

Session 10: Mitochondria and Degenerative Diseases.

II. Patients and Human Cell Lines



10-01. Oxidative capacity, lipotoxicity and mitochondrial function in type 2 diabetes mellitus.

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Recent evidence points towards decreased oxidative capacity and mitochondrial aberrations as a major contributor to the development of insulin resistance and type 2 diabetes mellitus.

Type 2 diabetes mellitus is accompanied by accumulation of fatty acids in non-adipose tissues. In metabolically active tissues, such as skeletal muscle, fatty acids are prone to so-called oxidative damage: in addition to producing energy, mitochondria are also a major source of reactive oxygen species, and the latter can lead to lipid peroxidation. Especially the mitochondrial matrix, which contains DNA, RNA and numerous enzymes necessary for substrate oxidation, is sensitive to peroxides-induced oxidative damage and needs to be protected against the formation and accumulation of lipids and lipid peroxides.

One of the proteins thought to be involved in the protection of lipid-induced mitochondrial damage is the mitochondrial uncoupling protein 3 (UCP3). We and others have observed that the regulation pattern of UCP3 closely parallels changes in fatty acid metabolism: UCP3 is up regulated under conditions of an abundant fatty acid supply to the mitochondria and is down regulated when fatty acid oxidation is increased or plasma FFA levels are lowered. Under conditions where fatty acid delivery mismatched oxidative capacity, the surplus of fatty acids may reach the mitochondrial matrix and be responsible for the formation of lipid peroxides, leading to mitochondrial damage. Because UCP3 is able to export fatty acid anions across the mitochondrial membrane away from the matrix and is activated by 4-hydroxynonenal, a byproduct in lipid peroxidation [1], we have postulated the hypothesis that UCP3 is an exporter of fatty acid anions with the function to protect the mitochondrial matrix against lipid accumulation and lipid peroxidation-induced mitochondrial damage [2,3].

Interestingly, type 2 diabetic patients are characterized by a 50 % reduction in UCP3 protein content [4], and are also characterized by smaller and damaged mitochondria [5] and increased levels of lipid peroxidation [6]. Thiazolidinedione treatment and life style interventions, known to improve muscular insulin sensitivity, restore UCP3 levels in type 2 diabetic patients (unpublished observations), although the effect on mitochondrial function and lipid peroxidation is yet unknown. In addition, UCP3 protein content is already reduced in the pre-diabetic state of impaired glucose tolerance (IGT). These observations suggest that low levels of UCP3 may ultimately lead to increased levels of lipid peroxidation and lipid-induced mitochondrial damage [2], which can be of relevance in the etiology of diabetes.

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10-02. Dissipating excess energy in the liver is a potential treatment strategy for the metabolic syndrome.

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Much data indicates that lowering of plasma triglyceride levels by hypolipidemic agents is caused by a shift in the liver metabolism, toward peroxisome proliferator activated receptor (PPAR) α regulated fatty acid catabolism in mitochondria. Feeding rats with tetradecylthioacetic acid (TTA) leads to hypolipidemia possibly by increased channeling of fatty acids to mitochondrial fatty acid oxidation at the expense of triacylglyceride synthesis [1]. Our results suggest that a TTA-induced increase in hepatic fatty acid oxidation and ketogenesis drains fatty acids from blood and extrahepatic tissues and that this contributes significantly to the beneficial effects of TTA on fat mass accumulation and peripheral insulin sensitivity [2]. These effects are associated with altered energy state parameters of the liver at the tissue-, cellular, and mitochondrial level [3]. The hepatic phosphate potential, energy charge, and respiratory control coefficients were lowered, while rates of oxygen uptake and oxidation of pyridine nucleotide redox pairs. This is compatible with uncoupling of mitochondria due to increased proton conductance of the inner membrane. Thus, uncoupling activity of TTA was confirmed by measuring the proton electrochemical potential. The data suggested that TTA influences expression and/or activity of electrogenic ion transport systems in the mitochondrial membrane. A candidate protein is uncoupling protein 2 (UCP2) whose mRNA expression was induced after TTA treatment in rats as well as in wild type and PPAR α -deficient mice. TTA also activates the other PPARs (e.g. PPAR δ), and this may compensate for the deficiency of PPAR α .

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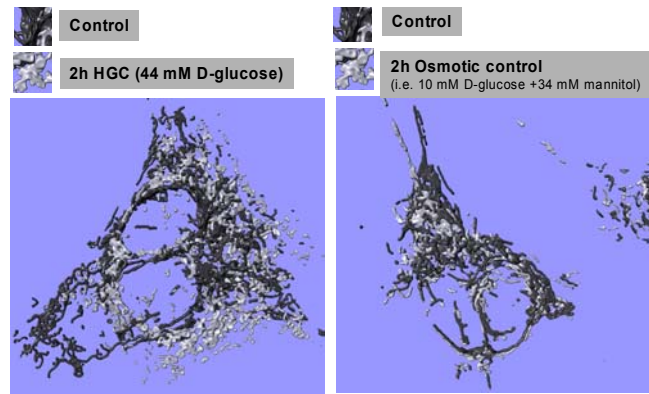
10-03. Endothelial mitochondria exhibit an initial target of hyperglycemia.

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Diabetes mellitus is associated with vascular disorders that are thought to be causally linked to endothelial dysfunction initiated by elevated high D-glucose, glycated (lipo-) proteins and advanced glycation end products [1]. However, the molecular mechanisms responsible for the development of endothelial malfunction in human diabetes is only

fractionally clarified. Recent findings suggest that endothelial mitochondria may represent a causal link between high blood glucose levels and the appearance of vascular diseases [2]. We showed that under hyperglycaemic conditions (HGC) endothelial mitochondrial shape changed from a mainly tubular, highly interconnected network towards multiple, isolated singular structures within several hours. This striking and fast alteration in



mitochondrial architecture was accompanied by enhanced mitochondrial free radical formation and a prolonged mitochondrial Ca^{2+} accumulation upon cell stimulation with an IP_3 generating agonist. Notably, the changes in mitochondrial structure by HGC could not be correlated with altered cytosolic Ca^{2+} signaling, while cytosolic Ca^{2+} signaling under HGC was normalized with antimycin A, an inhibitor of the respiratory chain. These data suggest that although mitochondrial structure greatly changed during HGC, alterations in cytosolic Ca^{2+} signalling are more likely due to the enhanced energy status/metabolism of the mitochondria. As endothelial Ca^{2+} signalling represents a key regulator for many endothelial vascular functions [3], mitochondria related alterations of endothelial Ca^{2+} homeostasis may support the development of vascular diseases in human diabetes. In addition, the balance between the metabolic stimulation of mitochondria and their free radical production during HGC may represent the turning point of either cell adaptation response or the initiation of fatal pathways is decided in diabetes.

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10-04. Complex I and physiopathology of mitochondrial oxygen metabolism.

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Conditions leading to arrest of cell replication are associated with decline of the activity of complex I of the respiratory chain and increased level of ROS in a variety of cell cultures *in vivo*. Short term (60 min) activation of the cAMP cascade by cholera toxin results in marked enhancement of the rotenone sensitive NADH ubiquinone oxidoreductase activity of complex I, a rate limiting step of the respiratory chain, and disappearance of ROS from the cells. Experiments on fibroblast cultures from patients with pathological mutations of nuclear genes of complex I were also carried out.

The results showed that mutations in the NDUFS4 gene (18 kDa subunit), causing complete suppression of complex I NADH ubiquinone oxidoreductase activity, prevented ROS formation in the fibroblast cultures. Mutation in the NDUFS1 gene (75 kDa, FeS protein) causing severe depression of the NADH ubiquinone oxidoreductase activity of

complex I, was associated with production ROS, reversed by activation of the cAMP cascade.



10-05. Two components in pathogenic mechanism of mitochondrial ATPase deficiency: Energy deprivation and ROS production.

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Isolated defects of mitochondrial ATP-synthase (ATPase) due to diminished biosynthesis of the enzyme represent a new class of severe mitochondrial diseases, characterized by selective, 70-90 % decrease in cellular content of ATPase [1,2]. The primary cause is a mutation in nuclear gene(s) affecting the initial stage of enzyme biogenesis - assembly of the F₁ catalytic part of the enzyme [1,3]. Our studies of cellular respiration and ATP synthesis in fibroblasts from several unrelated patients with ATPase deficiency showed that the content of the enzyme is insufficient, or without any reserve capacity to maintain basal mitochondrial energy provision. The cells exhibited altered discharge of mitochondrial membrane potential $\Delta\psi_m$ (cytofluorometry with TMRM), which was increased at state 4 and especially at state 3-ADP. ATPase-deficient cells further showed several-fold increase in mitochondrial ROS production analyzed by CM-H₂DCFDA fluorescence (using fluorometry or confocal microscopy) that was fully abolished by uncoupler FCCP. Activated ROS production was associated with a variable increase of mitochondrial superoxide dismutase (MnSOD), and small changes in cellular content of glutathione, but inhibition of glutathione synthase with buthionine sulphoximide (BSO) significantly activated ROS production. Aurovertin and oligomycin titration studies and replacement of glucose by galactose in culture medium demonstrated a marked decrease in viability of ATPase-deficient cells when they depend on mitochondrial oxidative metabolism rather than on glycolysis.

Our studies demonstrate that altered discharge and high levels of $\Delta\psi_m$ activate ROS production in ATPase-deficient cells. The resulting oxidative stress can be counteracted by activity of MnSOD and glutathione levels, however, activated mitochondrial ROS production is lethal in patient cells.

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10-06. Trolox restores aberrant mitochondrial morphology and improves the assembly and activity of complex I in patients with an isolated deficiency.

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Mutations in nuclear-encoded subunits of NADH:ubiquinone oxidoreductase (complex I) cause complex I deficiency (OMIM 252010), which is associated with a broad spectrum

of clinical presentations. Currently, the cellular consequences of these disorders remain elusive and rational treatment strategies are lacking. We recently demonstrated that mitochondrial complexity is increased upon inhibition of complex I [1] and linearly correlated with residual complex I activity in patient cells [2]. This finding suggests that an increase in mitochondrial complexity might reduce complex I deficiency. Here, we investigated this idea in a collection of 13 patients carrying mutations in nuclear-encoded subunits of complex I (NDUFV1, NDUFS1, NDUFS2, NDUFS4, NDUFS7 or NDUFS8). Cluster analysis highlighted two classes of patients within this cohort displaying a relatively low (class I: 33 ± 3 % of lowest control) or high (class II: 65 ± 3 %) residual complex I activity. Mitochondrial complexity was significantly lower in class I (68 ± 4 % of control) than in class II (115 ± 3 %) and not related to the activity of complex III and IV. Under resting conditions, patient cells displayed a normal cytosolic calcium homeostasis but an increased rate of ROS generation. The latter was fully normalized by treatment with the vitaminic antioxidant Trolox. Both in vehicle and Trolox-treated patient cells complex I activity and assembly were linearly ($y=x$) correlated. This suggests that complex I, once fully assembled, displays normal catalytic activity. In class I, treatment with Trolox fully restored mitochondrial complexity and enhanced the expression and activity of complex I by 200 %. In class II, Trolox did not affect mitochondrial complexity and increased complex I expression and activity by 50 %. Such restoration was induced by treatment with the mitochondria-targeted antioxidant mitoquinone (MitoQ). We conclude that nuclear-encoded mutations in complex I affect the assembly of the complex but, once assembled, not its catalytic activity. The reduced expression of complex I results in enhanced levels of ROS that contribute to the cellular phenotype by affecting mitochondrial complexity and complex I expression/activity. The effectiveness of Trolox might indicate a potential beneficial role in the treatment of patients.

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10-07. Cytochrome c oxidase assembly defects.

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Mutations in SURF1, the cytochrome c oxidase (COX)-specific assembly factor, are the primary cause of Leigh syndrome associated with COX deficiency (LS^{COX}), one of the most frequent mitochondrial disorders with fatal consequences [1]. The Surf1p absence in LS^{COX} patient fibroblasts leads to severe reduction of the COX holoenzyme content and accumulation of COX assembly intermediates. Our previous studies showed that such deficiency manifests in decreased stability of the enzyme, impaired proton-pumping ability of COX, and decreased affinity of the enzyme for oxygen [2,3]. We suggested that these functional changes were due to altered properties of the COX assembly intermediates [2,3].

Recently, we extended our studies in the SURF1 knock-out mice model of LS^{COX} [4]. In mitochondria isolated from tissues of homozygous SURF1 -/- mice we observed similar functional alterations as in patient fibroblasts, e.g. decreased affinity of COX for oxygen and release of the enzyme downregulation. These changes, however, were not accompanied by the accumulation of COX assembly intermediates, only severe reduction of the holoenzyme content was found. Using high-resolution BN-PAGE and 2D-PAGE

techniques and the SURF1 knock-out mice model, we aim to further resolve whether the functional manifestations of the COX assembly defect are due to (i) accumulation of intermediates with altered functional properties; (ii) changes in subunit composition of the COX holoenzyme that has not been so far recognized due to low resolution of BN-PAGE, i. e. absence of one or several small nuclear-encoded subunits; and (iii) decreased content of otherwise structurally and functionally normal COX.

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10-08. Mitochondrial alterations during apoptosis induced by a thia fatty acid in leukemia cells.

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Some fatty acids and derivatives are known to induce cell death in cancer cells. Mitochondria may have important roles in the death process. The modified fatty acid tetradecylthioacetic acid (TTA) has in multiple studies been demonstrated to have anticancer properties [1, 2]. The objective in this study was to investigate the underlying mechanism behind TTA-induced apoptosis in IPC-81 leukemia cells. The first signs of apoptotic morphology were seen after 12 hours. Overexpression of the antiapoptotic protein Bcl-2 partially blocked the induction of apoptosis. Mitochondrial release of cytochrome c could be detected less than one hour after TTA administration. This was equally evident in Bcl-2 overexpressing cells, and in cells overexpressing the inducible cAMP early repressor (ICER), which are resistant to cAMP induced apoptosis. Intriguingly, caspase-3 activation in TTA-treated cells occurred in parallel with the appearance of apoptotic morphology, i.e. several hours after the release of cytochrome c. The broad-spectrum caspase inhibitor zVAD-fmk did not block induction of apoptosis; however, it appeared to suppress nuclear fragmentation. In addition to early cytochrome c release, the importance of mitochondria in TTA-induced apoptosis was further substantiated by depolarisation of the mitochondrial membrane potential ($\Delta\psi$). Finally, we observed an early depletion of mitochondrial glutathione and a reduction in the percentage of non-oxidized glutathione (GSH). This supports the understanding that the mitochondrial level of glutathione may be of importance early in the apoptotic process. It is concluded that TTA, as a modified fatty acid, seems to cause apoptosis possibly through a direct effect on mitochondria [3]. This type of compounds might therefore be candidates for new mitochondrion-targeting drugs that overcome apoptosis resistance.

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10-09. Mitochondrial derangement induced by PPAR-ligands in HEP-G2 cell lines. Pathophysiological and pharmacotoxicological implications.

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Thiazolidinediones, fibrate derivative drugs, have emerged as a treatment option in the pharmacotherapy of type 2 diabetes mellitus. Some drugs belonging to this class, however, are characterized by an intriguing pharmacotoxicological profile. The first thiazolidinedione drug, troglitazone, was rapidly withdrawn from the market because of liver toxicity. Moreover, also the 'newer' glitazones have been reported to provoke a dangerous hepatotoxicity [1]. Recently the American Heart Association and the American Diabetes Association contraindicated thiazolidinediones in patients at risk for heart failure. Our recent studies showed that some fibrates (clofibric acid, gemfibrozil and bezafibrate) and a thiazolidinedione derivative, ciglitazone, are all capable of inhibiting NADH cytochrome c reductase activity of the mitochondrial electron transfer chain in human myeloid and human muscular cell lines [1].

All these drugs are considered well-known PPARs (Peroxisome Proliferator Activated Receptors) ligands and their pharmacological activities are classically reported as consequences of their capacity to bind and activate these particular class of nuclear receptors [2]. In particular fibrates, interacting with subtype alpha of PPARs, seem mainly to control the transcription of genes encoding key enzymes related to fatty acid catabolism, and thereby are used as hypolipidemic-hypotrygliceridemic drugs while fibrate derivatives thiazolidinediones, interacting mainly with PPAR subtype gamma, seem to act as insulin sensitizers and because of this they have been introduced in therapeutic protocols of type 2 diabetes mellitus.

At present, clinical data about a moderate activity of glitazones as hypoglycemic agents together with unwanted noxious effects (episodes of acute liver insufficiency and/or heart failure) seem to confirm that the pharmacotoxicological profiles of these PPAR ligands cannot be fully ascribed only to receptor activation. Mitochondria may represent secondary but still important targets of these drugs and this aspect can not be underestimated in all its implications anymore. In fact, these organelles, characterized by a typical physical and chemical matrix *milieu* ($\Delta\psi_m$ and ΔpH), may accumulate some amphipatic xenobiotics like fibrates and glitazones at concentrations much higher than those measured in blood [3].

PPAR-ligands impairing NADH oxidation at the level of Complex I of the mitochondrial respiratory chain induce cells to respond to the energy demand by a series of metabolic shortcuts (i.e., stimulation of glycolysis and/or β -oxidation) which could explain some aspects of their pharmacological and toxicological profiles. In our opinion, a right emphasis on the interaction between synthetic PPAR-ligands and mitochondria is fundamental in order to reduce, in genetically or pathologically predisposed patients, further dangerous side effects.

Considering the role of liver in the pathophysiology of type 2 diabetes and its demonstrated vulnerability to the toxic effects of glitazones, the scope of this investigation was the characterization of the metabolic and toxicological effects of different PPAR-ligands in a human liver cell line.

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10-10. Cancer cell growth can be affected by different mitochondrial DNAs from subjects without cancer.

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The role of mitochondrial dysfunction in carcinogenesis has been shown in previous studies [1,2]. Most of the previous studies used pathogenic mutant mitochondrial DNA (mtDNA) to examine the effect of mtDNA on carcinogenesis. In this study, we made trans-mitochondrial hybrids (cybrids) containing 143B osteosarcoma cell nucleus and mtDNAs from platelets of normal subjects ($n = 24$) who did not have cancer at the time of study. Since mtDNA haplogroup D has been reported to be associated with longevity in Japanese [3], we included haplogroup D with other common Asian-specific haplogroups. The mtDNAs used in this study were haplogroup A, B, D, and F ($n = 6$ in each haplogroup). We compared cell counts at 72 h after seeding same number of cells and found that the cybrids harboring haplogroup D mtDNAs grew at the lowest rate ($P < 0.05$ compared to 143B rho+ parent cell). When comparing individual clones, we observed that cybrid A1 showed the highest growth rate and cybrids D1, D3, D6 showed the lowest rate. The thymidine uptake rate was higher in cybrid A1. By FACS analysis using propidium iodide staining, we found that the cells of sub-G1 phase were less than 40% in cybrid A1 and more than >50 % of cybrids D1, D3, and D6 remained at sub-G1 phase. There was no difference in the rate of apoptosis. In prostate cancer, it has been shown that reactive oxygen species (ROS) contributes as cell growth stimuli [1]. However, we could not find any difference among cybrids A1, D1, D3, and D6, although 143B rho+ cells showed significantly increased ROS levels. By sequencing of whole mtDNA, we found that cybrid A1 has Met490Thr mutation in COI gene and Thr127Ala mutation in ND5 gene. Among them, Thr127Ala is highly conserved amino acid across all species. Thus, it could be responsible for the functional difference of cybrid A1. From above results, we conclude that cancer cell growth is affected by different mtDNAs from subjects without clinically evident cancer and subjects harboring mtDNA haplogroup D could live longer by avoiding carcinogenesis.

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10-11. Importance of the mitochondrial genetic background in the mtDNA mutations expression: Implication in mitochondrial diseases.

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The mitochondrial genetic diseases can be caused by more than 50 mtDNA mutations and 200 rearrangements. It has been already observed that several distinct mtDNA mutations can product the same disease and reciprocally several diseases can be related to the same mutation. These observations highlight the problem of the variability of the expression of these mutations. Several hypotheses such as differences in the "threshold effect", or heteroplasmy levels have been proposed to explain this variability. However, differences in the mitochondrial genetic background is another hypothesis, but this notion is difficult to investigate with traditional genetic approaches.

The recent progress in molecular anthropology has led to the possibility of the definition of monophyletic groups of mitochondrial DNA. Indeed, the analysis of mtDNA sequences has shown a high degree of homogeneity among European populations with 99% of European mtDNAs fall into one of ten haplogroups (H, I, J, K, M, T, U, V, W or X). These "haplogroups" can be a useful tool for such genetic/epidemiology study. For the moment, only few studies are published on the relationships between haplogroups and mitochondrial diseases and concern few of these pathologies. In addition, these works often concern a small number of patients, which do not allow a clear statistical analysis.

Three laboratories of Bordeaux^{1,2,3} in collaboration with the French network of the mitochondrial diseases work to define the haplogroups of most of 500 French patients and to highlight the importance of the mitochondrial genetic background in the mtDNA mutations expression.

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10-12. A novel approach for rapid screening of mitochondrial D310 polymorphism.

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Mitochondrial DNA alterations have been suspected to play an important role in the development and progression of cancer. Several mutations have been identified in a wide variety of human tumors including breast, colorectal, ovarian, gastric, hepatic and esophageal cancers as well as hematological malignancies [1]. D-loop region of the mtDNA is the most potent accumulation site for many of these mutations and numerous polymorphisms have also been reported in this region. The sequence alterations of this region may contribute to altered replication or transcription properties.

Recently, Sanches-Céspedes and colleagues [2] have identified a polyC mononucleotide repeat located between 303 and 315 nucleotides within the D-loop region as a mitochondrial hot spot of deletion or insertion mutations. This region is part of the conserved sequence block (CSB) II and consists of a stretch of cytosines interrupted by a thymine nucleotide (CCCCCCTCCCC). Although the number of the cytosine residues at the first stretch of polyC is accepted as 7-C (GeneBank NC_001807), it is highly polymorphic ranging between 6-C to 9-C [3,4]. It is still questionable if there is any correlation between the number of the cytosine residues and development and progression of the cancer. Typically, time and money consuming methods such as sequencing and radioactivity based gel electrophoresis are required in order to evaluate this polymorphism among individuals. Also, gel electrophoresis remains ineffective unless confirmed with sequencing and these limitations are especially obvious with studying large populations.

In this study we established a restriction fragment length polymorphism (RFLP) assay for the first step rapid screening of the individuals if they carrying 7-C at their mitochondrial D310 region. We tested a total of 141 tissue samples including normal and cancerous tissues of 25 breast and 25 colorectal cancer patients and 41 blood samples of healthy individuals. By using this simple approach, 41 % of the studied samples were found that have 7-C in their mtDNA D310 region without need for sequencing and/or radioactive labelling. Furthermore, we compared the cases and normal samples for their RFLP status and found a statistically significant difference between colorectal cancer samples and healthy individuals.

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10-13. Reduced skeletal muscle mitochondrial O₂ flux capacity in type 2 diabetes.

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Hyperglycemia in insulin-resistant type 2 diabetes is associated with mitochondrial dysfunction characterized by diminished mitochondrial oxidative capacity and proton uncoupling, elevated membrane potential, increased ROS production and impaired lipid metabolism. While mitochondria are considered a central locus of altered metabolic pathways leading to pathogenic processes in type 2 diabetes, the mechanisms underlying these factors remains to be elucidated. Evidence for reduced oxidative capacity of skeletal muscle in diabetes is based on markers of oxidative enzyme levels and gene expression defects, yet there is a paucity of data reporting direct measures of mitochondrial O₂ flux capacity in cells. In this study, O₂ flux capacity of permeabilized muscle fibers from biopsies of the quadriceps in healthy humans ($n=5$) and patients with type 2 diabetes ($n=7$) was measured at 37 °C using high resolution respirometry (OROBOROS Oxygraph-2k). Oxygen flux was expressed per mg muscle fresh weight. Diabetic and control subject characteristics were; age = 62 ± 2 and 56 ± 2 yrs; body mass index = 33 ± 2 and 29 ± 1 kg/m²; fasting glucose = 8.7 ± 0.8 and 5.3 ± 0.2 mM; lactate = 1.3 ± 0.1 and 1.5 ± 0.4 mM, respectively.

In healthy controls and diabetics respectively, ADP-stimulated state-3 respiration with complex I substrate (glutamate) was 43 ± 4 vs. 33 ± 2 pmol O₂·s⁻¹·mg⁻¹, and state-3 O₂ flux with parallel electron input from complex I+II (glutamate+succinate) was 87 ± 8 vs. 70 ± 3 pmol·s⁻¹·mg⁻¹. Further increases in flux capacity were observed with uncoupling by FCCP, but were lower in type 2 diabetics (106 ± 12 vs. 84 ± 2 pmol·s⁻¹·mg⁻¹). Subsequent fluxes with rotenone were 73 ± 9 vs. 58 ± 3 allowing for an estimation of individual fluxes through complex I and II. The findings demonstrate serial blunting of state-3 O₂ flux with electron flux through either complex I or II, and a similar reduction with parallel electron input through both complexes. Furthermore, on the basis of similar uncoupled responses relative to state 3 (glutamate+succinate) in both healthy (1.22) and diabetic subjects (1.2), the results reflect an attenuation of mitochondrial oxidative capacity in skeletal muscle of type 2 diabetic patients indicative of an impaired electron transport capacity.

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