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Diabetes induces mitochondrial dysfunction and alters cholesterol homeostasis and neurosteroidogenesis in the rat cerebral cortex

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ABSTRACT

The nervous system synthesizes and metabolizes steroids (i.e., neurosteroidogenesis). Recent observations indicate that neurosteroidogenesis is affected by different nervous pathologies. Among these, long-term type 1 diabetes, together with other functional and biochemical changes, has been shown to alter neuroactive steroid levels in the nervous system. Using an experimental model of type 1 diabetes (i.e., streptozotocin injection) we here show that the levels of these molecules are already decreased in the rat cerebral cortex after one month of the initiation of the pathology. Moreover, decreased levels of free cholesterol, together with alterations in the expression of molecules involved in cholesterol biosynthesis, bioavailability, trafficking and metabolism were detected in the rat cerebral cortex after one month of diabetes. Furthermore, mitochondrial functionality was also affected in the cerebral cortex and consequently may also contribute to the decrease in neuroactive steroid levels. Altogether, these results indicate that neurosteroidogenesis is an early target for the effect of type 1 diabetes in the cerebral cortex.

1. Introduction

The nervous system has steroidogenic activity (i.e., neurosteroidogenesis) [1–4]. Indeed, neurons and glial cells express molecules, like for instance steroidogenic acute regulatory protein (StAR) and translocator protein-18 kDa (TSPO). These molecules are involved in the transport of cholesterol (i.e., the substrate for steroidogenesis) into the mitochondria [5,6]. They also express steroidogenic enzymes, like for instance cytochrome P450 side chain cleavage (P450scc), which converts cholesterol into pregnenolone (PREG), and 3beta-hydroxysteroid dehydrogenase, the enzyme that synthesizes progesterone (PROG) from PREG [1–3]. Another steroidogenic enzyme expressed in the nervous system is 5alpha-reductase. This enzyme converts PROG into dihydroprogesterone (DHP) and testosterone (T) into dihydrotestosterone (DHT). DHP and DHT are further metabolized by the enzymes 3α-hydroxysteroid oxidoreductase and 3β-hydroxysteroid oxidoreductase. DHT is metabolized into 5alpha-androstane-3alpha,17beta-diol (3α-diol) and in 5alpha-androstane-3beta,17beta-diol (3β-diol), while DHP is metabolized into tetrahydroprogesterone (THP) and isopregnanolone (ISOPREG) [1].

As extensively demonstrated in several experimental models, neuropathological events, including diabetic encephalopathy (i.e., damage induced by diabetes in the brain) and diabetic peripheral neuropathy (i.e., damage in the peripheral nervous system), affect neuroactive steroid levels [2,7]. Indeed, in line with the alterations induced by diabetes in the reproductive axis and consequently on the levels of sex

Abbreviations: HMG-CoA R, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; 3α-diol, 5alpha-androstane-3alpha,17beta-diol; 3β-diol, 5alpha-androstane-3beta,17beta-diol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol; 24(S)-OH, 24(S)-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; ATP5A1, α subunit of the mitochondrial ATP synthase F1 complex; APOE, apolipoprotein E; ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; mt-COX2, cytochrome c oxidase subunit 2; P450scc, cytochrome P450 side chain cleavage; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; DHT, dihydrotestosterone; HSL, hormone-sensitive lipase; ISOPREG, isopregnanolone; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LDLR, low density lipoprotein receptor; mt-DNA, mitochondrial DNA; TFAM, mitochondrial transcription factor A; NDUFB8, NADH ubiquinone oxidoreductase subunit 8; CTRL, non-diabetic rats; PREG, pregnenolone; PROG, progesterone; OXPHOS, respiratory chain complexes functional subunits; SOD2, superoxide dismutase 2; SREBP2, sterol regulatory element-binding protein 2; StAR, steroidogenic acute regulatory protein; SOAT1, sterol O-acyltransferase 1; STZ, streptozotocin; SDHB, succinate dehydrogenase complex iron sulfur subunit B; T, testosterone; THP, tetrahydroprogesterone; TBARS, thiobarbituric acid reactive substances; TSPO, translocator protein-18 kDa; UQCRC2, ubiquinol-cytochrome c reductase core protein II

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steroid hormones [8–11], we demonstrated that three months of diabetes (i.e., long-term diabetes) induced in rats by streptozotocin (STZ), decrease the levels of PREG (i.e., the precursor of all steroids) as well as of several neuroactive steroids in plasma, in different areas of the central nervous system and in the peripheral nervous system [12]. The decreased levels of these important modulators of nervous function, which exert protective effects in central [13–15] and peripheral nervous system of diabetic animals [16–22], may contribute to the neural damage caused by diabetes. In addition, long-term type 1 diabetes (i.e., three months) has been shown to alter cholesterol homeostasis not only in the peripheral nervous system [23] but also in cerebral cortex [13]. Furthermore, diabetes induced in rats by STZ injection also affects mitochondrial function in the cerebral cortex [24,25]. Mitochondrial function, cholesterol homeostasis and neurosteroidogenesis are interrelated and their modification in diabetes may represent a common pathophysiological mechanism. Since in the brain, functional and biochemical changes may already occur in short-term diabetes [26,27], in this study we intend to determine whether alterations in mitochondrial function, cholesterol homeostasis and neurosteroidogenesis can be detected in the cerebral cortex after one month of diabetes.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (175–200 g at arrival, Charles River Laboratories, Lecco, Italy) where used. Animals were housed in the animal care facility of the Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB). All animals were kept in standard rat cages (cage size: 59.5 × 38.0 × 20.0 cm) with food and tap water available ad libitum and under controlled temperature (21 ± 4° C), humidity (40–60%), room ventilation (12.5 air changes per h) and light cycles (12 – hour light/dark cycle; on 7 a.m./off 7 p.m.).

The rats were acclimated to the new environment for seven days, then randomly divided into two experimental groups: i) control non-diabetic rats (CTRL) and ii) diabetic rats (STZ). Animal care and procedures were approved by our institutional animal use and care committee and followed institutional guidelines that are following national (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011).

2.2. Diabetes induction and characterization

Briefly, diabetes was induced by a single i.p. injection of freshly prepared STZ (60 mg/kg body weight; Sigma-Aldrich, Milano, Italy) in citrate buffer (0.09 M pH 4.8) as previously described [12]. Diabetes was confirmed after 48 h by tail vein blood glucose measurement in fasting condition as previously described [28]. Only rats with glucose level above 300 mg/dl were considered as diabetic. Body weight was monitored every week. After one month, CTRL and STZ rats were sacrificed, the cerebral cortex and plasma were collected and stored at −80 °C until analysis.

2.3. Liquid chromatography tandem mass spectrometry analysis (LC–MS/MS) analysis

2.3.1. Neuroactive steroids

For the quantitative analysis of different neuroactive steroids, the cerebral cortex and plasma were extracted and purified as previously described [12,29]. Briefly, using $^{13}C_3$ -17β-E (2 ng/sample) $^{13}C_3$ – PROG (0.4 ng/sample) and $^{13}C_2$ – PREG (10 ng/sample), as internal standards, PREG, PROG, DHP, THP, ISOPREG, DHEA, T, DHT, 3α-diol and 3β-diol levels were assessed based on calibration curves by high-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) as described previously [12,29].

2.3.2. Cholesterol and oxysterols

The levels of free and total cholesterol as well as of its metabolites, such as 24(S)-hydroxycholesterol (24(S)-OH), 27-hydroxycholesterol (27-OH), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-keto), were assessed by LC–MS/MS in the cerebral cortex of non-diabetic and STZ rats.

Cholesterol and cholesterol-2,2,3,4,4,6-d_6 as internal standard (S.I) were purchased from Sigma-Aldrich; 7α-hydroxycholesterol, 7β-hydroxycholesterol were purchased from Steraloids (Newport, RI, USA), 24(S)-hydroxycholesterol, 27-hydroxycholesterol, 7-ketocholesterol were purchased from ResearchPlus (Barnegat, NJ, USA).

Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap-mass spectrometer (LTQ, ThermoFisher Co., San Jose, CA, USA) for quantitative determination of total cholesterol and oxysterol as previously described [23]. Briefly, cerebral cortex of CTRL and STZ rats, were weighted, added with internal standards and homogenized in 1 ml of MeOH/ACN (1:1, v/v) using the TissueLyser (Qiagen, Italy). After centrifugation at 12,000 rpm for 5 min, supernatant was divided in two aliquots: one for the analysis of free cholesterol and oxysterols and the other one, after acid hydrolysis, for total cholesterol. Quantitative analysis was performed based on calibration curves prepared and analyzed in the same day.

2.4. Quantitative real time PCR (RT-qPCR)

RNA was extracted from snap-frozen cerebral cortex using Direct-zol™ MiniPrep kit (Zymo Research, Irvine, Calif., USA) following manufacturing protocol. The quantification of RNA was performed by NanoDrop™ 2000 (ThermoFisher scientific, Milano, Italy). Gene expression was assessed by TaqMan quantitative real-time PCR using a CFX96 real-time system (Bio-Rad Laboratories, Segrate, Italy). Samples run in 96-well formats in duplicate as multiplexed reactions with a normalizing internal control, 36B4 (Eurofins MWG-Operon, Milano, Italy) using the iTaq™ Universal Probes One-Step Kit (Bio-Rad, Segrate, Italy). Specific TaqMan MGB probes and primers sequence for SREBP2, HMG-CoA R, SOAT1, HSL, APOE, LDLR, ABCG1, ABCA1, StAR, TSPO, TFAM, mt-COX2 were purchased from Eurofins MWG-Operon (Milan, Italy) and the sequence are available on request. Whereas specific primers and probe mix for P450scc (Rn00568733_m1), Cyp27A1 (Rn01427419_m1), SOD2 (Rn00690588_g1) and 5alpha-reductase type 1 (Rn00567064_m1) were purchased from Life Technologies Italia (Monza, Italy).

2.5. Western blotting

Respiratory chain complexes functional subunits (OXPHOS) were assessed in mitochondria enriched fraction of snap-frozen cerebral cortex as previously described [30]. Total OXPHOS Rodent WB antibody cocktail (Mitoscience, Abcam, Cambrdge, UK), recognizing NDUFB8 (one of the subunits of complex I), SDHB (one of the subunits of complex II), UQCRC2 (one of the subunits of complex III), MTCO2 (one of the subunits of complex IV), ATP5A (one of the sububits of complex V), was diluted in PBS buffer – 0.1% Tween 20–2.5% non-fat dried milk, washed for 1 h and incubated for 2 h with a horse anti-mouse secondary antibody conjugated to horseradish peroxidase (Cell Signaling, Leiden, The Netherlands). OXPHOS on filter were detected with the ECL method (Bio-Rad, Milan, Italy). Filter was then cut and stripped. To detected GAPDH signal as internal control the membrane was blocked at room temperature in non-fat dried milk, successively incubated with a primary monoclonal GAPDH antibody (Santa Cruz Biotechnology inc., Heidelberg, Germany), then washed and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Milano, Italy). Protein was detected on filter