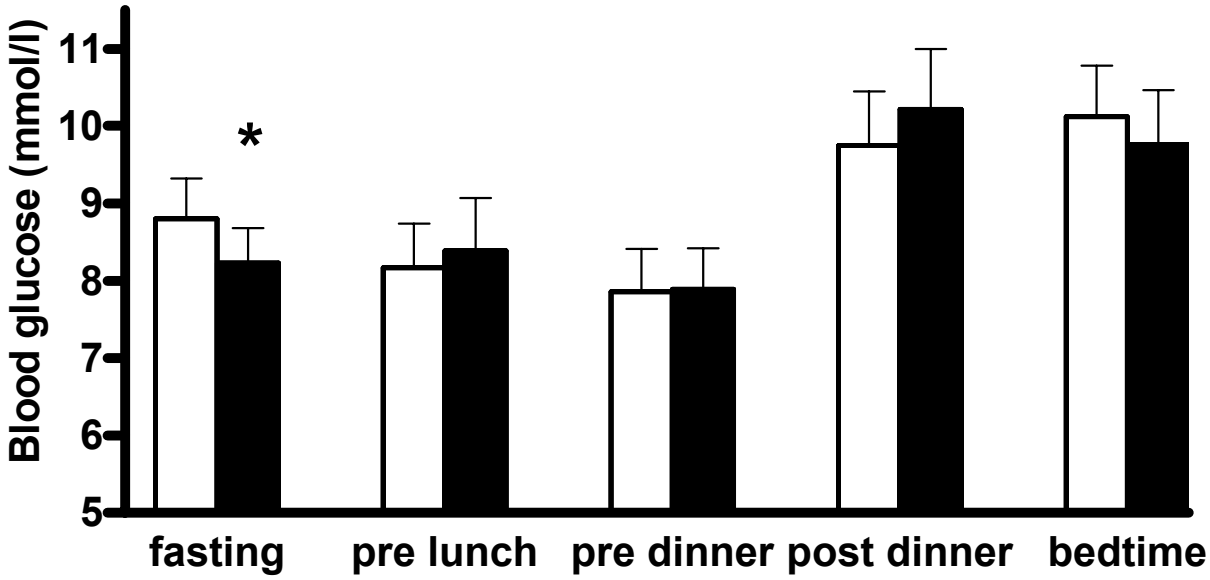
	Baseline all subjects n=19 median *(min-max)*	**Baseline men n=10** median *(min-max)*	**Baseline women n=9** median *(min-max)*	**P-value[11]**	**Intervention all subjects n=19** median *(min-max)*	**Intervention as % change of baseline n=19** median *(min-max)*	**P-value[12]**
Energy total MJ/d	7.9 *(5.3-13.2)*	9.1 *(6.5-13.2)*	7.0 *(5.3-9.9)*	0.009	6.6 *(5.3-13.3)*	89 % *(63-113)*	0.027
Protein (E%)[13]	18 *(12-26)*	17 *(12-20)*	19 *(14-26)*	0.142	23 *(18-30)*	129 % *(96-182)*	<0.0001
Fat (E%)	39 *(31-45)*	39 *(33-45)*	39 *(31-45)*	1.000	22 *(9-40)*	63 % *(23-90)*	<0.0001
Carbohydrates (E%)	41 *(33-55)*	43 *(33-55)*	40 *(36-48)*	0.935	50 *(37-68)*	125 % *(93-156)*	<0.0001
Alcohol (E%)	0 *(0-10)*	1 *(0-10)*	0 *(0-9)*	0.102	0 *(0-17)*	[14]	0.859
Sugar (E%)	4 *(0-16)*	6 *(0-16)*	3 *(0-4)*	0,003	3 *(0-10)*	96 % *(2-2873)*	0.277
Protein (g/d)	85 *(54-123)*	96 *(60-123)*	75 *(54-118)*	0.086	93 *(66-206)*	113 % *(69-192)*	0.016
Fat (g/d)	81 *(50-142)*	92 *(70-142)*	67 *(50-112)*	0,011	43 *(26-95)*	52 % *(20-89)*	<0.0001

[11] Mann-Whitney U-test was used to analyse differences between gender.
[12] Wilcoxon Signed Ranks Test was used to analyse the difference between baseline and intervention variables, n=19.
[13] E% = energy percentage
[14] Impracticable calculation because of zero intake of 10 subjects at baseline diet and zero intake of 15 subjects at intervention diet.

Carbohydrates (g/d)	187 *(127-344)*	225 *(145-344)*	166 *(127-224)*	0.022	210 *(125-450)*	115 % *(81-141)*	0.016
Alcohol (g/d)	0 *(0-30)*	5 *(0-30)*	0 *(0-21)*	0.085	0 *(0-77)*	[14]	0.515
Sugar (g/d)	22 *(1-92)*	33 *(1-92)*	13 *(1-23)*	0.003	13 *(1-64)*	92 % *(2-1847)*	0.159
Fibre (g/d)	20 *(12-30)*	21 *(12-30)*	20 *(14-24)*	0.935	27 *(16-48)*	142 % *(92-223)*	<0.0001
Beta-carotene (µg/d)	2748 *(413-4890)*	1969 *(413-4649)*	3658 *(2506-4890)*	0.007	3860 *(1345-10895)*	140 % *(49-2576)*	0.005
Alpha-tocopherol (mg/d)	7.7 *(5.3-18.9)*	9.5 *(6.5-17.7)*	7.2 *(5.3-18.9)*	0.369	6.8 *(3.7-16.0)*	90 % *(28-128)*	0.006
Vitamin C (mg/d)	71 *(29-296)*	73 *(50-296)*	63 (*29-169)*	0.744	111 *(35-268)*	130 % *(39-324)*	0.036
Bread (g/d)	163 *(87-277)*	176 *(87-277)*	159 *(96-193)*	0.060	218 *(105-320)*	123 % *(65-244)*	0.027
Potatoes (g/d)	85 *(29-290)*	76 *(29-290)*	130 *(55-169)*	0.142	118 *(58-429)*	131 % *(70-631)*	0.005
Vegetables (g/d)	115 *(0-364)*	90 *(0-266)*	126 *(79-364)*	0.165	252 *(69-405)*	200 % *(62-560)*	<0.0001
Fruit (g/d)	128 *(28-589)*	105 *(28-589)*	141 *(66-369)*	0.414	218 *(65-1015)*	172 % *(31-1337)*	0.016
Fish (g/d)	80 *(0-162)*	54 *(0-135)*	99 *(0-162)*	0.307	138 *(0-246)*	180 % *(0-355)*	<0.0001
Meat (g/d)	168 *(24-277)*	175 *(82-270)*	129 *(24-277)*	0.288	120 *(61-680)*	67 % *(36-535)*	0.494

Milk (g/d)	249 *(8-830)*	326 *(24-830)*	197 *(8-443)*	0.221	178 *(0-887)*	73 % *(0-333)*	0.469
Cheese (g/d)	19 *(0-82)*	19 *(0-58)*	29 *(5-82)*	0.462	29 *(0-88)*	104 % *(0-691)*	0.896
Eggs (g/d)	23 *(0-60)*	23 *(0-60)*	23 *(9-58)*	0.513	17 (0-57)	71 % *(0-397)*	0.055
Edible fats (g/d)	36 *(15-78)*	50 *(15-78)*	35 *(22-54)*	0.288	3 *(0-17)*	8 % *(0-115)*	<0.0001

Figure 1


Blood glucose (mmol/l)
11
10
9
8
7
6
5
*
fasting
pre lunch
pre dinner
post dinner
bedtime

LEGEND TO FIGURE 1

Concentrations of blood glucose obtained by home glucose monitoring. Bars depict mean values of measures at each day in the 6-days register period at baseline diet (open bars) and 3 days register period at intervention diet (filled bars), n = 19. “Post dinner” signifies values measured 2 h after the start of dinner. Results are mean ± SEM.

∗ $P<0.05$ (Wilcoxon Signed Rank Test).

NINE WEEKS OF BEDTIME DIAZOXIDE IS WELL TOLERATED AND BETA-CELL BENEFICIAL IN SUBJECTS WITH TYPE 2 DIABETES.

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Running title: Beta-cell rest in type-2 diabetes

SUMMARY

Aims. To test whether a single bedtime dose of diazoxide (D) would alleviate side effects and still improve beta-cell function in type 2 diabetes.

Methods. A double-blind randomised study was performed in 27 type 2 diabetic subjects (17M, 10F), negative for antibodies against glutamic acid decarboxylase and islet cell antigen-2, and treated with bedtime insulin (BTI) and metformin. Subjects received either bedtime D, 100mg, or placebo for 9 weeks. Duplicate C-peptide glucagon tests were performed before and at the end of intervention.

Results. No side effects of D were detected. Treatment with D did not incur any increase in BTI. C-peptide responses to glucagon were increased 0,15 ± 0,06nmol/l vs. -0,01 ± 0,04nmol/l for placebo, p<0,06 for difference. Corresponding effects on insulin were 66,2 ± 41,7pmol/l for D vs. –84,2 ± 51,5, for placebo p<0,03. Treatment with D decreased fasting glucagon levels by 41% vs. placebo, p<0,03. Glycated haemoglobin (HbA_{1c}) levels were not affected, whereas levels of blood glucose post breakfast were higher during D (1,34 ± 0,43mmol/l, p<0,01 vs. placebo). A breakfast test in the presence of 0.5mg repaglinide elicited a robust insulin response in D-treated subjects.

Conclusions. Bedtime treatment with D in type 2 diabetes on BTI and metformin has no measurable side effects, does not increase BTI supplementation, ameliorates beta-cell function but fails to improve metabolic control. Improvement of metabolic control could be contingent

upon combination treatment with daytime pharmacological enhancement of insulin secretion.

KEYWORDS:

bed time insulin, beta-cell rest, diazoxide, insulin secretion, type 2 diabetes.

ABBREVIATIONS

BMI body mass index, BTI bed time insulin, HbA1c glycated haemoglobin, HDL high density lipoprotein cholesterol, RIA radioimmunoassay

Hyperglycaemia impairs endogenous insulin secretion in type 2 diabetes [1-3] and in newly diagnosed type 1 diabetes [1]. In experimental studies over-stimulation plays an important role for the negative effects of hyperglycaemia. Thus, chronic hyperglycaemia in the rat abolished glucose-induced insulin secretion [4]; however secretion was upheld if insulin secretion was inhibited by diazoxide during the period of hyperglycaemia [5]. Also evidence obtained in vitro with diazoxide supports a role for over-stimulation [2,6].

Diazoxide inhibits glucose-induced insulin secretion by a molecular action opposite to that of glucose. By opening ATP-sensitive K^+ channels in the cell membrane of beta-cells [7], diazoxide antagonises the opposite effect by glucose. Diazoxide thereby prevents glucose-induced cell membrane depolarisation, opening of voltage-dependent Ca^{++} channels and ensuing insulin secretion. The inhibitory effects of diazoxide on insulin secretion are rapidly reversible [2,5]. The beneficial effects on insulin secretion that are demonstrable after stopping diazoxide can therefore be ascribed to diazoxide´s preventive effect on over-stimulation rather than to any effect of the drug per se.

Also clinical studies report beneficial effects of diazoxide on beta-cell function. Björk et al treated newly diagnosed type-1 diabetes patients with diazoxide for a period of 3 months in addition to their usual insulin treatment [8]. Insulin secretion was better preserved in diazoxide-treated compared with placebo-treated subjects, as assessed from higher levels of

C-peptide levels up to 12 months after the end of the treatment period. As to type-2 diabetes, patients treated with diazoxide for 7 days in an open trial displayed improved insulin secretion when diazoxide was discontinued [9].

Despite these promising findings the therapeutic use of diazoxide is in doubt. Side effects are one reason for this. Common side effects of diazoxide are oedema and lanugo hair growth [10]. Other side effects are nausea and hypotension. Oedema is treatable by diuretics and will subside like the other side effects once diazoxide treatment is discontinued. Hence, the side effects are not of a serious nature. They are however disturbing enough to create major obstacles to treatment. A further reason for not using diazoxide in type 2 diabetes is the perceived need to combine diazoxide with insulin treatment in patients not otherwise in need of insulin according to current therapeutic guidelines.

Clinical experience from treatment of insulinoma patients has shown that 100 mg diazoxide three times daily is usually required to suppress glucose-induced insulin secretion [11]. Such or similar doses were given in previous studies in diabetic patients [8,9] . A lower dose given three times daily could potentially diminish side effects but would at the same time diminish the inhibitory effects on insulin secretion while not eliminating, in type 2 diabetes, a potential need for 24h insulin treatment.

We reasoned that giving 100mg of diazoxide only once daily and then at bedtime to type 2 diabetic patients could have several advantages. First, the intermittent nature of diazoxide treatment could possibly produce less side effects than a three times daily regimen. Second, diazoxide given at bedtime could be added to bedtime insulin in combination with daytime peroral anti-diabetic treatment without any need for major adjustments of treatment. Third, it was intuitively sensible to let beta-cells “rest” during the night in order to perform better during daytime.

Against this background, a double blind randomised trial was designed in patients with type 2 diabetes to investigate the feasibility of treatment with diazoxide at bedtime combined with BTI and, furthermore, to test for improvement of endogenous insulin secretion.

PATIENTS AND METHODS

Subjects

Twenty-seven subjects with type 2 diabetes (17 males, 10 females) participated. The subjects were recruited from the Departments of Internal Medicine at Levanger Hospital and St Olav's University Hospital. Inclusion criteria were type 2 diabetes as defined by clinical criteria and by absence of islet cell antigen-2 and glutamic acid decarboxylase antibodies, and age between 35 and 80 years. For the purpose of the study, patients should be non-optimally controlled as defined by HbA1c values >7.0% for patients below 70 years and >8.0% for patients between 70 and 80 years (as measured at 2 different time points more than 5 weeks apart). The exclusion criteria were proliferative or pre-proliferative retinopathy, pregnancy or lactation, heart failure New York Heart Association grade III and IV, serum creatinine >150μmol/l, and alcoholism or other serious diseases affecting the possibility of the subject to participate.

At inclusion all but two patients were treated with metformin in doses varying from 500mg to 3000 mg/day (mean dosage 2060mg ± 170). Twelve subjects used sulphonylureas. Nine used glipizide, 10-15mg, two glimepiride, 3-4mg and one glibenclamide, 10,5mg. Ten of the subjects treated with metformin were on combination therapy with sulphonyureas. Ten of the subjects were already using BTI at the time of inclusion (dosage 24 ± 5IU, range 6-44). Eight of the patients received antihyperlipaemic treatment in the form of statins and thirteen patients received antihypertensive treatment. Two female participants were

receiving hormone replacement therapy. Other concomitant medications in one or more patients were salicylates, vitamin supplements, thyroxine, analgesics, antiastmatic medication, nitroglycerine, antihistamines, glaucoma medication, benzodiazepines and cox-2 inhibitors. No patient was on systemic steroid medication.

Experimental design

At the day of inclusion a physical examination was performed in all subjects. Then any sulphonylurea medication was discontinued and metformin treatment started if not already taken. The dose of metformin was adjusted according to recommendations [12], aiming at 2g daily. In those subjects not previously on BTI, such treatment (Insulin Insulatard, Novo Nordisk Inc.) was initiated at the date of inclusion and adjusted every third day following an algorithm based on fasting glucose measurements [13], where levels of fasting blood glucose between 4-7mM were aimed for. Other concomitant medications were kept at the same dosage throughout the study.

After a run-in period of 8 weeks, the patients performed 7-point home glucose monitoring for 3 days. Then, C-peptide glucagon tests [14] were performed in duplicate i.e., on two consecutive days, after which the patients were randomised to start with either diazoxide or placebo capsules at bedtime. Randomisation was done by a computerised minimisation procedure, Minimize [15]. The randomisation parameters that were weighted were (in decreasing order) HbA1c (2.0), body mass

index BMI (1.5), age and fasting C-peptide (1.0), sex and diabetes duration (0.5).

During the intervention period the participants were seen at outpatient visits by a study nurse or by one of the authors 1, 2, 4, 6, 8 and 9 weeks after the start of diazoxide or placebo treatment. At each visit body weight and blood pressure (sitting position) was measured, the presence of oedema checked for and the results of home glucose monitoring reviewed. The dosage of BTI was adjusted from measurements of fasting blood glucose [13]. At the end of the intervention period the patients again performed 7-point home glucose monitoring during 3 days. Then two C-peptide glucagon tests were again performed in duplicate on two consecutive days. Finally, thirteen diazoxide-treated patients and twelve placebo-treated patients underwent a standardised breakfast test together with 0.5mg repaglinide as detailed below.

The protocol was approved by the local ethics commission and by the Norwegian Drugs Control Authority. All subjects gave informed written consent.

Study medication

The production of diazoxide (Proglycem™, Schering Plough Inc.) and placebo capsules took place at the Hospital Pharmacy, St. Olav's University hospital. The quality control standards of the production were approved by the Norwegian Drugs Control Authority. The study pharmacist was responsible for allocation concealment.

C-peptide and breakfast tests

The patients fasted overnight and refrained from taking their morning medications. Standardised C-peptide glucagon tests were performed [14]. For the breakfast test, the patients ingested a standardised breakfast with 0.5mg repaglinide (Novonorm™). The breakfast contained 470kcal (47 % carbohydrates, 17 % protein and 36 E% fat). Following the start of the meal blood samples were collected every 15min for 2h.

After centrifugation, all samples were frozen and kept at –80°C degrees for later analysis.

Assays

Blood glucose in the hospital was determined by a reflectrometric device (HemoCue Ltd, Dronfield, UK). For home glucose monitoring, all patients were supplied with identical measuring devices for the trial (Glucometer Elite XL, Bayer Co, Tarrytown, NY). HbA1c was determined by DCA (Bayer Co, Tarrytown, NY). Human C-peptide and insulin were assayed by radioimmunoassay (RIA) (Linco Res. Inc, St. Louis, MO). Glucagon and proinsulin were also determined by RIA (Linco Res. Inc,).

Levels of fatty acids were determined by an enzymatic colorometric method (NEFA-C-kit, Wako Pure Chemical Industries Ltd, Osaka, Japan). Triacylglycerols, cholesterol and high density lipoprotein (HDL) were determined by standard laboratory techniques.

The presence of antibodies against glutamic acid decarboxylase and islet cell antigen-2 was determined by RIA (Dianova GmbH, Hamburg,

Germany). Insulin antibodies were determined by enzyme linked immunosorbent assay (Milenia-Biotec GmbH, Bad Nauheim, Germany).

Presentation of results

Values are given as mean ± SEM if not otherwise stated. Statistical analysis was done using SPSS/PC + 10.0 and based on comparisons between diazoxide and placebo treatment in the two test periods. Results obtained at the duplicate occasions of C-peptide-glucagon testing and the triplicate 7-point glucose measurements were averaged before being entered in tables and figures and before significance testing.

Two subjects tested positive for insulin antibodies at the end of the study (one from the diazoxide and one from the placebo group). These individuals were removed from the calculation of fasting and stimulated insulin data. Significance testing was done by Student's *t* test (paired or independent samples), or, for non-normally distributed variables, the Wilcoxon matched pairs signed-rank sum test or Mann-Whitney test. Pearson's and Spearman's correlation coefficients were used to evaluate bivariate correlations where appropriate.

RESULTS

Clinical characteristics

The average duration of known diabetes in these subjects was between 6 and 7 years (Table 1). Most subjects were obese, albeit to a varying extent. A majority of the subjects had n unsatisfactory, but not severely deranged metabolic control as assessed by HbA1c (Table 1). The levels of triacylglycerols were in the high normal range, according to current guidelines [16].

Three of the subjects were habitual smokers.

Randomisation

There were no significant differences in the clinical characteristics of patients randomised to diazoxide or to placebo (Table 1).

Dropouts and compliance

All randomised subjects completed the study. Two subjects did not participate in the breakfast test. Compliance with the treatment regimen appeared good. Thus, by blood glucose measurements in the clinic corresponded well with home glucose monitoring. Also, the amount of unused medication returned agreed with the prescribed doses of the study medications.

Bedtime insulin during the study

At the end of the run-in period the patients starting on diazoxide were treated with 32 ± 5 IU, whereas patients starting on placebo received 24 ± 5 IU. During the intervention period, the mean dose of intermediate acting insulin was increased to the same extent in both groups, i.e. by 7 ± 2 IU in the diazoxide treated subjects and by 7 ± 3IU in the placebo group (Fig 1A).

Adverse events

We did not detect oedema in any patient. Body weight increased marginally and non-significantly in both groups. The increase in the end of intervention amounted to 0,56 ± 0,29 kg in the diazoxide-treated group and 0,75 ± 0,42 kg in the placebo-treated group (Fig 1B). Blood pressure declined from 143/85 to 137/83 mmHg in diazoxide-treated subjects, $p<0,1$, and from 146/85 to 140/82 in placebo treated subjects, $p<0,005$), with no significant difference between the groups. There were no symptoms related to orthostatic hypotension. The youngest female subject (40 years) on diazoxide mentioned a possible increase in facial lanugo hair, an increase that was however not obvious on inspection.

Minor hypoglycaemic episodes during the night were experienced by four subjects in the diazoxide group. Three of these subjects were in the lower BMI range of the study population and had low fasting C-peptide levels at inclusion.

Effects of diazoxide on beta-cell function

Fasting levels of C-peptide decreased by 33% ($p<0,07$) in the diazoxide group, and by 13% ($p<0,2$) in the placebo-treated patients (Table 2). In the diazoxide group there was no correlation between individual patients of the C-peptide to insulin ratio on one hand and insulin doses on the other ($r= -0,28$, ns.). However, a negative correlation was seen in the placebo group ($r= -0,65$, $p<0,02$). These observations are compatible with an inhibitory effect of diazoxide on endogenous insulin secretion that overrides that of circulating insulin.

In contrast to the inhibitory of diazoxide on fasting C-peptide, the glucagon-stimulated levels of C-peptide were increased in the diazoxide-treated group vs. placebo by 95%, $p<0,06$ as were also insulin levels by 36% vs. placebo, $p<0,02$ (Fig 2). The increase in glucose levels during the C-peptide glucagon tests that were performed at the end of intervention, was similar between groups (+ 0,95 ± 0,20 mmol/l in the diazoxide and + 1,00 ± 0,10 mmol/l in the placebo group, NS).

As calculated from the duplicate testing, the coefficient of variation of stimulated C-peptide and insulin responses at the end of intervention was 4,3 in the diazoxide-treated and 7.3% in the placebo group. The corresponding coefficient of variation for stimulated insulin was 6,3% in the diazoxide-treated subjects and 6,8% in the placebo group.

A positive effect on stimulated C-peptide levels by diazoxide tended to correlate with fasting C-peptide levels at randomisation ($r= 0,37$, $p<0,17$) in the diazoxide group. Such tendency was not seen in the placebo group ($r= 0,19$, $p<0,55$). Diazoxide treatment also decreased levels of fasting proinsulin by 62% (Table 2) and the proinsulin /insulin ratio by 44%, both NS vs. placebo.

Effects of diazoxide treatment on glucagon secretion

Glucagon levels in the over-night fasted state were reduced after diazoxide treatment (by 41%, $p<0,03$, vs. placebo, Table 2).

Blood glucose and HbA1c

The 7-point blood glucose registration performed during 3 days at the end of the run-in period was compared with registrations performed in the end of the intervention period. Bedtime diazoxide increased blood glucose levels somewhat vs. placebo after breakfast (by $1,34 \pm 0,43$mmol/l, $p<0,01$, Fig 1C). Blood glucose levels recorded later during the day show a similar tendency but did not achieve statistical significance. Blood glucose levels during the evening were, on the other hand, not affected. The levels of fasting blood glucose recorded at the time of the C-peptide glucagon tests were not significantly affected in the diazoxide group but were decreased in the placebo group (Table 2). Similarly, the diazoxide treatment did not significantly decrease HbA1c (from 8.1 vs. 7.9%), whereas a decrease was seen after placebo (from 7,9 vs. 7,4, $p<0,03$, Table 2).

Other metabolic parameters

Plasma levels of fatty acids, cholesterol or HDL did not differ as result of intervention between the diazoxide and placebo groups during the study (results not shown). Triacylglycerol levels were marginally decreased by 0,09 ± 0,08mmol/l in the diazoxide treated subjects, and increased by 0,05 ± 0,10mmol/l in the placebo treated subjects, NS.

Standardised breakfast test

The study patients were not treated with sulphonylurea or similarly acting drugs. As a pointer for future studies it was of interest to establish whether diazoxide treated subjects were responsive to a breakfast in the presence of pharmacological enhancement of insulin secretion. A breakfast test was therefore performed in the presence of repaglinide at the end of the intervention period. This test induced a marked C-peptide and insulin response in diazoxide-treated subjects (Fig.3). The response was similar to that in placebo-treated subjects (data not shown).

DISCUSSION

Our study demonstrates that 9 weeks of treatment with a single 100mg dose of diazoxide combined with BTI in type-2 diabetes patients is without measurable side effects and perfectly acceptable to the patients. These findings are underscored by the very good compliance of patients and by the absence of dropouts in the study. The absence of side effects in comparison to other studies can likely be explained by the lower dosage and, perhaps, by the intermittent mode of administration. A theoretical possibility is that older subjects would be less susceptible to side effects than the young adults who participated in a previous study in type 1 diabetes patients [8]. However, we fail to find any evidence in the literature for age in adults being a factor for diazoxide´s side effects.

We found evidence for better insulin secretion during diazoxide treatment in terms of responses to glucagon in the C-peptide glucagon tests. The validity of these results was strengthened by performing tests always in duplicate and averaging results from each pair of tests. The finding of improved insulin secretion was obtained in spite of other evidence that some inhibitory effect of diazoxide on insulin secretion was still present, i.e. somewhat higher glucose levels in the morning. This possible discrepancy can be explained by the selectivity of diazoxide´s inhibitory effect on insulin secretion. By opening of potassium channels the drug counteracts the opposite and insulin-stimulating effects of glucose, but not that of insulin secretagogues that operate through other signal-secretion pathways [17,18]. These include the presently tested hormone glucagon as

well as meal-stimulated GLP-1, which both primarily activate the adenylcyclase - cyclic AMP pathway [19].

Also the tendencies for a decrease in proinsulin levels and proinsulin to insulin ratios during diazoxide treatment are in line with ameliorated beta-cell function. Elevated proinsulin and proinsulin to insulin ratios are commonly found in type 2 diabetes [20] and, in all likelihood, reflect a strain on beta-cells to produce insulin above their capacity [21].

There was some indication that a relatively preserved beta-cell capacity, as assessed from fasting C-peptide levels during the run-in phase of the study, may predispose for better insulin secretion after diazoxide. This finding agrees with other evidence demonstrating that beta cell capacity correlates to the beneficial effect that normalisation of blood glucose exerts on insulin secretion [22]. Hence, any intervention to improve and preserve insulin secretion should probably be instituted early during the course of diabetes.

It is interesting that the amount of BTI did not have to be increased during diazoxide relative to placebo treatment. This finding could not be explained by a lack of effect of the drug on insulin secretion since signs of residual inhibition of insulin secretion by diazoxide were present as late as in the morning following bedtime diazoxide. That BTI did not have to be increased therefore indicates increased insulin sensitivity. This finding is contrary to a 7 day previous study with diazoxide in lean type 2 diabetes

subjects [23]. However, it is in agreement with the study in type 1 diabetes patients by Björk et al. [8] that indicated no need to increase insulin dosage in diazoxide and insulin-treated type 1 diabetic patients. Likewise, diazoxide increases insulin sensitivity in rat models of diabetes [24]. An insulin-sensitising effect could, in part at least, be explained by a lowering of glucagon levels that we here document, to our knowledge for the first time, in diabetic subjects. An inhibitory effect on glucagon levels is likely to be a direct effect of diazoxide, since such an effect has been previously demonstrated in vitro [25].

Home glucose monitoring revealed that diazoxide treatment raised blood glucose levels after breakfast. Although these undesirable effects were small they may question the rationale for treatment with diazoxide, since any therapeutic regimen should in the end improve metabolic control. Two conditions could possibly ensure metabolic improvement. First, it is probably advantageous to combine bedtime diazoxide with short-acting pharmacological enhancers of insulin secretion at meals during daytime. A beneficial effect of such inclusion is suggested by the meal test with repaglinide in the present study. This test was intended only to reveal whether subjects on diazoxide could indeed respond with insulin release to a breakfast together with repaglinide, despite the evidence for lingering inhibition of glucose-induced insulin secretion at that time point. The results demonstrate a robust insulin response in the diazoxide-treated group. This finding is encouraging, since an even stronger effect of repaglinide and similar agents acting on the K^+-ATP-channels could be

expected at later meals when the counteracting effect of diazoxide is gone. This notion is supported by a study in dogs demonstrating that previous diazoxide actually enhances the subsequent insulin response to tolbutamide [26].

Second, selection of patients should be modified in future studies. Our patients had a rather long duration of known diabetes and were clinically in the stage of the disease in which some form of insulin treatment was considered in order to improve a deteriorating metabolic control. We selected such patients because they could be given diazoxide with minimal interference with a standard therapeutic regimen and also because a need for counteracting a potential increase of hyperglycaemia could to some extent be met by giving more BTI. With feasibility in terms of side effects now demonstrated and with no apparent need to balance effects of diazoxide with insulin, future studies could be performed in subjects with shorter duration of diabetes and with a documented good capacity of endogenous insulin secretion.

Beneficial effects on beta-cell function in humans have been found with somatostatin [27,28], which also inhibits insulin secretion, not by interaction with the ATP-sensitive channels, but by interaction with G-proteins in the beta-cell membrane [29]. However somatostatin must be administered by multiple sc. injections which reduces its potential as an everyday treatment. Hence, diazoxide treatment is probably advantageous to that of somatostatin. Diazoxide could also be advantageous to insulin

therapy since it depresses insulin secretion more than insulin alone [30 and present study].

In summary, bedtime treatment with diazoxide in type 2 diabetes on BTI and metformin has no measurable side effects, does not increase insulin supplementation, ameliorates beta-cell function but fails to improve metabolic control. Improvement of metabolic control could be contingent upon combination treatment with daytime pharmacological enhancement of insulin secretion and the selection of subjects with relatively preserved capacity for insulin secretion.

ACKNOWLEDGEMENTS

The help and advice of the following persons is gratefully acknowledged: H. F. Borgos for help during the screening and recruitment procedures, L. K. Myhre for the production of study medication and for doing the randomisation procedures, E. Tingstad and associates at the Dept. of Clinical Chemistry at Innherred Hospital for assistance during blood sampling and tests, Nurse M. Wæren at Innherred Hospital and the diabetes nurses at St. Olav's University Hospital for help during the trial visits, the Department of Immunology and Transfusions, St. Olav's University Hospital for antibody analysis and I. H. Jørgensen for C-peptide analysis.

We thank Novo Nordisk Inc., Norway for donating pre-filled insulin pens and Bayer Inc., Norway for donating the blood glucose measurement strips.

The study was supported by funds from the Norwegian Research Council (grant no141836/320).

REFERENCES

1. Rossetti L, Giaccari A, DeFronzo RA: Glucose toxicity. *Diabetes Care* 1990; **13**: 610-630.

2. Grill V, Bjorklund A: Dysfunctional insulin secretion in type 2 diabetes: role of metabolic abnormalities. *Cell Mol Life Sci* 2000; **57**: 429-440.

3. Donath MY, Gross DJ, Cerasi E, Kaiser N: Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. *Diabetes* 1999; **48**: 738-744.

4. Leahy JL, Cooper HE, Deal DA, Weir GC: Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic in vivo glucose infusions. *J Clin Invest* 1986; **77**: 908-915.

5. Sako Y, Grill VE: Coupling of beta-cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats. *Diabetes* 1990; **39**: 1580-1583.

6. Grill V, Bjorklund A: Overstimulation and beta-cell function. *Diabetes* 2001; **50** Suppl 1: S122-S124.

7. Trube G, Rorsman P, Ohno-Shosaku T: Opposite effects of tolbutamide and diazoxide on the ATP-dependent K+ channel in mouse pancreatic beta-cells. *Pflugers Arch* 1986; **407**: 493-499.

8. Bjork E, Berne C, Kampe O, Wibell L, Oskarsson P, Karlsson FA: Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. *Diabetes* 1996; **45**: 1427-1430.

9. Greenwood RH, Mahler RF, Hales CN: Improvement in insulin secretion in diabetes after diazoxide. *Lancet* 1976; **1**: 444-447.

10. Diazoxide: a review of its pharmacological properties and therapeutic use in hypertensive crises. *Drugs* 1971; **2**: 78-137.

11. Dollery C ed. *Therapeutic Drugs*. 2nd edn. Churchill Livingstone Edinburgh 1999: 84-87.

12. Garber AJ, Duncan TG, Goodman AM, Mills DJ, Rohlf JL: Efficacy of metformin in type II diabetes: results of a double-blind, placebo-controlled, dose-response trial. *Am J Med*1997; **103**: 491-497.

13. Makimattila S, Nikkila K, Yki-Jarvinen H: Causes of weight gain during insulin therapy with and without metformin in patients with Type II diabetes mellitus. *Diabetologia* 1999; **42**: 406-412.

14. Faber OK, Binder C: C-peptide response to glucagon. A test for the residual beta-cell function in diabetes mellitus. *Diabetes* 1977; **26**: 605-610,

15. Jensen CV: A computer program for randomizing patients with near-even distribution of important parameters. *Comput Biomed Res* 1991; **24**: 429-434.

16. Wood D, De Backer G, Faergeman O, Graham I, Mancia G, Pyorala K: Prevention of coronary heart disease in clinical practice: recommendations of

the Second Joint Task Force of European and other Societies on Coronary Prevention. *Atherosclerosis* 1998; **140**: 199-270.

17. Zunkler BJ, Lenzen S, Manner K, Panten U, Trube G: Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K+ currents in pancreatic B-cells. *Naunyn Schmiedebergs Arch Pharmacol* 1988; **337**: 225-230.

18. Mariot P, Gilon P, Nenquin M, Henquin JC: Tolbutamide and diazoxide influence insulin secretion by changing the concentration but not the action of cytoplasmic Ca2+ in beta-cells. *Diabetes* 1998; **47**: 365-373.

19. Gromada J, Holst JJ, Rorsman P: Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. *Pflugers Arch* 1998; **435**: 583-594.

20. Kahn SE: The importance of the beta-cell in the pathogenesis of type 2 diabetes mellitus. *Am J Med* 2000; **108** Suppl 6a: 2S-8S.

21. Bjorklund A, Grill V: Enhancing effects of long-term elevated glucose and palmitate on stored and secreted proinsulin-to-insulin ratios in human pancreatic islets. *Diabetes* 1999; **48**: 1409-1414.

22. Clauson P, Alvarsson M, Grill V: Enhancement of B-cell secretion by blood glucose normalization in type 2 diabetes is associated with fasting C-peptide levels. *J Intern Med* 1997; **241**: 493-500.

23. Olczak SA, Greenwood RH, Hales CN: Post-receptor insulin resistance after diazoxide in non-insulin dependent diabetes. *Horm Metab Res* 1986; **18**: 38-41.

24. Alemzadeh R, Slonim AE, Zdanowicz MM, Maturo J: Modification of insulin resistance by diazoxide in obese Zucker rats. *Endocrinology* 1993; **133**: 705-712.

25. Urdanivia E, Pek S, Santiago JC: Inhibition of glucagon secretion by diazoxide in vitro. *Diabetes* 1979; **28**: 26-31.

26. Anderson JH, Jr., Byrd GW, Blackard WG: Hyperresponsiveness to tolbutamide of dogs pretreated with diazoxide. *Metabolism* 1971; **20**: 1023-1030.

27. Bjork E, Berne C, Karlsson FA: Induction of beta-cell rest in type 1 diabetes. Studies on the effects of octreotide and diazoxide. *Diabetes Care* 1998; **21**: 427-430.

28. Laedtke T, Kjems L, Porksen N, Schmitz O, Veldhuis J, Kao PC, Butler PC: Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. *Am J Physiol Endocrinol Metab* 2000; **279**: E520-E528.

29. Sharp GW: Mechanisms of inhibition of insulin release. *Am J Physiol* 1996; **271**: C1781-C1799.

30. Puhakainen I, Taskinen MR, Yki-Jarvinen H: Comparison of acute daytime and nocturnal insulinization on diurnal glucose homeostasis in NIDDM. *Diabetes Care* 1994; **17**: 805-809.

LEGEND TO FIGURES

Figure 1. Change of A) insulin doses, B) blood glucose (home monitoring) and C) body weight after the intervention period.

Figure 2. Effects of intervention on stimulated A) C-peptide and B) insulin levels. # diazoxide, n=14 and placebo, n=11 due to insulin antibodies in two subjects.

Figure 3. Effects of a standardised test meal on incremental levels on A) blood glucose, B) C-peptide and C) insulin in diazoxide-treated subjects, n=13.

TABLE 1 *Clinical characteristics at inclusion in randomised groups. Mean ± SEM. No significant differences were seen between groups.*

	Diazoxide	Placebo
Age (years)	57,5 ± 2,6	60,4 ± 2,8
M/F	8/7	9/3
Diabetes duration (years)	7,5 ± 1,1	5,8 ± 0,9
BMI (kg/m^2)	29,6 ± 1,2	27,9 ± 0,9
Fasting blood glucose (mmol/l)	10,0 ± 0,7	8,9 ± 0,3
HbA1c (%)	8,6 ± 0,2	8,4 ± 0,3
C-peptide (nmol/l)	1,0 ± 0,2	0,8 ± 0,1
Triglycerides (mmol/l)	1,8 ± 0,4	2,5 ± 0,7
Cholesterol (mmol/l)	4,7 ± 0,5	5,4 ± 0,4
HDL (mmol/l)	1,29 ± 0,09	1,09 ± 0,06

TABLE 2*:Fasting levels of blood glucose,* HbA1c*, and islet hormones. Concentrations are given as mean ± SEM. *p<0,03, for change from run-in to intervention, diazoxide vs. placebo. # diazoxide, n=14 and placebo, n=11 due to insulin antibodies in two subjects.*

	Run-in		Intervention	
	Diazoxide	Placebo	Diazoxide	Placebo
Glucose (mmol/l)	6,7 ± 0,4	6,3 ± 0,3	7,1 ± 0,5	5,6 ± 0,3*
HbA1c (%)	8,1 ± 0,2	7,9 ± 0,4	7,9 ± 0,2	7,4 ± 0,3
C-peptide (nmol/l)	0,64 ± 0,13	0,46 ± 0,05	0,42 ± 0,06	0,40 ± 0,06
Insulin (pmol/l)#	188 ± 32	117 ± 23	217 ± 34	211 ± 54
Proinsulin (pmol/l)	26,0 ± 6,8	16,9 ± 2,8	21,2 ± 5,2	19,9 ± 3,9
Glucagon (ng/l)	81 ± 6	76 ± 5	74 ± 5*	86 ± 7

Figure 1

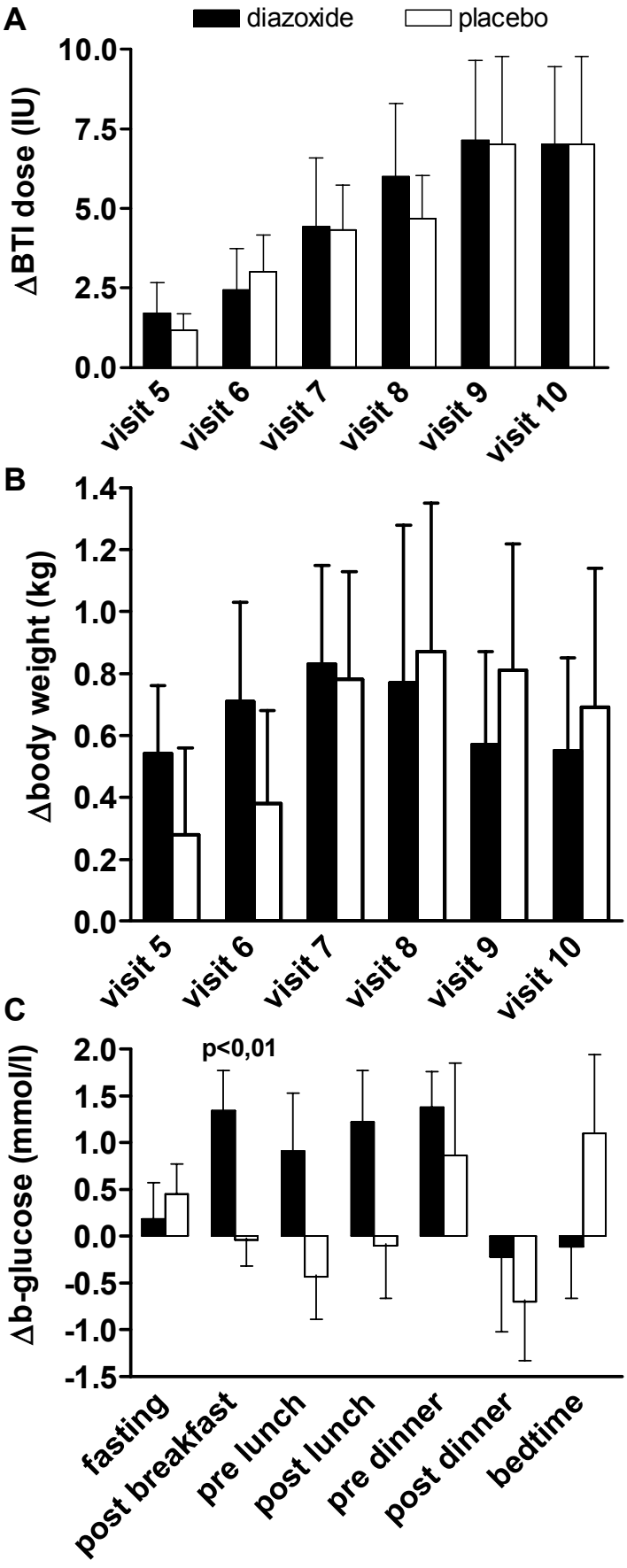

A
diazoxide
placebo
ΔBTI dose (IU)
10.0
7.5
5.0
2.5
0.0
visit 5
visit 6
visit 7
visit 8
visit 9
visit 10
B
Δbody weight (kg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
visit 5
visit 6
visit 7
visit 8
visit 9
visit 10
C
Δb-glucose (mmol/l)
p<0,01
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
fasting
post breakfast
pre lunch
post lunch
pre dinner
post dinner
bedtime

Figure 2

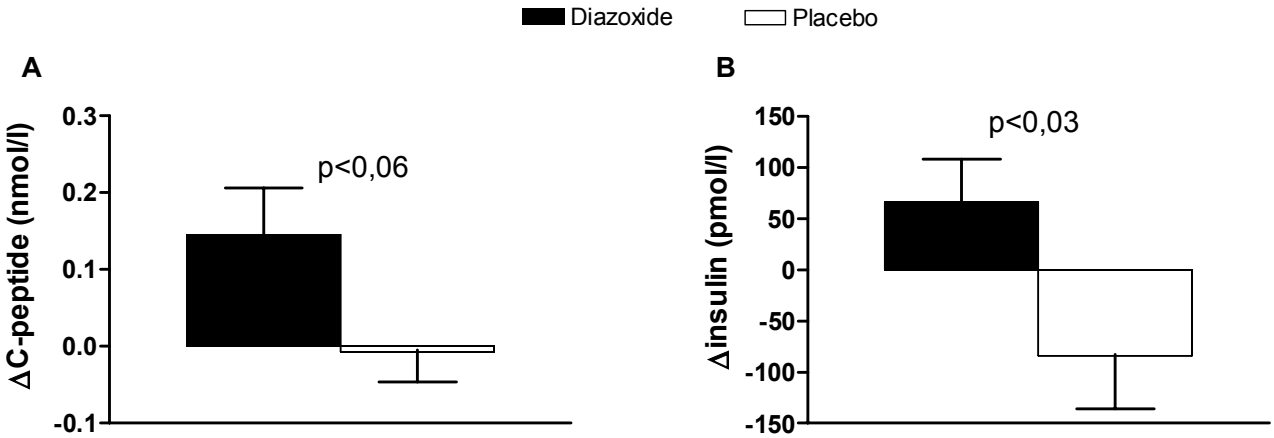
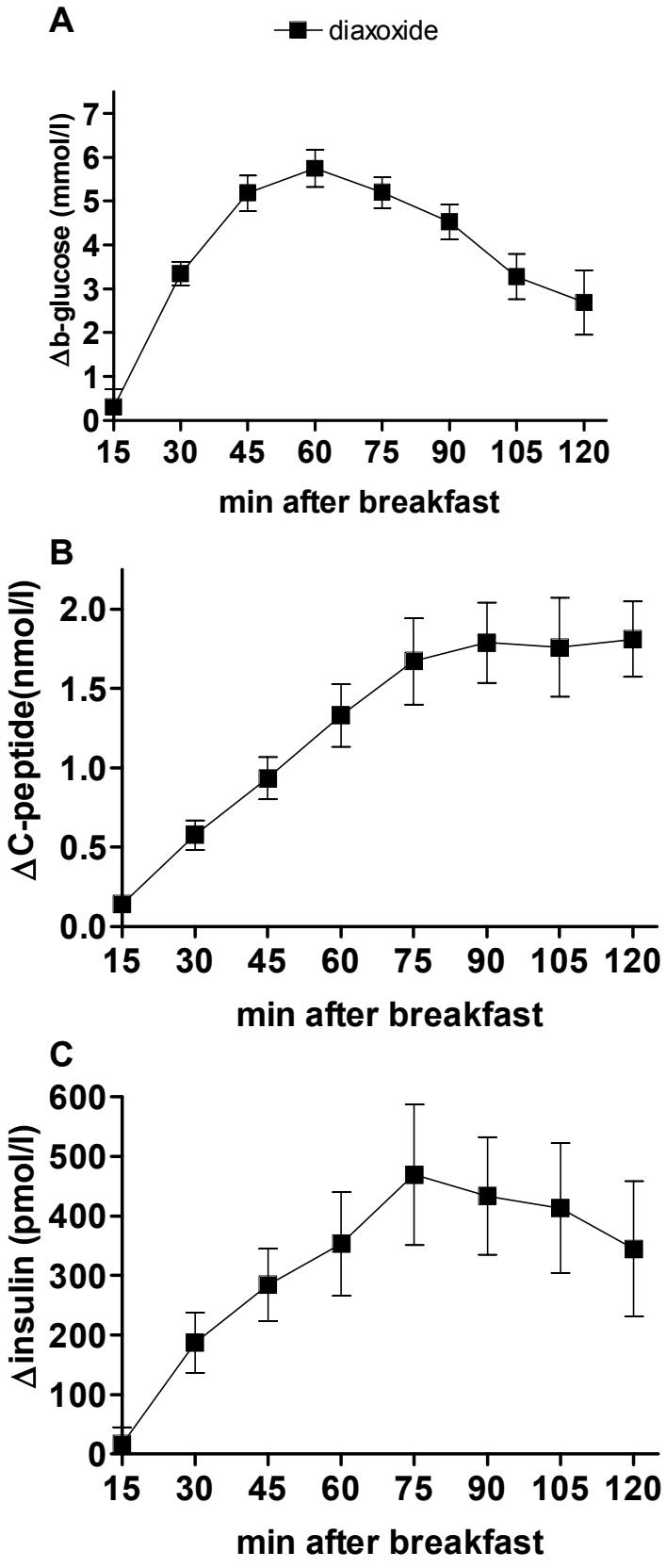

Diazoxide
Placebo
A
ΔC-peptide (nmol/l)
0.3
0.2
0.1
0.0
-0.1
p<0,06
B
Δinsulin (pmol/l)
150
100
50
0
-50
-100
-150
p<0,03

Figure 3


A
diaxoxide
Δb-glucose (mmol/l)
0
1
2
3
4
5
6
7
15 30 45 60 75 90 105 120
min after breakfast
B
ΔC-peptide(nmol/l)
0.0
0.5
1.0
1.5
2.0
15 30 45 60 75 90 105 120
min after breakfast
C
Δinsulin (pmol/l)
0
100
200
300
400
500
600
15 30 45 60 75 90 105 120
min after breakfast