Published in final edited form as:

Exp Clin Endocrinol Diabetes. 2012 April ; 120(4): 248–251. doi:10.1055/s-0031-1285833.

Platelet Mitochondrial Dysfunction is Evident in Type 2 Diabetes in Association with Modifications of Mitochondrial Anti-Oxidant Stress Proteins

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Abstract

Objective: Mitochondrial dysfunction and oxidative stress in insulin responsive tissues is implicated in the pathogenesis of type 2 diabetes. Whether these perturbations extend to other tissues and contribute to their pathophysiology is less well established. The objective of this study was to investigate platelet mitochondria to evaluate whether type 2 diabetes associated mitochondrial dysfunction is evident in circulating cells.

Method: A pilot study of mitochondrial respiratory function and proteomic changes comparing platelets extracted from insulin sensitive ($n=8$) and type 2 diabetic subjects ($n=7$).

Results: *In-situ* platelet mitochondria show diminished oxygen consumption and lower oxygen-dependent ATP synthesis in diabetic *versus* control subjects. Mass spectrometric identification and confirmatory immunoblot analysis identifies induction of the mitochondrial anti-oxidant enzymes superoxide dismutase 2 and thioredoxin-dependent peroxide reductase 3 in platelets of diabetic subjects. As oxidative stress upregulates anti-oxidant enzymes we assessed mitochondrial protein carbonylation as an index of oxidative-stress. Platelets of diabetic subjects exhibit significantly increased protein carbonylation compared to controls.

Conclusions: As platelets are anuclear fragments of megakaryocytes, our data suggest that the bone marrow compartment in type 2 diabetic subjects is exposed to increased mitochondrial oxidative stress with upregulation of nuclear-encoded antioxidant mitochondrial enzymes. This ‘stress-signature’ in platelets of diabetic subjects is associated with a diminution of their mitochondrial contribution to energy production and support that mitochondrial perturbations in type 2 diabetes extends beyond the classical insulin responsive tissues. Platelets, as “accessible human tissue”, may be useful to measure the mitochondrial modulatory effects of emerging anti-diabetic therapeutics.

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[ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00833846) Identifier: NCT00833846

Conflict of interest The authors declare that there is no conflict of interest associated with this manuscript.

Keywords

Mitochondria; platelets; reactive oxygen species; respiration; diabetes

Introduction

Type 2 diabetes mellitus (T2DM) is an evolving global pandemic with deleterious health consequences. Although the pathophysiology associated with the development of T2DM is heterogeneous, mitochondrial perturbations are shown to contribute to disease progression in the pancreas and in insulin responsive metabolic tissues [1,2]. Whether mitochondrial dysfunction contributes to T2DM pathophysiology in other tissues is unclear.

Although platelet dysfunction is evident in diabetes [3], whether mitochondrial perturbations contribute to this pathophysiology is similarly unknown. Furthermore, as platelets are anuclear viable fragments of bone marrow megakaryocytes the study of platelet mitochondria should provide a unique 'window' into the bone marrow. This concept is particularly pertinent to mitochondria as intergenomic regulation between the nucleus and mitochondria is required to maintain mitochondrial homeostasis [4]. Hence, the investigation of functional and protein changes in platelet mitochondria probably reflect, in large part, the 'historical' nuclear-mitochondrial regulation within the intact megakaryocytes.

This pilot study evaluated mitochondrial respiration and the 'oxidative-stress profile' in platelets comparing healthy insulin sensitive to T2DM subjects. We hypothesized that platelets from subjects with T2DM would exhibit altered mitochondrial characteristics.

Research Design and Methods

All subjects were enrolled after informed consent, approved by the NHLBI Internal Review Board. Eighteen subjects were enrolled into this pilot study at the NIH Clinical Research Center - [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00833846) Identifier: NCT00833846. Three subjects were excluded from analysis as their glucose tolerance tests showed an intermediate phenotype of impaired glucose tolerance. Exclusion criteria included the use of insulin or thiazolidinediones due to their known mitochondrial effects [5]. Additional exclusion criteria included pregnancy, concurrent acute/chronic illnesses or age greater than 60 years.

Fresh blood was collected and subjected to centrifugation, after which the upper 2/3 of the platelet-rich plasma was separated and re-centrifuged. The platelet pellet was washed three-times with 0.9% NaCl. The purified platelets were then resuspended and platelet respiration was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse BioScience) [6]. Platelets were adhered to 24-well respiration plates using Cell-Tak (BD Biosciences) and uniform plating was confirmed by direct microscopic visualization. As previously described [6], maximal oxidative capacity was calculated as the difference in oxygen consumption between dinitrophenol (200 μ M) administration and the basal oxygen consumption rate (OCR). ATP synthesis was calculated as the difference in OCR between basal and oligomycin (1 μ M) mediated inhibition of the ATP synthase. The mitochondrial proton leak was calculated as the OCR difference between administration of oligomycin and

rotenone (1 μ M) plus antimycin A (1 μ M). The cytosolic utilization is represented as the residual oxygen consumption following rotenone/antimycin A administration. The platelet respiratory profile was repeated in triplicate and data normalized to protein content.

Platelet isolation for protein analysis was performed similarly to that described for respiratory studies. Platelet purity was confirmed using CD61 positive immunofluorescence labeling antibody (BD Biosciences) by flow cytometry (Data not shown). The isolation of the crude mitochondrial fraction was performed using differential centrifugation as described [7].

The crude mitochondrial pellet was suspended in lysis buffer containing 15 mM Tris-HCl pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS. Two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometric (MS) analysis was performed as previously described [8]. Twenty μ g of crude mitochondrial protein from each subject was then employed for Western Blot analysis. Antibodies directed against manganese superoxide dismutase (SOD2; Santa Cruz Biotechnology), thioredoxin-dependent peroxide reductase 3 (PRDX3; Abcam) and β -actin (Sigma) were employed. Protein carbonylation (PC) modification on protein samples was determined using a PC test kit (BioCell Corp). The results expressed as total ELISA absorption for each participant.

Experimental data was analyzed by unpaired 2-tailed *t*-Test and the subject gender distribution using the Fisher's exact test. Results are expressed as mean $\pm$ SEM with differences significant for *p*-value <0.05.

Results

Participants were matched for age, gender, body mass index and lipid profiles. Significant elevations in fasting glucose levels (6.4 ± 0.2 versus 5 ± 0.2 mmol/L), glucose levels after the 2-hour oral glucose tolerance test (12.1 ± 1.8 versus 5.3 ± 0.5 mmol/L) and HbA1c levels (7.1 ± 0.6 versus $5.6\pm0.1\%$) were evident in T2DM participants versus controls.

Representative tracings of oxygen consumption in a paired control and diabetic subject at baseline and in response to electron transfer chain site-specific inhibitors are shown in Fig. 1. The composite data of all subjects is depicted in Fig. 1C. The results show that: i) basal mitochondrial oxygen consumption is reduced by $\approx 40\%$ in diabetic *versus* control subjects ($p=0.02$); ii) the relative contribution of oxygen consumption for ATP production is similarly reduced in the diabetic subjects ($p=0.03$) and iii) interestingly, the diabetic platelet mitochondria exhibited an $\approx 50\%$ reduction in proton leak ($p=0.01$). The relative utilization of cytosolic oxygen is similar in platelets from control and diabetic subjects.

Using fluorescent labeled 2D-DIGE, multiple differences in mitochondrial protein expression was evident comparing control to T2DM subjects (data not shown). In keeping with the known association between redox stress and mitochondrial dysfunction in diabetes [9], an intriguing finding was the induction of protein levels of two mitochondrial antioxidant enzymes, SOD2 and PRDX3 in diabetic subjects as shown by 2D-DIGE (Fig. 2A) and additional post-translational modifications of SOD2 are similarly evident comparing control to T2DM subjects (Fig. 2B).

To confirm the relative abundance of these anti-oxidant proteins identified, immunoblot analyses using mitochondrial protein was performed. There were a greater than 2-fold induction in the levels of SOD2 ($p=0.01$) and PRDX3 ($p=0.001$) in T2DM compared to controls (Fig. 2C and 2D). To assess whether these reflect changes in association with increased redox stress, we measured protein carbonyl content as a marker of oxidative modification of proteins. T2DM subjects had ≈ 1.6 times higher carbonyl levels than controls ($p=0.0002$, Fig. 2E).

Conclusion

The data from this pilot study shows blunted mitochondrial respiration with upregulation of mitochondrial anti-oxidant proteins and concomitant evidence of increased oxidative stress. Additionally, post-translational modifications evident in the two-dimensional gel analysis of SOD2 similarly may reflect alterations in the antioxidant regulatory program [10]. These data suggest more widespread disruption in mitochondrial function beyond the classical insulin responsive metabolic organs and support the oxidative stress hypothesis in the pathogenesis of T2DM [9].

The attenuation of mitochondrial oxygen consumption in insulin responsive tissues and in the pancreas during diabetes is well established and this is often associated with the downregulation of oxidative phosphorylation proteins [11]. Although we show that this 'respiratory profile' extends to platelets we did not see changes in mitochondrial oxidative phosphorylation protein levels by 2-D-DIGE (data not shown). This may represent a sensitivity issue due to the low number of mitochondria in platelet fragments or could reflect a change in respiratory function due to post-translational modifications not identified by 2D-DIGE. An intriguing respiratory observation in this study is the attenuation of proton leak in the diabetic subjects' platelet mitochondria. This has been previously shown in skeletal muscle mitochondria from older insulin resistant *versus* young insulin sensitive rats [12]. Whether a reduction in inner mitochondrial membrane proteins in diabetes changes the membrane physicochemical properties to attenuate this leak is an intriguing concept that would need to be validated. Additionally, whether a smaller proton leak exacerbates mitochondrial ROS generation is similarly plausible [13].

Mitochondrial anti-oxidant enzymes are nuclear-encoded and the integration of an intact nuclear-mitochondrial regulatory axis would be necessary to upregulate mitochondrial protective programming. Redox-stress itself initiates the upregulation of genes encoding mitochondrial anti-oxidant proteins to facilitate mitochondrial attenuation of subsequent oxidative damage [14]. In this study we show upregulation of anti-oxidant mitochondrial defenses against increased levels of oxidative stress in platelet mitochondria in T2DM subjects compared to controls. Specifically we note that T2DM subjects have increased levels of mitochondrial SOD2 and PRDX3. Although, we do not have data to dissect out the temporal course of events, the absence of the nuclear-mitochondrial intergenomic system in platelets supports that oxidative stress evoked upregulation of mitochondrial antioxidant enzymes preceded anucleation during platelet release into the vasculature. The increased carbonylation modifications confirm redox-stress although we cannot exclude extra-mitochondrial sources. Interestingly, oxidative stress is hypothesized to contribute to end-

organ damage in diabetes and to mitochondria damage during the development of diabetes [9]. A unifying hypothesis of our findings would be that the oxidative stress evident in the platelets of the diabetic subjects contributes to subsequent mitochondrial respiratory perturbations.

In conclusion, the findings in this pilot study generate a hypothesis suggesting that T2DM is associated with an increased redox-stress milieu within the bone marrow megakaryocytes which initiates oxidative stress-damage with the concomitant induction of mitochondrial anti-oxidant regulatory programs. As a possible consequence platelet mitochondrial respiratory function is attenuated which, may in turn, exacerbate the generation of mitochondrial reactive oxygen species. Whether megakaryocyte redox-stress and associated platelet mitochondrial perturbations contribute to diabetic platelet dysfunction is an intriguing hypothesis that would need to be explored. Furthermore, the evaluation of mitochondrial platelet respiratory function may be a useful peripheral blood ‘signature’ to measure reversibility of diabetic sequelae in response to emerging ‘mitochondrial-modulatory’ diabetic therapies [5,15].

Acknowledgements

This research is funded by the Division of Intramural Research of the NHLBI. CA and RJH were funded by an NIH-Pfizer Clinical Research Training Fellowship. We thank the NHLBI Flow Cytometry Core Facility for assistance in the confirmation of platelet purity and Stephanie A French, Darci Phillips and Dr. Marjan Gucek of the NHLBI for helpful discussion regarding mitochondrial protein isolation and proteomic analysis.

Abbreviations:

T2DM	Type 2 diabetes mellitus
SOD2	Superoxide dismutase 2
PRDX3	Thioredoxin-dependent peroxide reductase 3
OCR	Oxygen consumption rate
2D-DIGE	Two-dimensional differential in-gel electrophoresis
MS	Mass spectroscopy

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Fig. 1.

Mitochondrial respiration is perturbed in diabetic subject platelets. A. Representative tracing of basal oxygen consumption and maximal oxygen consumption induced by the uncoupler dinitrophenol comparing platelets from a control and diabetic subject. B. Representative tracing of oxygen consumption in response to inhibition of the F₁/F₀ ATP synthase with oligomycin, and in response to the subsequent inhibition of electron transfer chain activity with the inhibitors rotenone and antimycin A. The reduction in oxygen consumption following oligomycin represents oxygen utilization for ATP production. The difference in oxygen consumption between the two pharmacologic interventions represents the proton leak, and the residual oxygen utilization represents cytosolic oxygen consumption. C. Histograms showing the absolute differences in oxygen consumption comparing groups normalized to total protein content. The asterisk represents a $p < 0.05$ versus respective control subjects. Abbreviation: Mt - Mitochondria

Fig. 2.

Protein analyses showing altered reactive oxygen species regulatory proteins and consequences in diabetic subject platelets. **A.** Magnification of 2D-DIGE with Progenesis SameSpot software after mass spectrometry which located and analyzed protein spots assigning statistical confidence and difference in anti-oxidant protein levels. **B.** Post-translation modification of SOD2 on 2D-DIGE that would be compatible with change in the phosphorylation status between control and diabetic samples. **C.** Immunoblot confirmation of expression of anti-oxidant proteins. β -actin shows protein loading control. **D.** Histogram shows protein expression levels from all subjects. **E.** Relative carbonylation levels from platelet mitochondrial proteins from each subject. Asterisks highlight $p < 0.05$ versus relative control subjects.