



Amyloid β (1–42) peptide impairs mitochondrial respiration in primary human brain microvascular endothelial cells: impact of dysglycemia and pre-senescence

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Abstract Diabetes increases the risk of Alzheimer’s disease (AD). We investigated the impact of glucose concentrations on the β -amyloid ($A\beta$)-induced alteration of mitochondrial/cellular energetics in primary human brain microvascular endothelial cells (HBMECs). HBMECs were grown and passaged in media containing 15 mmol/l glucose (normal) based on which the glucose levels in the media were designated as high (25 mmol/L) or low (5 mmol/L). HBMECs were treated with $A\beta$ (1–42) (5 μ mol/l) or

a scrambled peptide for 24 h and mitochondrial respiratory parameters were measured using Seahorse Mito Stress Test. $A\beta$ (1–42) decreased the mitochondrial ATP production at normal glucose levels and decreased spare respiratory capacity at high glucose levels. $A\beta$ (1–42) diminished all mitochondrial respiratory parameters markedly at low glucose levels that were not completely recovered by restoring normal glucose levels in the media. The addition of mannitol (10 mmol/l) to low and normal glucose-containing media altered the $A\beta$ (1–42)-induced bioenergetic defects. Even at normal glucose levels, pre-senescent HBMECs (passage 15) displayed greater $A\beta$ (1–42)-induced mitochondrial respiratory impairments than young cells (passages 7–9). Thus, hypoglycemia, osmolarity changes, and senescence are stronger instigators of $A\beta$ (1–42)-induced mitochondrial respiration and energetics in HBMECs and contributors to diabetes-related increased AD risk than hyperglycemia.

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Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by the accumulation of extracellular plaques containing β -amyloid ($A\beta$) peptides in the

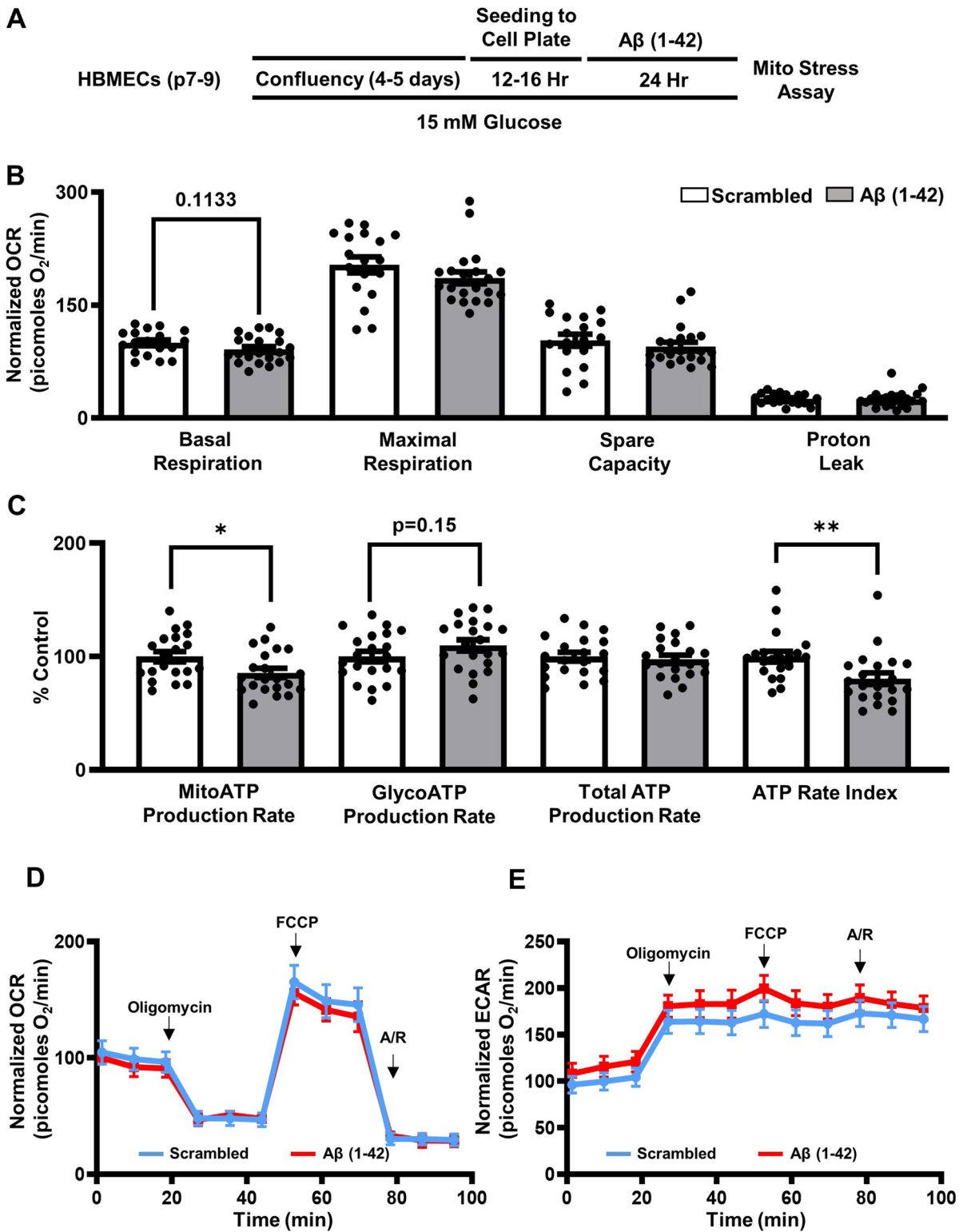


Fig. 1 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates at normal glucose levels (15 mmol/l). HBMECs were treated in presence of 15 mmol/l glucose and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student’s *t*-test. Data were pooled from 3 independent experiments. *N*=18–21 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

brain [1]. Aging and diabetes are major risk factors for the development and progression of AD [2–4]. Hyperglycemia [2, 3] accompanying diabetes as well as hypoglycemia, [5, 6] a common side-effect of insulin therapy, have been shown to increase the risk of AD. However, the mechanisms underlying the hyper- or hypoglycemia-related increased AD risk are still not well understood.

According to the vascular hypothesis, cerebral microvascular dysfunction characterized by impaired neurovascular coupling, cerebral hypoperfusion, and the blood–brain-barrier (BBB) disruption occurs long before the onset of AD [7]. Microvascular dysfunction preceding AD is accelerated by diabetes [7–10]. Altered vascular functions have been shown to result from increased production and decreased clearance of A β in the brain [11]. A β (1–42) is the major amyloid peptide present in the plaque and was shown to be highly cytotoxic among the amyloid peptides [12, 13]. A β (1–42) disrupts the function of neurons, astrocytes, and endothelial cells including pericytes [14–19]. Mitochondria are one of the major intracellular targets for A β peptide action [20]. A β (1–42) is transported into mitochondria and was shown to reduce mitochondrial respiration by decreasing the expression of tricarboxylic acid cycle enzymes and electron transport chain complexes in neurons [20], astrocytes [21], and brain microvascular endothelial cells (BMECs) [22–24] thereby inducing

mitochondrial apoptotic pathways. Recently, A β (1–42) has been shown to increase mitochondrial respiration and ROS production in mouse BMECs [25]; however, the effects of A β (1–42) on cellular energetics of the BMECs at various glucose concentrations in the media have not been investigated before.

Mitochondria play a critical role in the endothelial cell function including proliferation, migration, apoptosis, and blood–brain-barrier (BBB) function [26] along with tissue-specific functions [27]. Mitochondrial contribution to endothelial cell energy has been considered negligible but is deemed indispensable as the signaling center [28]. Interestingly, higher mitochondrial numbers exist in endothelial cells from the brain compared to other vascular beds [28]. Recently, we established that mitochondria contribute significantly to the cellular ATP levels in mouse brain microvasculature (60–70% of total ATP) and in the primary human brain microvascular endothelial cells (HBMECs) (30–45% of the total ATP) [29, 30]. Notably, for the first time, we characterized the impact of aging and pre-senescence on mitochondrial and glycolytic energy pathways in freshly isolated brain microvessels (BMVs) from aged mice and the cultured HBMEC high-passage numbers [29, 30]. Very few studies reported the mitochondrial dysfunction in other brain cells including glial cells and endothelial cells in Alzheimer’s disease [31, 32]. Interestingly, both hyper and hypoglycemia have been shown to induce mitochondrial dysfunction in neurons [33]. Considering the significance of hyperglycemia and hypoglycemia in the pathogenesis of diabetic complications and the related increased AD risk, we investigated the effect of A β (1–42) on mitochondrial energy metabolism in HBMECs at various glucose levels in the media. We used HBMECs grown and passaged in the media containing 15 mmol/l (normal) based on which the glucose levels in the media were designated as high (25 mmol/l) or low (5 mmol/l). Subsequently, we treated the cells with the A β (1–42) (5 μ mol/l) for 24 h and measured the mitochondrial respiratory parameters using the Seahorse XFe24 analyzer. In addition, ATP production rates from OXPHOS and glycolysis were also calculated using the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values that were measured during the XFe Mito Stress Test.

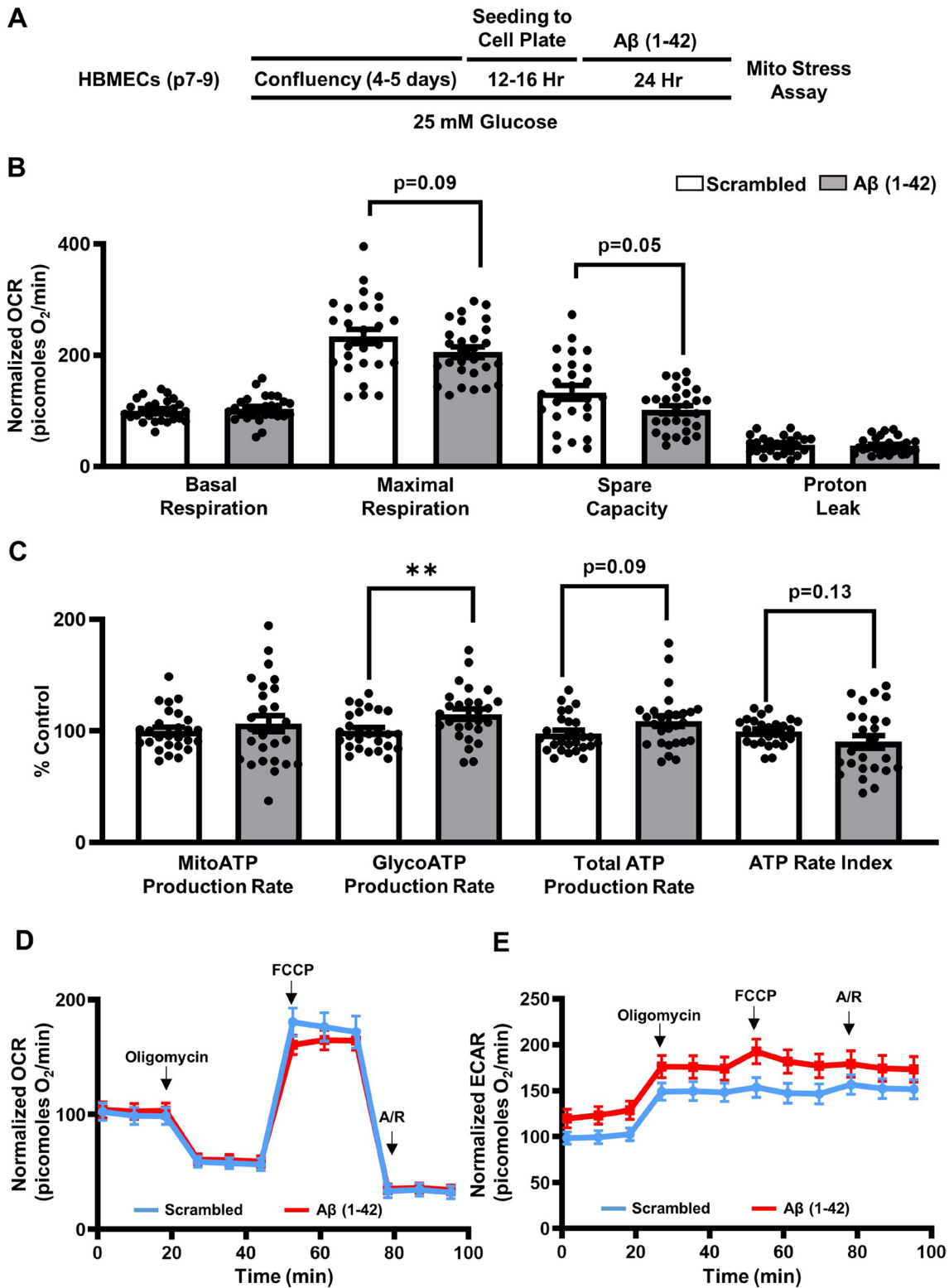


Fig. 2 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates at high glucose levels (25 mmol/l). HBMECs were grown in presence of 25 mmol/l glucose and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h, and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student’s *t*-test. Data were pooled from 4 independent experiments ($N=27-28$ wells/treatment). *, **, ***, and **** indicate $p < 0.05, 0.01, 0.001,$ and $0.0001,$ respectively

Materials and methods

Chemicals and reagents

Human β -amyloid (1–42), HCl (A-1166–2), and β -amyloid (1–42), scrambled (A-1004–2), were purchased from rPeptide, Watkinsville, GA. Seahorse analyzer reagents were purchased from Agilent Technologies (Santa Clara, CA) including XFe24 FluxPak (102,340–100), Seahorse XF RPMI media (103,576–100), Seahorse XF Cell Mito Stress Test kit (103,015–100), and XF 1.0 M Glucose Solution (103,577–100). The following chemicals were purchased from Millipore Sigma, St. Louis, MO, including sodium pyruvate (P8574, Millipore Sigma, St. Louis, MO), d-(+)-glucose (G7528, Millipore Sigma, St. Louis, MO), protease inhibitor cocktail (P8340, Millipore Sigma, St. Louis, MO), and phosphatase inhibitor cocktail (P0044, Millipore Sigma, St. Louis, MO). Other chemicals include L-glutamine (25,030,081) and Pierce BCA Protein Assay Kit (23,227) from Thermo Scientific, Waltham, MA, and NP40 lysis buffer (FNN0021, Invitrogen, Waltham, MA).

Cell culture experiments

Primary human brain microvascular endothelial cells (HBMECs) were purchased from Cell Systems (ACBRI 376, Kirkland, WA). HBMECs were woken up, grown, and passaged in the complete classic media

(4ZO-500, Cell Systems, Kirkland, WA) containing 15–16 mmol/l glucose as per the manufacturer’s protocol (we report as 15 mmol/l). Passages “7 to 9” were used for the seahorse experiments and were considered young cells [29]. HBMECs were grown in the media containing high glucose (25 mmol/l) and low glucose (5 mmol/l) in addition to the regular glucose concentration (15 mmol/l). In addition, 10 mmol/l mannitol was added to the cells that were grown at 15 mmol/l glucose as an osmotic control group for hyperglycemia. Addition of glucose to media increases the osmolarity and hence the observed effects of high glucose on cells could be due increased osmolarity or due to specific actions of the glucose. To discriminate glucose-specific effects from the effects of osmolarity changes, mannitol will be added to media at a similar concentration to induce similar osmolarity (similar molecular weight as glucose and poorly metabolized). Furthermore, cells that were grown at low-glucose conditions (5 mmol/l) were switched back to regular media containing 15 mmol/l glucose or added 10 mmol/l mannitol to achieve normal osmolarity. Along with the young cells, pre-senescent cells (passages between p14 and p15) were also grown in the media containing 15 mmol/l glucose to investigate whether the pre-senescent HBMECs are more prone to the A β (1–42) insult or not. At 90–95% confluence, cells were passaged to the seahorse cell culture plates (40,000 cells/well). After 12–16 h, HBMECs were treated with “A β (1–42) peptide” or “A β (1–42) scrambled peptide” at 5 μ mol/l concentration for 24 h and immediately proceeded to the seahorse experiments. A β (1–42) concentration was chosen based on the previous literature that reported significant alterations in brain endothelial cell functions [34, 35]. In total, this study contains seven experimental groups that include HBMECs (p7–p9) grown at (i) 15 mmol/l glucose, (ii) 25 mmol/l glucose, (iii) 5 mmol/l glucose, (iv) 15 mmol/l glucose+10 mmol/l mannitol, (v) 5 mmol/l glucose switched to 15 mmol/l glucose, (vi) 5 mmol/l glucose switched to 5 mmol/l glucose plus 10 mmol/l mannitol, and pre-senescent cells (p14–15) [29] grown at 15 mmol/l glucose (vii). The study design was given in the figures.

Mitochondrial respiration

Mitochondrial respiratory parameters were analyzed by Seahorse XFe24 analyzer using the XFe Mito

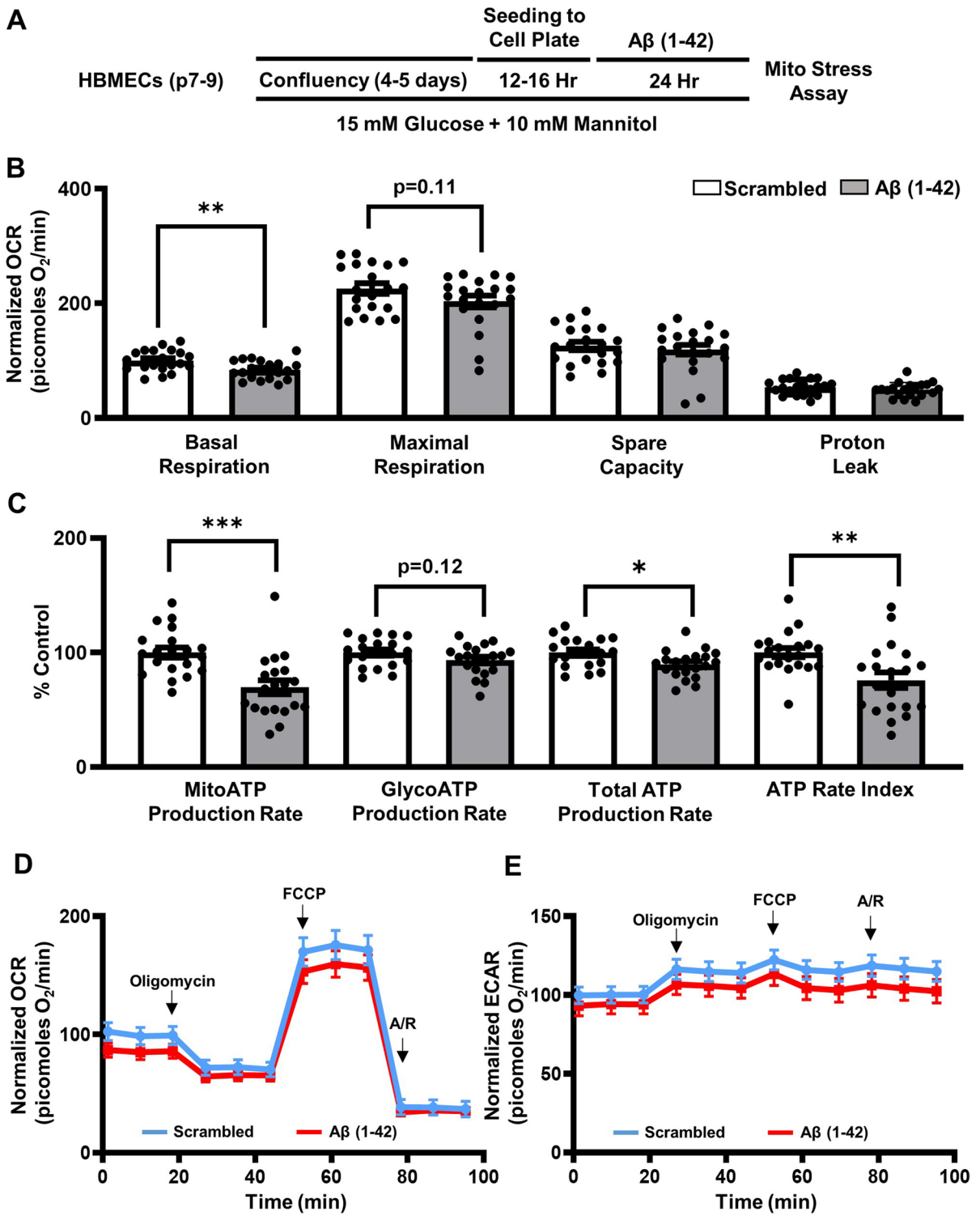


Fig. 3 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates at high-osmolar conditions (15 mmol/l glucose + 10 mmol/l mannitol). HBMECs were grown in presence of 15 mmol/L glucose plus 10 mmol/L mannitol and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h, and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student’s *t*-test. Data were pooled from 3 independent experiments and *N*=20 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

Stress Test kit as per the manufacturer’s instructions and the standardized protocols in our laboratory [29]. Briefly, after the A β (1–42) treatment, HBMECs were incubated in the seahorse media (RPMI media, pH 7.4) containing 25 mmol/l glucose, 10 mmol/l sodium pyruvate, and 2 mmol/l glutamine for 60 min at 37 °C in a non-CO₂ incubator. Meanwhile, seahorse cartridges that were incubated overnight in a non-CO₂ incubator with the calibrant were loaded with oligomycin, 2-[2-[4-(trifluoromethoxy)phenyl] hydrazinylidene]-propanedinitrile (FCCP), and antimycin A/rotenone mixture in the respective ports. Cartridges were calibrated in the seahorse analyzer, before inserting the cell plate for OCR and ECAR measurements. Basal OCR readings were taken followed by the measurements after the sequential injection of oligomycin (2 μ mol/l), FCCP (1.1 μ mol/l), and rotenone/antimycin A (0.5 μ mol/l). After the seahorse analysis, media were removed, and cells were digested with NP40 lysis buffer for protein estimation. All the OCR data was initially normalized for the respective protein content in each well. Basal respiration, maximal respiration, and spare respiratory capacity were measured along with the proton leak and non-mitochondrial oxygen consumption based on OCR using Wave Seahorse Desktop software (Agilent Technologies)[29]. To minimize the day-to-day variations, the mean of the basal OCR values from the control group (scrambled A β (1–42) treated) in

each seahorse experiment was taken as 100, and all OCR values of the control and treated groups were adjusted for it. The number of observations (wells) was pooled from the 2 to 4 independent experiments (*n*=12–28 wells/treatment). ATP production rates from OXPHOS and the glycolysis were measured manually from the OCR and proton efflux rates (PER) obtained during the XFe Mito Stress Test.

Mitochondrial and glycolytic ATP production rates

ATP production rates were calculated manually from the OCR and proton efflux rate (PER) from the Mito Stress Test using the classical stoichiometry.

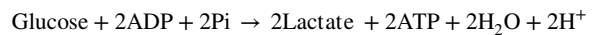
Mitochondrial ATP production rate



MitoATP production rate (picomoles of ATP/min) = OCR ATP (picomoles of O₂/min) \times 2 (number of oxygen atoms) \times 2.75 (P/O ratio, i.e., every oxygen atom consumed results in production of 2.75 ATP molecules)

$$OCR_{ATP} = (\text{BasalOCR} - \text{OligoOCR}) - \text{OCR}_{\text{after A/R}}$$

Glycolysis ATP production rate



According to the glycolysis equation, the rate of lactate (proton) generated is equal to the rate of ATP produced. GlycoATP rate is equal to the PER due to glycolysis (GlycoPER).

GlycoATP production rate (picomoles of ATP/min) = GlycoPER (picomoles of H⁺/min).

GlycoPER = Basal PER – PER due to OXPHOS (deduced by subtracting OCR after A/R from basal OCR). Note: PER due to OXPHOS: The amount of oxygen consumption decreased due to A/R which is equal to the amount of CO₂ released, which is converted to H₂CO₃ and releases one H⁺ in the extracellular media.

Statistics

Data were represented as the mean \pm S.E.M. Student *t*-test or Mann–Whitney test was used to measure the statistical significance for the selected parameter

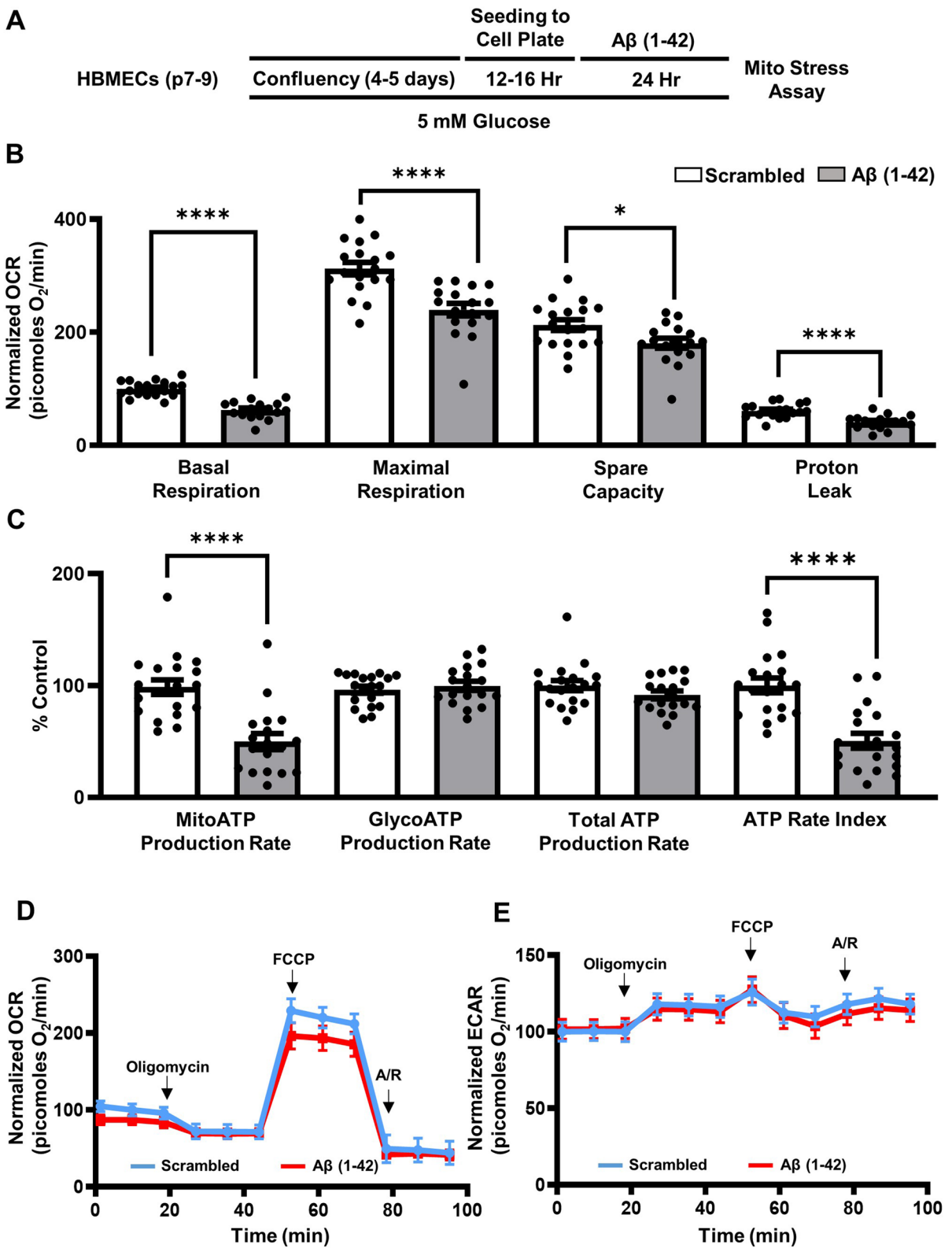


Fig. 4 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates at low glucose levels (5 mmol/l). HBMECs were grown in presence of media containing 5 mmol/L glucose and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student’s *t*-test. Data were pooled from 3 independent experiments. *N* = 16–20 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

(GraphPad Prism 8.4.3, San Diego, CA). *P*-value less than 0.05 was taken as statistically significant.

Results

Effect of A β (1–42) on energetics of HBMECs at normal glucose levels (15 mmol/l)

A β (1–42) treatment resulted in a trend toward a decrease in basal respiration in the HBMECs but did not alter the other respiratory parameters including maximal respiration, spare capacity, and proton leak (Fig. 1B). Mitochondrial ATP production rates were significantly decreased by A β (1–42) without altering the ATP production rates from the glycolysis and the total ATP production rates (Fig. 1C). ATP rate index, the ratio between mitochondrial (MitoATP) and glycolytic (GlycoATP) ATP production rates, was significantly decreased by A β (1–42) treatment in HBMECs (Fig. 1C).

Effect of A β (1–42) on energetics of HBMECs at high glucose levels (25 mmol/l)

At high glucose concentration, A β (1–42) treatment of HBMECs resulted in a significant reduction in spare respiratory capacity and a strong trend toward a decrease in mitochondrial maximal respiration but without altering the basal respiration, and proton leak (Fig. 2B). In contrast to the observations at normal

glucose levels, A β (1–42) significantly increased the glycoATP production rates but did not affect mitochondrial ATP production (Fig. 2C). A β (1–42) promoted a trend toward an increase in total ATP production rates along with decreased ATP rate index, possibly due to elevated glycoATP levels (Fig. 2C).

Effect of A β (1–42) on energetics of HBMECs at high-osmolar media (15 mmol/l glucose + 10 mmol/l mannitol)

In the presence of high-osmolar media containing 10 mmol/l mannitol in addition to 15 mmol/l glucose, A β (1–42) treatment of HBMECs significantly decreased basal respiration and induced a decreasing trend in the maximal respiration without altering the spare capacity, and proton leak (Fig. 3B). Furthermore, mitochondrial ATP and total ATP production rates as well as ATP rate index were significantly decreased by A β (1–42) treatment. However, A β (1–42) promoted a trend toward decreased glycoATP production (Fig. 3C). These observations indicate that A β (1–42) peptide may worsen HBMEC energy metabolism in high-osmolar conditions than at normal or high glucose levels.

Effect of A β (1–42) on energetics of HBMECs at low glucose levels (5 mmol/l glucose)

At low glucose, A β (1–42) significantly reduced basal and maximal respiration in HBMECs along with reduced spare capacity and the proton leak (Fig. 4B). Mitochondrial ATP production rates were significantly decreased by A β (1–42) resulting in a significantly lower ATP rate index without change in glycoATP and total ATP production rates (Fig. 4C).

Effect of A β (1–42) on energetics of HBMECs after switching from low to normal glucose levels (5 to 15 mmol/l glucose)

As A β (1–42) significantly impaired the energy metabolism at low glucose concentrations, we investigated whether switching HBMECs from low to normal glucose levels reverses the effect of A β (1–42) on mitochondrial respiration. Interestingly, restoring the glucose levels to normal did not impact A β (1–42)-induced mitochondrial respiratory dysfunction in HBMECs grown with low

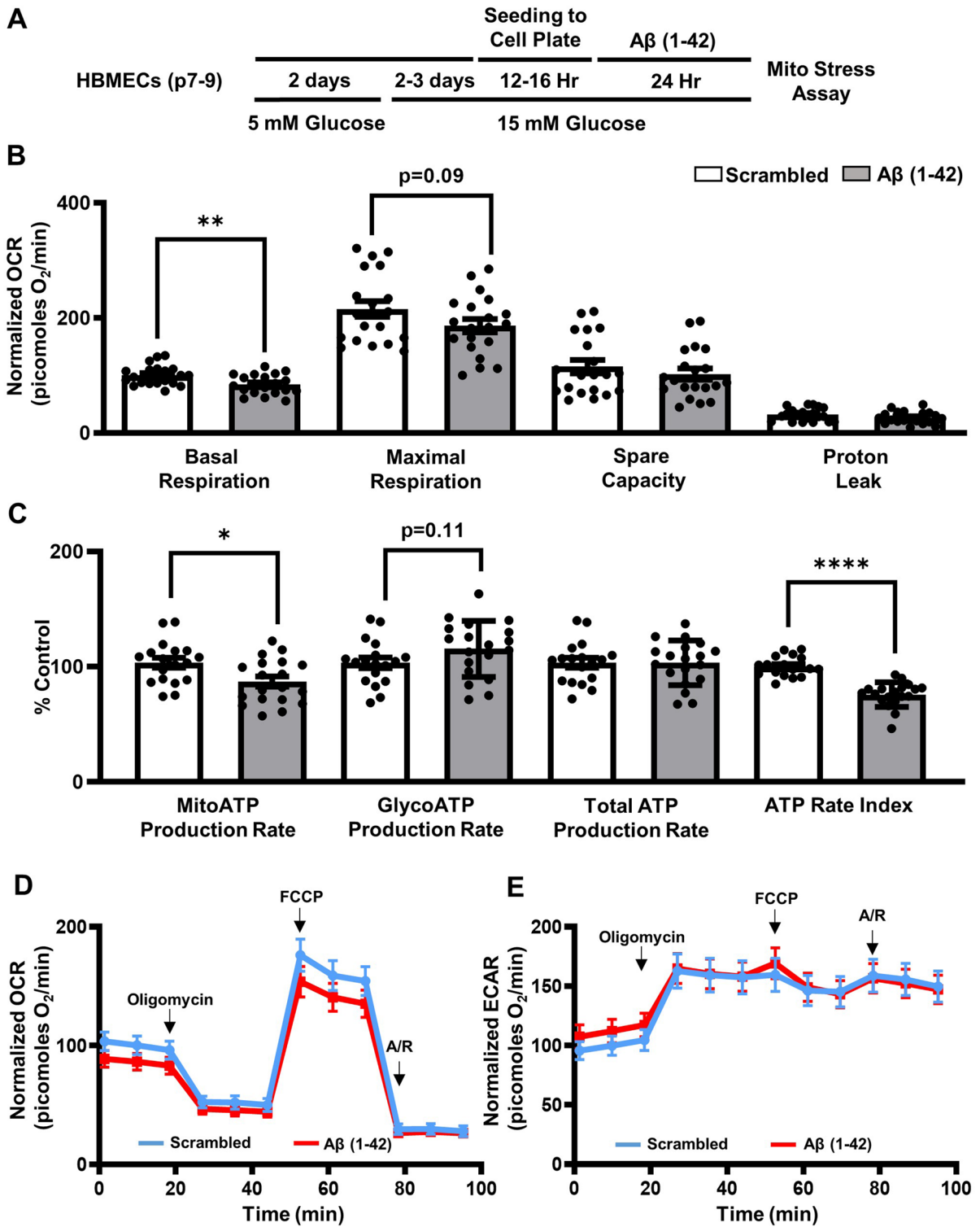


Fig. 5 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates after switching from low to normal glucose levels (5 to 15 mmol/l). HBMECs were initially in media containing 5 mmol/l glucose for 2 days and later 10 mmol/l glucose was added and grown until the confluency. HBMECs were seeded in cell culture plates and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student's *t*-test. Data were pooled from 3 independent experiments. *N*=18–20 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

glucose levels in the media. Specifically, basal respiration was significantly reduced by A β (1–42), whereas the maximal respiration showed a trend toward a decrease but the spare respiratory capacity and proton leak were unaffected (Fig. 5B). The mitochondrial ATP production rate was decreased significantly by A β (1–42) and a trend toward a decrease in glycoATP production rate was induced by A β (1–42) treatment in HBMECs resulting in an unaltered total ATP production rate and significantly reduced ATP rate index (Fig. 5C).

Effect of A β (1–42) on energetics of HBMECs after switching from low glucose levels to osmotic control media (5 to 5 mmol/l glucose plus 10 mmol/l mannitol)

As HBMECs are more prone to A β (1–42) effects on energy metabolism at low glucose levels and even after glucose levels were restored to normal, we investigated whether the observed changes were due to the increased osmolarity. Interestingly, adding mannitol (10 mmol/l) to HBMECs after culturing the cells initially at low glucose levels nullified all the mitochondrial respiratory defects induced by A β (1–42) at low glucose levels (Fig. 6B & C). In addition, A β (1–42) treatment significantly increased the glycoATP production rates and resulted in a decreased ATP rate index (Fig. 6C).

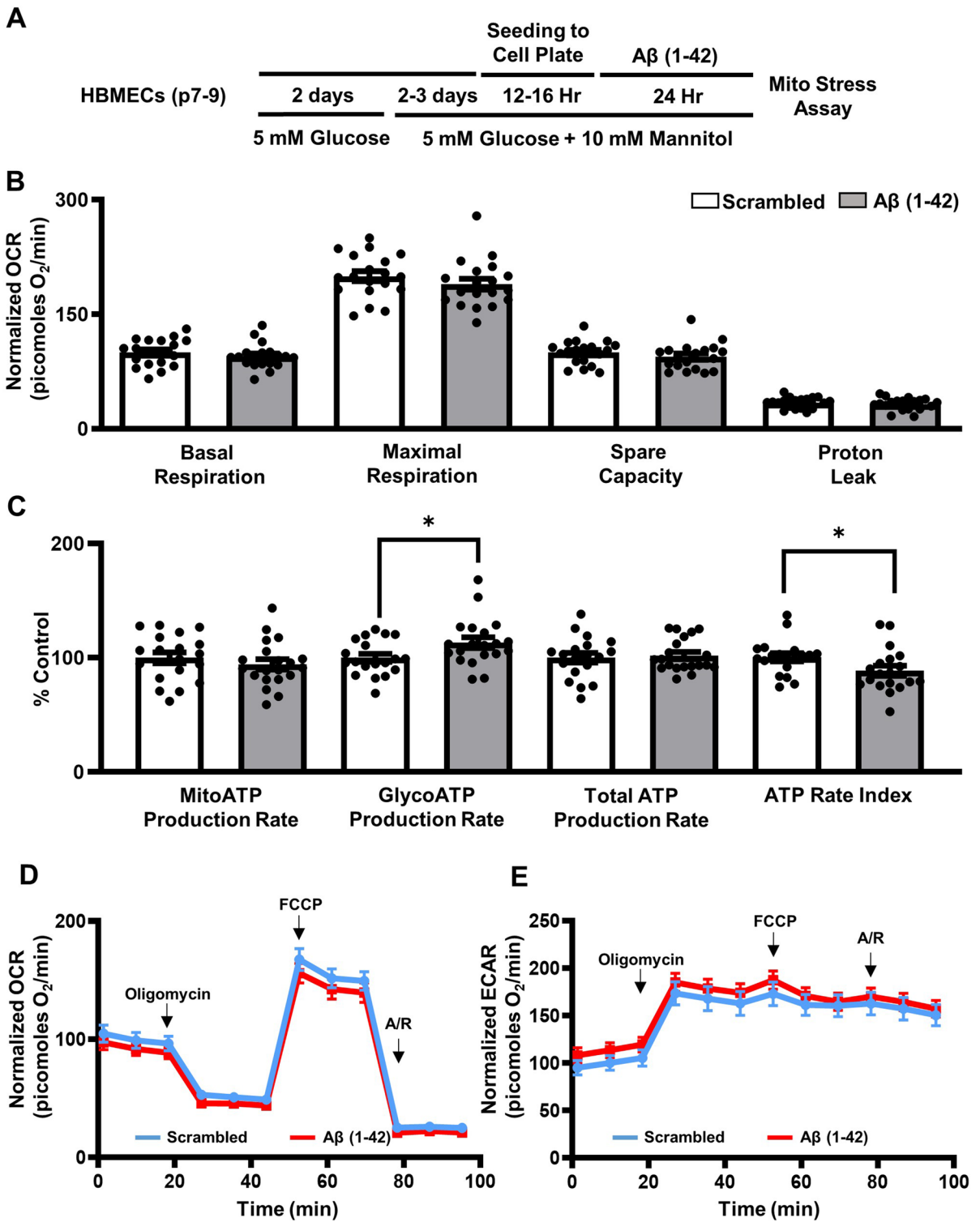
Effect of A β (1–42) on energetics of pre-senescent HBMECs at normal glucose levels

In a recent study, we proposed an *in vitro* aging model utilizing HBMECs and comprehensively characterized energetics of pre-senescence and senescence based on the passage numbers [29]. We observed that HBMECs at higher passage numbers (after passage 13 and higher) exhibit diminished glycolysis and impaired mitochondrial spare capacity. As aging is the major risk factor for AD development, we investigated whether A β (1–42) alters the mitochondrial function in the pre-senescent HBMECs at passages #14–15. At normal glucose concentration (15 mmol/l), A β (1–42) significantly decreased basal respiration without altering maximal respiration, spare capacity, and proton leak (Fig. 7B). The mitochondrial ATP production rate was significantly decreased whereas the glycoATP production rates were significantly increased by A β (1–42) treatment in the pre-senescent HBMECs (Fig. 7C). ATP rate index was also significantly decreased by the A β (1–42) in the pre-senescent HBMECs (Fig. 7C). These results indicate that the pre-senescent cells are more prone to A β (1–42)-induced alterations of mitochondrial and glycolytic energetics than the young cells at normal glucose concentration.

All the significant bioenergetic changes in response to A β (1–42) treatment in HBMECs are summarized in the Table 1.

Discussion

The present study, for the first time, reports the effect of A β (1–42) peptide on mitochondrial respiration and ATP metabolism in HBMECs in the presence of various glucose conditions to understand the molecular mechanisms regulating the mitochondria and energetics that link dysglycemia to the increased AD risk in diabetes. Major observations of the study include the following: (1) A β (1–42) minimally alters the mitochondrial OXPHOS function under normoglycemic conditions whereas it markedly impairs the mitochondrial spare respiratory capacity under hyperglycemic conditions. (2) Low glucose levels promote more severe A β (1–42)-induced impairments in mitochondrial respiration and mitoATP production rates compared to hyperglycemia. (3) Prior exposure to low glucose levels



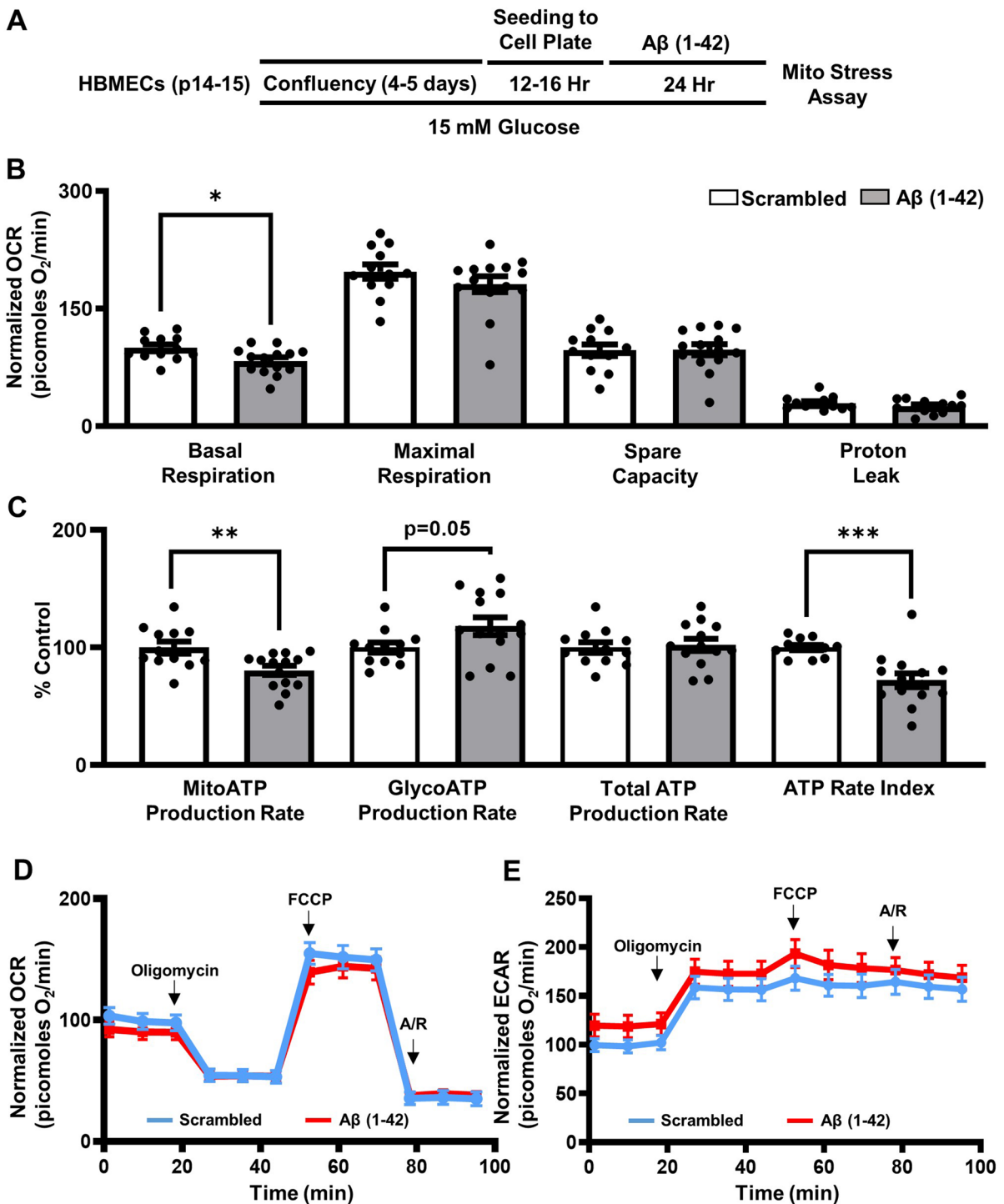
◀Fig. 6 Effect of A β (1–42) on HBMEC mitochondrial respiration and ATP production rates after switching from low glucose to normal osmolarity (5 mmol/l glucose plus 10 mmol/l mannitol). HBMECs were initially in media containing 5 mmol/l glucose for 2 days and later 10 mmol/l mannitol was added and grown until the confluency. HBMECs were seeded in cell culture plates and treated with “A β (1–42)” or “scrambled (A β 1–42)” peptides (5 μ mol/l) for 24 h and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μ mol/l), FCCP (1.1 μ mol/l), followed by antimycin/rotenone (0.5 μ mol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean \pm S.E.M and analyzed by Student’s *t*-test. Data were pooled from 3 independent experiments. *N* = 18–20 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

makes the HBMECs vulnerable to A β (1–42)-induced OXPHOS defects even after restoring the normal glucose levels. (4) Osmolarity is a critical factor in mediating A β (1–42) effects on mitochondrial energy metabolism in HBMECs both at low and high glucose concentrations. (6) Pre-senescent HMBECs are more prone to A β (1–42)-induced mitochondrial respiratory defects compared to young HMBECs under normal glucose conditions. (7) In the majority of the experimental conditions, A β (1–42) can decrease the ATP rate index mostly by decreasing the OXPHOS ATP production rates but in a few cases by elevating glycolytic ATP production rates. These observations reveal for the first time that brain endothelial OXPHOS is highly sensitive to A β (1–42) peptides under various glycemic conditions thus providing the novel mechanistic link between dysglycemia-associated risk in AD development and the progression in diabetic subjects.

The effect of A β peptides on mitochondrial respiration is well established in neuronal and non-neuronal cells. A β (1–42) is imported into mitochondria and accumulated in the matrix and cristae [36]. AD brain regions containing A β peptides are associated with decreased TCA cycle enzyme and mitochondrial complex activities [36]. A β peptides have been shown to decrease mitochondrial oxygen consumption and membrane potential, and induce apoptosis by activating permeability transition pore and cytochrome c release with increased ROS production

from neurons [36]. A β (1–42) has been shown to induce mitochondria dysfunction and oxidative stress in astrocytes thus leading to neuronal death [37]. Not many studies reported the effect of A β peptides on endothelial energy metabolism. Recently, A β (1–42) has been shown to increase mitochondrial respiratory parameters including ATP production in mouse brain endothelial cells that was accompanied by increased mitochondrial ROS production [25]. In contrast, under the conditions of our study, we observed that A β (1–42) treatment resulted in a slight but significant reduction in mitochondrial ATP production and a modest shift of cellular metabolism from mitochondrial OXPHOS to glycolysis without altering other respiratory parameters. These conflicting results could be due to differences in human versus mouse brain endothelial cells or the experimental design.

Diabetes is known to increase the risk of development and progression of AD, but the underlying molecular mechanisms are poorly understood. Microvascular dysfunction is suggested to be one of the links between diabetes and AD risk as both pathologies are associated with microvascular impairments. Hyperglycemia in combination with A β (1–42) has been shown to promote synaptic loss in neurons by inducing mitochondrial dysfunction [38]. Hyperglycemia promotes A β (1–42) secretion, and aggregation [39] resulting in decreased neuronal activity [40]. In endothelial cells, hyperglycemia increases the synthesis and secretion of A β (1–42) resulting in increased paracellular permeability [41]. To date, no studies reported the effect of A β (1–42) on endothelial bioenergetics in presence of hyperglycemia. Evidence shows that hyperglycemia increases the glycolytic flux due to increased glucose availability and shuttles substrates into other pathways including hexosamine, polyol, and glycation pathways [42]. In our experiments, in presence of hyperglycemia, A β (1–42) impaired mitochondrial spare capacity and stimulated the ATP production from glycolysis. The current observations suggest that under hyperglycemic conditions, A β (1–42) further increases the glycolytic flux and impairs the mitochondrial spare respiratory capacity. This increased glycolytic flux may elevate sorbitol, methylglyoxal, and glycosylated proteins resulting in altered cell function whereas the decreased mitochondrial spare capacity may limit the ability to meet increased energy demands of stress. Thus, cerebral endothelial energy metabolism is



highly sensitive to Aβ (1–42) under hyperglycemic conditions.

Similar to hyperglycemia, hypoglycemia has also been implicated in the development of AD. Insulin

therapy is the most common cause of hypoglycemia in diabetes [43] and the number of episodes of hypoglycemia has been shown to strongly correlate with the risk of dementia and AD in diabetic subjects [44,

Fig. 7 Effect of Aβ (1–42) on pre-senescent HBMEC mitochondrial respiration and ATP production rates at normal glucose levels (15 mmol/l). Pre-senescent HBMECs (passages 14–15) were cultured in media containing 15 mmol/l glucose and treated with “Aβ (1–42)” or “scrambled (Aβ 1–42)” peptides (5 μmol/l) for 24 h and were analyzed for respiratory parameters using Seahorse XF Cell Mito Stress Test. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured before and after treatment with oligomycin (1 μmol/l), FCCP (1.1 μmol/l), followed by antimycin/rotenone (0.5 μmol/l each), and data were analyzed by Wave 2.6 software. ATP production rates are manually calculated using Microsoft Excel. **A** Schematic for the experiment. **B** Mitochondrial respiratory parameters (after deducting the non-mitochondrial respiration). **C** ATP production rates and ATP rate index. **D** Representative OCR graph. **E** Representative ECAR graph. Data were represented as mean ± S.E.M and analyzed by Student’s *t*-test. Data were pooled from 2 independent experiments. *N* = 12–14 wells/treatment. *, **, ***, and **** indicate *p* < 0.05, 0.01, 0.001, and 0.0001, respectively

45]. Interestingly, recurrent hypoglycemia has been shown to accelerate the progression of learning and memory deficits in STZ-induced diabetic double transgenic mice expressing a chimeric mouse/human amyloid precursor protein and a mutant human pre-senilin 1 (APP/PS1) mice by decreasing the neuronal GLUT3 expression [46]. Molecular mechanisms connecting the low glucose levels to AD risk

are poorly understood. Insulin-induced hypoglycemia has been shown to increase the circulatory APP in type 2 diabetic subjects [47] and increase amyloid precursor protein (APP) expression in astroglial cells [48]. Furthermore, hypoglycemia has been shown to induce Tau protein phosphorylation in neurons [46]. However, no studies to date have reported the effect of low glucose levels on Aβ (1–42)-induced alterations of endothelial metabolism or function. Interestingly, the present study observed that detrimental effects of Aβ (1–42) on endothelial mitochondrial respiration are more pronounced at low glucose levels when compared to normal or high glucose levels. Aβ (1–42) treatment resulted in a more than 50% decrease in mitochondrial ATP production and impaired all the other respiratory parameters, without affecting the glycoATP production rates. These results indicate that hypoglycemic conditions might profoundly enhance the brain endothelial cell vulnerability to the detrimental effects of Aβ (1–42). Interestingly, once the HBMECs were exposed to low glucose levels, restoration of glucose to normal levels did not mitigate impairments of mitochondrial respiration, indicating that hypoglycemia-induced vulnerability lasted long after it was corrected. These observations have serious implications

Table 1 Summary of cellular energetics in human brain microvascular endothelial cells after Aβ (1–42) treatment

HBMEC	Experimental conditions	Basal respiration	Maximal respiration	Spare respiratory capacity	Proton leak	MitoATP production rate	GlycoATP production rate	Total ATP production rate	ATP rate index
Young (p7–p9)	15 mM glucose	-	-	-	-	↓	-	-	↓↓
	25 mM glucose	-	-	-	-	-	↑↑	-	-
	15 mM glucose + 10 mM mannitol	↓↓	-	-	-	↓↓↓	-	-	↓↓
	5 mM glucose	↓↓↓↓	↓↓↓↓	↓	↓↓↓↓	↓↓↓↓	-	-	↓↓↓↓
	5 to 15 mM glucose	↓↓	-	-	-	↓	-	-	↓↓↓↓
	5 to 5 mM Glucose + 10 mM mannitol	-	-	-	-	-	↑	-	↓
Pre-senescence (p14–p15)	15 mM glucose	↓	-	-	-	↓↓	-	-	↓↓↓

Note: “-” indicates no change, “↓” indicates decrease, and “↑” indicates Increase

Number of arrows represent “*p*” value, 1< 0.05, 2< 0.01, 3< 0.001, and 4< 0.0001

Basal Res. basal respiration, Max Res. maximal respiration

for cerebral microvasculature in diabetic patients receiving insulin therapy experiencing recurrent hypoglycemia. Thus, recurrent hypoglycemia could increase the risk of AD development and progression indirectly by sustaining the A β (1–42)-induced cerebral microvascular dysfunction.

Glucose levels in the plasma contribute to plasma osmolarity. Osmotic control experiments reveal whether the observed effects are due to direct effects of high glucose levels or due to increased osmolarity. Interestingly in our study, adding mannitol to normal glucose media to simulate hyperosmolarity during hyperglycemia resulted in A β (1–42)-induced impaired basal respiration and worsened mitochondrial ATP production, in contrast to the effects observed in the cells at hyperglycemia such as reduced spare capacity and the elevated glycolysis. Similarly, the addition of mannitol to low glucose media to achieve osmolarity of normal glucose levels (15 mmol/L) abrogated the detrimental effects of A β (1–42) on endothelial OXPHOS. Notably, A β (1–42)-induced effects on energetics at different glucose levels were different than the effects after correcting the osmolarity. These results indicate that osmolarity could be an important driver of the A β (1–42)-mediated effects on endothelial energy metabolism.

Aging impairs cerebral microvascular function and contributes to the development and progression of AD by promoting A β peptide accumulation and Tau phosphorylation [49, 50]. However, the impact of aging on the vulnerability of the aged brain cell types to A β (1–42) peptides has not been adequately studied. A β peptide binding and membrane plasticity are increased in aged neurons compared to young neurons [51]. A β (1–42) has been shown to induce senescence in HBMECs [52]. To our knowledge, the effects of A β (1–42) on bioenergetics of young and aged endothelial cells have never been reported. In the present study, the effect of A β (1–42) on decreased mitochondrial ATP production and metabolic shift from OXPHOS to glycolysis is more pronounced in the pre-senescent HBMECs compared to the young cells. Our recent studies revealed that aged brain microvasculature and HBMECs exhibit impaired bioenergetics [29, 53]. Our studies reveal that A β (1–42) further worsens mitochondrial OXPHOS in the pre-senescent HBMECs to contribute to the worsening of microvascular dysfunction and progression of AD.

Limitations Primary brain microvascular endothelial cells utilized in the majority of the studies grow the cells in glucose concentrations ranging from 15 [29, 54] to 25 mmol/l [55, 56] which represent severe hyperglycemia in diabetic patients. Thus, the designation of low and high glucose levels based on the 15 mmol/l glucose levels in the media used to grow cells over several passages is a limitation [57]. However, by employing the osmolarity controls for all glucose levels, we minimized the impact of higher normal glucose levels in the endothelial media.

In conclusion, A β (1–42) impairs mitochondrial OXPHOS in HBMECs at various glucose conditions. A β (1–42) effects on OXPHOS were more severe when accompanied by low glucose levels compared to high glucose levels, thus revealing a novel mechanism underlying the increased risk of AD development in diabetes. Considering the differences between the in vivo and in vitro glucose concentrations, further in vivo studies are needed to confirm the observations. Moreover, our findings also identified osmolarity as an important instigator of A β (1–42)-induced impairments of endothelial cell bioenergetics.

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Declarations

Conflict of interest The authors declare no competing interests.

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