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Proteome changes in the myocardium of experimental chronic diabetes and hypertension
Role of PPARα in the associated hypertrophy

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ABSTRACT

Diabetes with or without the presence of hypertension damages the heart. However, there is currently a lack of information about these associated pathologies and the alteration of linked proteins. For these reasons, we were interested in the potential synergistic interaction of diabetes and hypertension in the heart, focusing on the proteome characterization of the pathological phenotypes and the associated hypertrophic response. We treated normotensive and spontaneously hypertensive (SHR) rats with either streptozotocin or vehicle. After 22 weeks, type-I diabetic (DM1), SHR, SHR/DM1 and control left-ventricles were studied using proteomic approaches. Proteomics revealed that long-term DM1, SHR and SHR/DM1 rats exhibited 24, 53 and 53 altered proteins in the myocardia, respectively. DM1 myocardium showed over-expression of apoptotic and cytoskeleton proteins, and down-regulation of anti-apoptotic and mitochondrial metabolic enzymes. In both SHR and SHR/DM1 these changes were exacerbated and free fatty-acid (FFA) ß-oxidation enzymes were additionally decreased. Furthermore, SHR/DM1 hearts exhibited a misbalance of specific pro-hypertrophic, anti-apoptotic and mitochondrial ATP-carrier factors, which could cause additional damage. Differential proteins were validated and then clustered into different biological pathways using bioinformatics. These studies suggested the implication of FFA-nuclear receptors and hypertrophic factors in these pathologies. Although key ß-oxidation enzymes were not stimulated in DM1 and hypertensive hearts, peroxisome proliferator-activated receptors-α (PPARα) were potentially activated for other responses. In this regard, PPARα stimulation reduced hypertrophy and pro-hypertrophic factors such as annexin-V in high-glucose and angiotensin-II induced cardiomyocytes. Thus, activation of PPARα could reflect a compensatory response to the metabolic-shifted, apoptotic and hypertrophic status of the hypertensive-diabetic cardiomyopathy.

Abbreviations: DM1, type-I diabetic; STZ, Streptozotocin; SHR, Spontaneously Hypertensive Rats; FFA, Fatty Acid; ROS, Reactive Oxygen Species; PPAR, Peroxisome proliferator-activated receptors; HNF4α, Hepatocyte Nuclear Factor-4α; PGC1α, PPARγ coactivator-1α; MEF2, Myocyte-enhancing factor-2; ERRα, Estrogen-related receptor-α; CPT1b, Carnitine palmitoyltransferase 1B; ACADL, Acyl- Coenzyme A dehydrogenase,long chain; ACADM, Acyl-Coenzyme A dehydrogenase,medium chain; PDK4, Pyruvate dehydrogenase kinase-4; DIGE, Differential In-Gel Electrophoresis; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time Of Flight; MS, Mass Spectrometry.

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1. Introduction

Diabetes is currently a worldwide pandemic, which is expected to worsen in the coming years. More than 36 million people will have type-I diabetes by 2030, two thirds of whom may die because of cardiovascular complications [1]. In humans, cardiac damage can be directly induced by both type-I and type-II diabetes and exacerbated by the frequent coexistence of hypertension [2]. However, few studies have analysed the combined and comparative effects of chronic diabetes and hypertension on the heart. Knowledge of the altered proteomes linked to specific cellular mechanisms present in the pathological phenotypes may provide key information for the field.

Hyperglycemia alters metabolic, structural and contractile proteins activating cellular responses such as hypertrophy and apoptosis in the heart [1–3]. These same responses are also seen in hypertensive cardiomyopathy [4,5]. We previously described cardiac hypertrophy and apoptosis by using an experimental model of long-term DM1 and hypertension [3]. According to these studies, the combined effect of type-I diabetes and hypertension did not increase the fibrotic and inflammatory rates in the rats. However, given that hypertrophy and apoptosis were further stimulated when both pathologies coexisted, it follows that key information may be obtained by identifying altered hypertrophic/apoptotic proteins and related pathways in the diabetic heart, and whether or not it occurs when diabetes associated with hypertension. Proteomics allows the comparative expression analysis of hundreds of proteins in different biological samples at the same time [6]. The resulting data can be also included as input for bioinformatics studies carried out to predict molecular pathways. In previous data, proteomic studies revealed alteration of the cytoskeleton, metabolic and apoptotic proteins in short-term DM1 [7–11] and both short- and long-standing hypertension [12–14]. In particular, free fatty-acid (FFA) oxidation enzymes and pro-hypertrophic/apoptotic molecules were dysregulated. The peroxisome proliferator activated receptors (PPARs) are members of a nuclear receptor family of transcription factors. PPARα is the most abundant PPAR-isoform in the heart and FFA are putative endogenous ligands for these receptors. Under ligand stimulation, PPARα recruits specific co-activators (such as PGC1α) and other transcription factors to regulate fuel homeostasis genes, among others [15]. Impairment of FFA oxidation is a significant characteristic of cardiac hypertrophy [16], whose attenuation may be a promising therapeutic target for the prevention of cardiac apoptosis and failure [17]. In experimental diabetic cardiomyopathy, a PPARα-agonist treatment ameliorated the induced cardiac apoptosis and dysfunction [18,19]. In this work we have investigated the putative synergistic proteomes of chronic diabetes and hypertension in the heart, and the potential beneficial role of PPARα activation in the associated hypertrophy.

2. Methods

2.1. *Long-term hypertensive-diabetic model in rats; study design*

This work is a continuation of a previous study performed using an experimental model of chronic diabetes and hypertension in rats [3]. Six-week-old spontaneously hypertensive rats (SHR) and normotensive (Wistar) male rats as control, received either three streptozotocin injections (50 mg/Kg/day) or vehicle. As a result, there were four different groups: normotensive type-I diabetic (DM1), SHR, SHR/DM1 and Wistar rats. Blood glucose was monitored twice a week (10 a.m.) using a glucometer. Then, where needed, basic insulin (Insulatard NPH) was administrated (by intramuscular injection) in diabetic rats to prevent severe hyperglycemia, while maintaining the blood glucose between 400–600 mg/dl. 3 IU insulin were injected when blood glucose raised to 300–450 mg/dl, and 4 IU insulin when blood glucose reached 450–600 mg/dl. After 22 weeks, the rats were anesthetized (2.5% isofluorane) and the heart isolated. These investigations adhered to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and approval for these experiments was granted by the Ethics Committee of the hospital.

In this work we have used frozen pieces and paraffin slices of left ventricles to carry out proteomic, biochemical and histological studies. To determine the proteomic pattern, samples from DM1, SHR, SHR/DM1 and control rats were analysed using proteomic approaches (DIGE analysis; n=6, each group). Then, to predict related cellular pathways, the resulting lists of differentiated proteins were used as inputs in bioinformatics software for molecular prediction (IPA®, Pathway Architect®). To confirm protein implication, the expression/activation of key differentiated proteins and suggested factors involved mainly in hypertrophy were validated by histological (immunohistochemistry) and biochemical (Western B., EMSA) experiments in myocardium samples (n=6-8, each group). Next, to further investigate into the stimulated hypertrophic response, cultured cardiomyocytes (n≥3, independent experiments) were used for the characterisation of specific mediators (immunocytochemistry, Western B., QPCR).

2.2. *Differential in-gel electrophoresis (DIGE) and predictive analysis*

Left ventricle samples from DM1, SHR, SHR/DM1 and control rats (n=6, each group) were powdered and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris–pH 8.5) containing 50 mM DTT and additionally broken with the 2D Grinding Kit (GE Healthcare, Uppsala, Sweden), and the proteins were labeled with fluorescent dyes for DIGE analysis (see supplemental methods), as described previously [20]. After electrophoresis, the gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at 100 μm resolution using appropriate wavelengths and filters for Cy2, Cy3 and Cy5 dyes. Relative protein quantification across DM1, SHR, SHR/DM1 and control samples was performed using DeCyder software v 7.0 and multivariate statistical module EDA (Extended Data Analysis) (GE Healthcare) in a stepped process. Spots showing significantly altered expression were selected for excision, digestion and analysis by MALDI-MS (Ultraflex MALDI-TOF/TOF mass spectrometer). Mass data were used to search a non-redundant protein database (NCBInr; ~6.5×10^6 entries) using the Mascot software (see supplemental methods). 91 proteins were identified differentially expressed