Serum arylhydrocarbon receptor transactivating activity is elevated in type 2 diabetic patients with diabetic nephropathy

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ABSTRACT

Aims/Introduction: Evidence is emerging that exposure to persistent organic pollutants (POPs) is a risk factor for obesity-related diseases and for diabetes mellitus (DM). We found that POPs could be measured by a cell-based arylhydrocarbon receptor (AhR)-dependent reporter assay. We tested if serum AhR transactivating (AHRT) activities are a risk factor for diabetic nephropathy in people with type 2 diabetes.

Materials and Methods: We enrolled diabetic patients with normoalbuminuria (n = 36), microalbuminuria (n = 29), macroalbuminuria (n = 8) and end-stage renal disease (n = 31). Sera were tested for their AHRT activities, which were standardized by an AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and expressed as TCDD equivalents (TCDDeq pmol/L).

Results: Mean serum AHRT activities were higher in patients with microalbuminuria (40.1 ± 7.1 pmol/L), macroalbuminuria (37.4 ± 5.5 pmol/L) and end-stage renal disease (59.1 ± 20.0 pmol/L) than in subjects with normoalbuminuria (12.7 ± 5.4 pmol/L; $P < 0.05$ for all comparisons). Serum AhR ligands showed a correlation with estimated glomerular filtration rate (eGFR; $r = -0.663$, $P < 0.001$), serum creatinine level ($r = 0.635$, $P < 0.001$), systolic blood pressure ($r = 0.223$, $P = 0.026$), glycated hemoglobim ($r = 0.339$, $P < 0.001$) and diabetic duration ($r = 0.394$, $P < 0.001$). In a multiple regression analysis, diabetic nephropathy was found to be an independent risk factor for higher AHRT activity after controlling for the confounding factors.

Conclusions: The present findings suggest serum AHRT activity, thus serum AhR ligands, is a risk factor for diabetic nephropathy. Further studies are required to clarify if an accumulation of POPs in the body is causally related to diabetic nephropathy.
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KEY WORDS: Aryl hydrocarbon receptor ligands, Diabetic nephropathy, Persistent organic pollutants

INTRODUCTION

Diabetes mellitus has become a large global health burden. One of the most important impacts of diabetes is its macro- and microvascular complications. Epidemiological data show that the degree and duration of hyperglycemia is associated with the microvascular complications of diabetes[1]. Diabetic nephropathy is one of most feared and common long-term complications of diabetes[2]. Previous studies have shown a close relationship between poor blood glucose control and diabetic nephropathy; two prospective landmark studies, the Diabetes Control and Complications Trial (DCCT) in type 1 diabetic patients and the United Kingdom Prospective Diabetes Study (UKPDS) in type 2 diabetic patients found that the risk of developing diabetic nephropathy and other microvascular complications is significantly reduced by intensive glycemic control[3,4]. Nevertheless, if hyperglycemia *per se* was sufficient to induce diabetes-related kidney disease, all diabetic patients under poor glycemic control would eventually develop diabetic nephropathy. However, in fact, normal renal function is preserved and proteinuria does not develop in many diabetic patients, despite many years of poor diabetic control[3,4]. Inversely, proteinuria develops in some diabetic patients, despite very strict glycemic control[3,4]. In addition to hyperglycemia, other unknown factors are believed to play a crucial role in the pathophysiology of diabetic nephropathy.

Recently, emerging evidence has suggested that persistent organic pollutants (POPs), the garden variety of lipophilic chemicals accumulated in adipose tissue, might be crucially involved in the pathogenesis of type 2 diabetes[5]. POPs includes two kinds of compounds; halogenated hydrocarbons and polycyclic aromatic hydrocarbons; in particular, pesticides,

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herbicides and many byproducts of industrial processes are examples. They are resistant to environmental degradation, bioaccumulate, and persist in human and animal fat tissues, and have harmful impacts on human health. Lee *et al.*[5] first reported that individuals with high blood POPs levels were at an increased risk of insulin resistance and type 2 diabetes. Of note, individuals with low POPs levels did not show increased risk of type 2 diabetes, even though they were obese[5]. This result suggests the crucial role of the complex interplay between the POPs and obesity in the pathogenesis of type 2 diabetes. In a subsequent study, Lee *et al.*[6] found that organochlorine (OCs) pesticides among five classes of POPs show a dose–response relationship with the prevalence of diabetic neuropathy independently of the status of glycemic control. Furthermore, OCs showed a relationship with the status of glycemic control[6]. They suggested two mechanisms by which POPs have an influence on diabetic neuropathy; first, a direct neurotoxic effect; and second, an indirect effect through their impact on glycemic control[6].

Among the POPs, polyhalogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have the ability to bind to and activate the ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR)[7]. Structurally-related compounds that bind to the AhR and show biological actions similar to TCDD are commonly referred to as 'dioxin-like compounds'. Classical Ah ligands are planar, aromatic and hydrophobic compounds, with a structure close to that of TCDD. Among them, we can cite polychlorinated dibenzo dioxin/furan, polychlorinated biphenyl (PCB), poly brominated biphenyl, polybrominated diphenyl ethers, poly cyclohexylene dimethylene terephthalate, polychlorinated naphthalenes, poly aromatic hydrocarbons, aromatic amines, hexachlorobenzene and so on. The well-known Chemically Activated LUciferase gene eXpression (CALUX) assay is a cell-based measurement of AhR ligand mixtures using an AhR-dependent reporter assay[7]. This biological assay is used to screen and monitor TCDD and dioxin-like compounds in various samples, which is much easier and cheaper to use compared with the gas chromatography and mass spectrometry (GC/MS) methods[7]. One advantage of the CALUX assay is that it allows us to determine the cumulative biological effects of a mixture of AhR ligands, whether an individual ligand is agonistic or antagonistic, although an individual identification of the chemical is not possible. We had previously reported that arylhydrocarbon receptor transactivating (AHRT activity) is elevated in type 2 diabetes and metabolic syndrome by using modified CALUX assay[8].

Among the numerous kinds of POPs, the most widely investigated in relation to type 2 diabetes and metabolic syndrome are dioxins, PCBs and furans[5,9–12]. There are fewer studies reporting that several kinds of pesticides are also associated with diabetes[5,13,14]. However, none of these studies investigated the associations between POPs and diabetic nephropathy. We hypothesized that POPs would be associated with the risk of diabetic nephropathy. In particular, we focused on the dioxins and dioxin-like compounds, because these have been widely reported to be linked to diabetes and insulin resistance. To test this hypothesis, we measured the AHRT activity in sera of patients with diabetic nephropathy using a modified CALUX assay.

MATERIALS AND METHODS

Participants

We enrolled diabetic patients with normoalbuminuria (n = 36), microalbuminuria (n = 29), macroalbuminuria (n = 8) and end-stage renal disease (ESRD) who were on hemodialysis for renal replacement treatment (n = 31). Informed consent was obtained from all 104 participants. The institutional review boards of the hospitals approved the study (EMCIRB 10–48) in accordance with the Declaration of Helsinki.

Clinical Assessments

Blood pressure, height and weight were measured. The urinary albumin–creatinine ratio (UACR) in a spot urine sample was measured. According to the level of UACR, the stages of diabetic nephropathy were classified as normoalbuminuria (UACR < 30 mg/g creatinine), microalbuminuria (UACR 30–99 mg/g creatinine) and macroalbuminuria (UACR ≥ 300 mg/g creatinine). The stages of nephropathy were confirmed in two assessments. The presence of diabetic retinopathy and cardio- and cerebrovascular diseases (CVDs), and the use of medications were also investigated. Complete blood cell counts, liver profiles and lipid profiles were also routinely measured. FPG was measured using a glucose oxidase method, and total cholesterol, triglyceride and high-density lipoprotein cholesterol (HDL-C) levels were measured using enzymatic colorimetric procedures with an auto-analyzer (Hitachi-747; Hitachi, Tokyo, Japan). Serum aspartate aminotransferase and alanine aminotransferase (ALT) were analyzed using ARCHITECT c8000 (Toshiba, Tokyo, Japan). Glycated hemoglobin (HbA_{1c}) was measured with high-performance liquid chromatography using HLC-723G7 (Tosoh, Tokyo, Japan)[15]. Estimated glomerular filtration rate (eGFR) was calculated using the following formula: $eGFR\ (mL/min/1.73\ m^2) = 186 \times (creat/88.4)^{-1.154} \times (age)^{-0.203} \times (0.742, \text{if female}) \times (1.210, \text{if black})$[16].

Sample Preparation

Serum was prepared by allowing the blood to clot and then removing the clot. Each serum sample was heat-inactivated by incubation at 65°C for 30 min. Fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) was treated with activated charcoal (Sigma Co., St. Louis, MO, USA) overnight at 4°C, and filtered to remove all small molecules and AhR agonists that might have been present. Indole-3-carbinol and TCDD, positive controls for AhR agonists, were purchased from Sigma Co. TCDD was considered extremely hazardous, so appropriate personal protective methods and materials were used.

Cell-Based AhR-Mediated Luciferase Assay

Hepa-1c1c7 mouse hepatoma cells (CRL-2026) expressing pGL3-CYP1A1-luc reporter plasmid containing four dioxin-responsive element consensus sequences were used for the measurement of AHRT activity, as reported previously[7]. Briefly, the transfected stable cells seeded at 5×10^4/well in a 96-well plate were cultured for 24 h in α-modified minimum essential medium supplemented with 10% FBS and 1% penicillin and streptomycin. After media were changed to 90 μL phenol red-free Dulbecco's minimum essential medium, the cells were treated for 24 h with 10 μL of heat-inactivated human serum samples or charcoal-stripped FBS (control) to obtain a final concentration of 10% serum. The cells were harvested with 20 μL luciferase lysis buffer (Promega, Madison, WI, USA), and their luciferase activities were measured using a luciferase assay kit (Promega) and a luminometer (Berthold, Badwildbad, Germany). Protein concentrations were determined by the bicinchoninic acid method using 1 μL of cell lysate. The luciferase activity was normalized by protein concentration and converted as a fold induction of the charcoal-stripped FBS control. The cells were exposed to serially diluted TCDD (0–100 pmol/L) for 24 h in the presence of 10% charcoal-stripped FBS, and their luciferase activities (AHRT activity) were used to prepare a standard curve. The fold induction was converted to TCDD equivalents (TCDDeq pmol/L) by interpolating from the linear region of the standard curve (0–20 pmol/L of TCDD) and multiplying by the dilution factor of ten. All assays were carried out in triplicate in two independent experiments. The intra- and interassay coefficients of variation of the current method were 4.6 and 15.0%, respectively.

Statistical Analysis

Data are expressed as mean ± SD or median (interquartile range). Comparisons between frequencies were tested by the chi squared-test. Differences between groups were tested using the unpaired Student's *t*-test for normally distributed variables and Mann–Whitney *U*-test for variables with skewed distribution. Differences among the three groups were investigated by one-way analysis of variance (ANOVA) for normally distributed variables and the Kruskal–Wallis test for variables with skewed distribution. Pearson's correlation test was used to evaluate the relationship between TCDDeq and various metabolic parameters. The independent relationship of each variable on the AHRT activity was assessed by multiple regression equations, as presented in Table 3. The significance level was considered as $P < 0.05$. All analyses were carried out using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Clinical Characteristics of the Study Participants

The mean age of all participants was 60.5 ± 9.8 years, and 55 (52.9%) of the patients were men. At study enrollment, mean HbA_{1c} was 7.8 ± 1.5% and mean body mass index (BMI) was 24.8 ± 3.6 kg/m^2. Table 1 lists the clinical characteristics of the study groups classified according to the stage of nephropathy. Differences in baseline characteristics were detected in bodyweight, BMI, systolic blood pressure, diabetic duration, diabetic micro- and macrovascular complications, medications, creatinine, eGFR, cholesterol, HDL-cholesterol, LDL-cholesterol (low-density lipoprotein cholesterol), ALT, and hemoglobin levels. ESRD patients tended to have lower bodyweight, BMI, cholesterol, ALT and hemoglobin levels, and higher creatinine, eGFR and diabetic duration.

Serum TCDDeqs in Diabetic Patients With Normoalbuminuria, Microalbuminuria, Macroalbuminuria and ESRD

As shown in Figure 1, mean serum AHRT activities were higher in the microalbuminuria (40.1 ± 7.1 TCDDeq pmol/L), macroalbuminuria (37.4 ± 5.5 TCDDeq pmol/L) and ESRD (59.1 ± 20.0 TCDDeq pmol/L) groups than in the normoalbuminuria group (12.7 ± 5.4 TCDDeq pmol/L; $P < 0.05$ for all comparisons). Stepwise increases of serum AHRT activities were observed across the advancing stages of nephropathy; the ESRD group showed higher AHRT activity compared with the micro- and macroalbuminuria groups. Serum AHRT activities were correlated with eGFR ($r = -0.663$, $P < 0.001$) and serum creatinine level ($r = 0.635$, $P < 0.001$) within the pool of all participants. In the cohort excluding ESRD patients, serum AHRT activity correlated with albumin creatinine ratio ($r = 0.677$, $P < 0.001$) and with creatinine ($r = 0.677$, $P < 0.001$), but not with eGFR ($r = 0.192$, $P = 0.104$; Figure 2). Furthermore, systolic blood pressure ($r = 0.223$, $P = 0.026$), HbA_{1c} ($r = 0.339$, $P < 0.001$) and diabetic duration ($r = 0.394$, $P < 0.001$), which were known risk factors for the progression of nephropathy, were found to be the correlates of the AHRT activities (Figure 2). When we compared the AHRT activities according to the presence of CVD and use of medications, those with CVD and who used calcium channel blockers or beta-blockers (BBs) showed higher AHRT activities than those without CVD and those not taking these medications (Table 2).

The independent relationship of each variable on AHRT activities was assessed by three separate multiple regression equations, as presented in Table 3. In model A, model B and model C, the stages of diabetic nephropathy, HbA_{1c}, BMI, CVD and BBs remained statistically significant variables in the equations (Table 3). In all models, the stages of diabetic nephropathy remained statistically significantly correlates of AHRT activity (Table 3).

Comparison of Toxicity Equivalent Values from Current CALUX Assay and High Resolution Gas Chromatography/Mass Spectrometry Method

AHRT activity (TCDDeq) values from 25 serum samples (100 mL) of healthy volunteers, who participated in a dioxin monitoring program carried out by the Seoul Municipal Government in 2010, were compared with toxicity equivalent (TEQ) values obtained by conventional high resolution gas chromatography/mass spectrometry (HRGC/HRMS) analysis. We found an excellent correlation of AHRT activities

Table 1 | Clinical characteristics of the study participants according to the stage of nephropathy

	Normal (n = 36) Mean ± SD	Microalbuminuria (n = 29) Mean ± SD	Macroalbuminuria (n = 8) Mean ± SD	ESRD (n = 31) Mean ± SD
Age (years)	57.1 ± 9.7	63.0 ± 9.2	60.4 ± 11.6	61.9 ± 9.4
Sex, male/female (n)	21/15	16/13	6/2	12/19
Bodyweight (kg)*	68.9 ± 10.4[a]	64.6 ± 12.5[a,b]	72.2 ± 11.6[a,b]	59.2 ± 9.0[b]
BMI (kg/m^2)*	26.0 ± 3.1[a]	25.0 ± 4.1[a,b]	25.4 ± 2.9[a,b]	23.1 ± 3.4[b]
SBP (mmHg)*	132.5 ± 10.8	131.9 ± 17.6	140.6 ± 7.8	140.5 ± 11.1
DBP (mmHg)	76.7 ± 6.3	75.2 ± 10.6	75.0 ± 12.0	72.3 ± 8.4
Diabetic duration (years)*	11.3 ± 8.2[a,b]	13.7 ± 8.3[a]	16.0 ± 4.0[a,b]	19.6 ± 7.9[b]
Diabetic retinopathy (n/%)*	3 (8.3%)	7 (24.1%)	4 (50.0%)	29 (93.5%)
CVD (n/%)*	1 (2.8%)	4 (13.8%)	2 (25.0%)	9 (29.0%)
Medications				
ARB or ACEi/CCB/BB/ statin/aspirin n (%)*	8/4/0/20/22 (22.2/11.1/0.0/55.6/61.1%)	16/6/0/19/12 (55.2/20.7/0.0/65.5/41.4%)	7/4/2/3/5 (87.5/50.0/25.0/37.5/62.5%)	14/11/14/8/8 (45.2/35.5/45.2/25.8/25.8%)
SU/MET/DPP4inh/TZD/ insulin n (%)*	20/15/1/7/7 (55.6/41.7/2.8/19.4/19.4%)	12/22/5/1/9 (41.4/75.9/17.2/3.4/31.0%)	2/3/0/0/7 (25.0/37.5/0.0/0.0/87.5%)	0/0/0/0/24 (0.0/0.0/0.0/0.0/77.4%)
Creatinine (μmol/L)*	81.0 ± 13.1[a]	89.3 ± 23.8[a]	100.6 ± 26.7[a]	737.7 ± 155.5[b]
eGFR (mL/min/1.73 m^2)*	71.4 ± 11.5[a]	64.7 ± 16.3[a]	60.8 ± 18.6[a]	5.3 ± 1.3[b]
HbA_{1c} (%)	7.4 ± 1.1	7.9 ± 1.2	8.1 ± 1.5	8.2 ± 2.1
Cholesterol (mmol/L)*	4.09 ± 0.73[a]	4.09 ± 0.86[a]	4.39 ± 1.72[a]	3.46 ± 0.81[b]
Triglyceride (mmol/L)	1.51 ± 1.04	1.63 ± 1.09	1.65 ± 0.70	1.26 ± 0.67
HDL-cholesterol (mmol/L)*	1.22 ± 0.29	1.24 ± 0.27	1.55 ± 1.31	1.01 ± 0.26
LDL-cholesterol (mmol/L)*	2.40 ± 0.71	2.29 ± 0.61	2.27 ± 1.38	1.81 ± 0.63
AST (IU/L)	22.3 ± 7.9	26.1 ± 8.8	21.8 ± 10.4	19.4 ± 11.4
ALT (IU/L)*	25.3 ± 18.0[a,b]	32.0 ± 16.9[a]	18.9 ± 8.5[a,b]	16.0 ± 15.2[b]
WBC (n/μL)	6560.0 ± 2435.5	7584.3 ± 1889.2	7502.5 ± 2107.1	6635.8 ± 1827.3
Hemoglobin (g/dL)*	14.2 ± 1.5[a]	13.8 ± 1.7[a]	12.5 ± 1.2[b]	10.4 ± 0.8[c]
AHRT activity (TCDDeq [pmol/L])*	12.7 ± 5.4[a]	40.1 ± 7.1[b]	37.4 ± 5.5[b]	59.1 ± 20.0[c]

ACEi, angiotensin converting enzyme inhibitor; AHRT, arylhydrocarbon receptor transactivating; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartase aminitransferase; BB, beta-blocker; BMI, body mass index; CCB, calcium channel blocker; CVD, cardiovascular and cerebrovascular diseases; DBP, diastolic blood pressure; DPP4inh, DPP-4 inhibitor; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET, metformin; SBP, systolic blood pressure; SU, sulfonylurea; TCCDDeq, 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents; TZD, thiazolidinedione; WBC, white blood cell count. Values are expressed as mean ± SD. *Significant at $P < 0.05$ by ANOVA and Tukey's test or χ^2-test (a, b, c; groups containing the same character did not differ according to the *post-hoc* test).

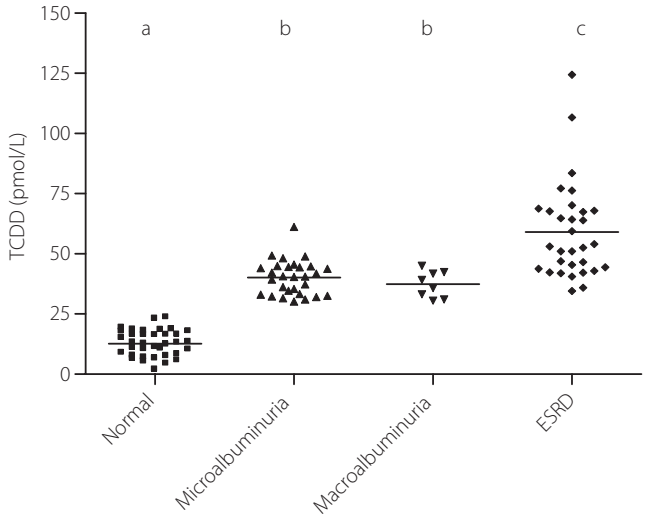

Figure 1 | Comparison of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) concentrations among participants according to the stage of nephropathy (a) (normoalbuminuria), (b) (microalbuminuria), (c) (end-stage renal disease [ESRD]); groups containing the same character did not differ according to the *post-hoc* test.

(TCDDeq values) with TEQ values, as determined by conventional chemical detection methods ($r = 0.931$, $P < 0.0001$; Figure 3).

Table 2 | Comparison of arylhydrocarbon receptor transactivating activities according to the presence of cardiovascular and cerebrovascular diseases and medications

	Yes		No		*P*
	n	Mean ± SD	*n*	Mean ± SD	
CVD	16	54.0 ± 30.4	88	32.8 ± 19.0	0.015
ARB or ACEi	45	40.4 ± 19.6	59	32.8 ± 23.8	0.085
CCB	25	44.1 ± 21.9	79	33.6 ± 22.0	0.040
Beta-blocker	16	50.8 ± 14.3	88	33.4 ± 22.5	0.004
Statin	50	32.3 ± 19.1	54	39.7 ± 24.5	0.090
Aspirin	47	31.4 ± 21.6	57	40.0 ± 22.3	0.052

ACEi, angiotensin converting enzyme inhibitor; ARB, an angiotensin receptor blocker; CCB, calcium channel blocker; CVD, cardiovascular and cerebrovascular diseases. Values are expressed as mean ± SD. Significant at $P < 0.05$ by Student's *t*-test.

DISCUSSION

In the present study, we found that serum AHRT activities are elevated in diabetic patients with nephropathy. Furthermore, diabetic nephropathy was found as an independent risk factor for higher AHRT activity after controlling potential covariates. To our knowledge, this is the first study to show the association between serum AHRT activity and diabetic nephropathy.

Diabetic nephropathy is a leading cause of ESRD[17]. In addition, the majority of elderly patients with diabetic nephropathy

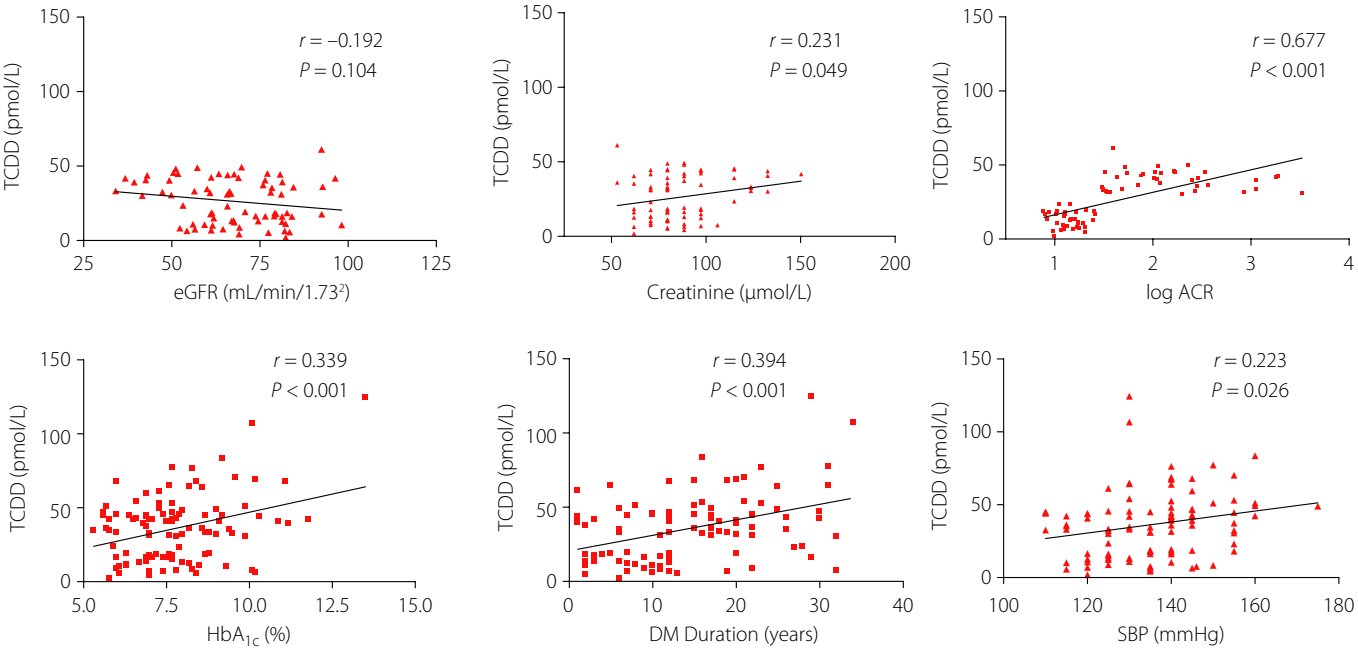

Figure 2 | The correlation between 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) concentrations and various metabolic parameters. The correlation between arylhydrocarbon receptor transactivating (AHRT) activities and creatinine, estimated glomerular filtration rate (eGFR), and log albumin to creatinine ratio (logACR) were analyzed in the cohort excluding end-stage renal disease patients. Glycated hemoglobin, diabetic (DM) duration and systolic blood pressure (SBP) were analyzed in all participants. Pearson's coefficient *r* and *P*-values are presented.

Table 3 | Standardized regression coefficients (Beta) for multiple regression models testing the relation between arylhydrocarbon receptor transactivating activity and other potential variables

	Beta		
	Model A	Model B	Model C
Stages of nephropathy	0.788*	0.798*	0.931*
Age	0.016		
Sex	–0.086		
BMI		0.129*	
HbA_{1c}		0.164*	
SBP		–0.052	
Diabetic duration		0.071	
CVD			0.147*
CCBs			0.002
Beta-blockers			–0.231*
ACEi or ARBs			–0.028
Statins			0.097
Aspirin			0.066

ACEi, angiotensin converting enzyme inhibitor; ARB, an angiotensin receptor blocker; BMI, body mass index; CCB, calcium channel blocker; CVD, cardiovascular and cerebrovascular diseases; HbA_{1c}, glycated hemoglobin; SBP, systolic blood pressure. *$P < 0.05$. The dependent variable is arylhydrocarbon receptor transactivating activity and several of the independent variables in the analysis are presented by dummy variables: stages of nephropathy (0 = normoalbuminuria, 1 = microalbuminuria, 2 = macroalbuminuric, 3 = end-stage renal disease), sex (0 = female, 1 = male).

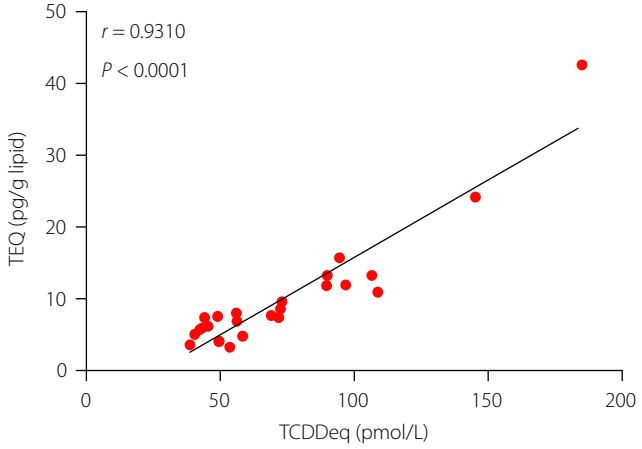

Figure 3 | The correlation between the results obtained by the current chemically activated luciferase gene expression assay and high-resolution gas chromatography and mass spectrometry (HRGC/HRMS) using the same human sera sample. A linear correlation between serum 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents (TCDDeq) concentration and toxicity equivalent (TEQ) by HRGC/HRMS.

have concommitant vascular diseases, further worsening the overall prognosis[18]. Thus, halting the progression of diabetic nephropathy in patients with type 2 diabetes is important to improve the prognosis. Tight glycemic control in diabetes is one of the cornerstones of management of diabetes to prevent diabetic microvascular complications[3,4]. Tight blood pressure control, preferably with angiotensin converting enzyme inhibitors or angiotensin receptor blockers, is also advantageous in reducing the risk of developing diabetic nephropathy[19,20]. However, if environmental exposure to POPs is involved in raising the risk of this complication, the current therapeutic approach might be insufficient. The study results suggest that finding ways to prevent exposure to POPs or to attenuate their actions at molecular levels might play a role in preventing diabetic microvascular complications; of note, POPs are accumulated in animal fats, so minimizing consumption of animal fats might reduce the risk.

The natural course of diabetic nephropathy is well characterized by a progressive rise in urinary albumin excretion from normoalbuminuria through microalbuminuria to clinical proteinuria, accompanied by declining glomerular filtration rate, eventually leading to ESRD. There are three major changes occurring in the glomeruli of patients with diabetic nephropathy; mesangial expansion, perhaps through increased matrix production or glycosylation of matrix proteins; glomerular basement membrane (GBM) thickening; and glomerular sclerosis, caused by intraglomerular hypertension that is induced by renal vasodilation[21–23]. Renal hemodynamic changes, induced in part by hyperglycemia, vasoactive hormones and mechanical factors, result in glomerular hyperperfusion and hyperfiltration[24]. These changes facilitate albumin leakage from glomerular capillaries and overproduction of the mesangial cell matrix, as well as thickening of the GBM. Metabolic abnormalities associated with diabetic kidney disease are as follows: oxidative stress and reactive oxygen species (ROS) production; non-enzymatic glycosylation of macromolecules, particularly basement membrane; activation of glucose metabolizing enzymes; and cytokines and other humoral imbalances (in particular, angiotensin II, transforming growth factor-β, insulin-like growth factor-1, platelet-derived growth factor, vascular endothelial growth factor)[25–29].

There are several possible mechanisms to link serum AHRT activity and diabetic nephropathy. We have found that serum AHRT activity correlated with its inhibitory function on cell respiration[8]. In the previous report, we showed that serum AHRT activities had a positive correlation with ROS production and a negative correlation with adenosine triphosphate (ATP) levels. Furthermore, sera of diabetic and prediabetic patients that showed higher AHRT activities, reduced intracellular ATP levels and increased ROS generation of co-cultured C2C12 mouse myoblast cells to a greater degree compared with those of normal controls[8]. These data suggest that POPs in sera measurable through CALUX assay would exert harmful effects on patients.

Dioxin and dioxin-like compounds are well-recognized ligands for AhR. Therefore serum AHRT activity reflects serum levels AhR ligands, which are both agonists and antagonists. AhR is known to mediate diverse toxicity of dioxins. Several

cytochrome P450 genes are modulated by AhR; specifically, CYP1 genes have been suggested to link dioxin-induced toxicity. Altered gene expression and signal transduction induced by dioxin have been reported to be linked to increased oxidative stress characterized by increased levels of ROS, DNA damage and lipid peroxidation, which could play a crucial role in the pathogenesis of diabetic nephropathy along with numerous cytokines[30–34]. A recent study reported that dioxin alters genes associated with mitochondrial function, which might contribute to dioxin-induced mitochondrial toxicity[35]. We believe that mitochondrial dysfunction is an important link between toxicity of POPs, type 2 diabetes and diabetes-related complications[36,37].

Recent evidence has suggested that AhR signaling modulates the expression of genes involved in matrix metabolism, in particular the matrix metalloproteinases (MMPs)[38]. MMPs play a crucial role in remodeling the extracellular matrix[39]. Altered activity of MMPs has been reported in numerous diseases, including tumors and cardiovascular disease, and decreased MMP activity (in particular MMP-2) was reported in diabetic nephropathy[40–42]. We believe that the altered MMP activity is another possible link between dioxin and diabetic nephropathy.

In addition, poor glycemic control caused by POPs might also increase the risk of diabetic nephropathy. The positive association between POPs and HbA_{1c} in the present study supports this possibility.

The CALUX assay we used has several advantages over the standard GC/MS method. First, the requirement for sample volume has been greatly reduced; 10 μL for CALUX assay vs 100 mL for the GC/MS method. Second, the cost and time for assay have also been reduced. Third, high throughput test is possible. The discrepancy between the results of CALUX and GC/MS has been narrowed, as we directly treated cells with heat-inactivated serum instead of hexane extracts of serum, and thereby reduced the chance of variable loss of serum. This modified CALUX technology would make possible various large-scale epidemiological studies to be undertaken.

The present study had several limitations. First, the cross-sec tional study design did not allow a causal relationship between POPs and diabetic nephropathy to be established. Further studies with longitudinal study designs will draw conclusions about the causal association. Second, this was a single-center study with a small sample size. Nationwide and multinational cooperation to clarify the association is urgently warranted. Third, there are several 'non-classical' AhR ligands besides dioxins, and dioxin-like compounds. These include tryptophan, and its metabolites, and phytochemicals, and the research to identify AhR ligands is still continuing[43]. Some of them are AhR agonists and the others are inhibitors[44]. The AhR ligand levels we determined should be the collective sum of the agonists and antagonists of AhR in serum. So the interpretation of AHRT activity in relation to dioxins and dioxin-like compounds should be cautious. There is also a need to evaluate the correlation between AHRT activity and TCDD TEQ by GC/MS in ESRD patients, because unknown uremic toxins might have an effect on AHRT activity. We also suggest study into the effect of interventions to lower the body burden of POPs, specifically a vegetarian diet or an application of drugs, on their relationship with the level of POPs, and the development of type 2 diabetes and diabetic complications.

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