CORRELATIONS FOR CARDIAC HYPERTROPHY WITH OXIDATIVE STRESS AND INSULIN RESISTANCE MARKERS IN TYPE 2 DIABETIC PATIENTS

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Growing evidence highlights oxidative stress and HOMA-IR as important mechanisms in cardiac hypertrophy. The aim of this study was to evaluate the relationship between plasma markers of oxidative stress and insulin resistance in diabetic patients with and without cardiac hypertrophy. Dislipidemia and other cardiovascular risk factors were assessed. Two groups of subjects were selected: one with type 2 diabetes mellitus (group DM, n=60) and a control group (group M, n=30). The first group was subdivided in two subgroups, according to the presence (n=18) or absence (n=42) of left ventricular hypertrophy (LVH). Cardiac hypertrophy was determined by ECG and oxidative stress parameters were measured by spectrophotometry. Microalbuminuria was determinated by measuring of urinary albumin excretion rate, while insulin and proinsulin levels were determined by ELISA method. The determined parameters were modified significantly in the diabetic patients versus control. Comparing the diabetic patients subgroups, increased values for blood pressure($p<0.05$), smoking status($p<0.01$), HOMA-IR($p<0.04$), HbA1c ($p<0.02$), total cholesterol ($p<0.05$), urinary albumin excretion rate($p<0.01$), MDA($p<0.04$) and erythrocyte glutathione($p<0.05$) were measured in LVH subgroup vs non-LVH subgroup. Plasma total antioxidant activity (TEAC) was similar in subgroups and HDL was reduced in LVH patients ($p<0.04$). The precence of LVH was correlated with smoking status($r=0.28$), duration of diabetes and blood pressure ($r=0.37$, respectively $r=0.39$), with HbA1c and HOMA-IR ($r=0.32$, respectively $r=0.37$) and with microalbuminuria ($r=0.67$), with TEAC and erythrocyte glutathione ($r=0.34$, respectively 0.37). MDA was positively correlated with monocyte NADPH oxidase activity ($r=0.29$) and duration of blood pressure ($r=0.27$). This study emphasizes the presence of clustering factors (high blood pressure, low HDL, dysglycaemia, increased plasma lipid peroxidation, albuminuria) involved in left ventricular hypertrophy in diabetes mellitus type 2. Knowing their tight correlations and their synergistic action will help us for a better management of diabetes mellitus complications.

Key words: cardiac hypertrophy; insulin resistance; oxidative stress; microalbuminuria; diabetes.

INTRODUCTION

It is widely acknowledged that diabetes mellitus is a premier risk factor for heart failure and this association is partly mediated by its effect on left ventricular structure. Considerable clinical evidence supports a role for insulin resistance in the pathogenesis of left ventricular hypertrophy (LVH) [1,2,3]. Rutter *et al.* demonstrated in 2623 of subjects, involved in a Framingham study, that LV mass and wall thickness increased with worsening glucose intolerance, an effect that was more striking in women compared with men[1]. A recent clinical study, done in a large population-based sample (n=820), showed a strong relation between HOMA-IR and left ventricular mass independent of intra arterial blood pressures, antihypertensive medication and hypertension status[2]. It seems that insulin may act as a growth

hormone for cardiomyocytes, maily through the extracellular signal regulated kinase (ERK) and/or protein kinase C (PKC) pathways.

Insulin also stimulates collagen synthesis in cardiac fibroblasts. Hyperglycemia may in itself promote left ventricular hypertrophy, mainly involving transforming growth factor beta1 and collagen synthesis by cardiac fibroblasts, likely via the phosphatidylinositol 3-kinase and PKC-beta pathways. Insulin interacts with neurohormonal systems and activates the sympathetic nervous system, increases the pressor response to angiotensinII and increases the stimulating effects of angiotensin II on ERKs involved in cellular proliferation and extracellular matrix deposition[2].

In short, insulin resistance can influence cardiac structure through several mechanisms: increased cellular lipids, non-enzymatic glycated end products, altered myocardial protein degradation, insuline-like growth factors mediated effects, altered matrix remodeling, altered vascular compliance, sympathetic activation , increased renal sodium reabsorbtion[1].

Also, knowedays, there are evidence, based more on experimental data, for the role of oxidative stress on cardiac remodeling. ROS stimulate myocardial growth, matrix remodeling, and cellular dysfunction. ROS activate a broad variety of hypertrophy signaling kinases and transcription factors.

In rat neonatal cardiomyocytes, H_2O_2 stimulates the tyrosine kinase Src, GTP-binding protein Ras, protein kinase C, mitogen-activated protein kinases and Jun-nuclear kinase. Also, ROS directly influence contractile function by modifying proteins central to excitation–contraction coupling[4].

Cardiomyocyte apoptosis is another important contributor to hypertrophic remodeling and cell dysfunction. Apoptosis is inhibited in cells at low levels of ROS stimulation, whereas the opposite occurs at higher levels. Mechanisms include DNA and mitochondrial damage and activation of proapoptotic signaling kinases[4,5].

Oxidative stress and metabolic shift are intimately linked with myocardial hypertrophy, but their interrelationship is not clearly understood. In a recent research, the conclusion underlines that investigations aimed at prevention of hypertrophy should address reduction of oxidative stress[6].

The aim of this study was to evaluate the relationship between plasma markers of oxidative stress and insulin resistance in diabetic patients with and without cardiac hypertrophy. Dyslipidemia and other cardiovascular risk factors were also assessed.

MATERIALS AND METHODS

Study Subjects

The study was approved by the ethical commission of the „N. Paulescu" National Institute of Diabetes, Nutrition and Metabolic Disease and informed consent was obtained from all participants.

Blood was collected from 60 (29 male/31female) diabetic patients attending of the "N. Paulescu" National Institute of Diabetes, Nutrition and Metabolic Disease after 12 hours of overnight fasting. Morning urine sample was collected in a container for analysis of creatinine and albumin excretion rate. Blood samples were also taken from a group of 30 sex-matched nondiabetic healthy volunteers recruited from the university and medical hospital staff (control group, M).

Type 2 diabetic patients were divided into two groups according to ECG left ventricular measurements as follow: group 1, with left ventricular hypertrophy (LVH, n=18) and group 2 (n=42), without LVH.

The *inclusion criteria* in the group with type 2 diabetes mellitus were: positive diagnosis of type 2 diabetes, age 40–70 years, fasting blood glucose level >7mmol/l (>126mg/dl), HbA_{1c}≥6,5%, duration of diabetes > 3years, previous oral anti diabetic therapy or appropriate diet therapy. Type 2 diabetes was defined according to the criteria of American Diabetes Association[7].

The *exclusion criteria* included the following: urinary infection, congestive heart failure, myocardial infarction, or stroke in the past 6 months; epilepsy or other severe disease; liver disease, serum creatinine concentration >120µmol/L (1.36mg/dL), or macroalbuminuria, proliferative retinopathy or severe maculopathy; excessive alcohol consumption (>20 g/day) and night work as previously described.

The BMI was calculated as weight in kilograms divided by height in square meter. Albumin excretion rate was determined by measuring the albumin excretion rate from three non consecutive first morning urine samples. Normal albumin excretion rate was <30mg/g creatinine and microalbuminuria was defined as AER in the range of 30 and 299 mg/day[7]. The ACR (albumin to creatinine ratio) was calculated as follows: urinary albumin concentration (mg/L)/urinary creatinine concentration (mg/dL). The mean value of

each patient's three ACRs was used to indicate the level of albumin excretion[7].

Reagents

PBS (Dulbecco's Phosphate Buffered Saline), Ficoll-Paque™ Plus, D-glucose, Trypan Blue, lucigenin (N,N'-dimethyl-9,9'-biacridinium dinitrate, LG), PMA (phorbol 12-Myristate 13-Acetate), thiobarbituric acid, ortho-phosphoric acid, butylhydroxytoluene, ABTS (acid 2,2'–azinobis 3-etilbenzotiazolin-6-sulfonic), potasium persulphate, TROLOX (acid 6-hidroxi-2,5,7,8-terametilcroman-2-carboxilic), DTNB (acidul 5,5'-ditiobis-(2-nitrobenzoic)) were purchased from Sigma Chemical Co., St.Louis, MO, USA.

Analytical metdods

Routine blood tests including total cholesterol, glucose, HDL-cholesterol (high density lipoprotein; HDLc), triglycerides (TG), serum creatinine, urea, uric acid were measured by colorimetric methods, while glycosylated hemoglobin (reference range 4.0-6.0%) was measured using standard HPLC techniques on the day of sampling from fasting venous blood samples. LDL-cholesterol (low density lipoprotein; LDLc) was calculated according to the Friedewald equation[8]. Insulin and proinsulin were determined by ELISA method (EIA-2935, EIA-4156-DRG Instruments GmbH, Germany). CV was 5.99% and 8.3% respectively.

Oxidative stress status was evaluated by measuring the plasma malonyldialdehyde (MDA), plasma TEAC, erythrocyte glutathione (nonprotein SH) and monocyte respiratory burst. *Isolation of peripheral blood mononuclear cells (PBMC) and Respiratory Burst (RB):* PBMC were isolated by density centrifugation on Ficoll-Paque™ Plus (1.0077g/mL). After centrifugation at 630g for 30 min the mononuclear cells (PBMC) were collected, washed twice and resuspended in 1mL PBS. Cell viability by Trypan Blue exclusion was ≥ 90%.

The ability to produce a respiratory burst was monitored by lucigenin-enhanced chemiluminescence[9]. In short, to PBMC (0.3×10^6 cells) resuspended in phosphate-buffered saline, dark-adapted lucigenin (final concentration 0.143µmol/L) was added. After monitoring spontaneous chemiluminescence for 15 min, the respiratory burst was initiated by adding of 100µL PMA (final concentration 5.4 µmol/L) and the maximum chemiluminescence peak was recorded (Luminometer TD 20/20, Turner Designs).

Chemiluminescence production was expressed as the relative chemiluminescence units (RLU).

Total plasma antioxidant capacity was evaluated by the ABTS decolourization assay (TEAC). The etalonation curve is done with TROLOX and the results are expressed in mmol/L TROLOX[10,11].

Plasma total malondialdehyde was analysed by pretreating plasma samples with thiobarbituric acid in ortho-phosphoric acid containing butylhydroxytoluene as antioxidant. The pink-coloured product was measaured spectrophotometricaly at 532nm[12]. Reduced glutathione (GSH) in whole blood was measured by a colorimetric method using Ellman's reagent[13]. Assessment of Insulin Resistance. Insulin resistance was assessed from fasting insulin and glucose levels and the previously validated homeostasis model assessment (HOMA-IR),thus: HOMA-IR=fasting glucose (mmol/L) × fasting insulin (µU/mL)/22.5.

Statistical Analysis

Results were expressed as mean ± SD (standard deviation)/SEM (standard error of the mean). Data were analysed using the statistical package SPSS version 16.0. Differences between groups were analysed using Student's t-test and two-tailed p-values<0.05 were considered as statistically significant. Differences between groups were calculated using two-way ANOVA with Bonferroni post-hoc tests. Multiple linear regression and Pearson correlations were applied to identify the relationship between the various parameters.

RESULTS AND DISCUSSION

Subject characteristics

The clinical characteristics and routine biochemistry of the healthy subjects and the diabetic patients are shown in Table I, oxidative stress markers are shown in table II and in Table III, the correlations between LVH and the studied parameters were calculated.

Parameters	Healthy subjects (lot M) (n=30)	Diabetic patients (n=60)	Diabetic patients		p-value group 2 vs group 1	p-value diabetic patients vs martor
			Group 1 (n=18)	Group 2 (n=42)		
Age (years)	52.28±7.16	55.63±8.13	58.83±6.26	54.9±8.06	NS	NS
Body mass index (kg/m2)	23.1±2.24	33.29±3.78	32.88±3.41	33.47±3.9	NS	<0.001
Duration of diabetes (years)	-	5.88±3.08	7.0±2.64	5.14±2.68	<0.01	-
Duration of treatment for HBP	-	7.56±8.1	10.5±8.2	5.73±4.1	<0.005	-
Sex (female/male)	14/16	31/29	8/10	23/19	NS	NS

Smokers/Nonsmokers	12/18	17/43	7/14	10/30	<0.01	NS
Systolic blood pressure (mmHg)	118±5.21	135±7.74	137.0±5.7	132.8±6.8	<0.04	<0.001
Diastolic blood pressure (mmHg)	70±4.7	82±5.45	84.3±4.7	80.7±5.3	<0.01	<0.001
Serum total cholesterol (mg/dL)	179±20	201.95±34.07	189.2.±41	201.65±36	NS	<0.001
Serum HDL cholesterol (mg/dL)	54±4	45.25±9.01	41.3±7.5	46.5±9.06	<0.04	<0.001
Serum LDL cholesterol (mg/dL)	99±19	122.12±32.96	114.3±40	126.7±25	NS	<0.001
Serum triacylglycerol (mg/dL)	129±14	172.86±72.49	179.3±55.4	170.0±53	NS	<0.001
Atherogenic Index	3.3±0.33	4.6±1.07	4.39±1.11	4.57±0.8	NS	<0.05
FPG (mg/dL)	84±9	155±23	158.8±22	154.2±27	NS	<0.001
HbA1c(%)	4.94±1.4	7.86±0.84	8.11±0.78	7.62±0.78	<0.02	<0.001
HOMA-IR (arbitrary units)	1.26±0.33	5.39±3.4	6.12±2.5	6.15±3.76	<0.04	<0.05
Proinsulin μU/ml	-	29.8±14.7	33.37±12.8	26,38±12.6	<0.05	-
Urine ACR (mg/g)	5.36±1.75	22.77±14	29.2±10.4	19.9±14.2	<0.01	<0.001
Urea (mg/dL)	30.88±5.36	34.29±8.36	33.2±6.9	34.7±8	NS	<0.05
Uric Acid (mg/dL)	4.18±0.62	5.96±1.43	6.02±1.69	5.93±1.32	NS	<0.05

Table 1. Clinical characteristics of the healthy subjects and diabetic patients. Group 1 is with LVH, while group2 is without LVH. Data are expressed as mean ± SD. NS = not significant; FPG = fasting plasma glucose; ACR = albumin to creatinine ratio; HOMA-IR = homeostasis model assessement of insulin resistance.

Parameters	**Healthy subjects (lot M) (n=30)**	**Diabetic patients (n=60)**	**Diabetic patients**		**p-value group 2 vs group 1**	**p-value diabetic patients vs martor**
			Group 1 (n=18)	**Group 2 (n=42)**		
MDA (nmol/g protein)	180.26±10.07	208.1±51.1	226.9±53.7	195. 0±43.3	<0.05	<0.001
TEAC (mM Trolox/L)	0.87±0.09	1.4±0.15	1.44±0.13	1.38±0.15	NS	<0.001
Nonprotein SH (μmol/g Hb)	7.6±0.67	4.55±0.55	5.07±1.62	4.35 ±0.98	<0.05	NS
NADPH oxidase (RB) RLU	0.452±0.032	1.0±0.3	1.0±0,6	0.87±0.3	NS	<0.001

Table 2. Oxidative stress parameters. Group 1 is with LVH, while group2 is without LVH.

Low levels of reactive oxygen species (ROS) are thought to play a role in normal cardiac signaling, growth adaptations and matrix changes. Higher levels play a role in pathophysiologic remodeling, apoptosis and chamber dysfunction[4].

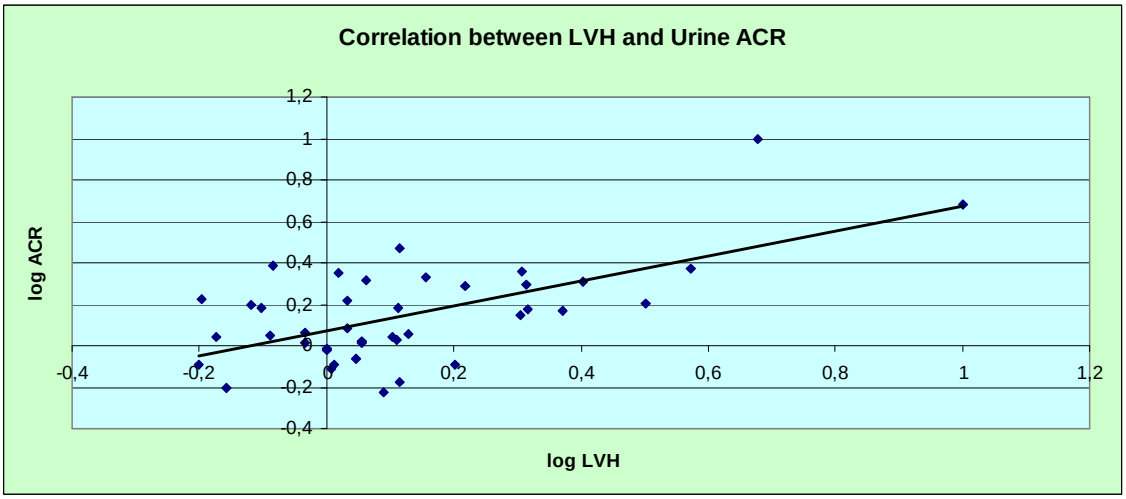
Furthermore, blood pressure level is one of the most important determinants of left ventricular mass[2]. Between the mechanisms which play a key role in the adverse cardiac remodeling resulting from chronic pressure overload (present in high blood pressure) we mention uncoupled NOS3 and activation of the enzymes NADPH oxidase and xanthine oxidase[4].

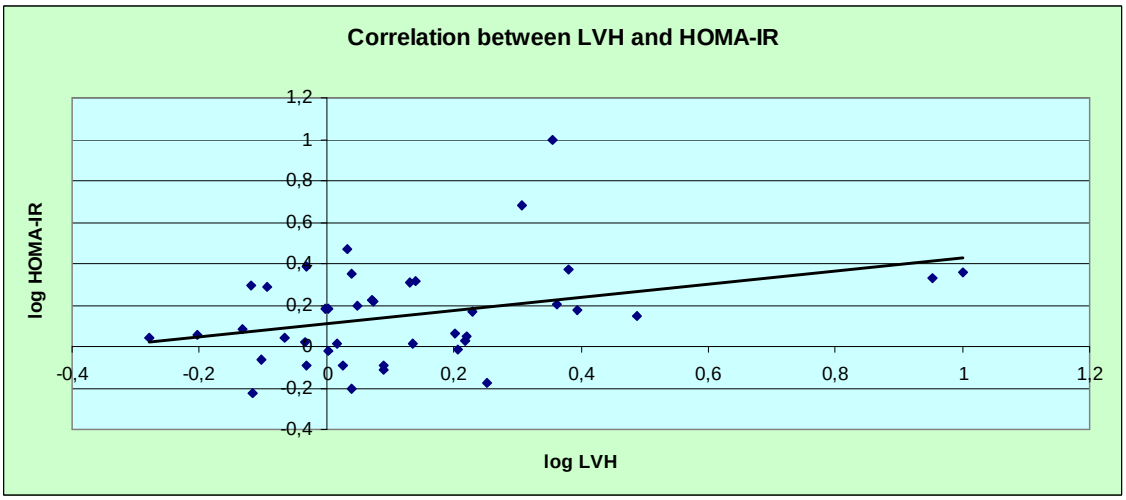
NOS3, or endothelial NOS is expressed in endothelial cells and also in myocytes. When exposed to oxidative or nitrosative stress or when deprived of BH4 or arginine, NOS3 becomes structurally unstable and anion superoxide is formed. Combination of anion superoxide with NO forms peroxinitrite radical which augments free radicals formation. Increased ROS determine metalloproteinase (MMP) activation and hypertrophy signaling activation which determine fibrosis, matrix remodeling and ventricular hypertrophy[4].

In our study, it can be noticed that the average for blood pressure (systolic and diastolic) is in normal ranges, but not at the target value for diabetics, which means under 130/80 mmHg.

Even the values for blood pressure are close to each other in the two diabetic groups, in those with LVH, the values are increased significantly. All 60 diabetic patients have taken conversion enzyme inhibitors, but it can be noticed that a better blood control, even with only few mmHg units, has a great impact on cardiac remodeling and contractility.

Taking into account the blood pressure pathogenesis it should be mentioned that also angiotensinII, alfa-adrenergic agonists and tumor necrosis factor-alfa activates NADPH oxidase (cardiac isoforms Nox2 and4). This enzyme increases the ROS production which determine hypertrophy, fibrosis and MMP activation[14].





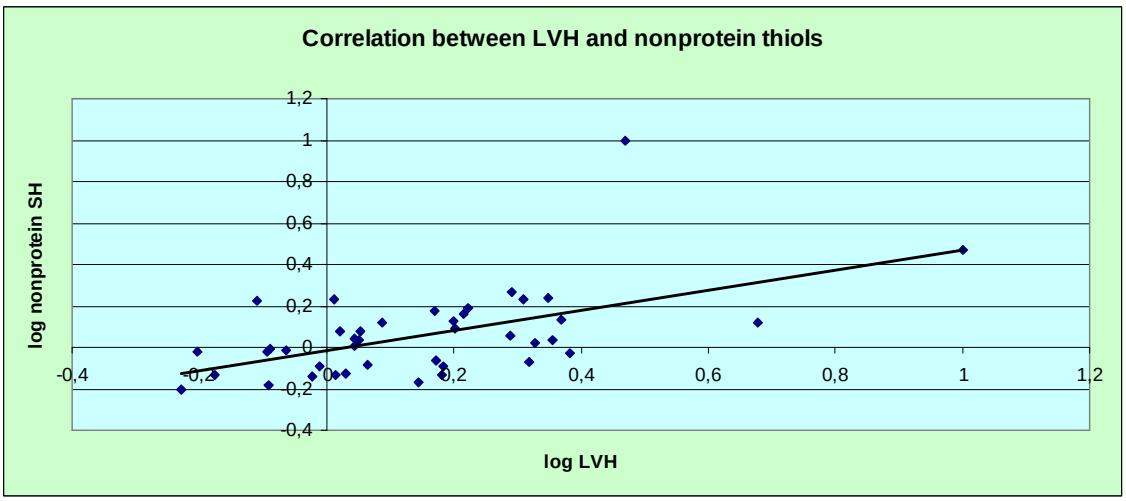


Fig. 1. Correlations for LVH with HOMA-IR, ACR and nonprotein thiols.

	Smoking status	Duration of diabetes	Duration of HBP	HbA1c	Systolic blood pressure	Diastolic blood pressuure	Insulinemia	HOMA-IR	ACR	TEAC	Nonprotein thiols
LVH	0.28	0.38	0.31	0.37	0.29	0.31	0.32	0.35	0.67	0.34	0.47

Table 3. The correlations between LVH and the studied parameters. The correlations lower than -0.26 and higher than 0.26 are considered significant.

Also, pressure overload activates xanthine oxidase (the enzyme lies in the proximity in the sarcoplasmic reticulum of cardiac myocytes) which increases anion superoxide production with contractile dysfunction consequently. Another argument for this association, between xantin oxidase system and cardiac contractility, is sustained by chronic treatment with allopurinol which significantly reduced adverse left ventricular remodeling[15].

In this study, plasma uric acid (the product of xanthine oxidase from many tissues) was not correlated with left hypertrophy, but was correlated with visceral fat ($r=0.30$, $p<0.05$), waist circumference ($r=0.32$, $p<0.05$), and trygliceridemia ($r=0.30$, $p<0.05$).

It is worth to mention that, in our study, blood pressure values were correlated with LVH (table III) but also with ACR, known also as an important cardiovascular risk factor (with $r=0.31$, for systolic pressure and 0.40 for diastolic pressure , $p<0.05$) .

Albuminuria, reflecting systemic microvascular damage, and left ventricular (LV) geometric abnormalities have both been shown to predict increased cardiovascular morbidity and mortality. In hypertensive patients with ECG LV hypertrophy, abnormal LV geometry and high LV mass are associated with high urine ACR independent of age, systolic blood pressure, diabetes, and race, suggesting parallel cardiac and microvascular damage[16].

We obtained a very strong correlation between urine ACR and LVH (table III). We calculated also correlations (for $p<0.05$), for ACR with markers of dysglycaemia: HbA1c($r=0.57$), glycaemia($r=0.50$), HOMA-IR($r=0.30$), proinsulin($r=0.30$). These correlations are strong arguments that dysglycaemia is involved in endothelial dysfunction and that endothelial modifications are associated with cardiac remodeling.

Oxidative stress and metabolic modulation have to be considered for studies that focus on the regression of hypertrophy and it seems that oxidative stress precedes the development of hypertrophy[6]. Experimental data supports this hypothesis.

As examples, plasma MDA was elevated in cardiac overload rat model[17] and acetyl-L-carnitine attenuates aortic stiffening and cardiac hypertrophy, possibly through its decrease of lipid oxidation-derived MDA/TBARS in the rats with insulin deficiency[18].

In our study, plasma MDA was increased in LVH group and the values were correlated with respiratory burst ($r=0.30$) and with the duration of high blood pressure treatment ($r=0.33$).

Reducing oxidative stress or enhancing intrinsec antioxidants provide effective approaches for reversing cardiac remodeling. In rats heart, inhibition of the endogenous antioxidant thioredoxin 1, primarily stimulated hypertrophy[19], while overexpressed glutathione peroxidase (important for removing H_2O_2 and detoxifying lipid hydroperoxides) ameliorated postmyocardiac remodeling[4].

Antioxidant properties of many antihypertensive drugs[20] and statins[4] may prevent cardiac remodeling dysfunction. In our study, the plasma total antioxidant capacity was not modified between the groups, but the erythrocyte nonprotein thiol was increased in LVH (maybe because the erythrocyte GPx is more glycated and with low activity). Both TEAC and erythrocyte nonprotein -SH were correlated with LVH(table III). Erythrocyte glutathione was also correlated with systolic blood pressure ($r=0.27$).

CONCLUSIONS

In conclusion, there are a lot of factors (high blood pressure, low HDL, dysglycaemia, increased plasma lipid peroxidation, microalbuminuria) involved in left ventricular hypertrophy in type 2 diabetes mellitus. These markers are tight correlated between them and help us to have an open eye on the multiple pathogenic factors involved in cardiac hypertrophy in diabetics, in order to get a better management.

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