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Full Length Article

Short-term effects of diabetes on neurosteroidogenesis in the rat hippocampus

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ABSTRACT

Diabetes may induce neurophysiological and structural changes in the central nervous system (i.e., diabetic encephalopathy). We here explored whether the levels of neuroactive steroids (i.e., neuroprotective agents) in the hippocampus may be altered by short-term diabetes (i.e., one month). To this aim, by liquid chromatography–tandem mass spectrometry we observed that in the experimental model of the rat raised diabetic by streptozotocin injection, one month of pathology induced changes in the levels of several neuroactive steroids, such as pregnenolone, progesterone and its metabolites (i.e., tetrahydroprogesterone and isopregnanolone) and testosterone and its metabolites (i.e., dihydrotestosterone and 3α-diol). Interestingly these brain changes were not fully reflected by the plasma level changes, suggesting that early phase of diabetes directly affects steroidogenesis and/or steroid metabolism in the hippocampus. These concepts are also supported by the findings that crucial steps of steroidogenic machinery, such as the gene expression of steroidogenic acute regulatory protein (i.e., molecule involved in the translocation of cholesterol into mitochondria) and cytochrome P450 side chain cleavage (i.e., enzyme converting cholesterol into pregnenolone) and 5α-reductase (enzyme converting progesterone and testosterone into their metabolites) are also affected in the hippocampus. In addition, cholesterol homeostasis as well as the functionality of mitochondria, a key organelle in which the limiting step of neuroactive steroid synthesis takes place, are also affected. Data obtained indicate that short-term diabetes alters hippocampal steroidogenic machinery and that these changes are associated with impaired cholesterol homeostasis and mitochondrial dysfunction in the hippocampus, suggesting them as relevant factors for the development of diabetic encephalopathy.

1. Introduction

Diabetic disease is a chronic metabolic syndrome characterized by hyperglycemia and alterations in carbohydrate, lipid and protein metabolism. Diabetes may induce neurophysiological and structural changes in the central nervous system (CNS) (i.e., diabetic encephalopathy) [1]. These complications are associated with acute alterations in mental status due to poor metabolic control, decline in cognitive function, increase risk of dementia, depression and eating disorders [2,3]. Diabetes also influences the plasma levels of sex steroids due to the dysfunction in the reproductive axis [4,5]. In addition, the pathology also affects the levels of neuroactive steroids in the nervous tissue [6,7]. Indeed, as demonstrated in an experimental model of this pathology (i.e., rats

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; 5α-R, 5alpha-reductase; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol; DHCR24, 24-dehydrocholesterol reductase; 24(S)-OH, 24(*S*)-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; P450scc, cytochrome P450 side chain cleavage; DHEA, dehydroepiandrosterone; (DHP), dihydroprogesterone; DHT, dihydrotestosterone; HSL, hormone-sensitive cholesteryl ester hydrolase; ISOPREG, isopregnanolone; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PREG, pregnenolone; PROG, progesterone; OXPHOS, respiratory chain complexes functional subunits; SOD-2, superoxide dismutase 2; StAR, steroidogenic acute regulatory protein; SOAT1, sterol *O*-acyltransferase 1; STZ, streptozotocin; T, testosterone; THP, tetrahydroprogesterone; (TBARS), thiobarbituric acid reactive substances; TSPO, translocator protein-18 kDa.

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treated with streptozotocin), the brain levels of pregnenolone (PREG), progesterone (PROG) and testosterone (T), as well as of their metabolites are decreased after three months of diabetes [6,7]. Brain neuroactive steroid levels depend on the levels of peripheral steroids as well as on local synthesis and local metabolism [8,9]. As in other steroidogenic tissues, brain steroidogenesis is a highly compartmentalized process of reactions. The first, and limiting, step is the translocation of cholesterol from the cytoplasm to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) and translocator protein–18 kDa (TSPO) [10,11]. Cholesterol is then cleaved into PREG by the enzyme cytochrome P450 side chain cleavage (P450scc). PREG is further transformed into PROG, dehydroepiandrosterone (DHEA) and T in the endoplasmic reticulum [12]. PROG and T are then converted by the enzyme 5alpha-reductase (5α-R), into dihydroprogesterone (DHP) and dihydrotestosterone (DHT), respectively. These steroids are then further metabolized by the action of 3alpha- or 3beta-hydroxysteroid oxidoreductase. In particular, DHP is converted in tetrahydroprogesterone (THP) or in isopregnanolone (ISOPREG), while T is converted in 5alpha-androstane-3alpha,17beta-diol (3α-diol) or in 5alpha-androstane-3beta,17beta-diol (3β-diol) [12].

The brain is the most cholesterol-rich organ and accounts for the 25% of the total body cholesterol. However, the blood brain barrier prevents cholesterol uptake from the circulation [13,14]. *De novo* synthesis of cholesterol implies the rate limiting reaction of sterol synthesis by the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA R), which converts HMG-CoA into mevalonate as well as the conversion of desmosterol into cholesterol, by 24-dehydrocholesterol reductase (DHCR24). Interestingly, a link between DHCR24 and neuroactive steroids has been reported. Indeed, as demonstrated in human hepatoma cells (HepG2), PREG and PROG are potent inhibitors of DHCR24, leading to the accumulation of desmosterol [15].

The homeostasis of cholesterol is finely regulated by several enzymes, like for instance the sterol O-acyltransferase 1 (also known as Acyl-CoA cholesterol acyltransferase) (SOAT1 or ACAT1), which controls the equilibrium between free and esterified cholesterol [16,17]. The esterification step is crucial to prevent the excess of free cholesterol. Indeed, an excess of this molecule may become toxic for the cell [18]. SOAT1 activity generates a storage pool of cholesteryl ester that is readily mobilized by hormone-sensitive cholesteryl ester hydrolase or hormone sensitive lipase (HSL), to produce free cholesterol for the synthesis of steroid hormones [19].

Recent findings have demonstrated that HSL plays a vital role in regulating StAR expression in adrenal and gonadal cells [20]. Indeed, the increase of hydrolytic activity, promoted by the activation of cAMP/PKA signaling, is tightly connected with StAR expression [21]. In agreement, deficiency of HSL affects StAR and steroid levels [22,23].

The cholesterol turnover in the brain is also balanced by the formation of oxygenate derivatives. Cholesterol is enzymatically converted into 24(*S*)-hydroxycholesterol (24(S)-OH) by the cholesterol 24-hydroxylase (i.e. CYP46A1) [24]. Moreover, 24 (S)-OH is not the only metabolite of cholesterol produced in the brain. Indeed, it is well known that the CNS expresses the enzymatic equipment to produce 25- and 27-hydroxycholesterol (25-OH and 27-OH). In addition, oxidative stress may oxidize cholesterol producing 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-keto) [25,26].

Evidence in literature suggests that diabetic encephalopathy may already occur after short-term period of diabetes [27,28]. Therefore, on the basis of our previous data obtained in long-term diabetes (i.e., 3 months) on neuroactive steroid levels [6,7], we presently assessed in the hippocampus and in plasma of STZ rats whether the levels of neuroactive steroids were already altered after one month of diabetes. On the basis of the results obtained we measured in the hippocampus the gene expression of molecules involved in synthesis and metabolism of neuroactive steroids as well as of cholesterol. Moreover, because the limiting step of steroidogenesis occurs in the mitochondria, and their function is affected by oxidative stress occurring in diabetic encephalopathy [29,30] we assessed the effects of short-term diabetes on the mitochondria functionality in the hippocampus.

2. 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–h light/dark cycle; on 7 a.m./off 7 p.m).

The rats were acclimated to the new environment for 7 days before being randomly assigned to one of the experimental groups described below. Animal care and procedures were approved by our institutional animal use and care committee and followed institutional guidelines that are in compliance with 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

The animals were randomly divided into two experimental groups: i) non-diabetic animals (CTRL) and ii) streptozotocin diabetic animals (STZ). To induce diabetes, rats received a single i.p. injection of freshly prepared streptozotocin (60 mg/kg body weight; Sigma-Aldrich) in citrate buffer (0.09 M pH 4.8) as previously described [7]. After 48 h, diabetes was confirmed by tail vein blood glucose measurement in fasting condition using a commercial glucometer (One Touch UltraMini®, Johnson and Johnson Healthcare, USA). Only rats with blood glucose above 300 mg/dl were considered as diabetic. Body weight was assessed every week. After one month from the determination of hyperglycemic status, CTRL and STZ rats were sacrificed and the hippocampus and plasma were collected and sorted at −80 °C until analysis. In particular, blood samples were first collected in heparin tubes, then placed in centrifuge tubes and centrifuged at 2500*g* for 15 min at 4 °C to obtain plasma.

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

2.3.1. Neuroactive steroids

For the quantitative analysis of different neuroactive steroids, the hippocampus and plasma were extracted and purified as previously described [31]. 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 on the basis of calibration