A novel mutation in the NADH dehydrogenase (ubiquinone) 1 alpha sub-complex 4 (*Ndufa4*) gene links mitochondrial dysfunction to the development of diabetes in a rodent model

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Author summary

We report for the first time on a major inherited mutation in a diabetes-prone animal model that adversely affects mitochondrial function and leads through oxidative stress to the development of diet-induced diabetes.

Abstract

The mechanisms underlying diabetes remain unresolved. The Cohen Diabetic rat represents a model of diet-induced diabetes in which the disease is induced after exposure to a diabetogenic diet (DD) in the diabetes sensitive (CDs/y) but not in the resistant (CDr/y) strain. Diet imposes a metabolic strain that leads to diabetes in the appropriate genetic background. We previously identified through whole genome linkage analysis a diabetes-related QTL on RNO4 which incorporates NADH dehydrogenase (ubiquinone) 1 alpha sub-complex 4 (*Ndufa4*), a nuclear gene that affects mitochondrial function. We currently sequenced the gene and found a major deletion in CDs/y that leads to lack of expression of the NDUFA4 protein that is reported to be involved in the activities of mitochondrial complexes I and IV. In the absence of NDUFA4 in the diabetic CDs/y on DD, complex I activity is reduced in comparison to the non-diabetic CDs/y on regular diet and CDr/y on either diet; complex IV activity is reduced in both strains provided DD, and thus as result of diet and unrelated to the gene mutation. Tissue ATP fails to increase in diabetic CDs/y in response to DD, in comparison to non-diabetic CDr/y on DD. Plasma MDA levels are elevated in CDs/y on DD, while SOD1 and SOD2 levels fail to increase, indicating increased oxidative stress and inability of the pancreas to generate an appropriate anti-oxidative stress response. These findings suggest that the *Ndufa4* mutation in CDs/y on DD is directly associated with mitochondrial dysfunction which we attribute to the lack of expression of NDUFA4 and to diet, and which prevents the anticipated increase in ATP production. The resulting enhanced oxidative stress impairs the ability of the pancreas to secrete insulin, leading to the development of diabetes. This is the first demonstration of an inherited mutation in a nuclear gene that adversely affects mitochondrial function and promotes diet-induced diabetes.

Introduction

The pathophysiology of diabetes remains incompletely understood. Multiple studies over the past decades in humans and in animal models have indirectly implicated the mitochondria in the pathogenesis of diabetes and its complications (Chow et al. 2017;Lowell and Shulman 2005). We currently provide novel evidence of an inherited mutation in a candidate gene that links mitochondrial dysfunction to the development of diet-induced diabetes.

The Cohen diabetic rat is an experimental model reminiscent of type 2 diabetes in humans. The model consists of two sub-strains, the Cohen Diabetic sensitive (CDs/y) and the Cohen Diabetic resistant (CDr/y) rats (Weksler-Zangen et al. 2001). Both sub-strains exhibit a normal metabolic profile while fed regular diet (RD), but when provided a custom-prepared diabetogenic diet (DD), CDs/y invariably develop diabetes whereas CDr/y do not (Weksler-Zangen, Yagil, Zangen, Ornoy, Jacob, & Yagil 2001). Genetic linkage studies in this model have led us to uncover a genomic locus on rat chromosome 4 (RNO4) that incorporates in the rat and in syntenic regions in the mouse and humans several candidate genes, including *Ndufa4* (NADH dehydrogenase (ubiquinone) 1 alpha) (Barkalifa et al. 2010). *Ndufa4*, a nuclear gene, encodes for the NDUFA4 protein that makes up at least one of the subunits of the mammalian oxidative phosphorylation (OXPHOS) complexes and which exerts its effect within the mitochondria (Mishmar et al. 2006).

In the current study, we investigated in CDs/y and CDr/y the *Ndufa4* gene sequence, the gene transcript, its expression at the protein level and mitochondrial function, with a focus on complexes I and IV in which this gene has been previously implicated (Balsa et al. 2012;Garbian et al. 2010;Hayashi et al. 2009;Mishmar, Ruiz-Pesini, Mondragon-Palomino, Procaccio, Gaut, & Wallace 2006;Pitceathly et al. 2013;Sinkler et al. 2017). We present the results which demonstrate in CDs/y a novel mutation within the *Ndufa4* gene that accounts for lack of expression of the intact NDUFA4 protein which, in the appropriate environment, leads to impaired mitochondrial function, increased oxidative stress and the development of diet-induced diabetes mellitus.

RESULTS

Sequencing of the *Ndufa4* gene

Exon sequencing We amplified *Ndufa4* cDNA from pancreatic extracts of the two strains and found a difference in size of the PCR product: CDs/y 330bp versus CDr/y 390bp (Fig.1A), regardless of whether the animals were on DD or RD, or expressed the diabetic phenotype or not (Fig. 1B). We found the same shorter PCR product size in CDs/y also in the heart, liver, kidney and muscle (Fig. 1C), but not in other non-diabetic rat strains, including the Sabra hypertension prone (SBH/y), the Sabra hypertension resistant (SBN/y) and the spontaneously hypertensive rat (SHR) rats (Fig. 1D). We sequenced the gene and found that the short PCR product in CDs/y was due to skipping of exon 3, whereas exons 1, 2 and 4 were identical in CDs/y and CDr/y (Fig. 2). The size difference in the PCR products between the two strains was accounted for in full by the 61 bp size of *Ndufa4* exon 3 in the rat.

Genomic sequencing In CDr/y, we successfully amplified the entire *Ndufa4* gene, consisting of 4 exons and 3 introns. In CDs/y, we were able to amplify only exons 1, 2 and 4 and introns 1 and 2, but were unsuccessful in amplifying exon 3 or intron 3. To investigate further the lack of amplification of exon 3 and intron 3 in CDs/y, we used Phusion High-Fidelity DNA Polymerase to amplify a big PCR product from the middle of intron 1 and up to 110 bp downstream from exon 4, covering thereby exon 3 and intron 3. In CDr/y, the PCR product was ~ 2800 bp larger than in CDs/y. We sequenced the big PCR product and confirmed its belonging to the published *Ndufa4* gene sequence. We then designed primers for small PCR products to cover the gene components demarcated by the large PCR products. We successfully amplified several small PCR products in CDr/y but not in CDs/y. Using "gene walking" and primers designed to regions proximal and distal to the unamplified genomic segment, we confirmed the size difference between CDs/y and CDr/y. The amplified PCR product from CDs/y revealed that the gene sequence jumped from the end of intron 2 (bp# 1859 – the last part of the 5' spliceosome of exon 3) directly to the middle of intron 3, thereby skipping 2793 bp.

Expression of the NDUFA4 protein

We identified by western blot the NDUFA4 protein in CDr/y fed RD and DD but were unable to demonstrate the protein in CDs/y on either diet (Fig. 3). We confirmed these results in whole cell homogenates, in membranous enriched samples and in soup protein extracted from the pancreas, liver, kidney and muscle. In CDr/y, the level of expression varied with diet and was double to triple the amount found in animals on RD compared with DD (Fig. 3).

Localization of NDUFA4 within the electron chain complex

The 1D blue native gel (Fig. 4A) placed the NDUFA4 protein at about 900 kD, corresponding to the Complex I region and at ~ 200kD, in proximity of Complex IV. The 2D blue native gel (Fig. 4B) confirmed the NDUFA4 protein MW ~ 10 kD location within Complex I, but also an additional band with MW between 75-100 kD, in the vicinity of Complex IV. The large size of the second molecule, well above that of NDUFA4, suggests either non-specific binding at that site or that NDUFA4 is bound to a subcomplex of complex I that has been dissociated in the denaturing conditions. We found the same pattern in CDr/y under RD and DD also in tissue extracted from liver and kidney (data not shown).

OXPHOS abundance

We found similar abundance of the proteins composing each of the complexes I, II, III and V, with no difference between CDr/y and CDs/y, whether on RD or DD (Fig. 5A). Within complex IV, however, we found lower OXPHOS protein abundance during DD compared to RD, irrespective of animal strain. This finding was confirmed by studying specifically COX4 expression (a component of complex IV) that was reduced in CDs/y and CDr/y provided DD, as compared to RD (Fig. 5B). In parallel, NDUFA4 abundance in CDr/y was reduced while on DD when compared to RD, whereas protein expression was totally lacking in CDs/y (Fig. 5B).

Mitochondrial function

Complex I activity To compare activity between CDs/y and CDr/y under RD and DD, we focused our studies on the liver, muscle, kidneys and pancreas (Fig. 6A). In the liver, muscle and kidney, complex I activity was similar in CDs/y and CDr/y while on RD; on DD, however, the activity was higher by 60-100% in the liver and muscle of CDr/y, but did not change in CDs/y, whereas in the kidney, such changes did not occur. In the pancreas, complex I activity showed a different pattern of response to diet than in the other organs: While on RD, complex I activity was ~80% higher in CDs/y than in CDr/y, whereas on DD, the activity in CDs/y was ~40% lower than in CDr/y

Complex IV activity Complex IV activity was similar in CDr/y and CDs/y provided RD in the liver, muscle, kidney and pancreas (Fig. 6B). When provided DD, complex IV activity declined significantly in both CDr/y and CDs/y.

ATP production In animals provided RD, ATP levels in the liver, muscle, kidney and pancreas were not different between CDr/y and CDs/y (Fig. 7). When provided DD, ATP levels increased significantly in CDr/y in the liver and in the pancreas, but only marginally in muscle and not in the kidney. In CDs/y fed DD, ATP levels failed to increase within muscle, kidney, liver and pancreas.

Oxidative stress

MDA serum levels MDA serum levels were significantly elevated in CDs/y-DD compared to all other groups (Fig 8A). Interestingly, serum MDA levels also rose significantly in CDr/y when fed DD, as compared to levels when fed RD – but the increment was less than half of that observed in CDs/y.

SOD1, SOD2 Pancreatic SOD1 and SOD2 levels were higher in non-diabetic CDr/y on DD when compared to non-diabetic CDr/y provided RD (Fig. 8B and C). In CDs/y fed RD, SOD1 and SOD2 levels were not different than in CDr/y provided RD; in contrast to CDr/y, however, in CDs/y fed DD, SOD1 and SOD2 protein levels failed to rise and even tended to decrease (Fig. 8B and C).

Discussion

The current in-depth exploration of *Ndufa4* as a mitochondrial functional candidate gene involved in the pathogenesis of diet-induced diabetes was driven by our previous localization of the gene by linkage analysis to a diabetes-related quantitative trait locus (QTL) on rat chromosome 4 (RNO4) and the differential expression of the gene between the diabetes prone CDs/y and resistant CDr/y strains (Barkalifa, Yagil, & Yagil 2010;Yagil et al. 2007;Yagil et al. 2016). We now report on a novel mutation in the *Ndufa4* gene in CDs/y that is not present in CDr/y and that consists of a large deletion, resulting in skipping of exon 3 and part of intron 3 and creating a frame shift mutation and a stop codon (Fig. 2). We confirmed this deletion in the *Ndufa4* gene by next generation sequencing and determined that this sequence variation was unique to CDs/y among 25 other diabetic and non-diabetic rat strains (Yagil, Markus, Kohen, & Yagil 2016).

Based on the difference in the gene structure between CDs/y and CDr/y, the predicted translation of *Ndufa4* leads to a 45 amino acid peptide in CDs/y (up to the stop codon), instead of the intact 81 amino acid protein in CDr/y. As result, the intact NDUFA4 protein is expressed in CDr/y under both RD or DD, but the *Ndufa4* gene mutation in CDs/y prevents altogether the expression of the intact NDUFA4 protein.

The NDUFA4 protein is a 9kDa subunit that has NADH dehydrogenase and oxi-reductase activities and transfers electrons from NADH to the respiratory chain, the immediate electron acceptor thought to be ubiquinone. The protein has previously been identified as part of the 45 different subunits that compose the mammalian complex I of the respiratory mitochondrial respiratory chain (GeneCards, Entrez (http://www. genecards.org/cgi-bin/carddisp.pl?gene=Ndufa4)) (Garbian, Ovadia, Dadon, & Mishmar 2010;Hayashi, Ishimori, Takahashi-Niki, Taira, Kim, Maita, Maita, Ariga, & Iguchi-Ariga 2009;Mishmar, Ruiz-Pesini, Mondragon-Palomino, Procaccio, Gaut, & Wallace 2006;Pitceathly, Rahman, Wedatilake, Polke, Cirak, Foley, Sailer, Hurles, Stalker, Hargreaves, Woodward, Sweeney, Muntoni, Houlden, Taanman, & Hanna 2013). More recent data suggest that the NDUFA4 protein may be related to the mitochondrial cytochrome c oxidase (COX, complex IV) activity, as the terminal component of the mitochondrial chain that catalyzes the reduction of oxygen to water and that it is required for complex IV maintenance

(Generads, UniProtKB/Swiss-Prot) (Balsa, Marco, Perales-Clemente, Szklarczyk, Calvo, Landazuri, & Enriquez 2012;Pitceathly, Rahman, Wedatilake, Polke, Cirak, Foley, Sailer, Hurles, Stalker, Hargreaves, Woodward, Sweeney, Muntoni, Houlden, Taanman, & Hanna 2013). Irrespective of whether NDUFA4 locates to complex I or IV, we reasoned that the absence of its expression in CDs/y would carry functional implications and affect mitochondrial function that might be associated with the development of diabetes. We set out, therefore, to investigate mitochondrial function in CDs/y and CDr/y fed RD or DD.

Prior to studying the effect of the lack of expression of the NDUFA4 protein on mitochondrial function, we sought to determine if the mutation in CDs/y affects the integrity of the components making up the mitochondrial respiratory chain. We demonstrated through OXPHOS studies that size and abundance of the individual mitochondrial complexes I, II, III and V were unperturbed in CDs/y compared to CDr/y on either diet, attesting to their intactness in CDs/y, despite the *Ndufa4* mutation. We did observe reduced abundance of complex IV in both CDs/y and CDr/y fed DD, which we attributed to the effect of diet and not to the *Ndufa4* mutation or to the diabetic phenotype which do not appear in CDr/y. We then attempted to clarify further the controversy whether NDUFA4 locates within the mitochondrial respiratory chain to complex I (Garbian, Ovadia, Dadon, & Mishmar 2010;Hayashi, Ishimori, Takahashi-Niki, Taira, Kim, Maita, Maita, Ariga, & Iguchi-Ariga 2009;Mishmar, Ruiz-Pesini, Mondragon-Palomino, Procaccio, Gaut, & Wallace 2006;Pitceathly, Rahman, Wedatilake, Polke, Cirak, Foley, Sailer, Hurles, Stalker, Hargreaves, Woodward, Sweeney, Muntoni, Houlden, Taanman, & Hanna 2013) or IV (Balsa, Marco, Perales-Clemente, Szklarczyk, Calvo, Landazuri, & Enriquez 2012;Pitceathly, Rahman, Wedatilake, Polke, Cirak, Foley, Sailer, Hurles, Stalker, Hargreaves, Woodward, Sweeney, Muntoni, Houlden, Taanman, & Hanna 2013). Our findings indicate that in the rat, NDUFA4 locates to complex I, as originally determined. On the other hand, we cannot rule out with certainty its location also within complex IV, even though in the latter complex, NDUFA4 appears with a molecular weight between 75-100 kd, well above the ~9kd attributed to the protein, suggesting either non-specific binding at that site or that NDUFA4 is bound to a subcomplex of complex I that has been dissociated in the denaturing conditions. Our findings thus reposition with a great degree of

confidence NDUFA4 to complex I, but the possibility that NDUFA4 or a similar protein might also localize to complex IV remains open and requires additional investigation.

As we could not rule out the possibility that the two mitochondrial complexes might involve NDUFA4, we studied the activity of both complex I and IV activities in CDs/y (in which NDUFA4 is not expressed) and compared to that in CDr/y (in which the intact protein is fully expressed). In the basal state during which the animals are provided RD, complex I activity in CDs/y is not different from CDr/y in the liver, muscle and kidney, indicating that in these organs, the *Ndufa4* mutation and the lack of expression of the NDUFA4 protein do not affect basal mitochondrial function. In the pancreas of CDs/y fed RD, however, complex I activity is unexpectedly elevated, compared to CDr/y provided RD, which could be interpreted as an organ-specific requirement for increased activity to compensate for the absence of the NDUFA4 subunit. In contrast to the basal state when animals are fed RD, when CDr/y are fed DD, the dietary diabetogenic stimulus, complex I activity increases significantly in the liver, muscle and pancreas, indicating an increase in mitochondrial activity, most likely in response to enhanced demand induced by DD. Interestingly, complex I activity in the kidney does not increase, suggesting that the kidney, as opposed to liver and muscle, may not actively participate in the regulation of energy metabolism. Contrary to what we find in CDr/y on DD, CDs/y significantly fail to increase complex I activity in the liver and muscle in response to DD. Moreover, in the pancreas of CDs fed DD, complex I activity drops to levels comparable to basal levels in CDr/y provided RD. These finding suggest that when exposed to DD, the liver, muscle and pancreas of CDs/y with the *Ndufa4* mutation are unable to achieve the high level of mitochondrial activity that is normally required in response to increased demand, most likely because of the absence of the intact NDUFA4 protein.

While investigating complex IV, we found similar activity in CDr/y and CDs/y provided RD. When the animals are fed DD, however, we find a reduction in complex IV activity, similarly in both strain. These findings suggest that the reduction in complex IV activity is primarily related to the DD and not to strain differences, and therefore not to the *Ndufa4* mutation or to the occurrence diabetes which develops in CDs/y but not in CDr/y. A possible explanation for the reduction

in the activity of complex IV may be related to the low copper content in DD, copper being an essential co-factor for normal complex IV activity (Horn and Barrientos 2008).

To complement the study of mitochondrial function, we studied ATP levels, one of the major end-products generated by mitochondria. We examined ATP levels in the liver, muscle, kidney and pancreas of animals in the basal non-diabetic state when they were provided normal diet and when they were challenged with a metabolic load imposed by the DD. We found that ATP levels increase or tend to increase when CDr/y are provided DD when compared to RD in the metabolically active organs, liver, muscle and pancreas, but not in the kidneys. We interpret these findings as reflecting increased mitochondrial activity in response to the metabolic load imposed by DD. In CDs/y fed DD, however, ATP levels do not increase upon demand or even tend to be lower than in animals provided RD, suggesting that the *Ndufa4* mutation and the absence of the NDUFA4 protein prevent the mitochondrial ATP response to the diabetogenic challenge that is normally expected when the protein is expressed, as is the case in CDr/y.

As one of the important functions of complex I is regulation of mitochondrial induced oxidative stress (Sharma et al. 2009), we investigated whether the reduced complex I activity we observed in CDs/y fed DD is associated with increased oxidative stress, an important and uncontested culprit in the pathophysiology of diabetes (Kwak et al. 2010;Lowell & Shulman 2005;Ma et al. 2012). The increase in serum MDA levels we observed in both CDr/y and CDs/y when fed DD when compared to regular diet indeed suggests enhanced oxidative stress that is induced by exposure to this type of diet per-se. It is notable, however, that the increment in MDA levels is in fact significantly higher in CDs/y on DD than CDr/y on a similar diet, suggesting that the absence of the NDUFA4 protein and the enhanced oxidative stress are associated with the development of diabetes.

The anticipated response to an increase in oxidative stress is activation of anti-oxidant defenses. The increase in SOD1 and SOD2 levels in the pancreas that we observed in CDr/y fed DD compared to regular diet is indicative of an appropriate response in this strain to oxidative stress generated by the diet. The lack of such response in the pancreas of CDs/y on DD, on the other hand, suggests that this strain is unable to recruit the necessary defense mechanisms during the increased

oxidative stress that is induced by the DD in the presence of mitochondrial respiratory chain dysfunction that is caused by the absence of the NDUFA4 protein. The CDs/y strain that is fed DD and develops diabetes generates, therefore, a higher level of oxidative stress that is poorly dealt with by the anti-oxidative defenses, inadequate disposal of reactive oxygen species by detoxifying enzymes such as SOD1 and SOD2 and the imbalance between production and detoxification which determines the intracellular ROS concentration (Hasnain et al. 2016).

How do these findings further our understanding of the pathophysiology of diabetes in the Cohen Diabetic rat model of diet induced diabetes? Key to integrating all our findings is the realization that there are at least two central players that induce diabetes in our model of complex disease: One is the DD that comprises a high metabolic load that is deficient of copper, and the second is the *Ndufa4* mutation. When CDs/y, which carry the *Ndufa4* mutation, function in a normal environment and are fed regular diet, they are able to maintain complex I activity to a level that is sufficient so as not to develop diabetes. When fed DD with low copper content, however, CDs/y that carry the *Ndufa4* mutation demonstrate mitochondrial dysfunction, as evidenced by a lack of increase in complex I activity and a reduction in ATP generation in response to the dietary load, as well as enhanced oxidative stress with an impaired response of anti-oxidant enzymes. Although the exact sequence of events is yet unclear, the mitochondria in CDs/y are most likely affected through multiple pathways. Complex I is presented with lack of the NDUFA4 protein in its trans-membrane domain, which adversely affects mitochondrial complex I function in face of increased energetic demand imposed by the DD. Impairment in complex I function due to the absence of the NDUFA4 protein induces extensive free radical generation which need to be effectively dealt with. The important enzymes that hydrolyze these radicals is superoxide dismutases (Cu-Zn-Sod) or SOD, in which copper (Cu) and zinc (Zn) fulfill a major role; in the absence of copper, these enzymes are unable to respond appropriately to the increased level of oxidative stress in CDs/y. The reduced copper in DD is disadvantageous, but not sufficiently by itself to prevent the rise in SOD levels, since CDr/y fed the same copper poor diet respond adequately with a rise in SOD levels. An alternative explanation for the lack of response of CDs/y which we tested

after 1 month of DD, at which time CDs had already developed the full blown diabetic phenotype, is that the persistent enhanced oxidative stress in the absence of a constant supply of copper in diet exhausts over time the ability of the pancreas to respond. We conclude that the development of diabetes during exposure to DD in CDs/y is induced by a multiple-pathway hit, as most complex traits are. The *Ndufa4* mutation adversely affects complex I function, leading to increased oxidative stress that is further aggravated by the inability of CDs/y to generate an adequate anti-oxidative response with SOD1 and SOD2. Additionally, the low copper content in diet adversely affects complex IV function. The end result is a reduction in ATP levels in CDs/y. Together, these multiple hits adversely affect the function of the pancreas and impair insulin release, leading to the development of diabetes. Finally, to the best of our knowledge, our findings consist of the first demonstration of a direct link between a mutation in a gene that is associated with mitochondrial function and the pathophysiology of diet induced diabetes.

Materials and Methods

Animals

We obtained the animals from the colony inbred at the Israeli Rat Genome Center (IRGC) of the Barzilai University Medical Center in Ashkelon, Israel (www.irgc.co.il). (Weksler-Zangen, Yagil, Zangen, Ornoy, Jacob, & Yagil 2001;Yagil et al. 1996). We housed the animals in accordance with institutional and governmental regulations, principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and guidelines of the American Society of Physiology for the Care and Use of Laboratory Animals. We maintained climate-controlled conditions and light adjusted to regular timed diurnal cycles. The study protocol was reviewed and approved by the Barzilai University Medical Center Institutional Animal Care and Use Committee (Animal Experimentation Committee).

To elicit the diabetic phenotype in CDs/y, we fed the animals after weaning with standard rat chow and tap water ad libitum until age 6-7 weeks. We then switched to DD (DD) and copper-poor water ad libitum. We custom-prepared DD, as previously described (Weksler-Zangen, Yagil, Zangen, Ornoy, Jacob, & Yagil 2001). After 4 weeks on DD, the diabetic phenotype, which consists of an exaggerated

hyperglycemic response to glucose loading with impaired insulin response, invariably develops in CDs/y but not in CDr/y (Weksler-Zangen, Yagil, Zangen, Ornoy, Jacob, & Yagil 2001;Yagil, Barkalifa, Sapojnikov, Wechsler, Ben Dor, Weksler-Zangen, Kaiser, Raz, & Yagil 2007).

Sequencing of the *Ndufa4* gene

We initially sequenced the gene transcript, followed by whole genome sequencing.

Exon sequencing We surgically dissected out the entire pancreas in anesthetized animals, snap-froze the organ and extracted RNA by homogenizing the tissue in Tri Reagent (Molecular Research Center, Cincinnati Inc., Ohio). To derive cDNA, we used 1 µg total RNA to create the first strand with the Reverse-IT kit (AB gene), applying the random decamer option. We amplified the exon transcripts with custom-designed primers (http://frodo.wi.mit. edu/cgin/primer3/primer3 _www.cgi) from the 5' to the 3' untranslated sequence, 5' TGGCTGTGCTGG-AACTCACTCTGT and 3' AGACCTGAGGTTTTCCATCTC, and sequenced the PCR products.

Since we found a difference in size of the PCR products between CDs/y and CDr/y, we tested whether our findings were unique to the pancreas as an organ system, and to the Cohen diabetic rat as a model organism. We therefore derived cDNA from the heart, liver, kidney and muscle of CDs/y and of other non-diabetic rat strains, including the Sabra hypertension prone (SBH/y), the Sabra hypertension resistant (SBN/y) and the spontaneously hypertensive rat (SHR) rats (Friedman et al. 2003;Yagil et al. 1995;Yamori and Lovenberg 1987), amplified the transcripts and ran the PCR products in these samples as well.

To sequence the exons, we excised the PCR products by taking 50ul from the agarose gel, cleaned it with NucleoSpin Extract II and sequenced it using an ABI sequencer at the Crown Human Genome Center of the Weizmann Institute of Science, Rehovot, Israel.

Genomic sequencing We subsequently sequenced the entire gene, including its introns, exons and the promoter region 70 bp upstream to the genes and ~300 base pairs in the 3' transcribed but untranslated region. We extracted genomic DNA by salt precipitation, phenol-chloroform cleaning and amplification by PCR reaction in small 300-700bp PCR products. We cleaned the PCR products by the mini prep kit

and ran them on low melting point agarose gel. Using Primer 3 software, we designed primers designed to cover exons 1-4 and introns 1-3. The PCR reaction consisted of 1 min denaturing 98°C, 1 min annealing 53-58°C, and 72°C extension for 1 min x 35 cycles. For large PCR products of DNA (1000-4000 bp), we used Phusion High-Fidelity DNA polymerase, with 2 min denaturing at 98°C annealing 30 sec and 51-56°C extension for 3 min x 35. We sequenced the PCR products using an ABI sequencer (Weizmann Institute, Crown Human Genome Center). We used the NCBI-Blast software (http://www.ncbi.nlm.nih.gov/BLAST/) to identify sequence variations between CDs/y and CDr/y. To identify promoter regions motifs, we used the web-based database http://www.cbrc.jp/htbin/nphtf-search.

Expression of the NDUFA4 protein

We studied the expression of *Ndufa4* at the protein level in the liver, muscle, kidney and pancreas of CDs/y and CDr/y. NDUFA4 is a small sized (~9KD) membranous bound protein.

We surgically extracted the organs in the anesthetized animal and froze them at -80°C until processed further. As NDUFA4 resides in the mitochondria, we aimed to enrich the cell membrane fraction. We washed 50-100 ug frozen tissue and minced in PBS-PI (protease inhibitor cocktail from Sigma), dounce homogenized on ice and centrifuged the homogenate at 4000 rpm, obtaining a membrane-rich pellet and the supernatant consisting of the cytosolic fraction. We lysed the content in RIPA buffer and used the Bradford method to quantitate the amount of protein extracted.

To extract the protein in the pancreas, we used either the entire organ or obtained islet-enriched homogenates as described by Carter et al (Carter et al. 2009). For the latter, we anesthetized the animals, exsanguinated them, ligated the common bile duct proximal and distal to the pancreatic duct, cannulated the isolated bile duct segment with PE-50 and perfused the pancreas through the pancreatic duct with ~5 ml of 0.2% collagenase type 4 (Worthington). We then surgically excised the pancreas, inserted the organ into a tube containing collagenase and incubated at 37°C for 11 min with gentle shaking. We diluted the individual samples with ice cold HBSS buffer and spun for 2 min at 1300 rpm. We re-suspended the pellet in HBSS, layered the suspension on sieve size 40 gauge and centrifuged the resulting sieve

for 2 min at 1400 rpm. We separated the islets by gradient of a mixture of Sigma Histopaque 1119 and 1077, washed twice with HBSS, centrifuged at 3000 rpm for 5 min and moved the samples to 1.5 ml tubes containing PBS with proteinase inhibitors (Sigma). We homogenized the islet-enriched solution and froze the homogenates at -80^{0}C until further analysis.

To demonstrate protein expression in the processed tissues, we warmed 50-70 ug protein to 95^{0}C which we loaded on Any kD denatured gels (Bio-Rad), ran the gels and transferred to PVDF membrane. As antibody for NDUFA4, we used Ab133698 (epitope 38-81) (Abcam) diluted 1:500-1:1000. The antibody targets mainly exons 3 and 4 and recognizes the protein at ~10kD. After blocking the membrane with 5% milk in PBST (0.1%) for 1hr, we incubated with the NDUFA4 1st antibody for 1hr at room temperature or overnight at 4^{0}C. We then washed the membrane with PBST 0.1% Tween and incubated for 1 hr with the 2nd antibody - Goat anti rabbit 1:10,000 (Jackson Immuno Research). We developed the membranes with Clarity Western ECL Substrate (Bio-Rad).

NDUFA4 localization within the mitochondrial complexes

This protein has previously been reported to be incorporated as a protein subunit in complex I of the mitochondrial respiratory chain (Garbian, Ovadia, Dadon, & Mishmar 2010;Hayashi, Ishimori, Takahashi-Niki, Taira, Kim, Maita, Maita, Ariga, & Iguchi-Ariga 2009;Mishmar, Ruiz-Pesini, Mondragon-Palomino, Procaccio, Gaut, & Wallace 2006). Subsequent studies, however, have pointed to the possibility that NDUFA4 might localize to complex IV (Balsa, Marco, Perales-Clemente, Szklarczyk, Calvo, Landazuri, & Enriquez 2012;Pitceathly, Rahman, Wedatilake, Polke, Cirak, Foley, Sailer, Hurles, Stalker, Hargreaves, Woodward, Sweeney, Muntoni, Houlden, Taanman, & Hanna 2013;Sinkler, Kalpage, Shay, Lee, Malek, Grossman, & Huttemann 2017). It became thus incumbent upon us to first sort out these conflicting reports and re-explore the localization of the NDUFA4 protein within the mitochondrial complexes in our model. To achieve this goal, we utilized 1D (n=3) and 2D (n=5) blue native gels, onto which we loaded 60-90ug protein samples extracted from the liver and kidney of CDr/y with Native Sample Loading Buffer (Bio-Rad) and 5% Coomassie Blue G-250 (Bio Rad). We ran the native gels for 4 hrs in

parallel with a Native Marker Unstained protein standard. In the 2D gels, we cut the protein lane consisting of complex aggregates, incubated it for 30 min in dissociation buffer (Tris HCl buffer containing 1% SDS and 1% mercapto-ethanol) and positioned it on top of the denaturing gel composed of 4% stacking acrylamide/bis gel and 10% acrylamide/bis resolving gel. Both 1D and 2D gel products were transferred to PVDF membranes.

Effect of the *Ndufa4* mutation on mitochondrial integrity We initially tested whether in CDs/y, the absence of expression of NDUFA4, a structural component of complex I and possibly IV, affects the relative abundance of the mitochondrial complexes when compared to CDr/y by using the Oxidative Phosphorylation (OXPHOS) blue native western blot antibody cocktail (Abcam ab 110412).

Effect of the NDUFA4 on mitochondrial function

Function of complexes I and IV In preparation for studying mitochondrial function, we surgically retrieved tissue from the liver, muscle, kidney, spleen, lungs, salivary glands and pancreas of anesthetized CDs/y and CDr/y provided RD or DD and snap-froze them until use. We obtained membranous mitochondrial enriched fractions from 100 mg aliquots of frozen tissue by gentle homogenization (10-30 strokes) in PBS on ice and centrifuging at 13000 RPM for 10 min 4^OC. The pellet, consisting of the membranous-rich fraction, was further processed by adding 1X RIPA, homogenization on ice, and centrifugation at 13000 RPM for 15 min at 4^Oc. The supernatant was assayed for complex I and IV activity.

To study complex I activity, we used the Complex I Enzyme Activity Microplate Assay Kit (Abcam-ab109721) that determines OXPHOS mitochondrial Complex I (NADH dehydrogenase) activity, as determined by measuring oxidation of NADH to NAD+. To study complex IV activity, we used the Complex 4 Rodent Enzyme Activity Assay Kit (Abcam-ab109911) which determines mitochondrial cytochrome c oxidase enzyme activity. In both assays, absorbance was measured colorimetrically in duplicates or triplicates.

When studying complex I and IV activities, we set the level of activity in CDr/y fed RD as reference standard for normal activity and compared the results in the other groups to the reference values.

ATP levels We studied ATP levels in CDs/y and CDr/y, representing the end-product of the mitochondrial respiratory chain, using the ATP Determination Kit (Life-Invitrogen), a bioluminescence assay for quantitation of ATP using firefly luciferase and its substrate D-luciferin. We homogenized liver tissues with PBSPI and lysed the cells using a non-denaturing low detergent buffer. After centrifugation, we measured ATP emission using a multi-detection microplate reader (BioTek- FLx800) and normalized the data for protein concentration.

Oxidative stress

Oxidative stress is a well-recognized pathophysiological mechanism in the development of diabetes (Hasnain, Prins, & McGuckin 2016), that has been associated with mitochondrial dysfunction. We studied the level of surrogates of oxidative stress in serum and in tissues of CDs/y and CDr/y provided RD and DD.

MDA We measured lipid peroxidation in serum, an indicator of oxidative stress, using malondialdehyde (MDA), a naturally occurring product of lipid peroxidation and the TBARS (TCA method) colorimetric assay kit (Cayman Chemical). We expressed the data as percent of MDA levels found in the reference non-diabetic CDr/y strain provided RD (CDr/y-RD).

SOD1, SOD2 As tissue indicators of oxidative stress, we measured the enzymes SOD1 and SOD2 protein levels in the pancreas. We extracted 50-80ug protein from each organ (as described above), ran the extracts on Mini-Protean TGX Stain-Free Any KD precast gels (Bio-Rad), transferred to PVDF membranes and blotted with the respective antibodies. For SOD1, we used Abcam ab13498 (1:4000) (18kDa), for SOD2 Abcam ab13534 (1:4000) (26kDa). We normalized the results by using beta-actin and the anti-beta actin antibody ab227387 (1:20,000), as housekeeping protein. We used densitometry with a GelAnalyzer 2010a (GelAnalyzer.jar) to quantitate the results which we expressed as percent of average values found in CDr/y-RD.

Statistics

Data are shown in the graphs as mean +/- standard error of mean. Between group analyses were carried out by one-way analysis of variance, and within group post-hoc analysis was by the Least Significance Difference (LSD) testing, using the Statistica software.

Competing interests

None

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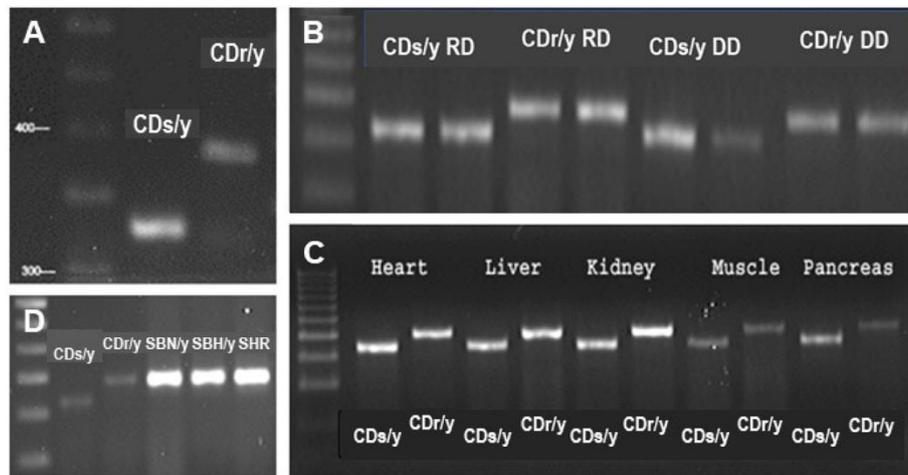
Figure 1 PCR products of *Ndufa4* full transcript cDNA amplification in (panel A) pancreatic extracts from CDs/y versus CDr/y provided RD, (panel B) pancreatic extracts from CDs/y and CDr/y provided RD or DD, (panel C) in extracts from other organs in CDs/y and CDr/y on RD and (panel D) pancreatic extracts from other unrelated rats strains.

CDr/y transcript:

ATGCTCCGCCAGATCCTCGGGCAAGCCAAGAAACATCCCAGC TTGATCCCTCTCTTCGTG
TTTATTGGAGCAGGGGGTACTGGAGCAGCACTGTATGTGATGCGCTTGGCATTGTTCAAT
CCAGATGTCAG CTGGGACAGGAAGAATAACCCAGAGCCTTGGAACAAACTGGGTCCCAAT
GAACAATATAAG TTCTATTCTGTGAACGTGGACTACAGCAAACTGAAAAAAGAAGGCCCA
GACTTCTAA

CDs/y transcript (with deletion of exon 3):

ATGCTCCGCCAGATCCTCGGGCAAGCCAAGAAACATCCCAGC TTGATCCCTCTCTTCGTG
TTTATTGG AGCAGGGGG TACTGGAGCAGCACTGTATGTGATGCGCTTGGCATTGTTCAAT
CCAG ATGTCAG CTGGGACAGG AAGAATAACCCAGAGCCTTGGAACAAACTGGGTCCCAAT
GAACAATATAAG TTCTATTCTGTGAACGTGGACTACAGCAAACTGAAAAAAGAAGGCCCA
GACTTCTAA

↑
STOP
CODON

Figure 2 Intact *Ndufa4* gene transcript in CDr/y (upper panel) and incomplete gene transcript in CDs/y with deletion of exon 3 (in light grey) causing a frame-shift mutation and leading to the formation of stop codon (lower panel).
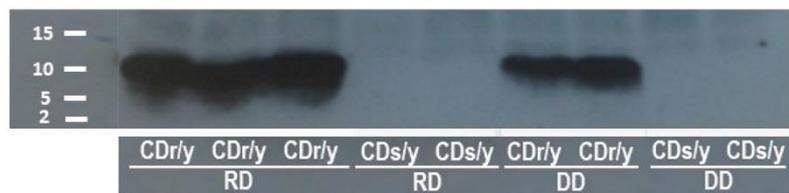

Figure 3 Expression of the NDUFA4 protein by western blotting. The protein is expressed in CDr/y but not in CDs/y; the level of expression is markedly greater in CDr/y fed RD than CDr/y provided DD.
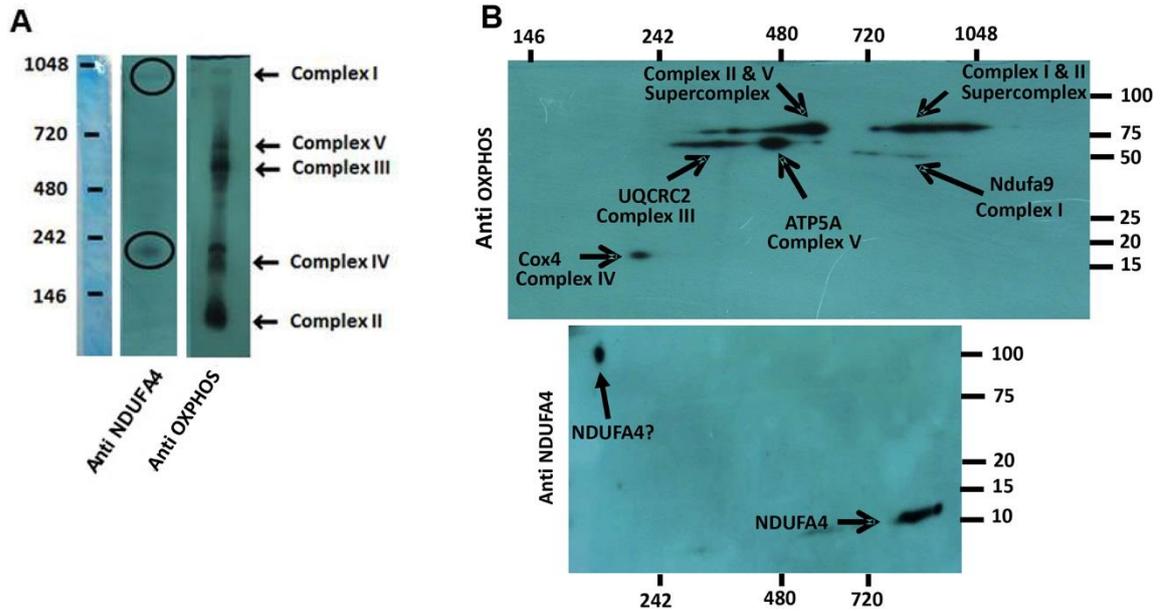
Figure 4 Localization of NDUFA4 by denaturing 1D western blot within the mitochondrial electron chain complexes I and IV (panel A) and by 2D blue native gels, with a MW of ~10 KD for NDUFA4 in complex I and a MW of 75-100 KD in complex IV (panel B).
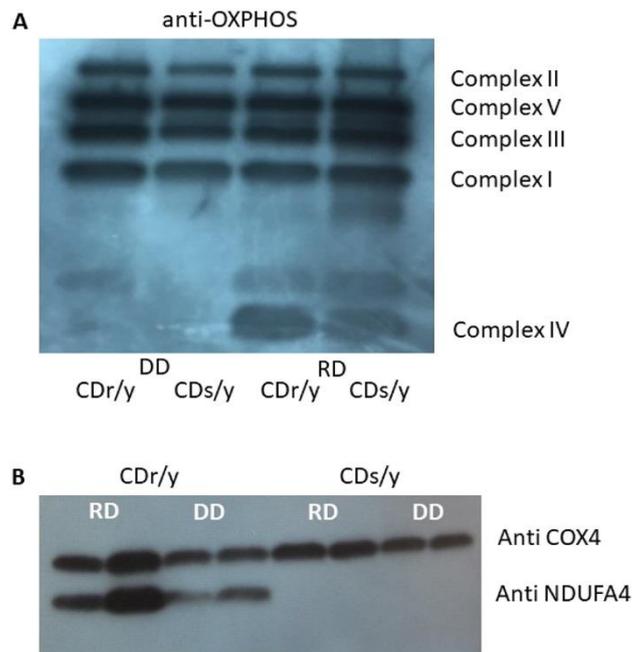
Figure 5 Anti-OXPHOS cocktail showing mitochondrial complex abundance in CDs/y and CDr/y provided RD or DD (panel A); there is similar expression of proteins in complexes I, II, III and V in CDs/y and CDr/y provided RD or DD, but lower abundance of complex IV expression in both strains when provided DD versus RD. This finding was confirmed with COX4, a component of complex IV, which expression is lower when fed DD versus RD, irrespective of strain (panel 5B).
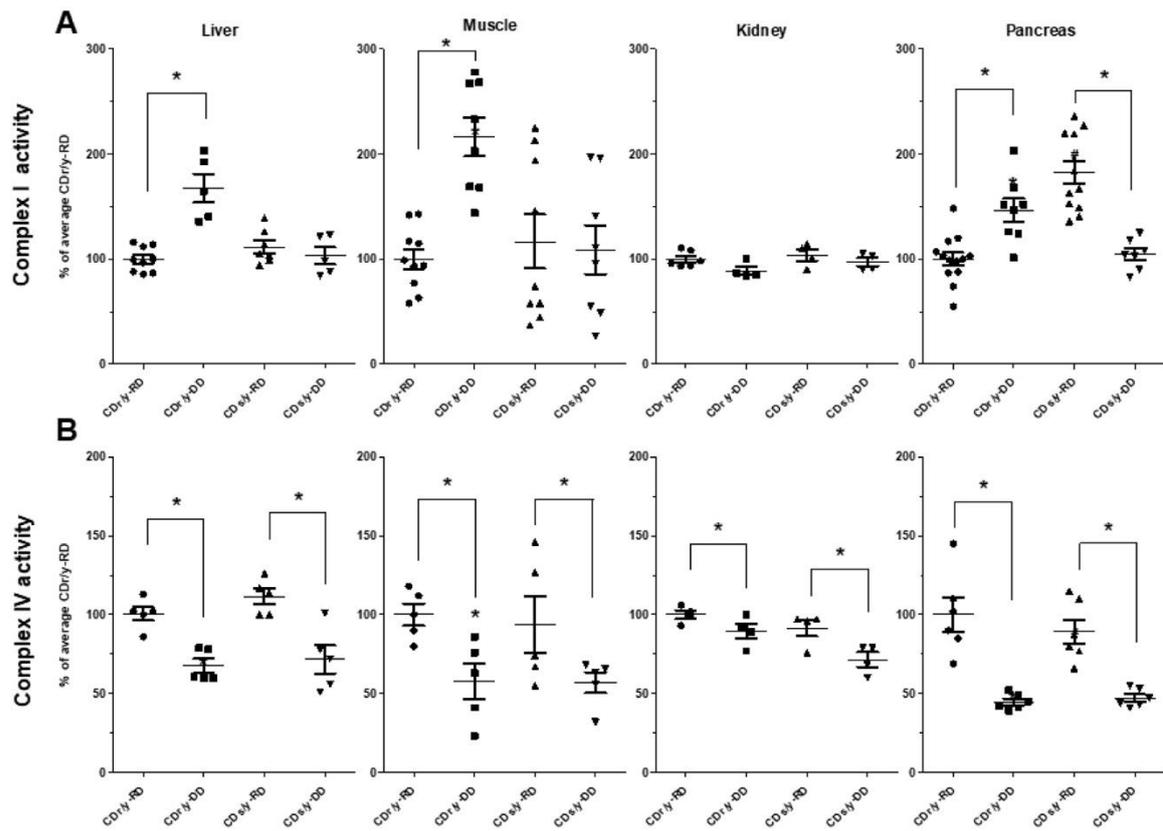
Figure 6 Activities of complex I (panel A) and IV (panel B) in the liver, muscle, kidney and pancreas of CDs/y and CDr/y on RD or DD. * p<0.01 by ANOVA and post-hoc LSD testing, DD versus RD within each strain; horizontal bars represent mean, and vertical bars SEM.
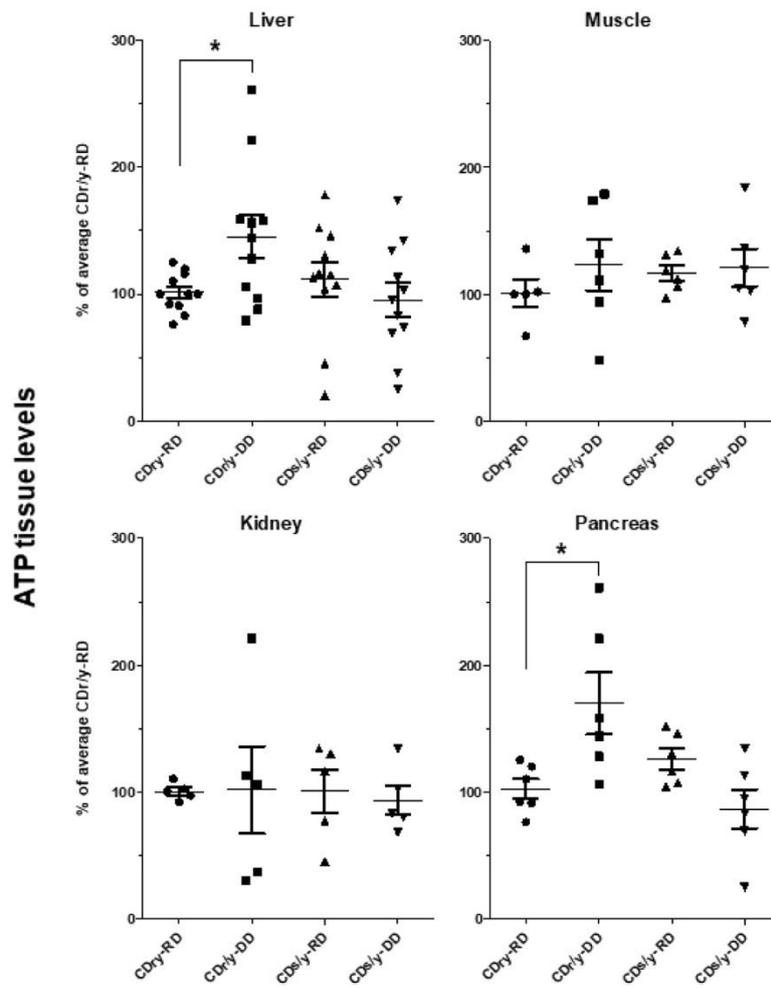
Figure 7 Tissue ATP levels measured in the liver, muscle, kidney and pancreas of CDs/y and CDr/y on RD or DD. * p<0.01 by ANOVA and post-hoc LSD testing, DD versus RD within each strain; horizontal bars represent mean, and vertical bars SEM.
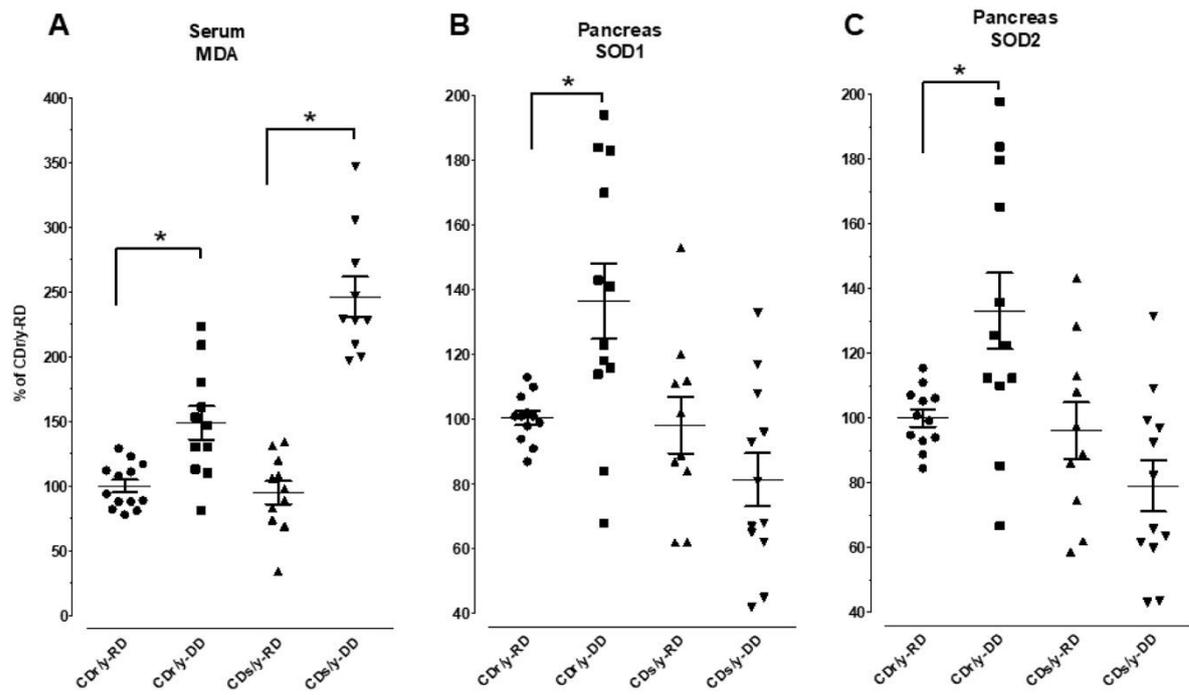
Figure 8 Serum MDA levels (panel A), and pancreatic protein levels of SOD1 (panel B) and SOD2 (panel C) in CDs/y and CDr/y on RD or DD. * p<0.01 by ANOVA and post-hoc LSD testing, DD versus RD within each strain; horizontal bars represent mean, and vertical bars SEM.