

Downregulation of Nrf2 and HO-1 expression contributes to oxidative stress in type 2 diabetes mellitus: A study in Juana Koslay City, San Luis, Argentina

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ABSTRACT

Oxidative stress is associated with diabetes mellitus, a condition characterized by increased prevalence and progression rate of cardiovascular disease. NFE2-related factor 2 (Nrf2) is a master regulator of cellular detoxification responses and redox status. The aim of this study was to examine associations between type 2 Diabetes Mellitus (T2DM), oxidative stress and the expression of NFE2-related factor 2 (Nrf2) in a population of diabetic patients living in Juana Koslay City, San Luis, Argentina. In addition, we evaluated the functional relevance of Nrf2 by measuring the HO-1 expression among persons with type 2 diabetes. We measured clinical and biochemical parameters related to lipid metabolism and oxidative stress in a population of Type 2 Diabetes Mellitus patients (T2DM, n = 40) and controls (Co, n = 30). Compared to Co, T2DM patients had higher fasting serum glucose, glycated hemoglobin, triglycerides, total cholesterol, low-density lipoprotein cholesterol, and thiobarbituric acid reactive substances and lower high-density lipoprotein cholesterol. T2DM individuals had also higher atherogenic index and body mass index than controls. We also founded that HO-1 mRNA in whole blood was lower in T2DM than controls, suggesting that T2DM may have an altered antioxidant response

to oxidative stress. Interestingly, we found reduced Nrf2 mRNA in whole blood from T2DM compared to Co. The results from this study provide novel evidence that genes associated to antioxidant defense mechanisms are markedly reduced in patients with type 2 diabetes, and that the reduction in the expression of these genes could be associated to hyperglycemia and increased levels of MDA. Linear regression analysis revealed that there was a strong and positive correlation between the changes of Nrf2 and HO-1 expression levels.

Keywords: Type 2 Diabetes Mellitus; HO-1; Nrf2 and TBAR'S

1. INTRODUCTION

The pathogenesis of Type 2 Diabetes Mellitus (T2DM) has not been fully elucidated; however, there is growing evidence linking this disease to oxidative stress [1-3]. Oxidative stress, resulting from increased production or decreased removal of reactive oxygen species (ROS), plays a key role in the pathogenesis of late diabetes complications [2] and insulin—stimulated glucose uptake [4].

Increasing evidence indicates that increased production of reactive oxygen or nitrogen species (ROS or RNS) and/or impaired endogenously protective mechanism is the major factor responsible for the development and progression of vascular complications in diabetic patients,

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although several other mechanisms were also proposed [5-7].

One of the most important antioxidant machineries is the Nrf2 system, with the transcription factor Nrf2 as the central component [8]. The transcriptional factor NFE2-related factor 2 (Nrf2), a member of the cap'n'collar family, is a master regulator of cellular detoxification responses and redox status [9]. Under physiological conditions Nrf2 locates in the cytoplasm and binds to its inhibitor kelch-like ECH-associated protein 1 (KEAP1) [10]. KEAP1 could mediate a rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome [10]. Upon exposure of cells to oxidative stress or electrophilic compounds, Nrf2 is released from KEAP1 and translocates into the nucleus. There, it binds to antioxidant-responsive elements (ARE) in the genes encoding antioxidant enzymes such as NADPH quinoneoxidoreductase (NQO1), heme oxygenase-1 (HO-1), glutathione S transferase, superoxide dismutase (SOD), catalase, and γ -glutamylcysteine synthetase, increasing their expression to play a role in the detoxification, antioxidant, and anti-inflammatory processes [9-11].

Among a panel of potential candidate genes related to oxidative stress, the heme oxygenase-1 (HO-1) gene has drawn much attention with its potent antiinflammatory, antioxidant, and antiproliferative effects [12]. Heme oxygenase is a microsomal rate-limiting enzyme responsible for the oxidation and degradation of heme into biologically active metabolites—biliverdin, which is rapidly reduced to bilirubin by biliverdin reductase, carbon monoxide, and iron [13]. Three mammalian heme oxygenase isoforms have been identified. Among them, the inducible isoform HO-1 is a stress-responsive protein ubiquitously distributed in mammalian tissues and can be induced by various oxidative agents [14,15]. The induction of HO-1 has been considered an adaptive cellular defense response protecting cells or tissues against injuries in pathophysiological states, attributed to the antioxidant properties of bilirubin and biliverdin, the extrusion and sequestration of cellular free iron by ferritin, and the antiapoptotic and anti-inflammatory effects of carbon monoxide [12,14-16]. The beneficial role of HO-1 in diabetes has been reported in animal models and in *in vitro* assays involving exposure to glucose [12,17], but little information on humans is available.

Our aim in this study was to examine associations between type 2 Diabetes Mellitus (T2DM), oxidative stress and the expression of NFE2-related factor 2 (Nrf2) in a population of diabetic patients living in Juana Koslay City, San Luis, Argentina. In addition, we evaluated the functional relevance of Nrf2 by measuring the HO-1 expression among persons with type 2 diabetes.

2. RESEARCH DESIGN AND METHODS

2.1. Subjects

The present study was carried out in accordance to the guidelines of the Helsinki Declaration. A total of 70 volunteers (40 patients with type 2 diabetes and 30 healthy age-matched controls) participated in this investigation. Criteria published by the American Diabetes Association were used to diagnose T2DM [18]. These patients reside in Juana Koslay, San Luis, Argentina. The protocol for this study was approved by the local Institutional Review Board, and a written informed consent was obtained from each patient to be enrolled. During an initial interview with each patient, they were asked for diseases, medication and smoking histories. Exclusion criteria included liver, kidney and thyroid diseases, as well as the use of anti-lipemic drugs.

2.2. Anthropometric and Clinical Data

For each subject enrolled, height (meters) and weight (Kg) measurements were acquired. Height and weight were measured to the nearest 0.5 cm and 0.1 Kg, respectively. The body mass index (BMI) was calculated as weight divided by height squared (Kg/m^2). Those patients with a BMI equal or greater than 30.00 Kg/m^2 were considered as obese.

2.3. Blood Sampling

Blood samples were obtained from patients that had fasted overnight for a minimum of 12 h and were drawn from the antecubital vein between 08:00 am and 09:30 am. Blood sample were obtained with or without anticoagulants to obtain plasma and serum, respectively. After separation of plasma, cell pellets were used to extract RNA (see below).

2.4. Biochemical Measurement

Fasting plasma glucose (FPG) was measured by using a glucose oxidase method with a commercial enzymatic kit (Wiener Lab, Rosario, Argentina) and Glycated hemoglobin (HbA1c) concentration was measured with a coupled ionic-exchange chromatography/spectrophotometric assay (BioSystems, Barcelona, Spain) in a Bayer Express Plus Chemistry Analyzer (Bayer Diagnostics, Siemens, Germany). Total cholesterol (TC), triglycerides (TG) and HDL-c concentrations were measured using commercial kits by following manufacturer's instructions (Wiener Lab, Rosario, Argentina) in a Bayer Express Plus Chemistry Analyzer (Bayer Diagnostics, Siemens, Germany). Low density lipoprotein-cholesterol (LDL-c) was calculated with the Friedewald formula: $\text{LDL-c} = \text{total cholesterol (mg/dL)} - \text{HDL-c (mg/dL)} - \text{triglycerides (mg/dL)}/5$ [19].

2.5. Lipid Peroxidation Assay

Plasma lipid peroxidation was determined as described by Jentzsch *et al.* (1969). Plasma protein was precipitated in trichloroacetic acid (TCA). MDA produced during lipid peroxidation, reacts with thiobarbituric acid (TBA) and generates a pink-colored complex. After some further steps, the absorbance of the supernatant was measured spectrophotometrically at 532 nm (Bayer Diagnostics, Siemens, Germany) using 1,1,3,3-Tetra ethoxypropane (Sigma Chemical, St Louis, MO) as standard. TBARS concentration was determined in triplicate and all results are presented in M.

2.6. Measurement of Gene Expression

Total RNA was isolated from cell pellets using the TRIzol reagent following manufacturer's instructions (Life Technologies, Carlsbad, CA). Agarose gel electrophoresis and ethidium bromide staining confirmed the purity and integrity of isolated RNA. RNA concentration was assessed by spectrophotometric measurements at 260/280 nm. Then, ten micrograms of total RNA were reverse-transcribed with 200 units of moloney-murine leukemia virus (MMLV) reverse transcriptase (Promega) using random hexamers as primers in 20 μ L reaction mixture following the instructions provided by the manufacturer. The reverse transcription reactions were performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) with conditions at 65°C for 10 min, 37°C for 60 min, and 90°C for 5 min. PCR was used to quantify human Nrf2, HO-1 and Cyclophilin A expression. PCRs were carried out in 35 μ L reactions. Each amplification reaction included 20 ng of cDNA, 20 pmol of each PCR primer and 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Waltham, MA). These reactions were performed in a buffer 1X containing 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), and 200 pmol/L of each deoxynucleotide triphosphate. cDNA and control preparations were amplified using the following conditions: 95°C for 2 min followed by 40 cycles of: denaturation for 1 min at 95°C, primer annealing for 1 min at 60°C, and extension for 1 min at 72°C; followed by a final extension at 72°C for 5 minutes. Deoxy-oligonucleotide primers were constructed from the published cDNA sequence of HO-1, Nrf2

and Cyclophilin A, and the PCR products resulted in 323, 201 and 166 bp fragments, respectively. Cyclophilin A controls were performed for all PCRs. The primers sequences are presented in **Table 1**.

The PCR products were electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) ethidium bromide. The image was visualized and photographed under UV transillumination. The intensity of each band was measured using NIH *Scion Image* 1.6.3 software and reported as the values of band intensity units.

2.7. Statistics

All results are presented as mean \pm SD. Student's t-test was used for the analysis of data with a Gaussian distribution. The Pearson's product moment and Spearman's correlation coefficients were used to determine the relationships between the studied parameters. Statistical significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Subject Characteristics

Seventy individuals were included in this study, 40 of them were diabetic and 30 were controls. **Table 2** shows the demographic characteristics of both groups. 54.3% were women and 45.7% were men, age distribution was not different between the groups with a media age of 56.3 years old. The average value of BMI was 27.1 kg/m² for the control group and 30 kg/m² for the diabetic one. There was a significant difference in weight and BMI between both groups. Diabetic female subjects were more obese than diabetic male subjects.

As shown in **Table 3**, fasting plasma glucose and HbA_{1c} concentrations were higher in the diabetic group when compared with age-matched control subjects. Total cholesterol, TG and LDL-c were increased in the diabetic group, while HDL-c was significantly lower in these patients, when compared to controls. LDL cholesterol was lower in diabetic female than in diabetic male subjects. The atherogenic index (AI) was two fold above the control value in the diabetic patients, what suggests a higher risk for cardiovascular diseases in these patients.

Abbreviations used: TG, triglycerides; TC, total cholesterol; LDL, low density lipoprotein; HDL, high den-

Table 1. Primers sequences for RT-PCR.

Gen/Gen Bank access	Primers	Fragment size
Nrf2 (<i>Homo sapiens NFE2-related factor 2</i>) NM_006164.3	Forward 5' AGATTCACAGGCCTTTCTCG 3' Reverse 5' CAGCTCTCCCTACCGTTGAG 3'	201 bp
HO-1 (<i>Homo sapiens heme oxygenase 1</i>) NM_002133.2	Forward 5' AGAGCTGCACCGCAAGGCTG 3' Reverse 5' GGGAGTTCATGCGGGAGCGG 3'	323 bp
Cyclophilin A (<i>Homo sapiens peptidylprolyl isomerase A</i>) NM_021130.3	Forward 5' TCCTAAAGCATACGGGTCCTGGCAT 3' Reverse 5' CGTCCATGGCCTCCACAATATTCA 3'	166 bp

Table 2. Anthropometric characteristics in control and T2DM patients.

	Control (n = 30)	T2DM (n = 40)	<i>p</i>
Gender (F/M)	23/7	15/25	
Age (years)	54.63 ± 10.63	58.13 ± 10.96	0.186
Weight (kg)	73.20 ± 16.08	85.98 ± 18.65	0.004
Height (m)	1.65 ± 0.08	1.67 ± 0.08	0.204
BMI (kg/m ²)	27.13 ± 5.29	30.00 ± 4.94	0.023

Data are shown as mean ± SD; Abbreviations used: BMI, body mass index.

Table 3. Biochemical characteristics in control and T2DM patients.

	Control (n = 30)	DMT2 (n = 40)	<i>p</i>
FPG (mg/dL)	85.10 ± 13.37	149.25 ± 51.74	<0.000 1
HbA1c (%)	5.51 ± 0.74	7.74 ± 2.04	<0.000 1
TC (mg/dL)	172.30 ± 30.28	233.55 ± 38.70	0.001
HDL-c (mg/dL)	41.60 ± 4.00	34.8 ± 6.30	0.04
LDL-c (mg/dL)	110.80 ± 26.20	142.1 ± 28.20	0.001
TG (mg/dl)	127.20 ± 28.16	190.68 ± 56.45	0.001
AI	3.41 ± 0.65	6.30 ± 1.82	<0.000 1

Data are shown as mean ± SD.

sity lipoprotein; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; AI: TC/HDL-c

3.2. Prooxidant Status

The malon-dialdehyde (MDA) concentration was assessed to evaluate the oxidative stress using the thio-barbituric acid-reactive substances (TBAR'S) method. The results obtained are consistent, with a significant increase in TBAR'S levels in the diabetic group, when compared to age-matched control subjects (**Figure 1(a)**). TBAR'S levels were lower in diabetic female than in diabetic male subjects (**Figure 1(b)**).

3.3. mRNA Expression

As shown in **Figure 2**, Nrf2 gene expression was significantly higher (0.90 ± 0.04 Nrf2/Cyclophiline A gene expression ratio) in leucocytes from controls when compared to type 2 diabetic patients (0.86 ± 0.04 , $p < 0.0001$). Similarly, HO-1 gene expression was significantly higher in controls (0.80 ± 0.03 HO-1/Cyclophiline A gene expression ratio) when compared to the T2DM patients (0.74 ± 0.07 , $p < 0.003$) (**Figure 3**). Nrf2 and HO-1 gene expression were not different between genders in both groups.

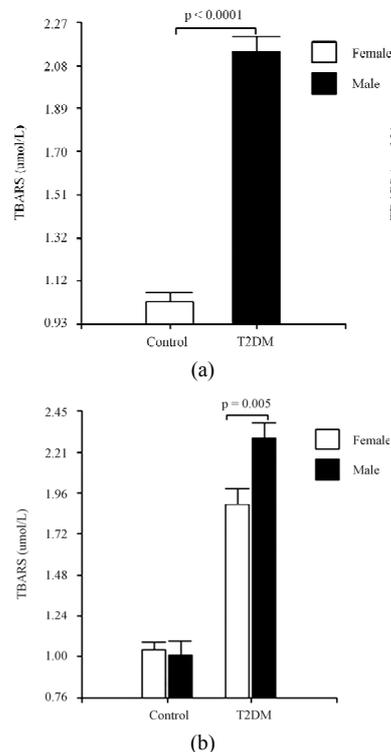


Figure 1. (a) Lipid peroxidation among healthy controls and diabetic subjects. (b) Lipid peroxidation among female and male in diabetic subjects. Data are expressed as mean ± SEM.

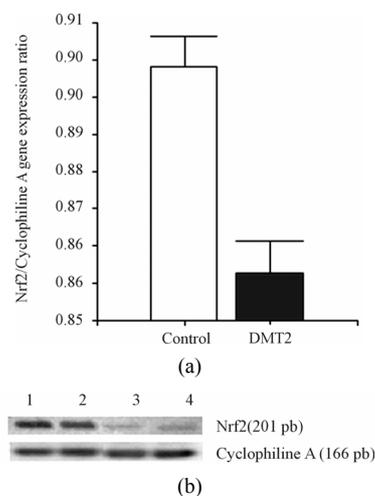


Figure 2. Expression of Nrf2. (a) The bar graphs represent the densitometric analysis of Nrf2 gene expression in leucocytes of controls (lanes 1 and 2) and type 2 diabetic patients (lane 3 and 4). (b) The expression of Nrf2 was assessed by RT PCR, using specific primers, and it was normalized against the expression of the housekeeping gene Cyclophiline A, as reported in *Material and Methods*. Data are expressed as mean ± SEM.

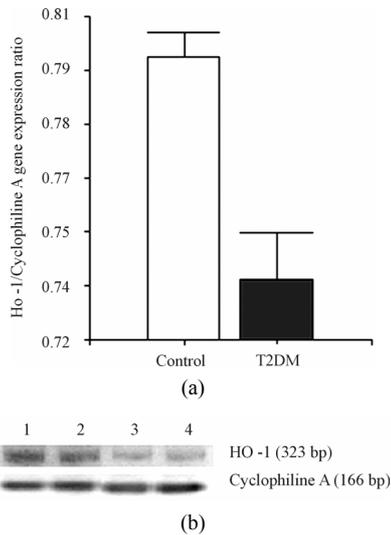


Figure 3. Expression of HO-1. (a) The bar graphs represent the densitometric analysis of HO-1 gene expression in leucocytes of controls (lanes 1 and 2) and type 2 diabetic patients (lane 3 and 4). (b) The expression of HO-1 was assessed by RT PCR, using specific primers, and it was normalized against the expression of the housekeeping gene Cyclophilin A, as reported in *Material and Methods*. Data are expressed as mean \pm SEM.

3.4. Correlation and Lineal Regression Studies

Because both Nrf2 and HO-1 mRNA were reduced in the diabetic group, we performed correlations to determine whether this reduction was associated with metabolic factors that are important for insulin resistance. When the data from all diabetic patients were pooled together, HbA1c was positively correlated with FPG and BMI ($r = 0.484$, $p = 0.001$; $r = 0.307$, $p = 0.04$, respectively).

A subsequent linear regression analysis revealed that there was a significant and positive correlation between the changes of the Nrf2 and HO-1 expression levels, independent of the changes of the other measured factors. **Figure 4** shows the linear regression analysis between the expression of Nrf2 and HO-1 in both groups.

4. DISCUSSION

Here we measured some anthropometrical, clinical and biochemical parameters in a population of men and women diagnosed to be T2DM-patients, which reside in Juana Koslay city, San Luis—a state located in the central region of Argentina. In this study we report for the first time that T2DM patients have lower Nrf2 and HO-1 mRNAs in blood, when compared to healthy controls.

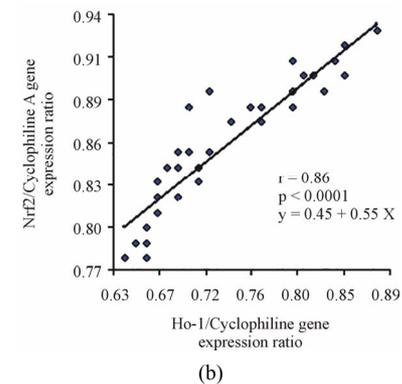
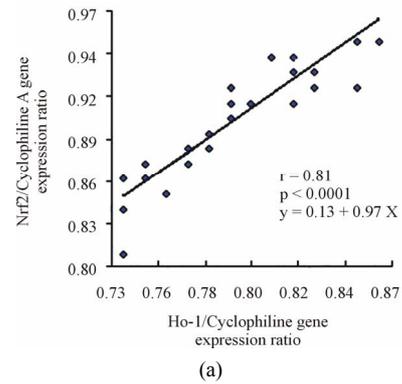


Figure 4. (a) Linear regression between Nrf2 and HO-1 in the control group. (b) Lineal regression between Nrf2 and HO-1 in T2DM.

This change was accompanied by an increased plasma concentration of TBAR'S.

Type 2 Diabetes Mellitus (T2DM) is common and is characterized by hyperglycemia, insulin resistance, and a relative impairment in insulin secretion. Abdominal obesity, specifically, is associated with resistance to the effects of insulin on the utilization of fatty acids and on peripheral glucose levels. Insulin resistance is integral in the pathogenesis of T2DM and is frequently accompanied by hypertension, high serum LDL, low serum high-density lipoprotein, and high serum triglyceride levels, which promote the development of atherosclerotic cardiovascular disease [20].

Hyperglycemia is a major factor in the development of diabetic complications, although the mechanisms of how increased glucose levels contribute to these changes have not been fully elucidated. Adverse biochemical changes associated to hyperglycemia include increased flux of glucose through the polyol pathway, enhanced nonenzymatic glycation and activation of the diacylglycerol-protein kinase C pathway. Hyperglycemia may also result in increased production of ROS within numerous biochemical pathways that have the potential to initiate adverse changes in the endothelial function [21]. Oxygen-free radicals had been suggested to be a contributory

factor in complications of diabetes mellitus. There are many reports that indicate that the changes in biochemical parameters are due to diabetes-induced oxidative stress.

Our results indicate the presence of higher levels of MDA in plasma of type 2 patients as compared to healthy controls. Increased levels of MDA in diabetic subjects are indication of lipid peroxidation. Increased production of MDA has also been demonstrated in the erythrocyte membranes of diabetic patients [22,23] and high-lipid peroxides levels are also observed in diabetic rats [24].

Oxidative stress is a consequence of an unbalance between the production of oxidants and antioxidants, which affects the structure, function and turnover of macromolecules leading to tissue dysfunction [25]. Oxidative stress has been suggested to be an important mechanism in the development of type 2 diabetes [1,2]. Nrf2 maintain the balances controlling the antioxidant defense in the body which are at stake in a variety of conditions including diabetes and diabetic neuropathy. In response to oxidative stress, Nrf2 escapes ubiquitination by dissociating from its negative regulator KEAP1 (Kelch-like ECH-associated protein 1), translocates to the nucleus, and activates the genes involved in the synthesis of antioxidant enzymes [26]. These genes carry a special Nrf2-binding site, called the antioxidant response element (ARE), in their promoter region [27]. Nrf2 itself contains an ARE in its promoter.

Lipid peroxidation leads to the formation of a number of short-length electrophilic fatty acid-derivatives and lipid peroxides, which are known to trigger Nrf-2 pathway activation and synthesis of antioxidant proteins and other proteins involved in the removal of electrophilic compounds [28,29]. Although acute activation of Nrf2 occurs *in vivo* in response to oxidized phospholipids' signaling, increased ROS production, hyperglycemia, and shear stress [30-32], in chronic disease states the antioxidant response is often insufficient to maintain redox balance and prevent disease progression [33,34]. Our T2DM patients have increased lipid peroxidation and lower Nrf2 mRNA in blood than controls.

Like many other antioxidant genes, HO-1 gene expression is triggered by the binding of Nrf2 to the antioxidant response element in its gene's promoter. In the anti-oxidative system, HO-1 acts as a key factor in mechanisms to mitigate oxidative stress because of its potent anti-inflammatory, antioxidant, and anti-proliferative actions [12]. In addition, impaired HO-1 protein expression/activity may be related to the pathogenic process induced by oxidative stress. Although accumulating evidence from animal studies supports a beneficial role of HO-1 in the diabetic state [12,17], it is still not clear whether HO-1 exerts a similar biologic effect in humans.

We studied HO-1 expression in leucocytes from control and T2DM patients. As expected, decreased levels of HO-1 mRNA expression were observed in diabetic patients when compared to healthy controls. These findings suggest that reduced HO-1 expression may contribute to the development of type 2 diabetes, consistent with the results of several small clinical studies which found decreased mRNA expression of HO-1 in peripheral blood mononuclear cells [35], skeletal muscle [36], and retinal pigment epithelium [37] among patients with type 2 diabetes.

Taken together our data indicate that the decreased expression of HO-1 and increased levels of TBAR'S observed in T2DM could be a consequence of a reduced expression of Nrf2. Continuous oxidative stress may be a consequence of a reduced capacity of T2DM patients to trigger Nrf2-dependent antioxidant response against a positive energy balance.

The results from this study provide novel evidence that genes associated to antioxidant defense mechanisms are markedly reduced in patients with type 2 diabetes, and that the reduction in the expression of these genes could be associated to hyperglycemia and increased levels of MDA. The linear regression analysis revealed that there is a strong and positive correlation between the changes of the Nrf2 and HO-1 expression levels.

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