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Insulin resistance is associated with high plasma ouabain-like immunoreactivity concentration in NIDDM

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Summary The aim of the present study was to elucidate the pathophysiologic significance of circulating ouabain as a link between insulin resistance (IR) and hypertension (HT) in NIDDM. Euglycaemic (4.5 mmol/l) hyperinsulinaemic (360–580 pmol/l) clamping was performed using an artificial endocrine pancreas. Plasma ouabain-like immunoreactivity (OLI) was determined by radioimmunoassay using a highly specific antibody to ouabain. HT was defined as systolic blood pressure > 140 mm Hg and/or diastolic > 90 mm Hg or being treated with antihypertensive agents. The values (mean ± SEM) of glucose infusion rate (GIR) and plasma OLI were compared among the four groups classified using IR and HT as factors. Group I (IR–/HT–, n = 15):GIR 7.20 ± 0.36 mg · kg^{-1} · min^{-1}, OLI 130.8 ± 20.9 pmol/l, which was not different from that in eight normal control subjects (7.69 ± 0.40 mg · kg^{-1} · min^{-1} and 142.6 ± 32.3 pmol/l, respectively); Group II (IR–/HT+, n = 13): 5.89 ± 0.36 mg · kg^{-1} · min^{-1}, 172.5 ± 35.0 pmol/l; Group III (IR+/HT–, n = 14) 1.91 ± 0.28 mg · kg^{-1} · min^{-1}, 576.6 ± 161.5 pmol/l ($p < 0.01$ vs Group I and II); Group IV (IR+/HT+, n = 15) 1.79 ± 0.22 mg · kg^{-1} · min^{-1}, 703.1 ± 170.1 pmol/l ($p < 0.01$ vs Group I and II), respectively. Six of 57 NIDDM patients studied exhibited very high (> 1500 pmol/l) plasma OLI concentrations, showed marked insulin resistance and were all hypertensive. When analysed as a whole, plasma OLI was negatively correlated with GIR ($p < 0.001$), but was not correlated with arterial blood pressure. These results demonstrate that plasma concentration of OLI is closely associated with the severity of IR but not with blood pressure elevation. It is, however, possible that in some fraction of NIDDM patients with insulin resistance, the elevation of blood pressure may be causally related to circulating OLI. [Diabetologia (1995) 38: 792–797]

Key words Ouabain-like immunoreactivity, insulin resistance, hypertension, non-insulin-dependent diabetes.

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Abbreviations: BSA, Bovine serum albumin; NIDDM, non-insulin-dependent diabetes mellitus; OLI, ouabain-like immunoreactivity; GIR, glucose infusion rate; BMI, body mass index.

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The mechanism(s) behind hypertension in non-insulin-dependent diabetes mellitus (NIDDM) are poorly understood, but some studies have indicated that sodium retention may play a major role [1–4]. Impaired natriuresis and resultant expansion of extracellular fluid volume have been reported to be associated with hyperinsulinaemia or insulin resistance [5, 6]. Furthermore, insulin resistance has been shown to accompany the increased sodium and decreased potassium content in erythrocytes [7], and recent evidence also suggests that high Na^+–Li^+ countertransport is associated with insulin resistance in patients with essential hypertension [8]. Thus, intracellular sodium accumulation, which in turn cou-

ples with increased cytosolic free calcium through Na^+–Ca^{++} exchange may cause contraction of resistant vessels or sensitization of vascular smooth muscle cells to vasoconstrictors [9], thereby leading to hypertension [10]. In this context, Na^+/K^+-ATPase activity has been reported to be low in a variety of tissues in insulin-resistant states including diabetes [11–14]. Therefore, the reduced activity of sodium pumps (Na^+/K^+-ATPase) may be a key mechanism linking hypertension and insulin resistance in NIDDM. Recently, a potent inhibitor of Na^+/K^+-ATPase was isolated from human plasma and found to be indistinguishable from ouabain [15–17], although this novel evidence has been challenged by recent studies [18, 19].

In the present study, therefore, we measured plasma ouabain-like immunoreactivity (OLI) concentrations in patients with NIDDM and severe insulin resistance or normal insulin sensitivity to elucidate whether ouabain is involved in the pathophysiology of hypertension associated with insulin resistance.

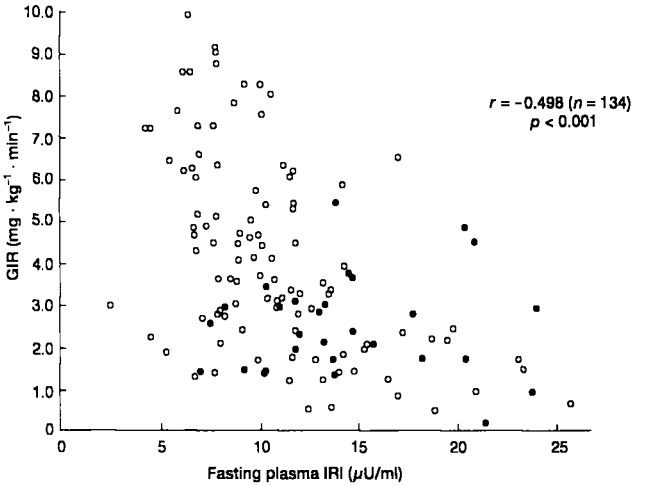

Fig. 1. Correlation between GIR values and fasting plasma IRI concentrations in 134 NIDDM patients without insulin therapy. There is a negative correlation between these two parameters ($r = -0.498$, $p < 0.001$) and also marked individual variation in GIR levels (GIR: 0.15–10.0 mg · kg^{-1} · min^{-1}). ● indicates obesity > 27 kg/m^2 (male), > 25 kg/m^2 (female)

Subjects and methods

Subjects. We studied 57 patients with NIDDM and eight healthy control subjects. None of these subjects had overt diabetic complications except early background retinopathy and intermittent proteinuria in some patients. All subjects were in stable health and they had no symptoms of congestive heart failure or oedema of any aetiology. Since NIDDM patients exhibit great variation in their individual sensitivity to insulin (GIR: 0.15–10.0 mg · kg^{-1} · min^{-1}) (Fig. 1), we randomly selected the subjects in the present study from both the highest quartile group (GIR > 5.2 mg · kg^{-1} · min^{-1}) and the lowest quartile group (GIR < 2.20 mg · kg^{-1} · min^{-1}). As shown in Table 1, NIDDM patients were divided into four subgroups according to the degree of insulin resistance and hypertension. Hypertension was defined as average systolic pressure of greater than 140 mm Hg and/or average diastolic pressure of greater than 90 mm Hg on at least three occasions preceding the euglycaemic clamp study or being treated with antihypertensive drugs. The four subgroups were matched for body mass index (BMI) and fasting blood glucose level. The mean ages (± SEM) of control subjects and Group I were significantly lower than those of other groups, but there was no difference among Groups II, III and IV. Glycaemic control (HbA_{1c}) and type of treatment for diabetes were similar among the groups. Calcium antagonists were used in 18 of 28 hypertensive patients either alone or in combination with other antihypertensive drugs, followed by beta-blockers in six patients and angiotensin-converting enzyme inhibitors in four patients. Diuretics were used in only two patients. No patient had been given any kind of digitalis treatment.

This study was approved by the Institutional Committee of the Diabetes Center, TWMC, and informed consent was obtained from each of the subjects studied.

Euglycaemic hyperinsulinaemic clamp technique. After an overnight fast and discontinuation of all the medications on the study morning, euglycaemic hyperinsulinaemic clamping was performed using an artificial endocrine pancreas (Nikkiso STG-22, Nikkiso Co., Tokyo, Japan). A primed-continuous infusion of short-acting insulin (Novolin R, Novo-Nordisk Pharma Co., Copenhagen, Denmark) at 1.12 mU · kg^{-1} · min^{-1} was started and peripheral venous plasma glucose was clamped at 4.5 mmol/l by infusing varying doses of 10% glucose according to the algorithm originally described by DeFronzo et al. [20]. A steady-state of euglycaemia was maintained over 60–90 min, and the average rate of glucose infusion (GIR, mg · kg^{-1} · min^{-1}) during the final 30 min of the study was used as an index for insulin resistance in the whole body. Steady-state plasma insulin concentrations were 498.7 ± 33.0 pmol/l. Blood samples were drawn for measurements of insulin (IRI), C-peptide (CPR) and OLI before and at appropriate intervals during euglycaemic clamping.

Radioimmunoassay of plasma OLI. Plasma OLI was measured by a recently developed highly specific radioimmunoassay [21]. In brief, blood samples collected in chilled tubes containing Na_2EDTA (4 mmol/l) were centrifuged at 4 °C, and plasma was stored at – 70 °C until assayed. One ml of plasma sample was applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, Mass., USA) and eluted with 75% ethanol. The extracts were evaporated, lyophilized, and re-dissolved in the assay buffer for radioimmunoassay. The antiserum used in the present assay was raised in New Zealand white rabbits against ouabain (Sigma Chemical Co., St. Louis, Mo., USA) conjugated to bovine serum albumin (BSA). The cross-reactivity of this antibody was 9.2% with digoxin, and those with BSA, rhamnose, hydrocortisone, and aldosterone were less than 0.007%. The sensitivity of the radioimmunoassay was 0.01 pmol/tube with 50% displacement at 0.24 pmol/tube. In this radioimmunoassay, the dilution curves of plasma extracts were parallel to the standard curve for authentic ouabain. On the reverse-phase HPLC with a linear gradient of acetonitrile/trifluoroacetic acid, plasma OLI was identified as a single major peak with the retention time corresponding to that of ouabain. The recovery rate of ouabain added to the plasma sample before extraction with Sep-Pak was approximately 95%. The intra- and interassay coefficients of variation at a concentration of 250 pmol/l were 11.5 and 17.5%, respectively.

Table 1. Clinical and metabolic characteristics of the groups of study subjects

Group (IR/HT)	NIDDM patients I (−/−)	II (−/+)	III (+/−)	IV (+/+)	Normal control subjects
n (male:female)	15 (12:3)	13 (9*4)	14 (10:4)	15 (10:5)	8 (4:4)
Age (years)	46.1 ± 2.3[a]	52.9 ± 2.6[ab]	54.2 ± 2.8[ab]	61.3 ± 2.8[ab]	37.8 ± 4.7
BMI (kg/m^2)	22.0 ± 0.7	24.2 ± 1.0[c]	23.2 ± 0.8	23.8 ± 0.9	20.8 ± 1.0
Duration (years)	5.6 ± 1.6	4.0 ± 0.9	6.6 ± 1.6	9.2 ± 1.9[d]	
FPG (mmol/l)	7.9 ± 0.9	7.0 ± 0.6	7.7 ± 0.5	9.2 ± 1.1	4.7 ± 0.1
HbA_{1c} (%)	9.2 ± 1.0	7.7 ± 0.5[e]	9.5 ± 0.6	9.1 ± 0.5	5.4 ± 0.2
SBP (mm Hg)	118.4 ± 2.9[f]	147.7 ± 4.4	124.6 ± 3.0[f]	154.6 ± 5.5	114.5 ± 3.5
DBP (mm Hg)	75.4 ± 1.5[g]	91.2 ± 2.5	72.9 ± 1.7[g]	87.8 ± 2.8	69.2 ± 2.0
FIRI (μU/ml)	6.8 ± 0.9	7.9 ± 1.2	11.8 ± 1.1[hi]	15.6 ± 2.9[hi]	6.1 ± 1.0
GIR (mg · kg^{-1} · min^{-1})	7.20 ± 0.36[j]	5.89 ± 0.36[k]	1.91 ± 0.28[l]	1.79 ± 0.22[l]	7.69 ± 0.40
Micro/Macro-AU	4/15	3/13	3/14	5/15	
Treatment					
Diet alone	6/15	7/13	7/14	6/15	
OHA	4/15	1/13	4/14	4/15	
Insulin	5/15	5/13	3/14	5/15	
Anti-HT		7/13		14/15[m]	

Values are means ± SEM. IR, insulin resistance; HT, hypertension; BMI, body mass index; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; FIRI, fasting plasma insulin; GIR, glucose infusion rate, Micro/Macro-AU, microalbuminuria (30–300 mg/day) or macroalbuminuria (> 300 mg/day); OHA, oral hypoglycaemic agents; Anti-HT, antihypertensive agents; IR−/HT+, for example indicates the absence of IR and the presence of HT; [a] $p < 0.05$–0.001 vs control; [b] $p < 0.05$–0.001 vs I; [c] $p < 0.05$ vs control; [d] $p < 0.05$ vs II; [e] $p < 0.05$ vs III and IV; [f] $p < 0.001$ vs II and IV; [g] $p < 0.001$ vs II and IV; [h] $p < 0.01$ vs control; [i] $p < 0.05$ vs II; [j] $p < 0.02$ vs II; [k] $p < 0.005$ vs control; [l] $p < 0.001$ vs I, II and control, [m] $p < 0.05$ vs II

Statistical analysis

Data were expressed as means ± SEM and statistically analysed by the Mann-Whitney test, the chi-square test, and the non-linear or linear regression analysis, as appropriate. The level of significance was set at $p < 0.05$.

Results

Plasma OLI concentrations were compared among the groups (Fig. 2). The mean level for eight normal glucose-tolerant subjects was 142.6 ± 32.3 pmol/l. The highest level was found in the Group IV with both insulin resistance and hypertension (703.1 ± 170.1 pmol/l). The lowest level was in Group I with neither insulin resistance nor hypertension (130.8 ± 20.9 pmol/l), and was not significantly different from that in the normal control group. Those in Groups II and III with either hypertension or insulin resistance were intermediate between these two values (172.5 ± 35.0, 576.6 ± 161.5 pmol/l, respectively). Very high plasma OLI levels (greater than 1500 pmol/l) were found in six patients, all of whom were in the insulin-resistant groups (Groups III and IV). The clinical features of these six patients are presented in Table 2. The mean plasma OLI level was elevated in the hypertensive groups compared with the normotensive counterpart, i.e. Group I vs II, and Group III vs IV, but the differences were not statistically significant in either pair. There was an inverse

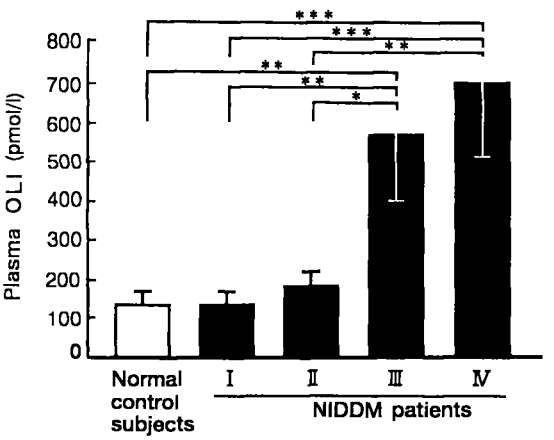

Fig. 2. Plasma OLI concentrations in normal control subjects and NIDDM patients. NIDDM patients were divided into four subgroups by the presence (+) or absence (−) of insulin resistance (IR) and hypertension (HT): Group I (IR−/HT−), II (IR−/HT+), III (IR+/HT−), and IV (IR+/HT+). The difference was significant between Group I and II and Group III and IV, while those between normal control subjects and Group I and II, between Group I and Group II, and between Group III and Group IV were not significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

correlation between plasma OLI levels and the GIR values when all subjects were combined ($p < 0.001$, $n = 65$, Fig. 3), and even when the extremely high values of OLI (> 1500 pmol/l) were excluded ($p < 0.01$, $n = 59$). However, no correlation was found between plasma OLI and blood pressure.

Table 2. Clinical profile of patients with very high (> 1500 pmol/l) plasma levels of OLI

	F.I.	Y.I.	N.T.	T.W.	H.T.	K.H.
Age (years): Sex (male/female)	71:Female	59:Male	65:Female	64:Female	60:Female	63:Female
BMI (kg/m^2)	23.4	25.6	24.7	21.7	26.3	21.9
HbA_{1C} (%)	10.0	6.0	9.9	10.6	10.1	12.1
Hypertension	+	+	+	+	+	+
Drug therapy	Ca^{++}-ant. diuretic	none (low salt diet)	Ca^{++}-ant. ACE-inh. diuretic	Ca^{++}-ant. diuretic	Ca^{++}-ant.	none (low salt diet)
GIR ($mg \cdot kg^{-1} \cdot min^{-1}$)	1.21	1.88	1.78	0.58	1.69	0.77
PRA ($ng \cdot ml^{-1} \cdot h^{-1}$)	2.2	2.4	1.7		0.8	1.1
PAC (ng/dl)	7.7	5.9	4.8		4.7	5.0
Diabetes therapy	SU	Diet	Insulin	SU	SU	Insulin
Past medical history			OMI	CHF	OMI	

Hypertension was defined by systolic blood pressure > 140 mm Hg and diastolic > 90 mm Hg or drug treatment. PRA, Plasma renin activity (normal: 0.5–3.0 $ng \cdot ml^{-1} \cdot h^{-1}$); PAC, plasma aldosterone concentration (normal: 2.2–15 ng/dl); SU, sulfonylurea; OMI, old myocardial infarction; CHF, congestive heart failure

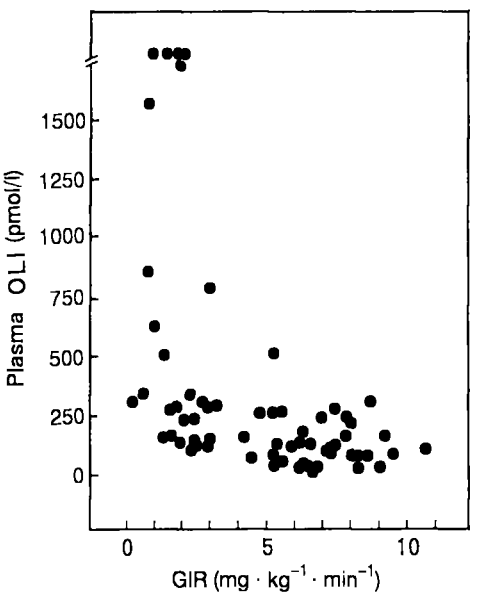

Fig. 3. Correlation between GIR and plasma OLI levels in all subjects studied (eight normal control subjects and 57 patients with NIDDM). A non-linear regression analysis showed a significant relationship between plasma OLI (Y) and GIR (X): $Y = 562.4 - 124.5X + 7.8X^2$, $r = 0.528$ ($p < 0.0001$). When the extremely high values of OLI (> 1500 pmol/l) were excluded, a linear negative correlation was found between them ($r = -0.487$, $p < 0.01$, $n = 59$)

Discussion

The present study demonstrated that plasma concentrations of OLI are significantly higher in insulin-resistant NIDDM patients than in insulin-sensitive patients or normal glucose-tolerant subjects, while there was no difference between the latter two groups. Furthermore, plasma OLI concentration showed a significant negative correlation with the GIR value, when analysed in each subject. To our knowledge, the relationship between insulin resistance and plasma levels of OLI has not been explored directly in patients with NIDDM.

Our values of plasma OLI concentration were comparable to those reported by other investigators [22, 23]. Hamlyn's group [22] reported that the plasma ouabain concentration ranges from below 200 to 700 pmol/l in most normal individuals, but in some patients to as high as 10 nmol/l or even higher. Similarly, a more than 50-fold variation in plasma levels of OLI was found in the present group, and in six patients plasma OLI levels exceeded more than 1500 pmol/l. These patients shared severe insulin resistance and hypertension, but otherwise there was no clinical marker that characterized this particular subgroup of patients. Since the concentrations of ouabain normally present in the circulation have been demonstrated to be active in modulating vascular contractility [24], this may have pathophysiologic implications in hypertension associated with insulin resistance.

Andronico et al. [25] estimated plasma concentrations of immunoreactive "endogenous digoxin-like factor (EDLF)", in 14 hypertensive and 12 normotensive subjects with obesity and glucose intolerance. In their studies, hypertensive subjects showed higher plasma EDLF levels and lower plasma glucose/insulin ratio than normal subjects, indicating a possible association between plasma EDLF levels and the degree of insulin resistance. The results of the present study are well in agreement with their findings. However, insulin resistance can be more reliably evaluated by the euglycaemic hyperinsulinaemic clamp method, and plasma digitalis-like substance can now be determined more directly with specific radioimmunoassay for ouabain. Our findings, therefore, provide direct and better evidence to support the close

relationship between plasma OLI and insulin resistance.

In our study, however, the relationship between hypertension and plasma OLI was not significant. Plasma OLI tended to be higher in the hypertensive group, but the difference was not statistically significant between the groups with comparable insulin resistance. Therefore, plasma OLI could not be concluded to play a role in the development of hypertension from the results of the present study. However, it has since been reported that plasma OLI levels are lowered after antihypertensive treatment in patients with essential hypertension [21], plasma levels of OLI in our patients could be underestimated, and, thus, may obscure the possible differences between the groups with and without hypertension.

Devynck et al. [26] reported that plasma potency of EDLF measured by its interfering activity with ^{3}H-ouabain binding to erythrocyte sodium pumps was significantly correlated with the rate of urinary sodium output in patients with essential hypertension. Therefore, elevated plasma OLI may be part of a compensatory mechanism for impaired natriuresis secondary to insulin resistance. This is in accordance with the observations that salt-loading and expansion of plasma volume induce a rise in plasma levels of ouabain in experimental animals and humans [27], and that elevated levels of ouabain are likely to be found in essential hypertension characterized by suppressed plasma renin activity [28, 29].

Ouabain possibly opposes the ability of insulin to stimulate tubular reabsorption of sodium in the kidney. On the other hand, in the cardiovascular muscle cells, ouabain causes an increase in intracellular sodium concentration, which in turn reduces the Na^+/Ca^{++} countertransport activity and results in increased cytosolic free calcium and decreased free magnesium, thus leading to potentiation of cardiovascular reactivity [9, 10, 30]. It has been reported that Na^+/K^+-ATPase activity is reduced in a variety of tissues in insulin-resistant states; human essential hypertension [11, 12], obesity [13], and experimental models of diabetes [14]. Therefore, our findings may partly explain the reduction of Na^+/K^+-ATPase activity accompanying insulin resistance. Moreover, reduced sodium pump activity has been proposed to exaggerate neural stimulation and norepinephrine overflow [31] which, together with increased peripheral vascular resistance, might explain the development of hypertension associated with insulin resistance. Recently, the parenteral administration of ouabain over a 4- to 6-week period was reported to cause chronic hypertension in rats [32]. However, recent studies cast doubts about the presence of ouabain in human plasma and its pathophysiological role in the development of hypertension [18, 19]. Therefore, further efforts should be made to elucidate whether an endogenous natriuretic and hypertensinogenic substance is a true ouabain, and whether ouabain could be a link between insulin resistance and hypertension in NIDDM.

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