



NEWS & VIEWS

Does Oral Supplementation of a Fermented Papaya Preparation Correct Respiratory Burst Function of Innate Immune Cells in Type 2 Diabetes Mellitus Patients?

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Abstract

Fermented papaya preparation (FPP) is a nutritional supplement reported to act as an antioxidant by scavenging reactive oxygen species (ROS) and removing “bad ROS,” while inducing “respiratory burst” production of necessary “good ROS.” We sought to investigate the safety of oral administration of FPP (9 g/day, 6 weeks) to T2D patients with regard to its effect on the hyperglycemia status of these patients. Peripheral blood was collected during a baseline visit, followed by subsequent collections both during and after supplementation. Induced “respiratory burst” ROS production was measured at each visit in addition to fasting blood glucose, lipid profile, glycated hemoglobin (HbA1c), and lipid/protein peroxidation. Oral FPP supplementation induced “respiratory burst” in peripheral blood mononuclear cells while not influencing other blood parameters studied. When human monocytic THP-1 cells were supplemented with sugar-based FPP, cellular ATP and NADPH concentrations were increased while matched glucose alone did not produce similar effects, suggesting a glucose-independent component of FPP to be responsible for increasing cellular energetics. THP-1 cells supplemented with FPP also exhibited higher mitochondrial membrane potential ($\Delta\psi/m$) and oxygen consumption as compared with cells treated with glucose alone. Taken together, our observations lead to the hypothesis that FPP corrects inducible “respiratory burst” function in type 2 diabetes patients. *Antioxid. Redox Signal.* 22, 339–345.

Introduction

FERMENTED PAPAYA PREPARATION (FPP) is a product of yeast fermentation of *Carica papaya* Linn (1). FPP, a granular substance available over the counter, is known to possess antioxidant properties (1). FPP is rich in amino acids and carbohydrates. Chronic wounds represent a major public health problem in diabetics (8). Recently, our laboratory reported the first experimental evidence demonstrating that FPP may improve diabetic wound outcomes by specifically influencing the response of wound-site macrophages and the subsequent angiogenic response (3). Furthermore, our *ex vivo* work demonstrated that FPP may correct deficiencies in respiratory burst capacity of peripheral blood mononuclear cells (PBMC) of type 2 diabetes mellitus (T2D) patients (6). The objectives of this study

Innovation

T2D patients are known to suffer from compromised respiratory burst function, making them vulnerable to infection-related complications. This work presents evidence from a clinical study demonstrating that a safe natural nutritional supplement may bolster respiratory burst function of peripheral blood mononuclear cells in T2D patients. Previous works show that as an antioxidant, fermented papaya preparation (FPP) may fight bad reactive oxygen species (ROS). This work demonstrates that FPP may bolster “good ROS” used to fight pathogens (7). Furthermore, this work supports the notion that FPP may fuel mitochondrial respiration and promotes cellular ATP generation while not adversely affecting the hyperglycemic status of T2D patients.

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TABLE 1. PATIENT POPULATION DEMOGRAPHICS

Total recruitment (<i>n</i>)	22
Completed study (<i>n</i>)	17
Age (years)	56.4 ± 12
Gender	
Males	12
Females	10
Ethnicity	
White	6
Black	12
Asian	4
HbA1c (%)	7 ± 0.8
BMI (kg/m ²)	32.8 ± 4.9

were twofold: (i) to test whether FPP (~90% carbohydrates) is safe for consumption by T2D patients without adversely affecting their hyperglycemia status; and (ii) to determine whether oral FPP supplementation may improve “respiratory burst” activity in PBMC of T2D patients.

FPP is safe for T2D patient consumption

FPP possesses potent antioxidant activity (1). Our studies with T2D subjects demonstrated a beneficial effect of the

supplement *ex vivo* on monocyte respiratory burst activity (6). This study was performed to determine whether FPP can confer a similar beneficial effect on monocytes when supplemented orally to T2D patients. In order to test the effect on monocytes *in vivo*, it is essential to determine first whether FPP (~90% carbohydrates) supplementation is tolerated well by T2D patients with regard to their hyperglycemic status and lipid profile. Peripheral blood was collected from T2D patients from the OSU Diabetes Clinics (Table 1). T2D patients were supplemented for 6 weeks with FPP (9 g/day; three sachets a day) followed by a 2 week washout period. Fasting glucose, glycated hemoglobin (HbA1c), and cholesterol levels were measured at baseline (0 weeks), during supplementation, and postwashout. FPP supplementation did not influence blood glucose or total lipid profile, suggesting that FPP can be safely consumed by T2D patients at three sachets per day for 6 weeks (Fig. 1). Long-term effects on the hyperglycemic status, as evident by HbA1c levels, remain unchanged during the period of the study (Fig. 1). FPP has a long track record of safe human consumption (1). This study is unique in that for the first time, the effects of FPP on blood glucose, HbA1c, and total lipids were investigated in T2D patients. These findings establish that despite its sweet taste and carbohydrate composition, FPP is well tolerated by T2D patients.

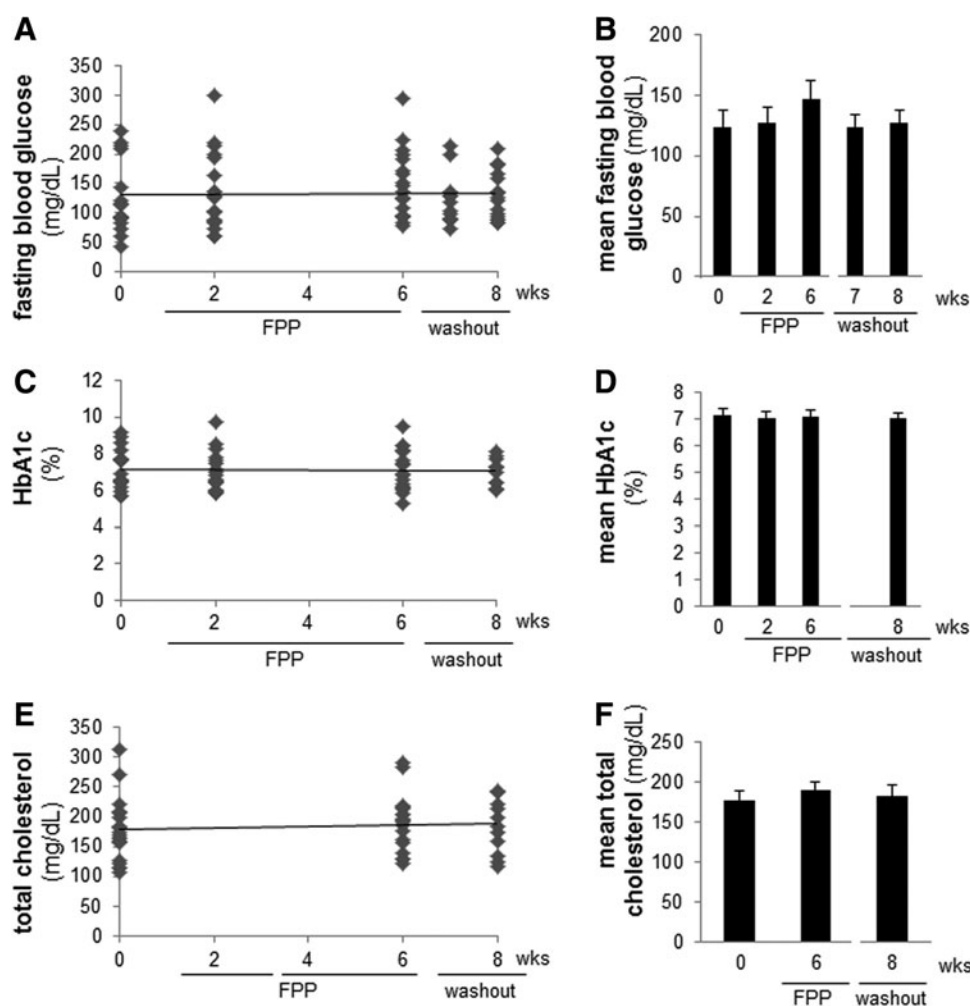


FIG. 1. FPP is safe for T2D patient consumption. Oral supplementation of FPP did not influence fasting blood glucose, glycated hemoglobin (HbA1c), or total cholesterol levels in T2D subjects. Human T2D patients were supplemented for 6 weeks with FPP (9 g/day) followed by 2 weeks of washout. Fasting glucose (A, B), glycated hemoglobin (HbA1c) (C, D), and cholesterol levels (E, F) were measured at baseline (BL), during supplementation and washout. Data are mean ± SEM (*n* = 15). FPP, fermented papaya preparation.

Improved inducible “respiratory burst” activity in T2D monocytes

Ex vivo treatment of FPP to PBMC has been shown to rescue compromised respiratory burst in PBMC of T2D patients (6). To determine whether oral supplementation of FPP may improve monocyte “respiratory burst” activity, PBMC were collected from T2D subjects at the baseline (0 weeks) visit, 2 and 6 weeks after the start of supplementation of FPP, and after 1 and 2 weeks of washout. Reactive oxygen species (ROS) production during “respiratory burst” in PBMC were measured *via* chemiluminescent detection of superoxide anion ($O_2^{\bullet-}$) after stimulation with phorbol 12-myristate 13-acetate (PMA; 1 μ g/ml). ROS production was significantly higher after FPP supplementation (Fig. 2A). Previously, the supplementation of antioxidant alpha-tocopherol has been reported to decrease unstimulated superoxide anion release by monocytes (5). To determine whether such increased NADPH oxidase-mediated ROS production by FPP resulted in augmented systemic oxidative stress, the effect of FPP on protein carbonyls and the lipid peroxidation marker 4-hydroxynonenal (4-HNE) were measured in blood plasma. Though FPP improved inducible “respiratory burst” ROS in T2D patients, supplementation of FPP did not adversely influence oxidative stress over the period of supplementation (Fig. 2B, C). Oral intake of 6 g FPP/day for a period of 14 weeks was observed to significantly lower the rate of hemolysis and accumulation of protein carbonyls in the blood plasma of prediabetics (9). Based on these observations, we hypothesize that FPP supplementation may improve innate immune response in diabetic patients.

FPP induces cellular ATP and NADPH production

Phosphorylation of the NADPH oxidase subunit p47(PHOX) may occur during the activation of NADPH oxidase in intact cells. We have previously demonstrated that FPP upregulates phosphorylation of p47(PHOX) in PBMC treated with FPP *ex vivo* (6). ATP is required for such phosphorylation reactions. ATP levels were observed to be 75% higher in human monocytic THP-1 cells that were cultured in normal glucose media [gluc(norm)] supplemented with FPP compared with a gluc(norm) alone group, suggesting that FPP may enhance ATP

generation in monocytes (Fig. 3A). Increased ATP levels were independent of extra glucose found in the supplement, as seen in the lack of observed increase in the high glucose [gluc(high)] group. Thus, a nonglucose component of FPP is responsible for the upregulation of intracellular ATP levels. The leukocyte NADPH oxidase catalyzes the reduction of oxygen to $O_2^{\bullet-}$ at

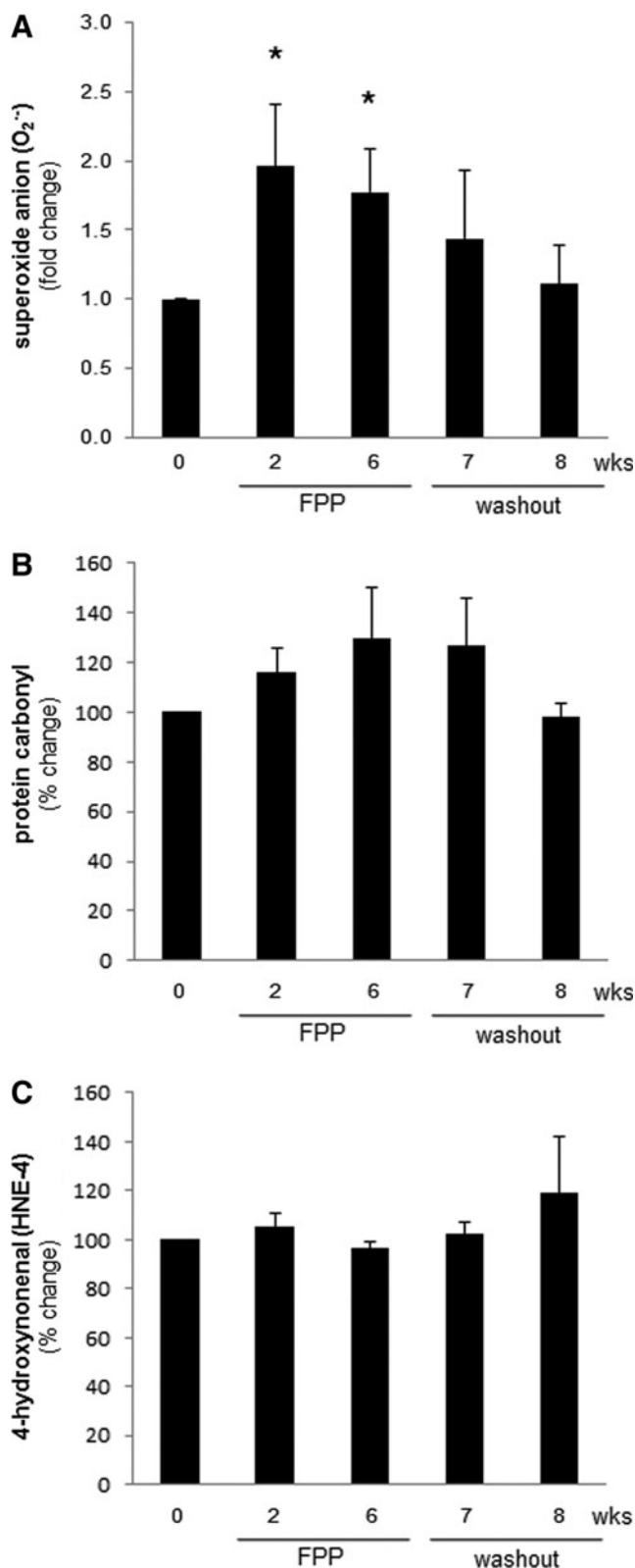


FIG. 2. Oral supplementation of FPP improved inducible “respiratory burst” activity in T2D monocytes. (A) PBMC were isolated from T2DM donors. PBMC were collected at baseline (0 weeks), at 2 and 6 weeks of FPP supplementation, and at 1 and 2 weeks of washout. Superoxide anion production was measured after PMA (1 μ g/ml) stimulation for 30 min. Data are expressed as fold change compared with the baseline (0 weeks). Data are mean \pm SEM ($n=14$). * $p<0.05$. Fresh plasma was flash frozen in liquid nitrogen for storage until the cohort was collected. All samples were measured at the same time. (B) Protein carbonyls were measured using ELISA-based assay. Each time point represents the average percent change for each visit as compared with the baseline (0 weeks) \pm SEM ($n=10$). (C) 4-HNE was measured using ELISA-based assay. Each time point represents the average percent change for each visit as compared with the baseline (0 weeks) \pm SEM ($n=17$). 4-HNE, 4-hydroxynonenal; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate.

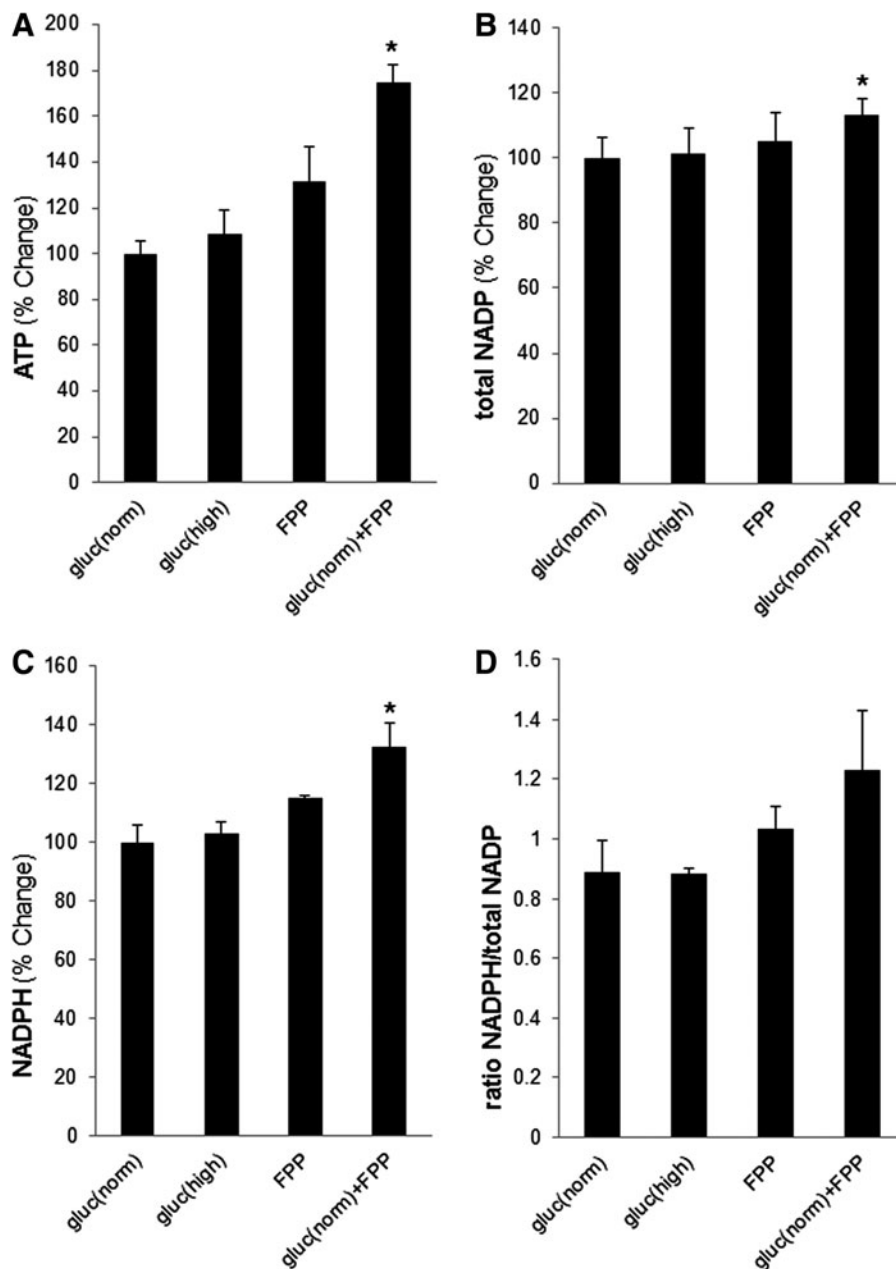


FIG. 3. FPP induces cellular ATP and NADPH production.

Human monocytic THP-1 cells were deprived of glucose for 48 h followed by incubation for 24 h under normoglycemia [gluc(norm), 2 mg/ml w/v glucose/GF media], FPP (2.9 mg/ml w/v FPP/GF media), hyperglycemia [gluc(high), 3.9 mg/ml w/v glucose/GF media], or gluc(norm)+FPP conditions. Cellular ATP (A), NADP (B), and NADPH (C), levels were determined via ELISA. Ratio of NADPH/total NADP (D) favors NADPH with FPP supplementation. Data represent percent change compared with gluc(norm) group \pm SEM ($n=4$). * $p<0.05$ compared with gluc(norm). GF, glucose free.

the expense of NADPH. Intriguingly, treatment of THP-1 cells with FPP resulted in increased (30%) levels in cellular NADPH levels while only marginally increasing total NADP (Fig. 3B, C). A marked shift in the ratio of NADPH/NADP toward NADPH was observed, suggesting that the increased NADPH oxidase-mediated “respiratory burst” dependent ROS production in T2D may be supported by increased cellular ATP and NADPH levels (Fig. 3D). Taken together, these observations indicate that FPP, by increasing cellular ATP and NADPH levels, promotes NADPH oxidase activity.

Elevated mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi/m$) measurements were performed using membrane-permeant JC-1 dye. Monocytic cells cultured under normoglycemic conditions supplemented with FPP had $\sim 20\%$ increase in $\Delta\psi/m$ compared with

cells cultured under normoglycemic conditions alone (Fig. 4B). To confirm our hypothesis that FPP supplementation resulted in increased ATP production because of augmented mitochondrial respiration, the oxygen consumption of monocytic cells in the presence and absence of FPP was determined using a Clark electrode and an oxygen monitor (Fig. 4C). By comparing the ratio of uncoupled respiration with the rate of state IV respiration, we observed that supplementation of FPP under normoglycemic conditions increased oxygen consumption (Fig. 4D).

In summary, FPP improved inducible “respiratory burst” in T2D PBMC, possibly as a result of increased availability of ATP and NADPH resulting from increased mitochondrial respiration. Due to the extensive history of safe human consumption of FPP and the current clinical data generated in this study, testing the significance of FPP in addressing T2D-related complications seems to be a prudent course.

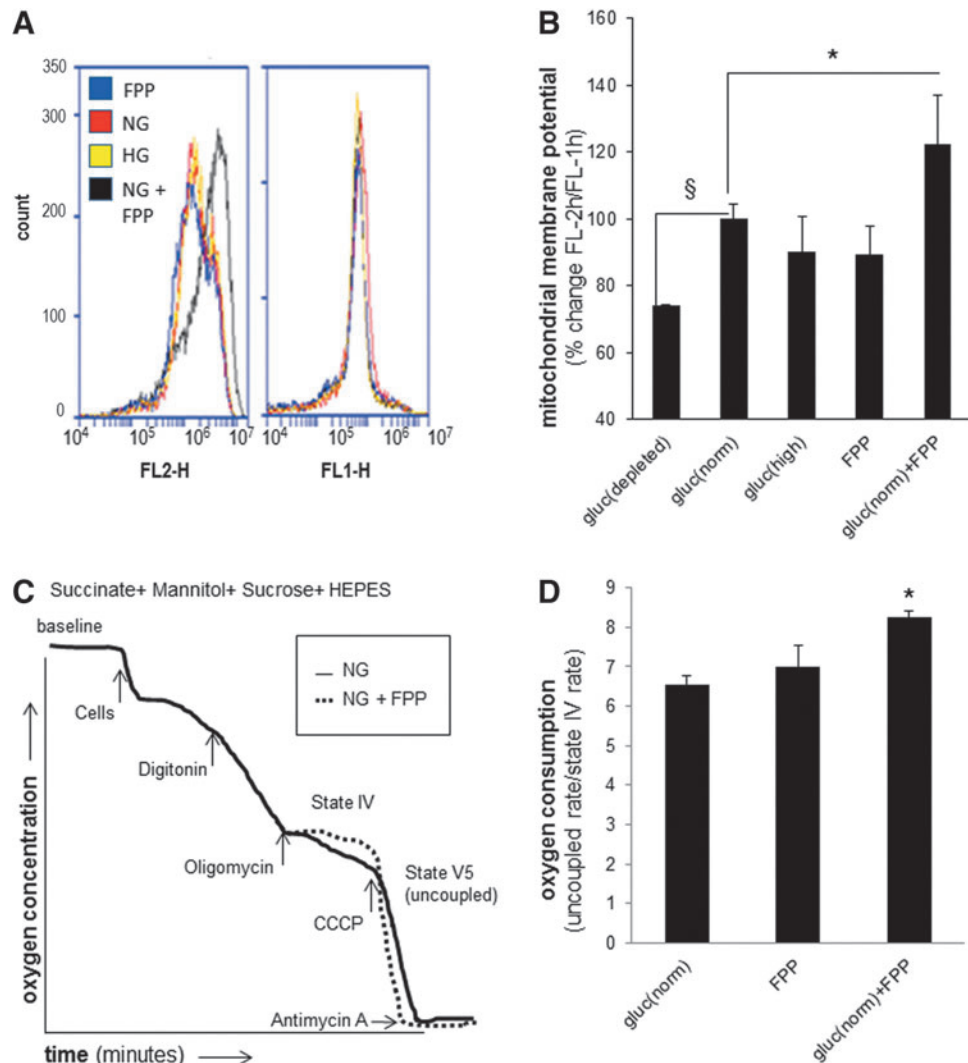


FIG. 4. Elevated mitochondrial membrane potential and O_2 consumption in monocytes treated with FPP. Human monocytic cell line THP-1 cells were deprived of glucose for 48 h. The cells were then incubated for 24 h with gluc(norm), gluc(high), FPP, and gluc(norm)+FPP. (A, B) Mitochondrial membrane potential ($\Delta\psi/m$) was measured using JC-1 and flow cytometry. (A) Increase in red fluorescence (FL2) in the treatment group of gluc(norm)+FPP, and (B) Ratio of polarized to depolarized cells calculated as a ratio between FL-2 and FL-1 fluorescence indicates an increase in mitochondrial membrane potential in the treatment group gluc(norm)+FPP. $^{\S}p < 0.05$ compared with glucose depleted. $^*p < 0.05$ compared with gluc(norm). (C, D) Mitochondrial oxygen consumption. (C) A schematic of oxygen electrode tracings measuring states IV and uncoupled respiration in gluc(norm) and gluc(norm)+FPP. Cells were “permeabilized” by adding digitonin (4 μ g/million cells). The respiration medium contained 230 mM mannitol, 70 mM sucrose, 3 mM HEPES; pH 7.4, 10 mM succinate. Oligomycin (2 mM) was used to initiate state IV respiration, and the uncoupled rate was determined by addition of carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 1.5 μ M) and stopped using antimycin A (1.5 mM). The oxygen consumption rate was measured using a Clark oxygen electrode and an oxygen monitor (D). Ratio of uncoupled respiration to State IV indicates higher mitochondrial respiration when cells are treated with gluc(norm)+FPP. $^*p < 0.05$ ($n=3$) compared with gluc(norm). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Notes

Human subjects and sample collection

Human studies were approved by The Ohio State University (OSU) Institutional Review Board (ClinicalTrials.gov Identifier: NCT01618045). Adult subjects clinically diagnosed with type 2 diabetes mellitus (T2D) with good glucose control, as defined by HbA1c $\leq 7\%$, were enrolled in the study. T2D subjects were recruited from the Diabetes Clinics at The Ohio State University Wexner Medical Center (Columbus,

OH). Subject demographics are presented in Table 1. T2D subjects, either immunosuppressed or taking peroxisome proliferator-activated receptor gamma medications, were excluded from the study. Peripheral blood (60 cc) was drawn by venipuncture. T2D patients were supplemented for 6 weeks with FPP (9 g/day; three sachets a day) followed by a 2 week washout period. Patient compliance, as determined by the return of empty FPP sachets, was estimated at 90%. Drawn blood (10 cc) was sent to The OSU Wexner Medical Center clinical laboratories for analysis of blood chemistry.

The remainder of the blood was utilized for isolation of PBMC and plasma. Freshly isolated PBMC were used for inducible "respiratory burst" assay. Peripheral blood plasma was snap frozen in liquid nitrogen before storage at -80°C until analysis.

Human PBMC isolation and culture

Fresh blood was diluted 1:1 with cold Dulbecco's phosphate-buffered saline (DPBS) (Gibco/Life Technologies, Carlsbad, CA). PBMC were isolated using Ficoll density centrifugation followed by sorting with anti-CD14 coated magnetic microbeads (Miltenyi Biotec, Auburn, CA) as previously described (6).

Induction and assay of respiratory burst

PMA-induced superoxide anion production was measured using the chemiluminescent LumiMax[®] superoxide anion detection kit (Stratagene, La Jolla, CA) according to the manufacturer's recommended protocol as previously described (6). At the time of measurement of ROS in PBMC, the experiment was run in duplicate using the human promyelocytic leukemia (HL-60) cell line to serve as a reference control for the effect of PMA in inducing respiratory burst.

Cell culture

Human monocyte THP-1 cells were deprived of glucose by incubation in RPMI 1640 glucose-free media containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (AA) (Life Technologies) for 48 h (4). To provide carbohydrate substrate to the cells, the previously mentioned glucose-free media cocktail was supplemented as follows: (i) [gluc(norm)]: with normoglycemic glucose levels (11 mM, 2 mg/ml w/v glucose in culture media); (ii) [gluc(high)]: with hyperglycemic glucose levels (20 mM, 3.9 mg/ml w/v glucose in culture media); (iii) FPP: with FPP (2.9 mg/ml w/v FPP in culture media); and (iv) gluc(norm) + FPP: with 11 mM, 2 mg/ml w/v glucose in culture media plus 2.9 mg/ml w/v FPP in culture media. Supplemented media were sterile filtered using a 0.22 μm vacuum-driven filtration system (Millipore, Billerica, MA) before being added to glucose-deprived THP-1 cells. Cells were incubated with their respective substrate group for 24 h before assay. HL-60 cells were cultured in high-glucose Dulbecco's modified Eagle's medium, with sodium pyruvate (Life Technologies), containing 20% FBS and 1% AA under standard culture conditions.

Metabolite and oxidative stress indices

NADP/NADPH concentration. NADP/NADPH concentration was measured using an NADP/NADPH quantitation kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's recommended protocol. After 24 h with the returned substrate, THP-1 cells were washed in cold DPBS. Cells were lysed by two rapid repetitive freeze-thaw cycles. To remove NADPH-consuming enzymes, lysate was filtered through a 10 kDa cut-off spin column (Amicon/Millipore). To detect NADPH, the extracted sample containing total NADP was heated at 60°C for 30 min to digest NADP, leaving only NADPH. Both total NADP and NADPH samples were incubated for 5 min at RT with NADP cycling enzyme, which converts NADP to NADPH. A colorimetric

developer was added, and the sample was incubated for 1 h at room temperature. The absorbance was measured at 450 nm using an automated microplate reader (model Synergy 2; BioTek, Winooski, VT).

ATP concentration. ATP concentration was measured using an ATP assay kit (Abcam, Cambridge, MA) according to the manufacturer's recommended protocol. After 24 h of replenishing the culture medium with glucose and/or FPP, THP-1 cells were washed in ice-cold DPBS. Washed cells were homogenized in assay buffer containing protease inhibitor. Samples were deproteinized with ice-cold 4 M perchloric acid (PCA). Excess PCA was precipitated by adding an equal volume of 2 M potassium hydroxide (KOH), which neutralized the sample to pH 6.8–7.2. Colorimetric assay was performed, with samples being incubated for 30 min before measurement at 570 nm.

Peripheral blood plasma was isolated from whole blood by centrifugation and snap frozen in liquid nitrogen before storage at -80°C for further analysis.

Protein carbonyls. Protein carbonyls were measured using a Protein Carbonyl Content Assay (Abcam) according to the manufacturer's recommended protocol.

4-hydroxynonenal. 4-hydroxynonenal (4-HNE) was measured using the OxiSelect HNE Adduct Competitive ELISA kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's recommended protocol.

Mitochondrial membrane potential

THP-1 cells were incubated (20 min under standard conditions) with the cationic JC-1 dye (Life Technologies), which is known to exhibit potential-dependent accumulation in mitochondria. An emission shift from green (~ 525 nm, FL1-H channel) to red (~ 590 nm, FL2-H channel) was recorded. Depolarization/lower membrane potential is indicated by a decrease in the red/green fluorescence ratio. The ratio of red/green is dependent on the membrane potential and is not influenced by mitochondrial size, density, or shape. Measurements were collected using the flow cytometry (model C6 Flow Cytometer; Accuri, Ann Arbor, MI) after 25K events per sample.

Oxygen consumption

To determine oxygen (O_2) consumption, we measured state IV and uncoupled respiration. Cells were permeabilized by adding digitonin (4 μg /million cells). The respiration medium contained 230 mM mannitol, 70 mM sucrose, 3 mM HEPES; pH 7.4, 10 mM succinate. First, succinate was oxidized through Complex II. Oligomycin (2 mM) was then added to inhibit complex V (ATP synthase), preventing ATP production from endogenous ADP (state IV respiration). Next, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1.5 μM), an uncoupler, was added to produce maximal respiration by chemically dissipating the membrane potential. Finally, the addition of Antimycin A (1.5 mM) inhibited complex III. This blocked oxygen consumption, because electrons were prevented from transferring to cytochrome C and subsequent reduction of O_2 to H_2O by complex IV. The rate of oxygen consumption was measured using a Clark

oxygen electrode and an oxygen monitor (Yellow Springs Instrument, Yellow Springs, OH). Oxygen consumption was calculated by dividing the uncoupled rate by the state IV rate.

Statistics

In vitro data are reported as mean \pm SEM of three to five experiments as indicated in respective figure legends. A comparison among multiple groups was tested using analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant. For human T2D studies, data from 17 human subjects ($n = 17$) have been presented (Table 1).

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Abbreviations Used

AA = antibiotic-antimycotic
ANOVA = analysis of variance
DPBS = Dulbecco's phosphate-buffered saline
FBS = fetal bovine serum
FPP = fermented papaya preparation
HbA1c = glycated hemoglobin
4-HNE = 4-hydroxynonenal
PBMC = peripheral blood mononuclear cells
PCA = perchloric acid
PMA = phorbol 12-myristate 13-acetate
ROS = reactive oxygen species
T2D = type 2 diabetes mellitus