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Aorta and muscle metabolism in pigs with peripheral hyperinsulinaemia

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Summary. Peripheral hyperinsulinaemia usually found in conventionally treated Type 1 (insulin-dependent) diabetic patients may have deleterious metabolic effects. We have used a hyperinsulinaemic model to examine intermediary metabolism in two key peripheral tissues, aorta and muscle. Nine pigs were immunized with crystalline insulin. Subsequently, they showed an insulin-binding capacity of 86.2 ± 25.0 pmol/l and fasting total serum insulin of 3.9 ± 3.1 nmol/l (control range 0.034–0.072 nmol/l), impaired glucose tolerance after oral glucose tolerance testing, significantly elevated levels of peripheral venous serum free insulin and C-peptide, and increased mean post-prandial free insulin/glucose ratios. The immunized pigs showed marked elevation of aorta and muscle triglycerides compared with control pigs (n = 15) but similar levels of non-esterified fatty acids. The glucose-6-phosphate-dehydrogenase, malic enzyme and 3-hydroxyacyl-CoA-dehydrogenase activities were all increased significantly (by 50%–300%) in both aorta and muscle. Phosphofructokinase was decreased in both tissues. Hexokinase was increased in muscle alone whereas pyruvate kinase was significantly decreased in aorta. Glyceraldehyde-3-phosphate dehydrogenase activity was not significantly different in aorta and muscle. Thus in insulin immunized pigs with normal β-cell function and pronounced peripheral hyperinsulinaemia there was increased peripheral lipogenic activity. These findings have potentially important implications with regard to macrovascular disease in diabetes.

Key words: Peripheral hyperinsulinaemia, aorta metabolism, muscle metabolism, tissue triglycerides, enzyme activities, macrovascular disease, pig.

Arteriosclerosis occurs earlier, more frequently and more extensively in diabetic than non-diabetic subjects and may be responsible for at least 60% of the mortality of diabetes [1–3]. In Type 1 diabetic patients treatment has little proven effect in preventing macrovascular disease and appears unable to correct established vascular complications [4]. Despite the commonly accepted concept that insulin deficiency and its metabolic consequences are the primary causes of diabetic complications, patients do not constantly show insulin deficiency, but, on the contrary, are hyperinsulinaemic for certain periods. In particular, peripheral circulating insulin levels may be elevated much of the time. In the hyperinsulinaemic state, vessel wall lipogenesis may well be increased [5]. The main aims of this study were to develop a non-diabetic model for hyperinsulinämia and to determine whether such peripheral hyperinsulinaemia has adverse effects on metabolism in key tissues, such as aorta and muscle.

Materials and methods

Animals

Insulin antibodies were induced in nine pigs (one male, eight females) by immunizing weekly with 20 U of twice crystallized bovine insulin (Novo) (with Freund's adjuvant). The pigs were fed a diet containing 17% protein, 2% fat and 81% carbohydrate. Fifteen age- and sex-matched non-immunized pigs of the same weight at the start of the experiment served as controls. The animals were killed under anaesthesia at the age of 3–6 years after 30 h of fasting and after removing biopsies (approximately 4–5 g) from the ascending aorta close to the heart and skeletal muscle (scapularis). Hybnodil and sedapane anaesthesia was used, these agents having minimal effects on intracellular enzyme activity [6].

Methods

Non-esterified fatty acid (NEFA) and triglyceride levels were determined in the tissue after extraction by the method of Folch et al. [7] as described earlier [8]. Re-extraction in 10 muscles and 10 aorta samples showed that all triglycerides had been extracted in the Folch extraction. The assays on aortic tissue were conducted on intima-media samples freed from surrounding connective tissue. Adventitia was removed totally as shown by microscopy. Activities of the following enzymes were measured in both aorta and muscle:
hydroxyacyl-CoA-dehydrogenase, EC 1.1.1.35 [9]; malic enzyme, EC 1.1.1.40 [10]; glucose-6-phosphate dehydrogenase (G-6-P), EC 1.1.1.49 [11]; hexokinase, EC 2.7.1.1 [12], phosphofructokinase, EC 2.7.1.40 [9]; pyruvate kinase, EC 2.7.1.40 [11], and α-glycerophosphate dehydrogenase, EC 1.1.1.8 [9].

The activities of the enzymes are calculated against wet weight per tissue, since both reference to DNA or protein gave poorer reproducibility of the analyses. Enzyme activity was re-estimated after renewed homogenization and centrifugation of the precipitate. In the rehomogenated precepitate no enzyme activity was measurable in either the immunized or control animals.

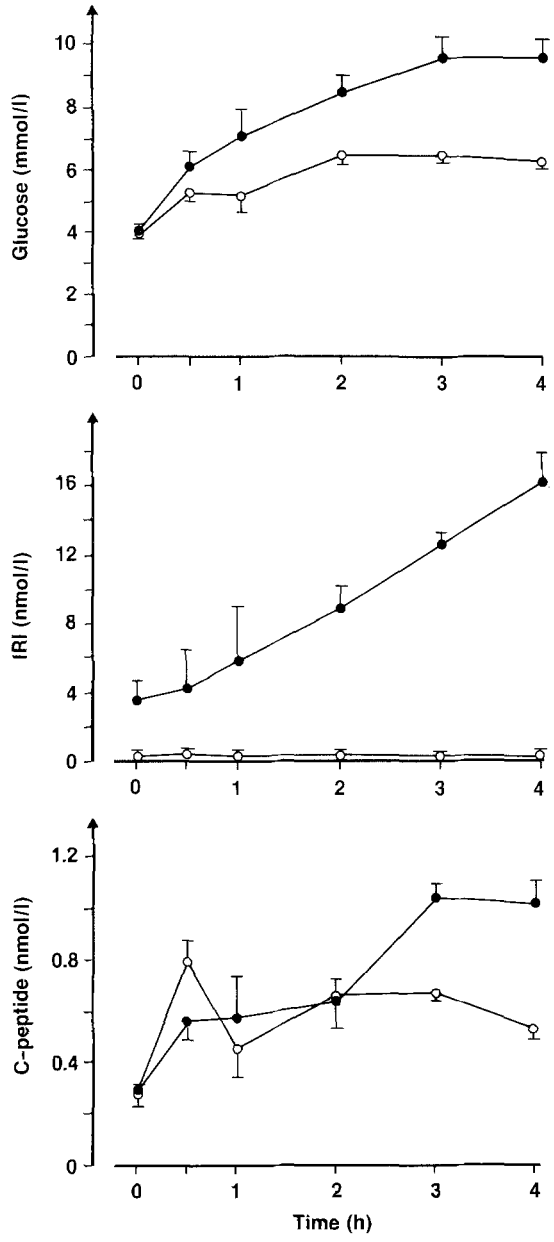

Fig. 1. Plasma glucose, total insulin, and C-peptide levels after a 100 g glucose load in nine immunized (●) and 15 normal pigs (○). (mean ± SD)

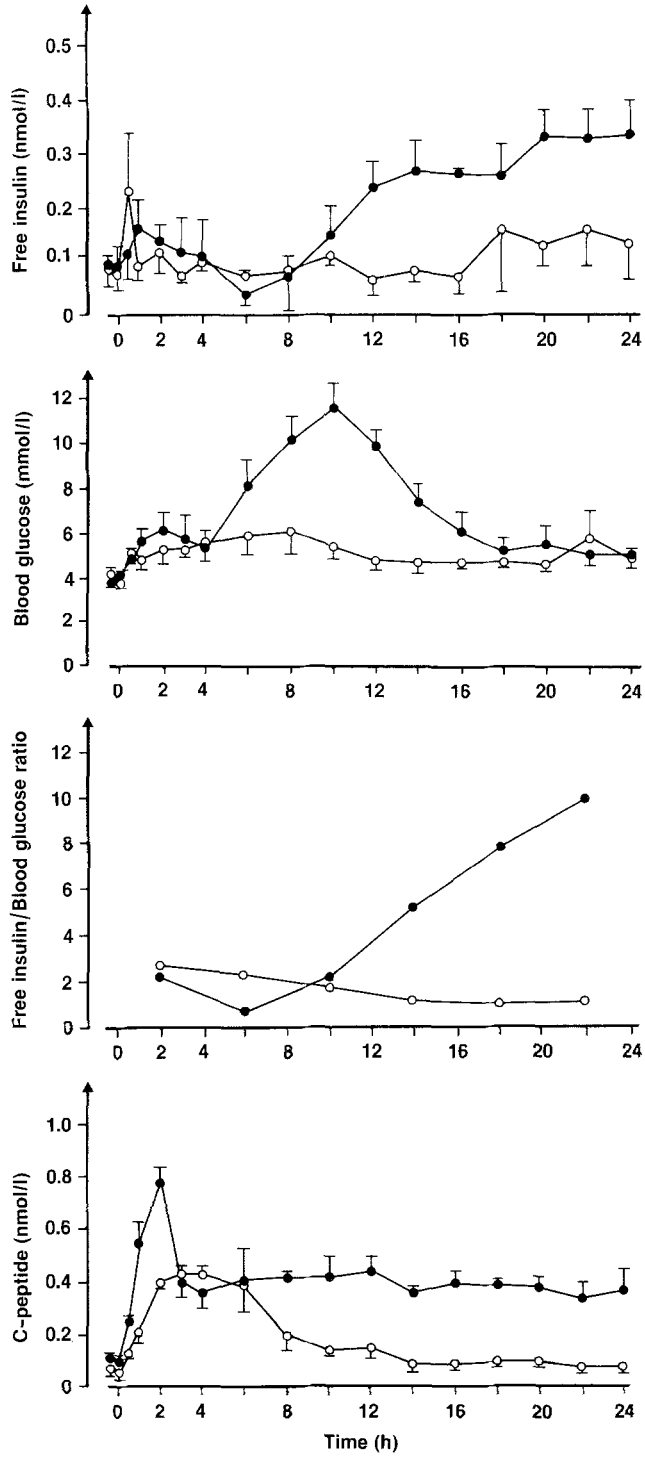

Fig. 2. Twenty-four-hour profiles of plasma free insulin, glucose, and C-peptide after a meal and ratios of the mean value of free insulin and blood glucose determined at 4-h intervals during the test meal in nine immunized (●) and 15 control pigs (○)

The animals were kept alive as long as possible, which resulted in the large variation in time from beginning of immunization until the experiments were carried out. There was no correlation between change in enzyme activity and content of triglycerides in aorta and muscle with the duration of insulin antibody production in the animals.

Antibody determination was performed according to the immunoelectrophoresis method of Christiansen [13]. Several studies were performed to characterize the in vivo metabolic status of the pigs. Profiles of blood metabolites (lactate, alanine, glycerol and 3-hydroxybutyrate [14], were measured every hour for 24 h after a meal, the pigs being starved for 24 h before the test. Samples for metabolites (0.5 ml) were taken in 2 ml 3% perchloric acid as described previously [14]. Plasma glucose [15], NEFA [16], triglyceride [17], and the hormones, C-peptide [18], glucagon [19], and total and free insulin [20] were also measured.

To obtain a reliable and reproducible measure for free insulin in pigs with high levels of antibodies, an optimized method was developed ensuring that no remaining antibody influence occurred.

Free insulin was measured after a preliminary step involving precipitation of these antibodies with polyethylene-glycol at neutral pH (PEG/MAKROGOL 6000). The samples were incubated for 2 h at 37 °C to reflect the equilibrium of free and antibody-bound insulin in

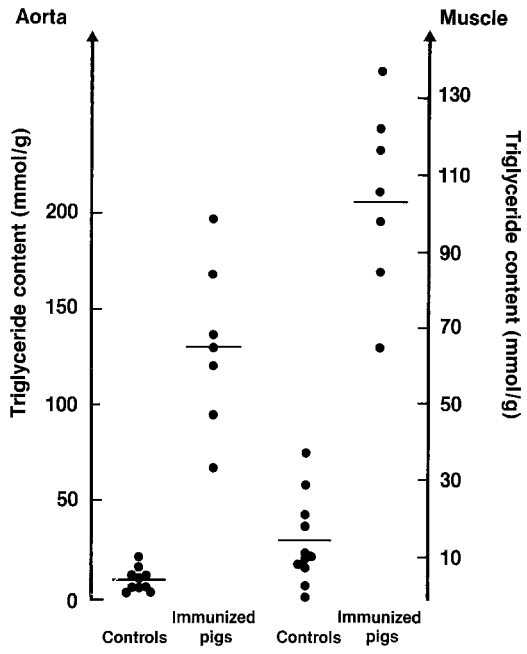

Fig. 3. Triglyceride content of aorta and muscle tissue in seven immunized and 11 control pigs. Horizontal bars represent mean values for triglyceride levels. They were compared using the Student's t-test for unpaired samples

Table 1. Plasma NEFA and triglycerides levels in the immunized and control pigs before, 12 and 24 h after a meal

	Time after a meal (h)		
	0	12	24
Plasma NEFA (mmol/l)			
Immunized pigs ($n=9$)	1.09 ± 0.30	0.57 ± 0.15	0.91 ± 0.26
Control pigs ($n=15$)	0.33 ± 0.10	0.29 ± 0.16	0.38 ± 0.12
p	0.001	0.005	0.001
Plasma triglyceride (mmol/l)			
Immunized pigs ($n=9$)	0.24 ± 0.09	0.43 ± 0.10	0.25 ± 0.07
Control pigs ($n=15$)	0.05 ± 0.04	0.11 ± 0.06	0.06 ± 0.04
p	0.001	0.001	0.001

Results expressed as mean ± SD

vivo; pH in sera increased during storage and thawing. To ensure complete precipitation by polyethylene-glycol, the samples were neutralized and precipitated using 30% polyethylene-glycol (wt/vol) dissolved in a 0.04 mol/l phosphate buffer (pH 7).

Sera with high IgG values (> 10 mU/ml) must be assayed after 1:1 dilution with a 0.04 mol/l phosphate buffer (pH 6.0; 0.250 ml + 0.250 ml), and precipitation with 30% polyethylene-glycol.

No difference was found in non-specific binding and standard curves between 30% polyethylene-glycol made up in buffer or water. Samples from 14 non-diabetic subjects were analysed in the direct insulin radioimmunassay after precipitation with 30% polyethylene-glycol; the results showed no significant difference (correlation coefficient 0.95). Plasma insulin and free insulin were measured with a sensitivity of 2.5 nmol/l and a coefficient of variation of 0.06 nmol/l. C-peptide had a sensitivity of 0.03 nmol/l and a coefficient of variation of 0.054 nmol/l, and glucagon a sensitivity of 0.01 mg/l and a coefficient of variation 0.05 mg/l. Metabolic profiles were performed twice in each pig at an interval of 2–4 weeks. Oral glucose tolerance tests (100 g glucose at time 0) were performed every 3 months in each pig after 24-h fasting. Samples were drawn at −30, 0, 30, 60, 120, 180 and 240 min. Plasma samples were analyzed for glucose, total insulin and C-peptide levels.

Statistical analysis

Results are presented as mean ± SD and range. They were compared, where appropriate, using the Student's two-tailed t-test for unpaired samples.

Results

Glucose tolerance testing

Immunization resulted in high insulin binding antibody levels (86.2 ± 25.0 pmol/l). The immunized pigs had normal basal glucose values but markedly impaired glucose tolerance (Fig. 1). Total insulin levels were significantly increased in immunized pigs (range 2.4–24.9 nmol/l) and remained elevated.

Meal testing

Plasma free insulin and glucose during 24 h following the ingestion of a meal are shown in Figure 2. The immunized pigs were clearly hyperinsulinaemic. The areas under the curve for the 24 h were 0.226 ± 0.069 nmol · l^{-1} · h^{-1} for the immunized pigs and 0.105 ± 0.029 nmol · l^{-1} · h^{-1} for the normal pigs ($p < 0.001$). Free insulin/glucose ratios were also increased, and so was the C-peptide (Fig. 2). Plasma NEFA and triglyceride levels were markedly elevated at all three times in the immunized pigs compared with the control pigs (Table 1). Other blood metabolites and plasma glucagon were not different between the two groups of pigs (data not shown).

Aorta and muscle substrates

In both aorta and muscle the triglyceride content was grossly elevated in the immunized compared with the control pigs ($p < 0.001$; Fig. 3). The tissue levels of NEFA were not statistically different in aorta (1.02–2.2 versus 0.7–3.3 μmol/g) or in muscle (1.4–7.1 versus 0.6–2.5 μmol/g) in the two groups.

Intracellular enzymes

The activities of the different enzymes in aorta and muscle are shown in Table 2. G-6-P, malic enzyme and hydroxyacyl-CoA-dehydrogenase activities were significantly increased ($p < 0.01$) in both aorta and muscle of immunized compared with control pigs. The activity of phosphofructokinase was significantly decreased in both aorta and muscle of immunized pigs ($p < 0.01$), while hexokinase was slightly elevated in muscle ($p < 0.05$). Pyruvate kinase was decreased in aorta ($p < 0.01$). The activity of α-glycerophosphate dehydrogenase was not statistically different between the two groups.

Table 2. Enzyme activities of aorta and muscle tissue of immunized and control pigs

Tissue		Enzyme activities (U/g wet weight)						
		G-6-P dehydrogenase	Malic	Hydroxyacyl-CoA-dehydrogenase	Phosphofructokinase	Hexokinase	Pyruvate kinase	α-Glycerophosphate dehydrogenase
Aorta	Immunized pigs ($n=9$)	0.56 ± 0.08	0.23 ± 0.03	1.9 ± 0.07	0.35 ± 0.03	0.39 ± 0.2	3.4 ± 0.5	0.46 ± 0.09
	Normal pigs ($n=15$)	0.40 ± 0.05	0.16 ± 0.02	0.8 ± 0.02	0.54 ± 0.09	0.54 ± 0.2	4.3 ± 0.2	0.56 ± 0.1
	p	< 0.01	< 0.01	< 0.05	< 0.001	NS	< 0.01	NS
Muscle	Immunized pigs ($n=9$)	0.21 ± 0.05	0.2 ± 0.04	5.5 ± 2.0	4.1 ± 1.0	5.7 ± 0.7	18.3 ± 4.0	39.9 ± 9.5
	Normal pigs ($n=15$)	0.07 ± 0.001	0.1 ± 0.02	1.7 ± 0.2	6.0 ± 0.6	3.7 ± 1.8	13.8 ± 3.1	37.8 ± 4.5
	p	< 0.01	< 0.05	< 0.01	< 0.01	< 0.05	NS	NS

Results expressed as mean ± SD

Discussion

Immunized pigs were characterized by high circulating insulin-binding antibodies and raised total and free insulin levels after the oral glucose tolerance tests and the meal tests. There was pronounced glucose intolerance presumably due to antibody binding of newly secreted insulin in response to the glucose, with slow release of insulin from the insulin antibody complex thereafter [21]. The high total insulin (25 nmol/l) is in the range observed in human insulin-treated diabetes [22]. The total and free plasma insulin were, as predicted, identical when the pigs did not have antibodies.

Plasma C-peptide levels also were elevated. This measurement allows β-cell secretion to be quantitated when insulin antibodies are present. The C-peptide secretion was elevated in the immunized pigs. It is unlikely that the removal of C-peptide is of quantitative importance. We feel that it is the increased secretion of C-peptide rather than the higher disposal rate that causes the elevated levels. In spite of this increased secretion, the animals developed hyperglycaemia. Consequently, the binding of insulin to the antibodies in the immunized pigs leads to a higher secretion from the β cell together with insulin resistance. Another effect of the insulin antibodies on the control of glucose in an animal with normal β-cell function is that the free insulin does not show the usual rapid decrease following a stimulus. It seems reasonable to conclude that the insulin antibody complexes slowly dissociate into free insulin and antibody thus continuing to supply tissues with free insulin. Ten hours after the ingestion of food and for the remaining period of the 24-h profile there was a constant supply of free insulin which maintained peripheral hyperinsulinaemia for at least 10 h in the immunized animals.

The positive association between atherosclerosis and plasma triglycerides shown by Carlson and Böttinger [23] has remained controversial, not being observed in other studies [24, 25]. Insulin has been suggested as an atherogenic factor [26, 27]. If both hypotheses are correct, two separate mechanisms could be operating: (1) an effect on circulating lipid metabolism; and (2) a direct effect on the arterial wall. An effect of insulin directly on the arterial wall is, however, a strong possibility.

Stout et al. showed that intravenous injection of 0.1 U/kg of insulin in rats resulted in increased incorporation of D-glucose-^{14}C into arterial wall triglycerides and, furthermore, increased proliferation of arterial smooth muscle cells with increasing levels of insulin concentrations in cultured, smooth muscle cells from aortas of monkeys [27]. A key question is whether the increased triglycerides in aorta and muscle observed in the present study are the result of a direct effect on the uptake of plasma triglycerides or stimulation by insulin of de novo synthesis of lipid on the arterial wall.

Our studies showed a 10- to 15-fold increase in the triglyceride content in both aorta and muscle of the immunized compared with the normal pigs. The NEFA content was the same in both aorta and muscle of immunized and control pigs, which, however, does not exclude the possibility that the increased triglycerides may be the result of esterification of NEFA derived from the circulation. Alternatively, it may result from increased uptake of triglycerides from plasma. However, this is hardly the case, as Falholt et al. [28] found an even more pronounced elevation of artery and muscle triglycerides in a hyperinsulinaemic dog model (pancreatectomized, receiving pancreatic autografts with drainage into the peripheral systemic circulation). These dogs had significantly lower levels of serum triglyceride and cholesterol levels than the control dogs. Thus, in the dog model, the high tissue triglycerides cannot be due to high levels of circulating triglycerides entering the arterial wall.

The increased activities of hydroxyacyl-CoA-dehydrogenase, indeed, suggest increased fatty acid oxida-

tion. The activity of the glycolytic enzymes measured in this study was lower (5- to 15-fold) in aorta than in skeletal muscle. However, the activity of G-6-P was threefold higher in aorta than in muscle and was higher than the activity of hexokinase in aorta, while in skeletal muscle, G-6-P activity was less than 5% of hexokinase activity indicating more pronounced metabolic disturbance in aorta. In rabbits with atherosclerosis induced by cholesterol feeding, the hexokinase activity of aortic tissue was decreased after 3 months [29]. In our pig model, there was also a tendency to lower hexokinase activity, in contrast to the grossly elevated G-6-P. The decreased activity of the glycolytic enzymes phosphofructokinase and pyruvate kinase in immunized pigs is consistent with the finding of lower values for several glycolytic enzymes (phosphofructokinase, glyceraldehydephosphate dehydrogenase, hexokinase) in atherosclerotic arteries compared with normal coronary arteries [30].

It is of interest that the activities of G-6-P and malic enzyme were increased in the immunized pigs. This may suggest an increased provision of reducing equivalents as NADPH, which is necessary for fatty acid synthesis. In aorta, however, de novo synthesis of lipid from glucose may be of quantitative importance [5]. This raises the question whether the increase in G-6-P in the immunized pigs, particularly in aorta where the activity is higher than that of hexokinase, may reflect, albeit indirectly, an increase in de novo synthesis of fatty acid.

In conclusion, immunized pigs had grossly elevated triglyceride content in aorta and muscle. This may be related to increased uptake of triglyceride from the circulation or increased uptake of fatty acid with esterification. Alternatively, it may be related to increased de novo synthesis of lipids as suggested by the increased activities of G-6-P and malic enzyme. These data are of potential importance in insulin-dependent and non-insulin-dependent diabetic man. Hyperinsulinaemia may be found in both states. Thus with peripheral administration of insulin consistently elevated free insulin levels have been reported [30–37], although there are still arguments about the accuracy of such measurements [38]. In non-insulin-dependent diabetes, there is a relative lack of insulin due to insulinresistance but absolute levels are often higher than in non-diabetic man, the problem being exacerbated by the oft-associated obesity. In both forms of diabetes, macroangiopathy is two to five times commoner than in non-diabetic man. The present study, albeit in an animal model, suggests that there may indeed be a causal relationship between hyperinsulinaemia and macrovascular disease.

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