Profiling of Thermostable Proteins in Diabetic Cardiovascular Rat Plasma

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***Abstract*- Animal models of diabetes coupled with proteome profiling have great potential not only to provide important insights to the mechanisms of the development of diabetes, its complications, but also help to identify new protein potential candidate biomarkers and to support for therapy of the disease. The aim of this study is to profile thermostable proteins in diabetic cardiovascular rat plasma. Diabetic cardiovascular rats were induced by high-fat diet and low-dose streptozotocin (STZ) injection. Diabetic cardiovascular rat plasma has been used for thermostable pre-fractionation. The thermostable proteins have been separated and identified by using two-dimensional electrophoresis and nanoLC-MS/MS. At least five proteins (fibrinogen alpha chain, antithrombin-III precursor, angiotensinogen 1, haptoglobin, haptoglobin alpha 1S) were significantly up-regulated and three proteins (apolipoprotein A-IV, apolipoprotein E, apolipoprotein A-I) were down-regulated in diabetic cardiovascular samples, in which, the concentration of the antithrombin-III increased most (2.87 folds), followed by fibrinogen alpha chain (2.02 folds), angiotensinogen 1 (1.42 folds), haptoglobin (1.97 folds), haptoglobin alpha 1S (1.59 folds), while apolipoprotein A-I decreased 1.37 folds, apolipoprotein A-IV and apolipoprotein E were not detected in diabetic cardiovascular rat's plasma, as compared with that of the control rats. The different expression level of thermostable proteins in STZ rat plasma could give us new and important evidence for the understanding of the mechanism of diabetic cardiovascular diseases.**

Keywords*- *Cardiovascular; Rat Plasma; Thermostable Proteins; Type 2 Diabetes Mellitus

I. INTRODUCTION

There is a strong relationship between type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [1]. Long-term complications of T2DM can include heart disease, strokes, diabetic retinopathy and kidney failure. Among them, CVD is a major chronic complication and the leading cause of early death among people with T2DM. It was estimated that people with T2DM have an approximately 2-4 folds increased risk of cardiovascular events as compared with those without diabetes, and about 65% of populations with T2DM have died of heart disease and stroke [2-4]. In T2DM disease, high blood glucose levels can affect the lining of the body's arterial walls. This increases the likelihood of furring up of the vessels (atherosclerosis) what causes a narrowing of the artery, reduces the blood flow and increases blood pressure. Moreover, people with T2DM also often have low HDL cholesterol and raised triglyceride levels, which both increase the risk of CVD [5-7]. Besides, high blood pressure, smoking, obesity and lack of physical activity are also risk factors for CVD [8-10]. Diabetic cardiovascular diseases are also at high risk of developing microvascular complications, the most clinically important being end-stage kidney disease or diabetic nephropathy [11, 12]. Therefore, the research on diabetic cardiovascular disease is urgently needed for diagnosis and treatment of this disease in order to achieve decreased T2DM patient mortality [13, 14].

Diabetes disease and its complications have been researched on animal models of disease coupled with proteomics studies in order to find pathological mechanisms, to identify new biomarkers for early diagnosis, and to search for targets for therapy [15, 16]. There are several advantages of studying diabetic complications on animal models. First, an animal model can perform intervention and prevention studies in a relatively short time span compared with the time needed for studies in humans. Secondly, they are genetically homogeneous within a particular animal model and possibility of tightly control of environmental factors like dietary intake. Furthermore, animal models allow studying tissues (heart, kidney and retina) that are difficult to obtain from human subjects. Animal models of diabetes coupled with proteome profiling have great potential to provide important insights to the mechanisms of the development of diabetes, its complications and changes after treatment with anti-diabetic drugs [16]. Moreover, the use of proteomics in animal models of diabetes may help to identify new protein markers and to support for therapy of diabetes disease. Nowadays, the most commonly used animal model of diabetes is high-fat diet and low-dose streptozotocin (STZ) injection [17, 18]. In this study, plasma of the diabetic cardiovascular rats developed according to the mentioned above model [19] has been used for thermostable fractionation and proteome profiling. The thermostable proteins have been separated by two-dimensional electrophoresis (2DE). The identification by nanoLC-MS/MS showed that at least eight proteins were up/down-regulated in plasma of diabetic cardiovascular rats. The change in the expression levels of the proteins can be used for the early diagnosis and prognosis of diabetes disease and its complications.

II. MATERIALS AND METHODS

A. Materials

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Plasma samples were collected at the time of diagnosis from male Wistar rats (170-190 g) that were randomly divided into two groups: control group and high-fat diet plus streptozotocin (STZ) injection group. Methanol and acetonitrile (ACN) were purchased from J.T Barker (J.T Barker, Pittsburgh, USA); formic acid (FA), trifluroacetate (TFA) were obtained from Fluka (Fluka Chemie GmbH, Buchs, Switzerland); dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3), 2-D Starter Kit, strips 7 cm (pH 4.7-5.9) were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA). Enzyme trypsin, streptozotocin (STZ), sodium cholate hydrate and sodium citrate were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). Coomassie Brilliant Blue G250 was purchased from MP Biomedicals (MP Biomedicals, Eschwege, Germany).

B. Methods

1) Rat model of diabetic cardiovascular disease

Rat model of diabetic cardiovascular disease is designed and developed according to the previous described method [19]. During the first week, all rats were provided with normal pellet diet and water ad libitum. After one week, control group (10 rats) was fed with regular pellet and experiment group (10 rats) was fed with high-fat diet (52.5% regular pellet, 35% lard, 6.25% sucrose, 5% casein, 1% cholesterol, 0.25% cholin hydrate, 60% calories from fat and total caloric value 5200 kcal/kg) for 6 weeks. Rats of high-fat diet group were then injected with a low dose of STZ (35 mg/kg) in a dose volume of 0.5 ml while rats of the control group were given vehicle sodium citrate buffer (pH 4.5). One week and six weeks after STZ injection, blood glucose of rats was measured. Rats with glucose < 16.7 mmol/l were injected again with a higher dose of STZ (40 mg/kg). All rats were then allowed to continue to feed on their respective diets and closely monitored for further 24 weeks until the end of the study. At the end of the experiment (24 weeks), the rats were killed and their hearts were excised and then fixed in 10% formalin solution for routine paraffin embedment. Sections were prepared, stained with hematoxylin and eosin or periodic acid-Schiff for microscopic evaluation. The sections were then examined by differential interference contrast microscope BX51 (Olympus, New York, USA) for the degree of vascular injuries.

2) Collection of plasma from blood

Blood was collected from retro-orbital plexus of the rats. Plasma samples were then separated at the time of diagnosis by centrifugation in 5 min at 5000 rpm. The protein contents of all plasma samples were determined by the Bradford protein assay [20]. The plasma was divided and stored at -80^o C until use.

3) Rat plasma thermostable pre-fractionation

The rat plasma was mixed 1:1 (v/v) with ETP buffer (PEG 6000 7%, EDTA 20 mM, Tris HCl 0.2 M pH 8.9) and incubated for 15 min at 100^{0}C, left for 15 min at room temperature. The samples were then centrifuged at 12,000 g for 15 min and supernatants were collected. Subsequently, thermostable protein concentrations in rat plasma samples were determined by Bradford protein assay [20].

4) Two-dimensional electrophoresis (2DE)

Isoelectrofocusing was carried out using immobilized pH gradient (IPG) strips pH 4.7-5.9 on a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) at 250 V for 20 min; 4,000 V for 1 h and then kept at 4,000 V until a total of 12,000 Vh. After isoelectrofocusing, strips were incubated for 10 min in reduction buffer (containing 6 M urea, 20% glycerol, 2% SDS, 37.5 mM Tris-HCl (pH 8.8), 2% DTT w/v) and then were alkylated by alkylation buffer (containing 6 M urea, 2% SDS, 37.5 mM Tris-HCl pH 8.8, glycerol 20% and 40 mM IAA) in 10 min. Subsequently, the equilibrated strips were transferred onto the second-dimensional SDS-PAGE, which was run on 12.6% polyacrylamide gels at 120 V for 2 h. In addition, 2DE gels were stained by Coomassive brilliant blue G250, and 2DE gel images were analysed by PDQuest v7.1 2D software package (Bio-Rad, Hercules, CA, USA). Protein spots were initially detected, matched between experiment and control samples. Only those significantly different spots were selected and indentified.

5) In-gel trypsin digestion

Protein spots were excised, washed and destained by using wash solution (50 mM NH_4HCO_3, 50% ACN). After hydrating with 100% ACN and drying in a SpeedVac, the gel pieces were reduced with 5 mM DTT solution at 56^{o}C for 30 min and alkylated for 1 h with 20 mM IAA solution in darkness at room temperature. Enzyme trypsin (1 µg enzyme per 50 µg substance) was then added and incubated for 16 h at 37^{o}C. Finally, peptides were extracted with extraction solution containing 60% ACN and 1% TFA (v/v). All extracts were concentrated, dried and dissolved in 0.1% FA.

6) Protein identification by NanoLC-MS/MS

The digested peptides were analysed by nanoLC system (LC Packing, Dionex, Netherland). Peptides mixture was desalted, concentrated on C18 TRAP column (PepMap100, LC Packing, Dionex, Netherland) and separated onto C18 reverse phase column (GraceVydac, Hesperia, CA, USA). The sample was loaded on capillary column with 0.1% TFA. After peptides were eluted from C18 column with gradient from 0% to 100% of solution containing 85% ACN with 0.1% FA for 60 min, they were identified by the QSTAR®XL mass spectrometer (Applied Biosystems, MDS SCIEX, Canada) with a nano-ESI ion source.

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Proteins were searched for the NCBInr protein database using Mascot v1.8 software (Matrix Science Ltd., London, UK). Species search was limited to *Rattus*. Peptide and MS/MS mass tolerance were set to ± 0.25 Da. For further verification, proteins were validated by MSQuant software, available at http:// msquant.alwaysdata.net/. The MSQuant software is used as a validation and quantitation tool that produces the Mascot peptide identifications (HTLM files) and allows manual verification against the raw MS data (QSTAR®XL raw files).

III. RESULTS

A. Rat model of diabetic cardiovascular disease

Rat model of T2DM with CVD has been designed and developed as described in previous paper [19]. Concentration of glucose and lipid in plasma of control and high-fat diet plus STZ injection rat group (STZ) were analysed. Fasting blood glucose levels of STZ group were much higher than that of the control rats and have reached appromixately 21.5 mmol/L at the end of the experiment. The levels of triglyceride and HDL-cholesterol of STZ rats increased slightly at the end of the experiment, while the levels of total cholesterol and LDL-cholesterol of STZ rats were many times higher than that of control rats. Besides, histological analysis of the aortic arch showed that all STZ rats had developed early atherosclerotic lesions, extensive aortic fatty streaks and advanced atherosclerotic plaques [19]. The obtained results have confirmed that cardiovascular complications of diabetes disease were observed in STZ rats. Plasma of the STZ rats has been used for thermostable fractionation and proteome profiling.

B. Separation and identification of thermostable proteins

Plasma is an exceptional and special specimen, which contains a lot of essential information for the study and disease diagnosis. However, the analysis of plasma is analytically challenging due to the high dynamic concentration range of constituent protein species. Albumin, alpha-2-macro-globulin, transferrin and immunoglobulins can represent as much as 80% of the total plasma protein. Therefore, it is very difficult to determine low abundance proteins in plasma by traditional proteomics approaches (such as 2DE). In this study, thermostable pre-fractionation method was used as a simple method to remove high abundance and thermolabile proteins in rat plasma. After treating rat plasma by high temperature (100^0C for 15 min), almost all thermolabile proteins were denatured and removed by centrifugation at 12,000 g for 15 min. The supernatant containing soluble thermostable proteins were collected, determined by Bradford protein assay [20] and used for further analysis. As a result, the percentage of proteins retained in the thermostable fraction is about 1.8 ± 0.02 mg/ml, approximately 3 % of total plasma protein concentration.

The plasma thermostable proteins of STZ rats and control individuals were then separated by 2DE to identify the differentially expressed proteins. Representative Coomassie brilliant blue G250 stained 2DE gels of control and STZ rats group are shown in figure 1. Each set of rat plasma from two groups was processed in parallel through protein thermostable pre-fractionation, 2DE and staining to be distinguished from sample handling. The gel images were analysed and evaluated by the PDQuest v7.1 software. More than 200 protein spots were identified in each gel of two groups, out of which five protein spots (spot 1, spot 2, spot 3, spot 5, spot 8) were up-regulated and three protein spots (spot 4, spot 6, spot 7) were down-regulated in plasma of STZ rats group as compared with control group.

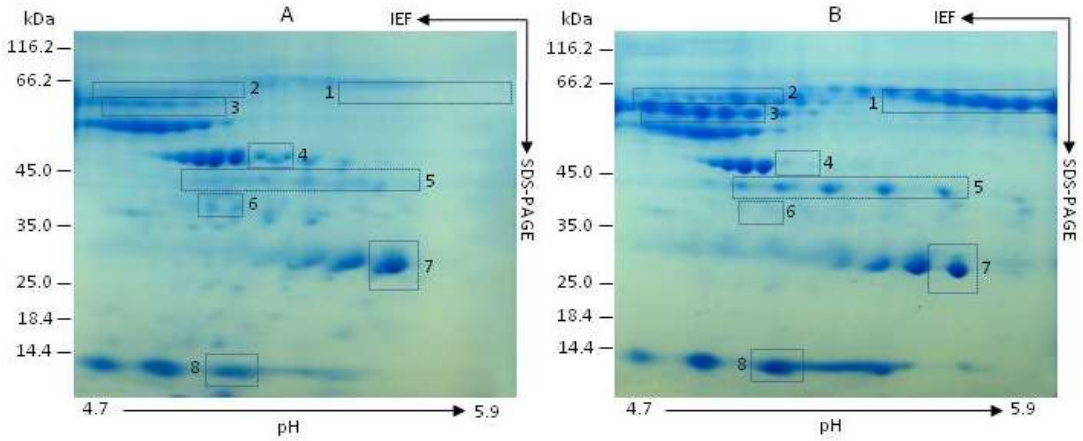

Fig.1 2DE gel images of thermostable proteins in plasma of STZ and control rats. (A): Control rat sample, (B): STZ rat sample. The thermostable proteins were separated in the first dimension with 7 cm length IPG strips, pH 4.7-5.9. The differentially expressed protein spots were circled and numbered. The identified protein spots included (1) Fibrinogen alpha chain isoform 1 precursor (FGA); (2) Antithrombin-III precursor (ATIII); (3) Angiotensinogen 1 (AGT); (4) Apolipoprotein A-IV (ApoA-IV); (5) Haptoglobin (Hp); (6) Apolipoprotein E (ApoE); (7) Apolipoprotein A-I (ApoA-I); (8) Haptoglobin alpha 1S (HpA1S).

The differentially expressed protein spots between STZ and control samples were then picked out, digested with trypsin and identified by nanoLC-MS/MS. Proteins were searched for the NCBInr protein database using Mascot v1.8 software

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(Matrix Science Ltd., London, UK). Results of protein identification are listed in Table 1. Spot 1 was identified as fibrinogen alpha chain isoform 1 precursor (FGA), which is 87.3 kDa; spot 2 as antithrombin-III precursor (ATIII), 52.7 kDa; spot 3 as angiotensinogen 1 (AGT), 52.2 kDa; spot 4 as apolipoprotein A-IV (ApoA-IV), 44.4 kDa; spot 5 as haptoglobin (Hp), 39.1 kDa; spot 6 as apolipoprotein E (ApoE), 35.8 kDa; spot 7 as apolipoprotein A-I (ApoA-I), 30.1 kDa; spot 8 as haptoglobin alpha 1S (HpA1S), 9.4 kDa.

TABLE 1. UP AND DOWN REGULATED THERMOSTABLE PROTEINS IN STZ RATS PLASMA

Spot No.	Protein name	Accession No.	Sequence coverage (%)	Mw* (kDa)	Score	Peptide match	Expression level in STZ rats plasma
1	FGA	Q7TQ70	16	87.3	55	3	Up
2	ATIII	Q5M7T5	20	52.7	108	9	Up
3	AGT	P01015	6	52.2	143	2	Up
4	ApoA-IV	P02651	67	44.4	1803	41	Not detectable
5	Hp	P06866	18	39.1	1092	45	Up
6	ApoE	Q6PAH0	50	35.8	595	20	Not detectable
7	ApoA-I	PO4639	27	30.1	1045	44	Down
8	HpA1S	Q6PEJ8	56	9.4	65	3	Up

(*) Mw is defined using NCBInr

C. The quantitative analysis of expression level of thermostable proteins

The expression levels of thermostable proteins in STZ and control rat plasma were calculated based on total spot volume and analysed by PDQuest v7.1 software. The comparative results of expression level of 8 proteins between two types of samples (STZ-treated and control) are shown in figure 2. It should be noted that the difference in the expression of thermostable proteins of STZ and control cases was statistically significant ($P<0.05$). ATIII increased most (2.87 folds), followed by FGA (2.02 folds), AGT (1.42 folds), Hp (1.97 folds), HpA1S (1.59 folds) while ApoA-I decreased 1.37 folds; ApoA-IV and ApoE could not be detected in STZ rats plasma.

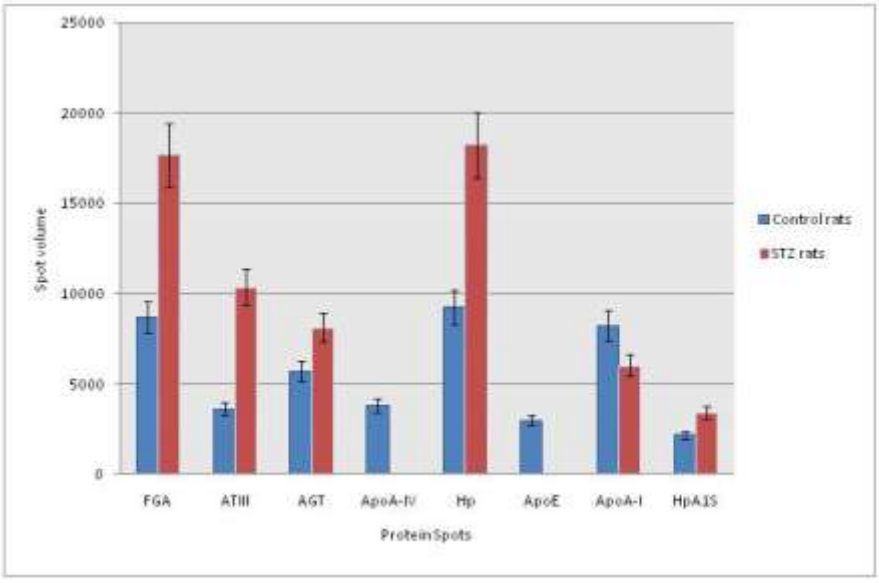

Fig. 2 The comparative chart of expression level of thermostable proteins between two groups of samples (control and STZ rats). STZ/control rat ratio of total volume of each spot: ATIII increased most (2.87 folds), followed by FGA (2.02 folds), AGT (1.42 folds), Hp (1.97 folds), HpA1S (1.59 folds) while ApoA-I decreased 1.37 folds; ApoA-IV and ApoE are not detectable in STZ rats plasma. The difference in the expression of thermostable proteins of STZ and control cases was statiscally significant ($P<0.05$).

IV. DISCUSSION

Cardiovascular diseases often are significant sequelae of diabetes. Risk factors for cardiovascular development are chronic hyperglycaemia, hypercholesterolemia, hyperlipidaemia and hypertension. Importantly, LDL cholesterol is a major risk factor [5]. In this study, both total and LDL cholesterol were very high in plasma of STZ rats. Moreover, remarkable lesions were determined in their aorta representing damage to large blood vessels. Our results have shown that STZ rats were developed cardiovascular complications of diabetes disease.

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This study provides the proteomic analysis of diabetic cardiovascular disease using 2DE and nanoLC-MS/MS. Eight differentially expressed proteins in STZ rat plasma were identified. Haptoglobin protein (Hp) is produced mostly in the liver and then released into plasma to combine with free plasma haemoglobin. Hp is as an antioxidant and prevents the oxidative tissue damage that may be mediated by free haemoglobin [21, 22]. While, diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defence [23]. Our results revealed that Hp content in the plasma of STZ rats was also higher than that of control individuals (Fig.1). Fibrinogen is a plasma glycoprotein synthesized in the liver. It is a hexamer containing two sets of three different chains (α, β, and γ), linked to each other by disulfide. Fibrinogen is converted into fibrin by thrombin during blood coagulation. Therefore, higher expression level of fibrinogen in plasma can increase thrombin-produced, leading to a greater risk of coronary thrombosis. Some previous publications had shown that higher expression level of fibrinogen was associated with cardiovascular, diabetes disease [24-26] and any form of inflammation [27]. Antithrombin is a small glycoprotein that inactivates several enzymes of the coagulation system and plays important role in regulating normal blood coagulation [28, 29]. In addition, antithrombin III refers to a substance in plasma that inactivates thrombin. Thus, different expression levels of fibrinogen and antithrombin III in plasma could be a risk factor of CVD. The obtained data from our experiment showed that both fibrinogen and antithrombin III were up-regulated in the STZ rat plasma (Table 1). Angiotensinogen or angiotensin precursor is produced in the liver and secreted in plasma. Plasma angiotensinogen level increases by the level of corticosteroid, oestrogen, thyroid hormone and angiotensin II in plasma. Elevated level of angiotensin II has been related to oxidative stress, inflammation and target organ damage in patients with T2DM [30]. Our data in figure 1 showed that angiotensinogen I increased in STZ rat plasma.

Apolipoproteins are proteins that bind lipids to form lipoproteins and their main function is to transport lipids. Apolipoproteins are keeping the structural integrity, solubility of lipoproteins and play an important role in lipoprotein receptor recognition [31]. Besides, they can regulate certain enzymes in lipoprotein metabolism. There are six classes of apolipoproteins (ApoA, ApoB, ApoC, ApoD, ApoE, ApoH) and several sub-classes. Among them, ApoA-I is a major protein component of high-density lipoproteins [32]. ApoA-IV carries out acting primarily in intestinal lipid absorption. ApoE is a plasma protein that mediates the transport and uptake of cholesterol and lipid by way of its high affinity interaction with different cellular receptors (the low-density lipoprotein receptor) [32-35]. Similarly, the uptake of cholesterol and lipid in STZ rat group must be higher than that of control group. This may explain the reason why the expression level of the proteins (ApoA-I, ApoA-IV, ApoE) could be changed. Recently, Bhonsle HS et al. also reported that downregulation of Apo AI may serve as an early predictive marker of diabetic complications [36]. In our study, ApoA-IV, ApoE and ApoA-I were clearly down-regulated in STZ rats plasma (Fig. 1). The results showed that different expression levels of ApoA-IV, ApoA-I and ApoE in plasma, probably, may contribute to diabetes-accelerated atherosclerotic cardiovascular disease. Therefore, further investigation on the expression level of different classes/subclasses of apolipoproteins, haptoglobins and other related plasma thermostable proteins might help to better understand the mechanism of the generation and progression of T2DM/CVD and its complications.

V. CONCLUSION

By using combination of thermostable protein pre-fractionation with 2DE and nanoLC-ESI-MS/MS methods, different expression levels of eight proteins in STZ rat plasma were indentified and analyzed. Among them, five proteins (FGA, ATIII, Hp, HpA1S, AGT) were significantly up-regulated and three proteins (ApoA-IV, ApoE, ApoA-I) were down-regulated in diabetic cardiovascular rats plasma, in which, ATIII increased most (2.87 folds), followed by FGA (2.02 folds), AGT 1 (1.42 folds), Hp (1.97 folds), HpA1S (1.59 folds), while ApoA-I decreased 1.37 folds and ApoA-IV and ApoE were not found in diabetic cardiovascular rats plasma. Those changed proteins gave us an evidence that might be used for the early diagnosis and prognosis of diabetic cardiovascular diseases.

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