Increased Urinary Excretion of Digoxin-like Immunoreactive Substance by Insulin-Dependent Diabetic Patients: a Linkage with Hypertension?

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Excretion of digoxin-like immunoreactivity (DLIS) was measured by RIA in timed overnight urine collections from 91 normotensive nondiabetic subjects and 104 normotensive insulin-dependent diabetic (IDDM) patients. The mean ± SD DLIS excretion rate for the diabetic patients significantly exceeded that for the controls (73 ± 41 vs 63 ± 36 pg/min, \( P = 0.024 \)). In both groups, the mean DLIS excretion rates for men were significantly higher (\( P = 0.0014, P = 0.0006 \)) than for women. In the controls, the DLIS excretion rate significantly correlated with the urinary excretion rate of creatinine (\( P < 0.01 \)), \( Na^+ \) (\( P < 0.05 \)), and \( K^+ \) (\( P < 0.05 \)), and with the subjects' body weight (\( P < 0.01 \)), body mass index (\( P < 0.05 \)), and systolic blood pressure (\( P < 0.05 \)). In the diabetics, the DLIS excretion rate was significantly correlated with body weight (\( P < 0.05 \)) and with urinary excretion rates for albumin (\( P < 0.01 \)), creatinine (\( P < 0.01 \)), \( Na^+ \) (\( P < 0.05 \)), and \( K^+ \) (\( P < 0.05 \)). Our data indicate that: (a) increased amounts of a cardiac glycoside-like substance (or a group of substances) are excreted in the urine of IDDM patients; (b) the urinary excretion of DLIS seems to depend on glomerular filtration rate and physicochemical properties of glomerular membrane, as well as on subjects' body mass; and (c) the presence of cardiac glycoside-like substances may increase peripheral vascular resistance, increased urinary excretion of DLIS by IDDM patients may indicate a tendency to develop hypertension.

Additional Keyphrases: albuminuria, sodium, potassium, creatinine, nephropathy, sex-related difference.

Considerable evidence (1, 2) indicates that increases in body sodium and expansion of extracellular volume lead to the release of humoral natriuretic factors. As recently reviewed (1–4), one of these factors is suggested to be an endogenous substance having immunological and biological activities similar to those of the cardiac glycosides. This endogenous factor inhibits \( Na^+ \)/\( K^+ \)-transporting ATPase (EC 3.6.1.37) in various tissues, including the kidney, and this causes natriuresis.

Insulin-dependent diabetes mellitus (IDDM) reportedly is associated with sodium retention, leading to increases of exchangeable body sodium \((5, 6)\) and extracellular volume and a suppression of the renin–angiotensin–aldosterone system.\(^1\) This has been recently confirmed by Feldt-Rasmussen et al. (7). Thus, the release of a substance with cardiac glycoside-like activity and \( Na^+ \)/\( K^+ \)-ATPase inhibiting activity could conceivably be triggered by volume expansion in IDDM. In fact, inhibition of \( Na^+ \)/\( K^+ \)-ATPase has been documented in tissues of experimental diabetic animals and in diabetic patients (8).

Several studies (for review, see references 1–4) indicate that, in addition to its natriuretic activity, this cardiac glycoside-like endogenous factor, by inhibiting \( Na^+ \)/\( K^+ \)-ATPase in arterioles, increases the intracellular \( Na^+ \), which in turn increases the intracellular calcium, leading to increased vascular reactivity. Hence, endogenous factors with activity similar to that of cardiac glycosides may play a role in the pathogenesis of some forms of hypertension, especially the low-renin forms (1–4). Such could also be the case in IDDM.

Hypertension and diabetes mellitus may occur together \((9, 10)\), and hypertension is more prevalent in diabetic patients than in the general population \((10, 11)\). Diabetic nephropathy is considered the main cause of hypertension in IDDM \((12)\), and blood pressure may begin to increase even in patients with slight albuminuria, who are at high risk of developing overt diabetic nephropathy \((13)\). However, the hypertension–diabetes association in IDDM patients cannot be entirely secondary to diabetic nephropathy, because hypertension can already be present in patients with recent onset of diabetes \((14)\) but without signs of renal involvement \((10, 11)\).

Because of abnormalities in the balance of body sodium and fluid volume in IDDM patients \((5–7)\), the release of an endogenous factor with cardiac glycoside-like activity could play a role in the pathogenesis of non-nephropathic hypertension in IDDM.

To test the hypothesis that production of a substance with cardiac glycoside-like activity might be increased in IDDM uncomplicated by overt diabetic nephropathy or hypertension, we used a sensitive RIA method \((15, 16)\) to measure the urinary excretion rate of a substance with digoxin-like immunoreactivity (DLIS) in 104 normotensive, non-nephropathic IDDM patients and 91 nondiabetic control subjects. In each of these groups (controls and diabetics), we also measured the urinary albumin excretion rate, an early and sensitive predictor of clinical diabetic nephropathy \((13)\), and some other clinical, metabolic, and renal characteristics of the subjects, to determine their relationship, if any, with urinary DLIS excretion rate.

Materials and Methods

Subjects. Ninety-one nondiabetic, normotensive subjects (46 men, 45 women) and 104 normotensive, Albustix-negative (Miles GmbH, Sparte Ames, Frankfurt, F.R.G.) IDDM patients (41 men, 63 women) participated in our study. No subject was being treated with digoxin. All subjects had a normal renal function, as assessed by endogenous creatinine clearance, and all, except for 10 diabetics, were normalalbuminuric. Some other pertinent clinical characteristics are reported in Table 1. For the slight ("pauci") albuminuria threshold, we chose the albumin excretion rate of 30 \( \mu g / \)min, in accord with Viberti et al. \((13)\). We verified by culturing that all subjects' urines were sterile. Of the diabetics, 62

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\(^1\) Nonstandard abbreviations: IDDM, insulin-dependent diabetes mellitus; DLIS, digoxin-like immunoreactive substance; HbA1c, glycated hemoglobin.

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(59.6%) had no signs of retinopathy, 29 (27.9%) had back-
ground retinopathy, and 13 (12.5%) had retinal proliferative
lesions. All patients were treated with insulin therapy only,
most of them with multiple insulin injections (the mean
daily doses are listed in Table 1).

Every subject collected urine specimens before going to
bed and upon arising, and the time of collection was exactly
recorded for the calculation of urinary DLIS excretion rate (in
pg/min) and albumin excretion rate (in µg/min). For most of
these subjects we also calculated the urinary excretion rate
for creatinine (mg/min) and electrolytes (Na⁺ and K⁺, 
mmol/min). When the same subjects collected more than one
urine specimen on consecutive nights (as did 28 of 104
diabetics and 43 of 91 control subjects), we used for statisti-
cal calculations the mean rate of urinary excretion of each
analyte from the several collections.

**DLIS assay.** We measured DLIS by a solid-phase RIA (15,
16) in which digoxin dissolved in a buffer containing human 
serum albumin, 40 g/L, is used as standard, 125I-labeled
digoxin is the tracer, and antibody-coated test tubes are 
used to separate bound from free digoxin (tracer and coated
test tubes were supplied by Byk Gulden Italia S.p.A.,
Corman, Italy). To improve the sensitivity and the repro-
ducibility of the assay as well as the stability of standard 
curves, we also included human serum albumin, 40 g/L, in
the assay buffer and added the same albumin-containing 
buffer to unknown urine samples, to equalize the effect of
albumin on the reaction volume. Results were expressed as
digoxin equivalents. We assayed 0.2-mL urine samples in
duplicate, without dilution or extraction. The mean limit of
detection (95% confidence limit), as determined in 21 sepa-
rate experiments, was 5.38 (SD 1.07) pg per tube. The mean
between-assay CV for DLIS in a urine pool, assayed over 10
months (n = 25), was 12.9% (mean DLIS concn, 281.9, SD
36.4 ng/L).

**Albumin excretion rate assay.** We used an RIA kit ("Albu-
mun RIA 100"; Pharmacia AB, Uppsala, Sweden) for assay
of albuminuria in normal subjects and diabetics. This RIA
involves purified human serum albumin as the standard,
125I-labeled human serum albumin as tracer, and a specific
antiserum to human serum albumin. Bound and free phases
were separated by a solid-phase system (sheep anti-rabbit
IgG, bound to Sepharose). We assayed, in duplicate, 50-µL
urine samples; the standard curve was based on assays of
solutions containing known amounts of purified human
serum albumin (0.8, 1.6, 4, 8, 16, 40, and 80 mg/L). The RIA
was not subject to interference from bovine serum albumin,
transferrin, or human immunoglobulins. The mean (n = 25)
limit of detection of this RIA was 0.28 (SD 0.13) mg/L. The
between-assay CV for a wide range of albumin concen-
trations in several pooled-urine specimens ranged between 5%
and 15%.

**Other methods.** We measured glucose in plasma by a
glucose oxidase (EC 1.1.3.4) method, in a glucose analyzer
(Beckman Instruments, Fullerton, CA). To quantify glycated
hemoglobin (HbA₁c), we used an automated HPLC analy-
zer system (HA-8110; Daiichi Kagaku, Kyoto, Japan).
Creatinine in serum and urine was measured by the Jaffé
reaction (Astra 8, Beckman), urinary sodium and potassium
by flame photometry (Model 435 flame photometer; Corning

**Statistical analysis.** All values for samples and the other
data for quality control of the two RIA systems were
calculated by use of previously described computer pro-
grams (17, 18).

The interpolation of the dose–response curves was calcu-
lated by using a four-parameter logistic function (18).

For statistical analysis for comparison of groups by para-
metric and nonparametric tests and calculation of simple
and multiple regression coefficients we used a Mackintosh
SE personal computer with the "512 + Stat-View" and
"Stat-View" programs. All data are reported as mean ± SD.

**Results**

Figure 1 shows the frequency distribution of values for
urinary DLIS excretion observed in the diabetic patients and
matched controls. The values for either group were not
normally distributed.

The mean DLIS excretion rate for men significantly ex-
ceeded that for women, both in control subjects and diabetics
(control subjects: women 51.6 ± 21.4 pg/min vs men 73.7 ±
42.7 pg/min, P = 0.0006 by the Mann–Whitney U two-tailed
test; diabetics: women 65.6 ± 39.2 pg/min vs men 86.7 ±
41.4 pg/min, P = 0.0014).

Diabetic patients (men and women) had a mean DLIS
excretion rate significantly higher than controls (73.09 ±
41.21 pg/min vs 62.80 ± 35.49 pg/min, P = 0.024 by the
Mann–Whitney U two-tailed test). Even taking into account
the sex of subjects, diabetics had a mean DLIS excretion rate
significantly higher than controls (diabetic vs control men:
P = 0.038; diabetic vs control women: P = 0.0316, Mann–
Whitney U two-tailed test) (Figure 2).

After the 10 microalbuminuric patients (albumin excre-

### Table 1. Clinical Characteristics of Subjects Studied (Means ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>IDDM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>91</td>
<td>94</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td>30.2 ± 10.1</td>
<td>31.7 ± 13.2</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>22.8 ± 3.0</td>
<td>23.1 ± 3.8</td>
</tr>
<tr>
<td><strong>Diabetes duration, y</strong></td>
<td>—</td>
<td>8.6 ± 7.1</td>
</tr>
<tr>
<td><strong>Insulin, units/day</strong></td>
<td>—</td>
<td>53.4 ± 21.2</td>
</tr>
<tr>
<td><strong>PPG, mg/L</strong></td>
<td>785 ± 118</td>
<td>1589 ± 667</td>
</tr>
<tr>
<td><strong>HbA₁c, %</strong></td>
<td>5.3 ± 0.4</td>
<td>8.4 ± 1.8</td>
</tr>
<tr>
<td><strong>AER, µg/min</strong></td>
<td>4.6 ± 2.7</td>
<td>6.9 ± 5.4</td>
</tr>
<tr>
<td><strong>Serum Cr, mg/L</strong></td>
<td>8.0 ± 1.0</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td><strong>Blood pressure, mmHg</strong></td>
<td>—</td>
<td>12.0 ± 15.0</td>
</tr>
<tr>
<td><strong>Systolic</strong></td>
<td>116.2 ± 6.8</td>
<td>75.6 ± 11.8</td>
</tr>
<tr>
<td><strong>Diastolic</strong></td>
<td>76.9 ± 6.4</td>
<td>122.1 ± 15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77.9 ± 7.0</td>
</tr>
</tbody>
</table>

*Body mass index: body weight/height². *Significantly different (P < 0.01) from control subjects. *Significantly different (P < 0.01) from IDDM subjects with AER <30 µg/min. Abbreviations: AER, albumin excretion rate; PPG, preprandial plasma glucose; Cr, creatinine.
In diabetic patients, the DLIS excretion rate significantly correlated with the excretion rates for urinary albumin, creatinine, Na⁺, and K⁺, and also with the body weight of patients, but not with their age, blood pressure, diabetes duration, HbA₁c, serum creatinine, or presence and severity of retinopathy (Table 2). In the multiple regression analysis, not only urinary creatinine excretion rate (the first variable entered), but also albumin excretion rate significantly contributed to the correlation with DLIS excretion rate in diabetic patients \( r = 0.517, P < 0.001, n = 63 \). The correlations between DLIS excretion rate and albumin excretion rate, and DLIS excretion rate and creatinine excretion rate, respectively, were still present also after the 10 paucialbuminuric patients had been excluded from the regression.

**Discussion**

Because DLIS concentrations in plasma of normal adults and hypertensive patients are often near the detection limit of our RIA method (about 25 pg/mL, digoxin equivalents), direct assay of plasma samples (i.e., without extraction and concentration of samples) is not indicated (19). On the contrary, because urine has three-to-10-fold higher concentrations of DLIS than those in blood, assay of urine samples seems preferable for clinical studies (19). The present study is the first one evaluating the urinary excretion of DLIS in a large number of normal subjects and IDDM patients. The timed overnight urine collection was chosen because it is easier to perform than 24-h collection, and because physical activity affects values for DLIS in both serum and urine (20, 21).

We measured DLIS values by a very sensitive RIA method specifically modified in our laboratory for the assay of digoxin-like immunoreactivity (16, 19). As previously reported for extracts of plasma of adults, pregnant women, and newborns, the results obtained with this RIA correlate well with those by a radioreceptor assay based on binding of [³H]ouabain to human erythrocytes (22). So this RIA can...
detect, to some extent, substances with both immunological and biological activity similar to that of cardiac glycosides.

Insulin-dependent diabetic patients, normotensive and with normal renal function, were chosen for the study because DLIS can be increased in biological fluids of patients with essential hypertension and with renal function impairment (3, 4).

In the present study, the urinary DLIS excretion rate was significantly smaller in women than in men, both in diabetic and controls, and was significantly related to subjects' body weight and body mass index. Evidently, DLIS urinary output depends closely on subjects' body mass and frame.

Urinary DLIS excretion rate was significantly higher in IDDM than in nondiabetic subjects. The overlap between the two groups (Figures 1 and 2) may be because we studied only normotensive diabetic patients, most of whom were in fair or good metabolic control and none of whom had clinical diabetic nephropathy. Preliminary findings from the 10 paucialuminuric patients seem to suggest that IDDM patients with overt or incipient (23) nephropathy and or in poor metabolic control could have greater DLIS excretion in urine. The 10 paucialuminuric diabetics tend also to have marginally increased blood pressure, although still within the normal range, in accord with previous reports (23). This marginally increased blood pressure conceivably could play a role in determining the increased urinary DLIS excretion.

Owing to its significant correlation with creatinine excretion rate in controls, and with creatinine excretion rate and albumin excretion rate in diabetic patients, the urinary excretion of DLIS seems conceivably to depend both on glomerular filtration and on physicochemical properties of glomerular membrane.

Among mechanisms that may contribute to the abnormality in the balance of body sodium and fluid volume reported in IDDM patients (5–7), peripheral hyperinsulinemia caused by exogenous insulin administration may be included (7). The hyperinsulinemia stimulates reabsorption of sodium by the kidney (7, 23–25), ultimately leading to an increase in exchangeable sodium and to an expanded extracellular volume (5–7, 26). In addition, diabetic patients, mainly insulin-dependent ones, tend to have high circulating concentrations of somatotropin (26), which itself stimulates tubular reabsorption of sodium (27, 28). Continued expansion of the body extracellular volume by sodium retention can thus stimulate the release of a circulating digitalis-like substance in IDDM patients (1–4). The significant correlation we found between urinary excretion rates of DLIS and Na is in harmony with this hypothesis. In addition, Na⁺/K⁺-ATPase activity has been found to be decreased in certain tissues of experimental diabetic animals—peripheral nerves, sarcolemma, blood–brain barrier (8, 29, 30, 31)—and in erythrocytes of type 1 diabetic patients (32). These findings suggest that, in IDDM, the tissues are probably in contact with increased amounts of an endogenous inhibitor of Na⁺/K⁺-ATPase.

Diabetes mellitus and hypertension are chronic conditions that frequently coexist (9). The increased urinary excretion rate of digoxin-like immunoreactivity in a group of normotensive, non-nephropathic IDDM patients could lead us to consider the increased urinary DLIS as a link with hypertension in IDDM.

As a working hypothesis (Figure 3), the release of an endogenous natriuretic factor with biological and immunological cardiac properties could cause a stable hypertension in genetically susceptible IDDM patients (33–35).

Further longitudinal studies are needed to definitively disclose and assess the pathophysiological and clinical relevance of increased urinary DLIS excretion in IDDM patients.

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References
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Table 2. Coefficients of Correlation between Urinary DLIS Excretion Rate and Other Clinical Characteristics in Control Subjects and Diabetic Patients

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.019</td>
<td>0.111</td>
</tr>
<tr>
<td>Body weight</td>
<td>0.235</td>
<td>0.256*</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.250</td>
<td>0.254*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.285</td>
<td>0.033</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.066</td>
<td>0.020</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>—</td>
<td>0.098</td>
</tr>
<tr>
<td>HbaA1c</td>
<td>—</td>
<td>0.045</td>
</tr>
<tr>
<td>Albumin excretion rate</td>
<td>0.026</td>
<td>0.264*</td>
</tr>
<tr>
<td>Na⁺ excretion rate</td>
<td>0.283*</td>
<td>0.281</td>
</tr>
<tr>
<td>K⁺ excretion rate</td>
<td>0.320*</td>
<td>0.296*</td>
</tr>
<tr>
<td>Creatinine excretion rate</td>
<td>0.567*</td>
<td>0.398*</td>
</tr>
</tbody>
</table>

Correlations are significant at *P < 0.01, **P < 0.05.