Relationship between PMN-endothelium interactions, ROS production and Beclin-1 in type 2 diabetes

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ABSTRACT

Type 2 diabetes is closely related to oxidative stress and cardiovascular diseases. In this study, we hypothesized that polymorphonuclear leukocytes (PMN)-endothelium interactions and autophagy are associated. We evaluated PMN-endothelial interactions, ROS production and autophagy parameters in 47 type 2 diabetic patients and 57 control subjects. PMNs from type 2 diabetic patients exhibited slower rolling velocity (p < 0.001), higher rolling flux (p < 0.001) and adhesion (p < 0.001) in parallel to higher levels of total (p < 0.05) and mitochondrial ROS (p < 0.05). When the protein expression of autophagy markers was analysed, an increase of Beclin-1 (p < 0.05), LC3I (p < 0.05), LC3II (p < 0.01) and LC3II/LC3I ratio (p < 0.05) was observed. Several correlations between ROS and leukocyte-endothelium parameters were found. Interestingly, in control subjects, an increase of Beclin-1 levels was accompanied by a decrease in the number of rolling (r = 0.561) and adhering PMNs (r = 0.560) and a rise in the velocity of the rolling PMNs (r = 0.593). In contrast, in the type 2 diabetic population, a rise in Beclin-1 levels was related to an increase in the number of rolling (r = 0.437) and adhering PMNs (r = 0.467).

These results support the hypothesis that PMN-endothelium interactions, ROS levels and formation of autophagosomes, especially Beclin-1 levels, are enhanced in type 2 diabetes.

1. Introduction

In recent years, a sustained global increase in the prevalence of obesity and metabolic syndrome [1] has provoked a rise in diseases such as type 2 diabetes. Currently, type 2 diabetes and its comorbidities are among the main health concerns worldwide because of their high prevalence and the associated cost related to public health services. Type 2 diabetes is characterized by hyperglycaemia and insulin resistance, which cause chronic subclinical inflammation [2,3]. Hyperglycaemia and inflammation produce cellular alterations, which are the molecular basis of diabetes and cardiometabolic diseases [3–5]. Previous studies have highlighted the relationship between diabetes and inflammation, pointing to circulating hyperlipidaemia and hyperglycaemia as triggers of inflammatory responses [5–7].

One of the consequences of chronic hyperglycaemia is the increased generation of reactive oxygen species (ROS), produced mainly by the mitochondrial respiratory chain [8,9]. This heavy load of ROS overwhelms antioxidant defences and can modify cellular molecules and organelles, disturbing cell homeostasis and inducing inflammation. Furthermore, mitochondrial dysfunction and oxidative stress have been closely related to cardiovascular diseases [10,11].

Hyperglycaemia, together with ROS production, leads to an increased presence of proinflammatory molecules that activate immune cells [8–10]. Moreover, endothelial cells are activated by ROS and proinflammatory cytokines thereby developing endothelial dysfunction [12–15]. This situation enhances a cascade of PMN-endothelium interactions, a process by which immune cells migrate to the site of inflammation [16]. The proinflammatory state and increased ROS content
characteristic of type 2 diabetes favour PMN-endothelial interactions throughout the vasculature, not only at the site of inflammation [17]. This process is enhanced in the comorbidities related to type 2 diabetes [17], but the cause and the pathways affected are still being investigated. One of the actions involves the interference of ROS with the β-cells [18], including mechanisms of protein homeostasis, such as protein folding and degradation [19]. It is known that ROS can damage various cellular components, which are degraded and recycled by a process named autophagy. It involves nonsel ective degradation of proteins, lipids and organelles [20], and occurs in response to internal or external stimuli such as oxidative stress, unfolded protein response (UPR) and malfunctioning of organelles (internal inducers), and growth factors, serum starvation or amino acid deprivation (external stimuli). In this sense, autophagy is a survival mechanism [20] and a strictly regulated process. Two key proteins in this process are microtubule-associated protein light chain 3 (LC3) and Beclin-1. The latter, together with other autophagy-related proteins, initiates the formation of the omegasome and the phagophore, thus priming the progression to autophagosome ([20]). In parallel, the cytoplasmic form of LC3I is lipidated to LC3II, and, in this form, is recruited to the inner and outer autophagosomal membrane in order to construct the autophagosome. In the case of selective autophagy, altered proteins and organelles are carried to the autophagosome via the ubiquitin- and LC3-binding protein SQSTM1 (p62). Ubiquitinated proteins or organelles are sequestered into the autophagosomes for their degradation. When autophagy is impared, p62 protein accumulates in the autophagosome; however, p62 is important not only in this process, but it also acts as a scaffold protein that intervenes in cell proliferation and survival/death signalling [21]. Autophagy has been shown to be enhanced and decreased in diabetic patients [22,23]. In fact, insulin influences autophagy regulation, in part through mTOR signalling. Yan et al. [24] described that the adipocytes of obese type 2 diabetic patients display increased autophagy and reduced mTOR signalling. Interestingly, they showed that this state leads is associated with an undermining of mitochondrial biogenesis and function. Furthermore, several studies have demonstrated that hyperglycaemia induces autophagy as a protective mechanism. For example, autophagy is active in diabetic mice podocytes with glomerular damage [25–27], a mechanism that may be modulated by heme oxygenase 1 (HO-1) and AMPK activation [28]. In mice, it has also been observed that defective autophagy in β-cells accelerates the progression from obesity to diabetes through enhancement of UPR, a mechanism also activated by hyperglycaemia [29]. In parallel to these observations, it has been established that the BCL2-Beclin-1 complex is dissociated in response to AMPK activation in cardiac muscle, thus enhancing autophagy and preventing cardiomyocyte death [30]. These observations have been confirmed in other tissues, such as endothelial progenitor cells [31]. Conversely, Qianrong et al. [32] reported that high glucose levels inhibit autophagy in cardiomyocytes, leaving cells unprotected and more prone to apoptosis. In summary, it is thought that autophagy is activated in situations of cellular stress such as hyperglycaemia, but the underlying mechanisms are unknown in most cell types.

In this context, we hypothesized that PMN-endothelial interactions, ROS and autophagy are altered in the PMNs of diabetic patients and that there is an association between all three. In this study, we analyse the link between Beclin-1, ROS production and PMN-

Table 1: Biochemical and anthropometrical parameters in control and type 2 diabetic populations. Data are expressed as mean ± SD for parametrical data and as median (25th percentile-75th percentile) for non-parametrical variables. Statistical significance (P < 0.05) was compared with T-test following a post-hoc test with BMI as covariate.
endothelium interactions, as well as the varying behaviour of autophagy in diabetic and control conditions.

2. Materials and methods

2.1. Study population

This cross-sectional observational study had a case-control design, and was conducted with 47 diabetic patients and 57 control subjects matched by age and sex. The patients were recruited at the Endocrinology and Nutrition Service of the University Hospital Dr. Peset, Valencia, Spain, and their characteristics are described in Table 1. A diagnosis of type 2 diabetes was determined according to the American Diabetes Association's criteria. Subjects aged 18 or older were eligible for inclusion in the study. The exclusion criteria were having an abnormal haematological profile, suffering any malignant neoplasm or autoimmune disease, consumption of any anti-inflammatory drugs in the two weeks previous to the analysis, and regular consumption of antioxidant nutritional supplements.

The procedures carried out in the study were approved by the Ethics Committee of the Hospital (ID: 97/16) and conducted according to the ethical principles stated in the Declaration of Helsinki. All subjects signed an informed consent document before the interventions. A physical examination was performed in all patients prior to blood extraction, which was conducted in a state of fasting. Body weight and height were recorded and body mass index (BMI) was calculated using the BMI formula (BMI = weight in kg/(height in m) 2).

2.2. Blood sampling

In order to determine biochemical parameters and obtain PMN, venous blood was collected from subjects in heparin, EDTA or citrate tubes after 12h overnight fasting. It was then centrifuged (1500g, 80 °C for subsequent analysis, or employed to determine antioxidant nutritional supplements.

2.3. Biochemical determinations

All the biochemical parameters were determined by the Hospital’s Clinical Analysis Service. An enzymatic method was employed to determine serum concentrations of glucose, total cholesterol, HDL-cholesterol and triglyceride levels with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA). Low density lipoprotein (LDL) cholesterol levels were calculated with Friedewald’s formula. An immunochromiluminiscent assay was used to determine insulin levels. Insulin resistance was determined employing the Homeostasis Model, calculated as [(fasting glucose in mg/dL x fasting insulin in μU/mL)/405]. Glycated haemoglobin (HbA1c) was assessed with an automatic glycohemoglobin analyser (Arkray, Inc., 73 KYOTO, Japan). Serum concentrations of high-sensitive C-reactive protein (hs-CRP) were determined by immuno nephelometry. Atherogenic Index of Plasma (AIP) was calculated using the formula (Total Cholesterol(mg/dL))/(HDL-Cholesterol (mg/dL)).

2.4. PMN-endothelium interaction assay

PMNs were isolated as previously described [33]. We employed a 1.2 mL aliquot of PMNs obtained from the peripheral blood of control and type 2 diabetic subjects with a density of 10⁶ cells/mL in complete RPMI (RPMI 1640 medium supplemented with 10% Fetal bovine serum,1% penicillin/streptomycin, 1% glutamine and 1% sodium pyruvate). Prior to this, primary cultures of human umbilical cord endothelial cells (HUVEC) were established. HUVEC were isolated as previously reported [32]. On the day of the experiment, PMNs were monitored through the endothelial monolayer at a speed of 0.3 mL/min during a 5-min period, which was recorded, and the number of rolling PMNs as well as their velocity and adhesion to the endothelial monolayer were determined. The number of rolling PMNs was measured as those rolling for 1 min, their velocity was assessed by determining the time in which 15 rolling PMNs covered a distance of 100 μm. Adhesion was analysed by counting the number of PMNs adhering to the endothelium for at least 30 s in 5 fields.

2.5. Protein extraction and quantification

PMN pellets were incubated for 15 min on ice with lysis buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 20% glycerol, 0.1 mM EDTA, 10 μM Na₂MoO₄, 0.5% NP-40) containing protease inhibitors (10 mM NaF, 1 mM NaVO₃, 10 mM PNP, 10 mM β-glycerolphosphate) and dithiothreitol 1 mM. Subsequently, samples were vortexed for 30 s and centrifuged at 13200 rpm for 15 min at 4 °C. The supernatant was then collected in a new tube and quantified with the BCA protein assay kit (Thermo Scientific, Rockford, USA). The protein extract obtained was stored for subsequent determinations at −80 °C.

2.6. Western blotting

25 μg protein samples were separated with SDS-PAGE (13% polyacrylamide gels) and transferred to a nitrocellulose membrane. The membranes were then blocked for 1h at RT with 5% skimmed milk in TBS-T or 5% BSA in TBS-T and incubated with primary antibodies overnight at 4 °C- anti-Beclin-1 (Millipore Iberica, Spain, Madrid), anti-LC3 (Millipore Iberica, Spain, Madrid), anti SQSTM/p62 (Abnova Corporation, Taiwan), anti-Actin (Sigma Aldrich, St. Louis, USA). The secondary antibody was HRP-goat anti-rabbit (Millipore Iberica, Spain, Madrid). The protein signal was revealed with SuperSignal West Femto (Thermo Scientific, Rockford, USA) and detected with a Fusion FX5 acquisition system (VilbertLourmat, Marne La Vallée, France). Densitometric quantification of proteins was performed with Bio1D software (VilbertLourmat, Marne La Vallée, France). Data were relativized with the Actin signal for each sample and also to an internal control. Each Western blot was performed and reproved several times, thus, cropped images are represented in Figs. 3 and 4.

Fig. 1. ROS levels in PMNs from control and type 2 diabetic populations. (A) Levels of total ROS measured in controls and type 2 diabetic patients with DCFH-DA fluorescence in arbitrary units; (B) Levels of mitochondrial ROS measured in control and type 2 diabetic populations with MitoSOX fluorescence in arbitrary units. Values were expressed as a percentage of an internal experimental control in both populations. *p < 0.05 vs Control group.
2.7. Quantification of total and mitochondrial ROS

Total and mitochondrial ROS were assessed with the fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and MitoSOX, respectively. Isolated PMNs were seeded in 48-well plates at a density of 150,000 cells/well and left to adhere in a 5% CO₂ incubator for 20 min. Cells were subsequently incubated with the specific nuclear stain Hoescht 33342 (4 μM) (Sigma-Aldrich, St. Louis, USA) and the fluorescent probes DCFH-DA (1 μM) or MitoSOX (5 μM) (Thermo Scientific, Rockford, USA) 30 min at 37 °C under gentle shaking. Cells were then washed twice with HBSS and were analysed with the static cytometry software “ScanR” (Olympus) which is coupled to an inverted microscope (IX81; Olympus). 12 fields per well were recorded and quantified. Measurements of fluorescence were referred as % of an external control for each sample.

2.8. Statistical analysis

SPSS was employed to perform statistical analyses. The data in Table 1 are expressed as mean ± standard deviation for parametric data, and median and 25th-75th percentiles for non-parametric data. The bar graphs in figures represent mean ± standard error. An unpaired Student’s t-test was performed to compare the control group and type 2 diabetic subjects, and adjustment by BMI was determined by means of a univariate general linear model. Correlations were calculated with Pearson’s correlation coefficient. Differences were considered significant when p < 0.05.

3. Results

3.1. Clinical and biochemical characteristics of the study subjects

We analysed 57 type 2 diabetic patients and compared them to 47 healthy control subjects with similar ages and sex distribution. Anthropometric and biochemical parameters were evaluated (Table 1). Type 2 diabetic patients showed higher BMI, fasting glucose, basal insulin, HOMA-IR index and glycated haemoglobin (HbA1c) compared to control subjects. Lipid metabolism parameters were also significantly enhanced compared to control volunteers, with higher VLDL and triglycerides, and lower HDL. Total cholesterol and LDL levels showed a slight decrease due to the treatment with statin (90% of patients). Furthermore, type 2 diabetic patients had a higher atherogenic index of plasma (AIP) and higher PCR levels.

Glucose, insulin, HOMA-IR, HbA1c, VLDL, HDL triglycerides and AIP maintained their statistical significance when data were adjusted by BMI, while differences in hsPCR and some lipid profile parameters – including total cholesterol and LDL - lost their statistical significance.

3.2. Total and mitochondrial ROS levels

Mitochondria can be severely damaged due to hyperglycaemia by releasing ROS. We measured total and mitochondrial ROS levels in
PMNs from type 2 diabetic patients and controls and found an evident enhancement of both total and mitochondrial ROS levels in type 2 diabetic subjects (p < 0.05) (Fig. 1) suggesting an oxidative stress condition.

### 3.3. PMN-endothelium interactions

Metabolic disorders are associated with increased levels of inflammatory markers. In the present study, we have observed that type 2 diabetic subjects had higher levels of TNFα and IL-6 levels, as well as increased NF-kB (p65) protein levels (Supplementary figure). This enhanced inflammatory background could be further confirmed analyzing the activation of the PMN cells and its interactions with the endothelial cells, using parallel-plate flow chamber experiments. This in vitro system reproduces physiological interactions between circulating cells and endothelium, and can quantify the frequency and stability of these interactions. Interestingly, PMNs from type 2 diabetic patients displayed lower rolling velocity through the endothelial monolayer (p < 0.001) (Fig. 2B), greater rolling number (p < 0.001) (Fig. 2A) and increased adhesion to the endothelial cells (p < 0.001) (Fig. 2C) with respect to those from the control population. This increase in PMN-endothelium interactions is reflected in the representative images obtained before and after the 5-min experimental period (Fig. 2D).

#### 3.4. LC3I, LC3II, Beclin-1 and p62 protein levels

We examined autophagy, a stress-activated cellular process that might be altered in type 2 diabetic population. PMNs were employed to analyse the protein expression of classical markers such as LC3, Beclin-1 and p62. Type 2 diabetic patients displayed an increased amount of LC3I (p < 0.05) (Fig. 3A and representative WB) and LC3II (p < 0.05) (Fig. 3B and representative WB), with a higher LC3II/LC3I ratio (p < 0.05) (Fig. 3C and representative WB). In addition, they showed enhanced Beclin-1 and decreased p62 protein levels (p < 0.05) compared to control subjects (Fig. 4), suggesting an increase in autophagy activation in the type 2 diabetic patient population.

#### 3.5. Correlations between ROS levels and autophagy markers

As we have mentioned before, excessive production of ROS can generate cellular stress that activates rescue pathways. In the present study, we have tried to highlight the relationship between autophagy and ROS production. We have evaluated correlations between the data obtained for ROS production and autophagic protein expression. We observed that total ROS levels correlated negatively with LC3II/I ratio in the control population (r = −0.714, p = 0.047) and positively with Beclin-1 levels in type 2 diabetic subjects (r = 0.911, p = 0.001). On the other hand, mitochondrial ROS was positively correlated with LC3II/LC3I ratio in the type 2 diabetic population (r = 0.416, p = 0.022). These data reinforce the hypothesis of a strong relation between autophagy and ROS production in type 2 diabetic patients.

#### 3.6. Correlation between autophagy proteins and PMN-endothelium interaction parameters

Once we had analysed the correlation between ROS and autophagy, we evaluated the correlation between PMN-endothelium interactions and autophagy markers. Interestingly, we observed that Beclin-1 protein levels were differentially correlated with PMN-endothelium interaction parameters (Fig. 5). In the control population, an increase of Beclin-1 was accompanied by a decrease in rolling number, a decrease in the number of adhered PMNs and a rise in the velocity of the rolling PMNs. In contrast, in the type 2 diabetic population, an increase in Beclin-1 was related to an increase in both rolling number and number of adhered PMNs and a trend towards a decrease in rolling velocity (Fig. 5). Additionally, a correlation between PMN adhesion, and LC3II
expression was observed in the type 2 diabetic population ($r = 0.386, p = 0.032$) while the rest of the parameters of PMN-endothelium interactions showed no correlation.

4. Discussion

In this cross-sectional study, we have shown that diabetic patients display enhanced PMN-endothelium interactions, ROS production, autophagy-related protein expression as well as proinflammatory cytokines TNFα and IL-6, and NF-κB activation. Moreover, we demonstrate a differential correlation between PMN-endothelium interactions and Beclin-1 expression in control subjects and type 2 diabetic patients.

With regard to the inflammatory basis of type 2 diabetic, high levels of circulating glucose and lipids increase the expression of adhesion molecules in both the endothelium and PMNs [13,14,16,34,35]. This has been corroborated by several observational studies of type 2 diabetic patients [14,35], but also in interventional studies in patients fitted with hyperglycaemic clamps and undergoing glucose challenge, in whom inflammatory cytokines increase after glucose input [36]. Hyperlipidaemia, another hallmark of type 2 diabetic, is also related to PMN function [17]; an increase in PMN ROS production has been described in hyperlipidaemic and hypertensive patients with respect to healthy controls, which can lead to the atherosclerotic complications [37]. Furthermore, it has been observed that PMN function is altered in patients with diabetic retinopathy; for example, in the case of enhanced extravasation [38]. In this sense, the close relationship between inflammation, ROS production and increase of PMN-endothelium interactions is widely recognised [18,35–37,39–42]. All these studies have concluded that the chronic inflammation characteristic of diabetes and hyperglycaemia promotes the production of inflammatory chemokines and ROS, which in turn alters the functions of the endothelium and PMNs, thus increasing their interaction. Although ROS have an important function as signalling molecules in physiologic processes, their overproduction causes damage of cellular components, which activates the inflammatory response of cells. In the present study, we have observed higher levels of total and mitochondrial ROS in the type 2 diabetic population compared to healthy controls. The relation between type 2 diabetes and ROS is well documented in the literature [8–10,18,28], and has even been directly related to the regulation of autophagy [19,28]. Interestingly, we have observed a differential pattern in the correlations found between total ROS production and LC3II/I ratio, suggesting a synergistic effect of ROS and autophagy in type 2 diabetic patients. These results suggest that autophagy is one of the mechanisms that mediate the link between ROS production and the increase of PMN-endothelium interactions in type 2 diabetes versus control conditions.

Several studies point to alterations in autophagy signalling in type 2 diabetic patients [21–23,25,26]. In the present study, type 2 diabetic subjects displayed enhanced protein markers of autophagy, such as LC3I, LC3II, LC3II/LC3I ratio and Beclin-1, which were related to a reduction in p62 protein levels. These results suggest an activation of autophagy in type 2 diabetic patients compared to healthy controls. Activation or alteration of autophagy has been reported in different situations of hyperlipidaemia and hyperglycaemia. For example, previous research has shown mitochondrial dysfunction and altered autophagy in adipocytes from obese type 2 diabetic patients [23], as well as in Goto-Kazikazi (type 2 diabetic) rats [24]. Furthermore, alterations in autophagic parameters in podocytes and leukocytes have been related to diabetic comorbidities such as diabetic nephropathy [26,27,43], cardiac complications [30,31] and neuropathy [31]. Interestingly, in diabetic Wistar rats, insulin exerted different effects on autophagy depending on the origin of the leukocytes [44]. In fact,
diabetic M1 bone marrow-derived macrophages (BMM) had their LC3 vesicle-bound content diminished while M2 BMM had enhanced LC3 levels, and insulin treatment failed to rescue autophagy to control levels. In endothelial cells, pro-inflammatory cytokines have been shown to induce autophagy, which enhances the production of adhesion molecules [45]. In other studies, autophagy has proved to be a crucial protective mechanism in beta cells [28,46].

Our study relates an increase in autophagy-related proteins with an increase of PMN-endothelium interactions in type 2 diabetic patients as well as an increase in NF-κB expression. We also show that Beclin-1 protein levels correlate differentially with PMN-endothelium interaction parameters depending on the health status of the subject. While an increase in Beclin-1 was related to a reduction in PMN-endothelium interactions in control subjects, it was associated with an increase in PMN-endothelium interactions in type 2 diabetic patients. This could mean that the increase in PMN-endothelium interactions is strongly influenced by Beclin-1, and that changes in its expression imply different signalling cascades depending on the status of the subject. Furthermore, we have observed a positive correlation between PMNs adhesion and LC3II in type 2 diabetic patients. Beclin-1 is implicated in different biological processes, including cytokinesis, immunity, adaptation to stress, development, ageing, tumorigenesis and cell death [47]. The effects described in the present study may be associated with the ability of Beclin-1 to exert several functions within the metabolism of the cell; for example, it interacts with BCL2 to form the BCL2-Beclin-1 complex, which is regulated by AMPK, provoking the dissociation of the complex and thus preventing apoptosis [29]. Another possible reason why only this protein is differentially regulated is that Beclin-1 interacts with VMP-1 upstream from all the other regulators of autophagy [48]; thus, variations in regulation could be due to differences at this level of the autophagy signalling.

5. Conclusions

In summary, this study demonstrates enhanced PMN-endothelium interactions, ROS production and autophagy activation in type 2 diabetic patients. Moreover, we show a differential behaviour of autophagy in control and type 2 diabetic subjects regarding ROS levels and PMN endothelium-interactions. These data endorse a connection between these three key mechanisms in type 2 diabetes, and highlights the changes in Beclin-1 as a possible linking mechanism between ROS production and PMN-endothelium interactions. Furthermore, we show that the pattern of autophagy markers differs depending on the presence or not of type 2 diabetes, perhaps pointing to metabolic pathways that need to be elucidated by future research.

Author contributions

Conceptualization: V.M.V, A.N., G.M. and R.M.; Methodology, A.M.M, A-J. Z, L-D.S, C.F and D-P.P; Resources: A.J, C. M., V.M.V and R.M; Data curation: A.M.M, F.I, V.M.V.; Writing-Original Draft: A.M.M and C.F; Writing-Review and Editing: V.M.V, L-D.S, A.N. and R.M.; Visualization: A.M.M and F.I; Supervision: C.M., A.N., V.M.V and R.M; Project administration: V.M.V and R.M.; Funding acquisition: V.M.V and R.M.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rexd.2020.101563.

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