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Influence of intermittent fasting on prediabetes-induced neuropathy: Insights on a novel mechanistic pathway

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ABSTRACT

Aims: Peripheral neuropathy (PN) is correlated with obesity and metabolic syndrome. Intermittent fasting (IF) has been described as the cornerstone in the management of obesity; however, its role in prediabetic complications is not well elucidated. Cytochromes P450 Monooxygenases (CYP450) are major sources of Reactive Oxygen Species (ROS) that orchestrate the onset and development of diabetic complications. One of the CYP-metabolites, Expoxyecosatetraenoic Acids (EETs), are considered to be negative regulators of ROS production. In this study, we elucidated the role of IF on ROS production and investigated its influence on prediabetes-induced PN.
Methods: C57/BL6 control mice, prediabetic, prediabetic that underwent alternate day fasting with different diet composition, and prediabetic mice treated with EET-metabolizing sEH-inhibitor, AUDA. Body mass composition, metabolic, behavioral, and molecular tests were performed.
Results: High-fat diet (HFD) led to an increase in NADPH-induced ROS production; that was due to an alteration in the epoxygenase pathway assessed by the decrease in CYP1a1/1a2 expression. IF reinstated the homeostatic levels of EETs in HFD-fed mice. Moreover, treatment with AUDA mimicked the beneficial effect observed with IF.
Conclusion: IF and EETs bioavailability have a protective role in prediabetes-induced PN, suggesting a novel interventional strategy in the management of prediabetes and its associated complications.

1. Introduction

Diabetes mellitus (DM) is among the leading causes of death in the world. Prediabetes, also known as intermediate hyperglycemia, is an alarming precursor of type 2 diabetes [1–3]. In the past decade, prediabetes has gained momentous attention because emerging evidence highlighted the pivotal role it plays in the development of DM. It is estimated that every year around 5–10% of prediabetic patients become diabetic [4,5]. Moreover, according to the American Diabetes Association, up to 70% of patients with prediabetes will eventually develop diabetes [6]. Diabetic complications are divided into macro-vascular and micro-vascular categories, and recent studies have suggested that the long-term complications can begin to develop during the prediabetic state [7,8]. Of these complications, diabetic peripheral neuropathy

(DPN), which is the focus of our study, falls under the category of microvascular complications. Diabetic PN is the most common form of PN and is seen to develop in 50–70% of diabetic patients [9]. Several studies confirmed that patients with prediabetes have an elevated risk of developing PN even before the onset of diabetes [8]. Diabetes is well known to cause PN, and numerous studies report hyperglycemic related mechanisms of nerve damage. However, clinical studies have revealed an association of dyslipidemia with PN independent of hyperglycemia [10]. In accordance, high-fat-diet fed mice, a model of prediabetes and dyslipidemia, have been shown by numerous studies to develop a form of PN without overt hyperglycemia [10–17]. Given the increased likelihood of PN developing in diabetics, and the independent associations found between prediabetes, dyslipidemia and PN, it is favorable to adopt practices that prevent the progression of prediabetes into DM to decrease the potential burden on the affected population.

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Abbreviations

PN	Peripheral Neuropathy
IF	Intermittent Fasting
ADF	Alternate Day Fasting
EET	Expoxyecosatetraenoic Acids
HETE	Hydroxy-Eicosatetraenoic Acids
sEH	Soluble Epoxide Hydrolases
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
CYP450	Cytochromes P450 Monooxygenases
ROS	Reactive Oxygen Species
AUDA	12-(3-adamantan-1-yl-ureido)-dodecanoic acid
HFD-60	High Fat Diet of 60% fat
TG	Triglycerides
TC	Total Cholesterol
FFA	Free Fatty Acids
NEFA	Hepatic Lipase

Recent data suggests that IF may provide a breakthrough in the management of metabolic disorders and obesity [18–22]. However, data regarding its role in prediabetes and prediabetic complications remains scarce thus far. Since IF is more tolerable than strict diets and calorie restriction regimens, daily IF has been slowly increasing in popularity as an alternative [20,23]. Increasing evidence has suggested that IF might serve as a more convenient and potentially therapeutic approach for the management of diabetes. IF was shown to be successful in restoring pancreatic beta cells, enhancing health metabolic parameters, decreasing cardiovascular disease risk factors and ameliorating diabetic complications [24–32]. The mechanism of action by which IF exerts its metabolic role has yet to be elucidated.

The human body is constantly undergoing various reactions through multiple pathways that could lead to the overproduction of reactive oxygen species (ROS). ROS pathway has been described as the final common signaling pathway orchestrating the onset and development of diabetic complications, specifically PN [2,33–38]. Furthermore, dyslipidemia and hyperglycemia have been shown to disrupt the oxidant-antioxidant equilibrium by inducing additional ROS production [39–41]. Subsequently, the identification of cellular sources of ROS is central to understanding its related pathophysiology in diabetes. However, the relation between prediabetes-induced-neuropathy and oxidative stress has yet to be elucidated.

Cytochromes P450 monooxygenases (CYP450) are reported to be major sources of ROS in numerous tissues [42–44] with implications in diabetic complications [45,46]. CYP450 enzymes can be classified as epoxygenases or hydroxylases, which convert arachidonic acids into expoxyecosatetraenoic acids (EETs) or hydroxy-eicosatetraenoic acids (HETEs) respectively [47]. Epoxygenases are sub-grouped into the CYP1 and CYP2 isozymes [48,49]. In cases of injury, EETs are converted by soluble epoxide hydrolases (sEHs) excessively into a 20-fold less metabolically active form called dihydroxyeicosatrienoic acids (DHETs). CYP450 enzymes, especially those involved in the epoxygenase pathways controlling EETs production, are considered to be negative regulators of ROS production. Many experiments have reported the variations of EETs and HETEs in diabetes. We and others have previously shown that, the bioavailability of EETs decreases while that of HETEs increases in diabetes. It has also been established that oxidative stress, followed by these fluctuations, could play a key role in the pathogenesis of the diabetic complications [46,50]. Moreover, some studies highlighted beneficial effects of EETs in diabetes by reducing insulin resistance and decreasing beta cells destruction which help in regulating insulin sensitivity in diabetes [51].

Metabolically stable drugs that inhibit sEH have begun to emerge in the field of diabetic neuropathy and have shown promising outcomes in restoring the homeostatic balance in CYP450 mechanisms and ROS production. AUDA (12-(3-adamantan-1-yl-ureido) dodecanoic acid) inhibits the enzyme sEH which converts EETs to DHETs, increasing the physiological bioavailability of EETs. This increase is thought to play a major role in stabilizing the negative consequences of oxidative stress-related vascular damages [52–57]. We herein want to explore the cellular and molecular mechanistic pathways through which IF exerts its effect. Furthermore, we investigated the influence of IF on prediabetes-induced PN.

2. Materials and methods

2.1. In vivo design

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (Beirut, Lebanon) following the National Institutes of Health (NIH) animal care guidelines. Animals were kept in a temperature-controlled room and on a 12/12 - dark/light cycle and had standard chow and water access. Body weight and blood glucose levels changes were monitored once a week via tail vein punctures and a glucometer.

6-weeks-old C57BL/6J male mice (n = 30) were divided into 6 groups (n = 5/group): Control group, fed normal chow for 18 weeks (duration of the study) (Control), Prediabetic group fed a high fat diet of 60% fat (HFD-60) for 18 weeks, (Prediabetic), Prediabetic group fed a high fat diet of 60% fat for 8 weeks followed by an alternate day fasting (ADF) protocol combined with dietary fat reversal (14.1% fat intake) for 10 weeks (Prediabetic + ADF 14.1% fat), prediabetic group fed a high fat diet of 60% fat for 8 weeks followed by an ADF protocol combined with dietary fat reversal (30% fat intake) for 10 weeks, (Prediabetic + ADF 30% fat), prediabetic group, fed a HFD-60 for 8 weeks then underwent an ADF protocol while keeping the 60% fat intake for 10 weeks, (Prediabetic + ADF 60% fat) Prediabetic group, fed a HFD-60 for 8 weeks then were treated with AUDA (20 mg/kg – oral gavage) while keeping the 60% fat intake for 10 weeks, (Prediabetic + AUDA).

2.2. Alternate day fasting

The specific diet for each group was removed every other day around 10 a.m. for 24 h, during which access to water was only allowed *ad libitum*. The next day, food was provided and mice had unrestricted access to food for the following 24 h.

2.3. Drug administration

AUDA was dissolved in methylcellulose and DMSO (5%). It was administered orally with a gavage needle at a 20 mg/kg dose daily for 10 weeks while maintaining the 60% fat intake. In order to decrease the variables that could affect our results, the control group and the prediabetic group were also treated with the dissolving solution (methylcellulose + 5% DMSO) by oral gavage.

2.4. Food intake and energy efficiency

Throughout the study, we recorded the average amount of food in Kcal consumed by the different groups of mice weekly. Energy efficiency was calculated as weight gained for 100 Kcal of food consumed throughout the study.

2.5. Body mass composition

Fat mass, lean mass and free body fluid of the mice were documented non-invasively prior to sacrifice using the minispec body composition analyzer LF110 (Bruker). It uses a magnetic resonance technology and is based on Time Domain NMR.

2.6. Behavioral tests

Thermal algesia was assessed using the Hind paw withdrawal test. The IITC plantar algesia was used according to the manufacturer's protocol. The test is characterized by a heating beam set at an idle intensity of 2% and active intensity of 25% with a cut-off time set at 20s. The platform was set at 32 °C. The heating beam was targeted at the hind paw of animals and the time to sense the heat and withdraw their paws was recorded for analysis.

Motor coordination and balance assessment were performed via the raised beam walking test. Animals were placed on a platform with a rod of 1.2 cm diameter and 100 cm length lying 70 cm above a flat surface. First, to allow the mice to adapt, they were trained for 2 days to cross. On the third day, the time acquired to cross the beam, and the numbers of slips were recorded for analysis.

2.7. Sacrifice

Mice were sacrificed following a 10-week treatment at the age of 6 months. At sacrifice, blood was collected and was centrifuged at 3000 rpm for 15 min at 4 °C, and plasma was stored at −80 °C for further analysis. Sciatic nerves were flash frozen in liquid nitrogen and stored at −80 °C for further molecular studies.

2.8. Lipid profile analysis

Non-Esterified Fatty Acids, total Cholesterol levels and Triglyceride levels were measured through High Performance Liquid Chromatography (HPLC) test from the serum collected at sacrifice at the laboratory at AUB-MC.

2.9. HbA1c measurement

HbA1c was measured from blood samples collected from the mice models using Mouse HbA1c Kit (Catalogue #80310, Crystal Chem, USA), according to the manufacturer's instructions and normalized against total hemoglobin.

2.10. Protein extraction and Western blot

To extract proteins, sciatic nerves were lysed using a RIPA buffer containing 0.1% sodium dodecyl sulfate, 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol, 1% of the protease and phosphatase inhibitors and 1 mM phenylmethylsulfonyl fluoride. The lysates were left to rotate overnight at 4 °C. The following day, lysates were sonicated for 10 cycles 30s/cycle at 4 °C and then centrifuged at 13,600 rpm for 30 min at 4 °C. Protein concentration was measured using the Lowry Assay.

For immunoblotting, 20–40 μg of proteins were separated on 15% polyacrylamide gel electrophoresis (Bio-Rad, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad). The blots were blocked with 5% BSA (Sigma-Aldrich, Darmstadt, Germany) in Tris-buffered saline for 1 h and then incubated overnight with mouse polyclonal *anti*-CYP 1a1/1a2 (1:500, Detroit R&D, Michigan, USA), and mouse polyclonal *anti*-HSC70 (1:1000, Abcam). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:10000, Bio-Rad). Bands were visualized by the stain-free chemidoc imaging system (Bio-Rad). Densitometric analysis was performed using Image J software.

2.11. ROS generation: Detection of superoxide via HPLC

The HPLC-based assay allows separation of superoxide-specific EOH from the nonspecific ethidium. Quantification of dihydroethidium (DHE), 2-hydroethidium (EOH), and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by ultraviolet absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

2.12. NADPH oxidative activity assay

Proteins were extracted from sciatic nerves and suspended in lysis buffer (20 mM KH_2PO_4 (pH 7.0), 1 mM EGTA, 1 mM phenyl-methylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). To start the assay, 20 μg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Photon emission expressed as relative light units was measured every 30 s for 5 min in a luminometer. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent.

2.13. Statistical analysis

Results were expressed as mean ± SD from multiple independent experiments. Statistical significance was assessed by one-way ANOVA and subgroup analysis was done using the Fisher-LSD method. *$p < 0.05$ *vs*. control and #$p < 0.05$ *vs*. prediabetic.

3. Results

3.1. ADF reverses metabolic parameters and lipid profile in prediabetic mice

Prediabetes is associated with a wide range of alterations in the metabolic parameters. Changes in glucose levels and lipid metabolism were evaluated in all mice. Our data show that ADF reduces the weight gain induced by high calories 60% fat feeding (Table 1). This was associated with a decrease in the random blood glucose levels along with a reduction in the prediabetes increased HbA1c levels. Furthermore, total cholesterol (TC) levels, triglyceride (TG) levels, and levels of the non-esterified fatty acids (NEFA), released from TG by the actions of lipoprotein lipase and hepatic lipase (NEFA), and are elevated in blood of prediabetic mice. ADF whether combined to a 14.1%, 30% or 60% fat diet reduces the levels of TC, TG and NEFA. Of interest, treatment of the prediabetic mice on 60% fat with AUDA did not show any effect on the measured metabolic parameters (Table 1).

3.2. Mice on the fat reversal regimen showed a decrease in food intake

The average amount of food in Kcal consumed by the different groups of mice was recorded weekly. Mice that underwent an ADF protocol with dietary fat reversal to 14.1% fat or 30% fat showed significant decrease in food consumption in comparison to the prediabetic mice, but no statistical significance was observed in mice that underwent an ADF protocol with 60% fat high fat diet regimen and those treated with AUDA. (Fig. 1A).

3.3. Mice on ADF protocol with 60% fat exhibited lower energy efficiency

Energy efficiency was calculated as weight gained for 100 Kcal of food consumed. Our results suggest that the total energy efficiency was significantly decreased in the prediabetic mice managed with ADF that were maintained on a high fat diet regimen with 60% fat while no statistical significance was observed in other groups (Fig. 1B).

Table 1
Functional parameters.

Metabolic Parameters +Lipid Profile Analysis	Control	Prediabetic	Prediabetic + ADF (14.1%)	Prediabetic + ADF (30%)	Prediabetic + ADF (60%)	Prediabetic + AUDA
Body weight (g)	27.5 ± 0.2	34.3 ± 3.0 *	24.6 ± 1.1 #	28.6 ± 2.2 #	29.2 ± 2.1 #	32.7 ± 1.9 *
Random Glucose (mg/dL)	151 ± 15.7	200 ± 11.7 *	144 ± 9.3 #	157 ± 5.0 #	171 ± 8.7	182 ± 13.5
HbA1c (%)	4.2 ± 0.1 (22 mmol/mol)	4.8 ± 0.1 * (29 mmol/mol)	4.6 ± 0.1 (27 mmol/mol)	3.8 ± 0.4 # (18 mmol/mol)	4.1 ± 0.1 # (21 mmol/mol)	4.4 ± 0.1 (25 mmol/mol)
Non-esterified Fatty Acids (mM)	0.68 ± 0.03	1.18 ± 0.05 *	0.66 ± 0.05 #	0.74 ± 0.05 #	0.72 ± 0.03 #	1.18 ± 0.05 *
Total Cholesterol (mM)	2.2 ± 0.1	6.36 ± 0.1 *	3.34 ± 0.1 #	3.52 ± 0.1 #	4.38 ± 0.2 #	5.84 ± 0.1 #*
Triglyceride levels (mM)	0.48 ± 0.03	0.97 ± 0.04 *	0.38 ± 0.03 #	0.52 ± 0.06 #	0.62 ± 0.03 #	1.95 ± 0.04 *

*p < 0.05 compared to control.
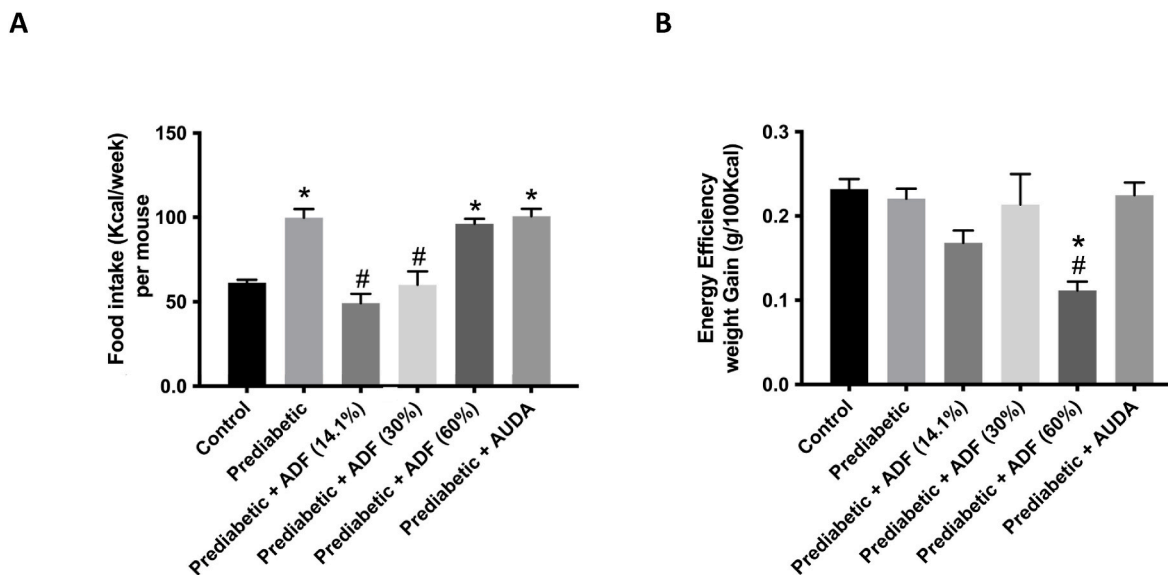#p < 0.05 compared to prediabetic.

Fig. 1. Effect of ADF on caloric intake and energy efficiency (A) Food consumption. **(B)** Energy efficiency. Values are the mean±SD from five different mice in each group (n = 5). *p < 0.05 vs control #p < 0.05 vs prediabetic.

3.4. Body mass composition ADF protocol reversed body mass composition parameters

Body composition assessment upon high fat feeding show a significant increase in body weight and in fat mass percentage in the prediabetic group when compared to their control littermates (Fig. 2A and B). In contrast, lean mass percentage was reduced in the prediabetic group of mice when compared to the control, while the percentage of free fluids did not differ between the two groups (Fig. 2C and D). Interestingly, when prediabetic mice followed the ADF protocol combined to a dietary restriction intake of 14.1% or 30% fat, or were kept on 60% fat, body weight and fat percentage (Fig. 2B) were reduced, while the lean mass percentage (Fig. 2C) was increased. Furthermore, the percentage of free fluids was only significantly decreased in the prediabetic mice that had a dietary intake reversal to 14.1% fat accompanied by ADF (Fig. 2D). As for the prediabetic mice that were treated with AUDA, we did not observe any change in all the body mass composition parameters assessed when compared to the prediabetic mice group (Fig. 2).

3.5. ADF protocol and AUDA treatment restored thermal algesia and motor dysfunction

Increasing evidence has shown that prediabetes is associated with the development of sensory deficits reflecting nerve injury in the peripheral nervous system [10–17]. We used the hind paw withdrawal test to assess thermal algesia in the prediabetic mice as well as the

prediabetic mice that underwent a 24 h ADF protocol with different fat percentages (14.1%, 30%, and 60%). The results of this test show that prediabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws. By contrast, prediabetic mice on ADF regimen with either 14.1%, 30% or 60% fat intake had a significantly lower latency suggesting that the ADF restored thermal algesia. Increase in the physiological availability of EETs using the pharmacological sEH inhibitor AUDA mimicked the effect of ADF on mice with or without dietary fat reversal (Fig. 3A).

PN is also associated with motor dysfunctions. To further confirm the severity of prediabetes-induced PN, we used the beam-walking test. The data from the test showed a relatively longer period of time for the prediabetic mice to cross the beam with an increased tendency to slip in comparison to their controls that seemed to cross with minimal setbacks. Notably, prediabetic mice that were subject to ADF regimen with (60% fat) or with ADF with dietary fat reversal (14.1% or 30% fat) showed a significant improvement in their assessed motor dysfunction and performed similar to the control group (Fig. 3B and C). A similar pattern was observed in mice treated with AUDA (Fig. 3A, B, C).

3.6. ADF protocol and AUDA treatment curbed superoxide generation and NADPH oxidase activity

ROS production was shown by our group and others to be associated with diabetes induced microvascular complications [46,50]. To determine if IF regulates ROS production through a NADPH-dependent
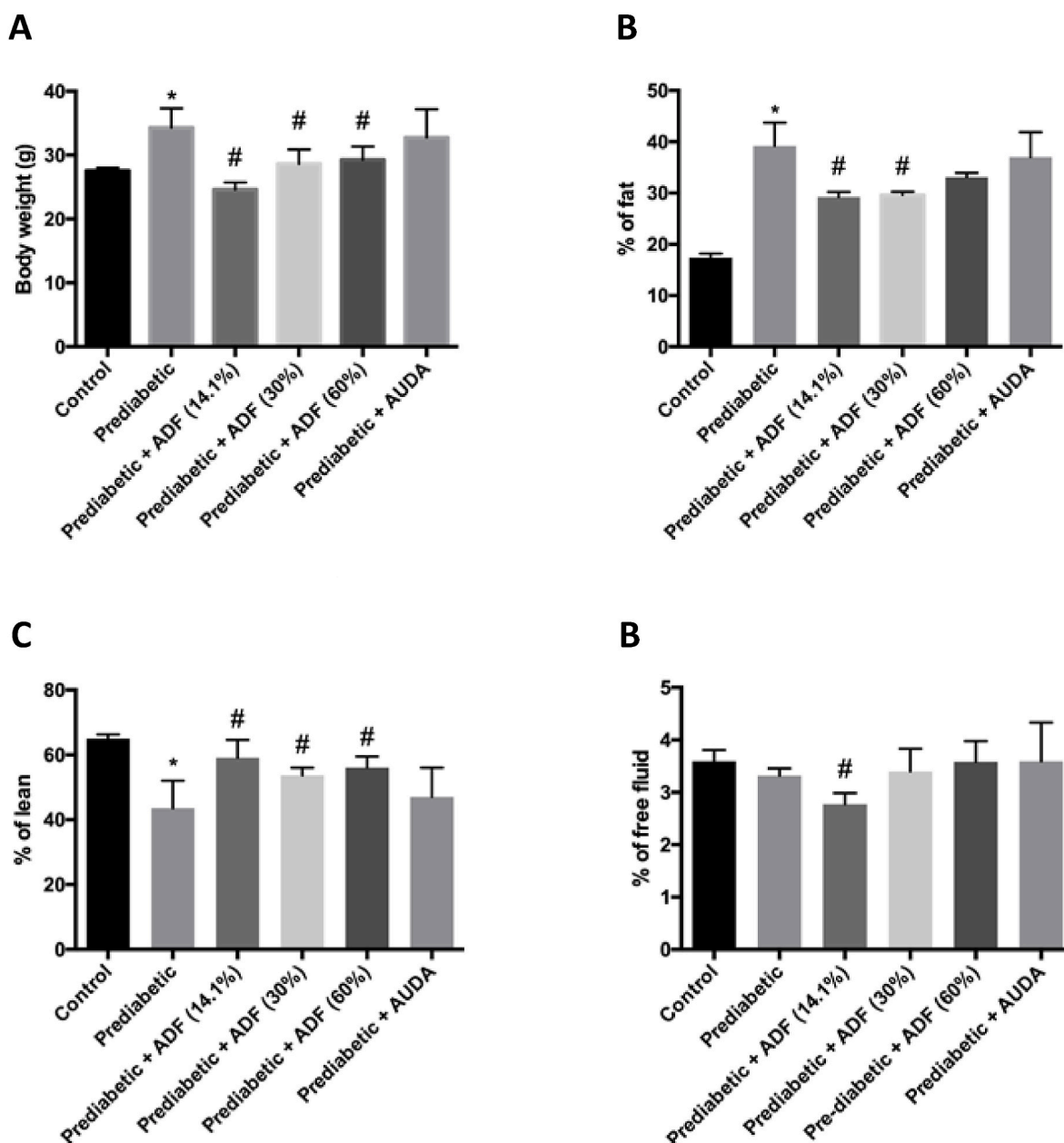
A

B

C

B

Fig. 2. Body mass composition analysis at the end of the study (A) Body weight (g). **(B)** Percentage of fat. **(C)** Percentage of lean mass. **(D)** Percentage of free fluid. Values are the mean±SD from five different mice in each group (n = 5). *$p < 0.05$ vs control #$p < 0.05$ vs prediabetic.

mechanism, we assessed superoxide production in the sciatic nerves isolated from the different groups of mice. Our data show that superoxide generation and NADPH oxidase activity are markedly increased in the prediabetic mice (Fig. 4A and B). These observations are reversed with the IF regimen whether accompanied with dietary fat reversal (14.1% or 30% fat intake) or not (60% fat intake) (Fig. 4A and B). In parallel experiments, our data show that prediabetic mice on a 60% fat diet have a decrease in ROS production and NADPH oxidase activity when treated with AUDA.

3.7. ADF regimen with 60% fat intake and AUDA treatment restored CYP1a expression

To further dissect the mechanism, CYP450 enzymes are known to require the cofactor NADPH as the source of electrons and an additional enzyme, CYP450 reductase. Our laboratory has previously identified CYPs as major source of ROS generation. However, the role of CYPs in

prediabetes-induced neuropathy is not yet described. Our data show that CYP1a1/1a2, a major cytochrome P450 subfamily of the EET-producing epoxygenase pathway, is significantly reduced in the isolated sciatic nerves of prediabetic mice when compared to their control littermates. This decrease in the protein expression of CYP1a1/1a2 is partially reversed with the ADF regimen accompanied with dietary fat reversal (14.1% or 30% fat intake) and significantly restored with 60% fat intake. As expected, treatment with AUDA, restored the homeostatic expression of CYP1a1/1a2 protein (Fig. 5).

4. Discussion

Prediabetes is associated with dyslipidemia, intermediate hyperglycemia and increased levels of HbA1c [58]. Our mice exhibited blood glucose concentrations and HbA1c levels that fit the diagnosis criteria for prediabetes. IF was successful in reversing these levels to values comparable to that of the controls. However, AUDA did not reverse
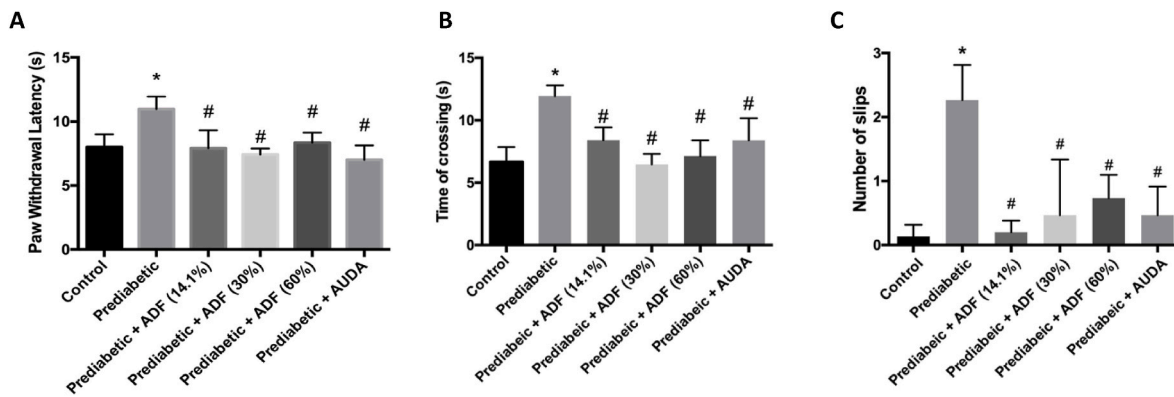
Fig. 3. Effect of ADF and AUDA on behavioral phenotypes using sensory and motor tests (A) Plantar algesia test presented as time to paw withdrawal in seconds. Beam walking test presented as **(B)** Time to cross the beam in seconds, and **(C)** Number of slips. Values are the mean±SD from five different mice in each group (n = 5). *p < 0.05 vs control #p < 0.05 vs prediabetic.
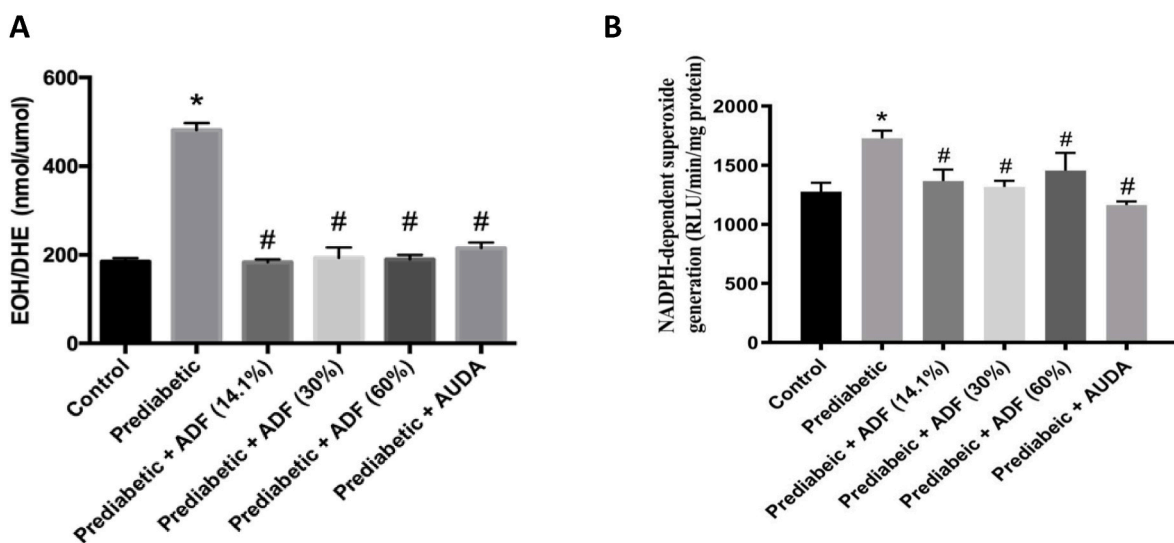

Fig. 4. Effect of ADF and AUDA on ROS and oxidative states (A) ROS production in sciatic nerve by HPLC (n = 3). **(B)** NADPH oxidase activity assay on sciatic nerve samples (n = 5). *p < 0.05 vs control #p < 0.05 vs prediabetic.

blood glucose levels which remained relatively high till the sacrifice. This may be due to AUDA which is described to lack hypoglycemic potential. This suggests that the improvements associated with AUDA are attributed to its physiological action in the CYP450 pathways rather than the effect of normalization of blood glucose levels.

Lipid profile analysis shows significant increases in the levels of NEFA, TC and TG levels in the prediabetic state, confirming the manifestation of dyslipidemia. These levels were restored when mice underwent a protocol of IF with 60% fat and dietary reversal with 14.1% and 30% fat. In line with these observations, body mass composition analysis showed a similar pattern. Body weight was significantly increased in our prediabetic model. Interestingly, all mice treated with ADF whether with a low-fat (14.1%), an intermediate-fat (30%) or a high-fat diet (60%) exhibited significant decrease in body weight when compared to the prediabetic mice. ADF successfully maintained this weight reduction even when the diet was sustained at 60% fat content.

The percentage of fat mass levels was also significantly increased in the prediabetic state. This increase was restored upon management with ADF implicating that the beneficial effects of IF are not prompted by the limitation of food intake and its associated weight decrease but rather to its action on physiological pathways. Given that the food intake of prediabetic and prediabetic animals on an ADF regimen with a high-fat diet (60%) was comparably similar, the observed decrease in fat mass

percentage compared to prediabetic mice implies that IF significantly increases fat metabolism. Additionally, prediabetes significantly decreased the percentage of lean mass which was restored upon management with ADF. Our findings lend support and show that prediabetic mice treated with AUDA exhibit similar phenotype with regards to food intake, body weight and energy efficiency compared to the prediabetic group, both of which receiving the same fat composition in their diet (consisting of 60% fat) [59]. This provides evidence that IF may be effective in restoring and maintaining lean mass during weight loss. Our findings are consistent with data from literature in which that pharmacological inhibition of sEH are ineffective in altering caloric intake. In summary, ADF was associated with a decrease in percentage of fat mass and an increase in percentage of lean mass, suggesting that on fasting days the body breakdowns fat first for energy and stores lean mass as a defense mechanism even with a high-fat diet.

Moreover, prediabetes can be associated with nerve injury and damage. In the Plantar Algesia Test, it is expected that mice with no peripheral nerve injury are capable of thermal sensation and withdrawal of the paw prior to tissue damage, while those with peripheral nerve injury lack sensation [17]. Indeed, the results show that prediabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws compared to the controls, in which the sensation was significantly restored upon management with ADF and sEH
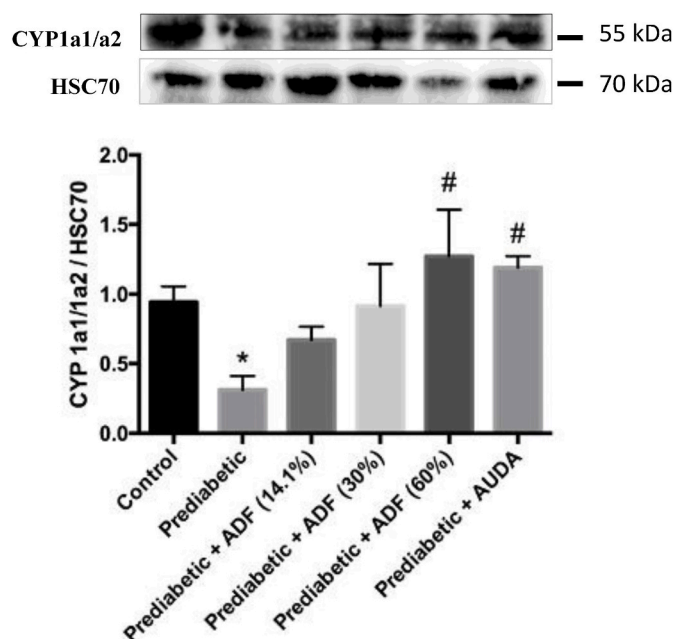
Fig. 5. Protein expression levels of CYP1a1/1a2 in mice sciatic nerve. Representative Western blot of CYP1a1/1a2/HSC70 with representative densitometric quantification in mice sciatic nerve (n = 5).

inhibition by AUDA. As for the Beam Walking Test, used to assess motor function and balance, the performance of prediabetic mice was poor relative to control mice, which was reversed after ADF and sEH inhibition by AUDA. The findings of the two tests imply impaired sensorimotor dysfunctions within the peripheral nerves. This is in line with what was previously reported in literature that prediabetes could also induce peripheral nerve injury [10–17].

Furthermore, growing evidence in the literature suggests a solid implication of oxidative stress and increased ROS production as prime instigators of diabetic complications [33,60,61]. To ensure this liability, we measured ROS generation levels by the HPLC test and NADPH oxidase activity assay. ROS production and NADPH oxidase activity were both significantly increased in the prediabetic state and reduced upon management and treatment. Moreover, numerous studies in the field reported considerable alterations in the arachidonic acid metabolism, mainly the CYP450 pathways. Our previous work in the lab linked diabetic nephropathy with a decrease in EETs [46,50]. Findings from our study were novel in that they suggest a significant decrease in CYP1a expression in prediabetic-induced PN models. ADF with dietary fat reversal to 14.1% or 30% fat or ADF with 60% fat were successful in reversing these molecular disturbances and restoring homeostatic balances. This entails that ADF succeeded in attenuating the alterations associated with ROS production which alleviated PNS injury and nerve damage. To further confirm the key role of EETs, prediabetic mice were treated with AUDA, an sEH inhibitor, that increases physiological availability of EETs. sEH inhibition has been previously described to reverse some of the alterations associated with oxidative stress in neuropathy [55–57]. We speculate that beyond its effect on potentiating CYP450 activity, IF can also inhibit the activation of other ROS generating enzymes. This is concomitant with data from the literature describing the role of IF in alleviating oxidative stress. Hence, this provides a plausible explanation of the observed non-significant difference of ROS production between IF groups while its effect on CYP1a1/a2 expression and other parameter differ significantly [62,63]. Our data using AUDA mimicked the beneficial effect observed with IF sustaining the role of ADF in a novel mechanistic pathway that involves CYP450

enzymes.

Taken together, these findings showed that prediabetes leads to increased ROS production through NADPH upregulation and CYP1a downregulation. Dyslipidemia-induced ROS generation and EET reduction resulted in PN manifested by abnormal sensorimotor response. ADF with high-fat diet of 60% fat or with dietary fat reversal to 14,1% or 30% fat show beneficial effects on the pathogenesis of prediabetes and reversed the associated damages. This suggests that the action of fasting in itself rather than the restriction of food intake was successful in restoring and normalizing the deleterious consequences of prediabetes.

5. Conclusion

To sum up, our study successfully described a novel pathway in the pathogenesis of prediabetic neuropathy. Dyslipidemia and intermediate hyperglycemia altered the CYP450 pathway by decreasing EETs bioavailability. These alterations might serve as plausible instigators of ROS overproduction. Moreover, our results suggest that oxidative stress is one of the final common signaling pathways orchestrating myelination alterations leading to nerve injury and damage in a prediabetic state. Furthermore, our study confirmed that upon management with IF, we are reconditioning metabolic, molecular and phenotypic modifications back to homeostatic balance.

CRediT authorship contribution statement

Maya Dannawi: Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Mansour E. Riachi:** Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Antony F. Haddad:** Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Mohamed El Massry:** Methodology, Visualization, Writing – review & editing. **Mary Haddad:** Methodology, Visualization, Writing – review & editing. **Pamela Moukarzel:** Writing – original draft, Writing – review & editing. **Frédéric Harb:** Formal analysis, Writing – review & editing. **Hilda E. Ghadieh:** Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Assaad A. Eid:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

Authors report no conflict of interest.

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