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Methods paper

The role of phosphoenolpyruvate carboxykinase in neuronal steroidogenesis under acute inflammation

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ABSTRACT

Phosphoenolpyruvate carboxykinase (PEPCK) is a key gluconeogenic enzyme found in many tissues throughout the body including brain. In the present study, we have investigated the effect of bacterial lipopolysaccharide (LPS) on PEPCK and its role in neuronal steroidogenesis. Adult female albino rats were administered LPS (5 mg/kg body weight) to induce acute inflammation. LPS administration resulted in a significant increase of PEPCK mRNA expression with concomitant increase in mRNA levels of steroidogenic acute regulatory (StAR) protein and other steroidogenic enzymes including 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase in brain tissue. Further, the inhibition of PEPCK expression by glipizide significantly decreased the mRNA expression of steroidogenic proteins and concurrently increased the mRNA levels of proinflammatory cytokines under LPS administration. The results of this study suggest a novel finding that PEPCK may have an important role in neuronal steroidogenesis; which serves as an adaptive response under inflammation.

1. Introduction

The brain synthesizes significant amounts of sex steroids besides testis and ovary. Steroid hormones in the brain exert important biological functions including neuroprotection, regulation of gene expression, neuronal survival, synaptic transmission, and neuronal and glial differentiation in many brain areas (Saldanha et al., 2009; Garcia-Segura, 2008; Stoffel-Wagner et al., 1999). Bacterial lipopolysaccharide (LPS) is a major component of the cell wall of gram negative bacteria contributing to the pathogenesis of bacterial infection, in particular to those diseases affecting central nervous system including cerebral malaria and bacterial meningitis. Proinflammatory cytokines generated in response to LPS exposure inhibit steroidogenesis in many tissue types including the ovary and testes (Magata et al., 2014; Reddy et al., 2006). In contrast to the ovary and testes, neurosteroid levels are increased in vivo after LPS treatment and result in the suppression of TNF production in brain (Ghezzi et al., 2000). LPS increases the expression and activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) in brain microglia (Gottfried-Blackmore et al., 2008). However, the mechanisms behind the increased production of neurosteroids in inflammatory conditions are not well understood.

Phosphoenolpyruvate carboxykinase (PEPCK) is found in many tissues throughout the body and catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis. Besides its role in gluconeogenesis, PEPCK has a number of other physiological roles such as glyceroneogenesis, anaplerosis and cataplerosis (Hanson, 2009). Most recently, it has been demonstrated that PEPCK and glucose-6-phosphatase (Glc-6-Pase) are required for steroidogenesis in testicular Leydig cells (Ahn et al., 2012). Further, Sugita et al. (2002) have shown that PEPCK mRNA expression levels were increased in liver in LPS administered rats. Interestingly, PEPCK is also expressed in the brain; however its physiological role in neurons is not well studied. With this background, the present work was designed to check whether PEPCK has any role in neuronal steroidogenesis under LPS induced acute infection.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide from *Escherichia coli* (serotype 055:B5) was purchased from Sigma Chemicals Company, Saint Louis, MO, USA. All of the other chemicals used were of analytical grade and were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; StAR, steroidogenic acute regulatory protein; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; LPS, lipopolysaccharide.

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2.2. Experimental animals

Adult female albino rats of Wistar strain weighing 220 ± 10 g (10–12 weeks old) were used in the study. The animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C), and light (12 h light/12 h dark). The animals were housed in large spacious cages bedded with husk and fed with a standard rat pelleted diet and had free access to water. Experimental animals were handled according to the university and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.3. Study design

The animals were randomly divided into three groups of six rats each. Saline treated animals served as controls (Group I). Group II (LPS) received intraperitoneal (i.p.) injections of LPS (dissolved in 0.5 ml of sterile saline) at a dose of 5 mg/kg body weight to induce acute inflammation. Group III (LPS + glipizide) received a single intravenous injection of glipizide (5 mg/kg body weight); 1 h prior to the administration of intraperitoneal injections of LPS (5 mg/kg body weight). The animals were fasted 12 h before commencement and also during the experimental period. The blood was collected and the brains were excised immediately after 24 h of treatment period.

2.4. Preparation of tissue homogenate

Briefly, the tissue was chopped and minced in 6–8 ml of 0.25 M sucrose/TKM buffer (50 mM Tris–HCl, 25 mM KCl and 5 mM $MgCl_2$). The tissue was then homogenized gently by using Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1500 rpm for 5 min. The supernatant was transferred and used as whole tissue homogenate for enzymatic analyses.

2.5. Estimation of protein

Protein content of the tissue fractions was estimated by the method of Lowry et al. (1951). Briefly, 0.1 ml of the diluted tissue homogenate was made up to 1 ml with water. To this, 4.5 ml of alkaline copper reagent (2% Na_2CO_3 in 0.1 N NaOH was mixed with 0.5% $CuSO_4$ containing 1% sodium potassium tartrate in the ratio of 50:1) was added, mixed and allowed to stand at room temperature for 20 min. Later, 0.5 ml of Folin–Ciocalteu reagent was added and shaken well. The blank and bovine serum albumin (BSA) standards were also treated in a similar manner. The blue color complex formed was measured at 640 nm after 15 min against the blank.

2.6. Assay of steroidogenic enzymes

2.6.1. 3β-Hydroxysteroid dehydrogenase (3β-HSD)

The activity of 3β-HSD was measured by the method of Shivanandappa and Venkatesh (1997). The enzyme was assayed in 0.1 M Tris–HCl buffer (pH 7.8) containing 500 μM NAD^+, 0.5 ml of color reagent (1% Tween 20 containing 0.08% iodonitrotetrazolium chloride) and the substrate (100 μM pregnenolone) in a total volume of 3 ml. The reaction was started by adding the enzyme (50 μl) and incubated at 37 °C for 60 min. The reaction was stopped by the addition of 2 ml of phthalate buffer (2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml 0.1 N HCl and 2.5 ml Tween 20; pH was adjusted to 3.0 and the volume made up to 250 ml). The turbidity was removed by centrifugation at 3000 rpm for 20 min and the supernatant was read at 490 nm in a spectrophotometer. For standard curve, aliquots of graded concentrations of freshly prepared NADH (0–150 nM) were reacted with 0.5 ml of color reagent (1% Tween 20 containing 0.08% iodonitrotetrazolium chloride and 0.02% phenazine methosulfate) and after color formed, 2 ml of phthalate buffer was added and the absorbance was read at 490 nm. The enzyme activity was calculated from the standard curve of NADH and expressed as nmol of NADH formed/min/mg protein.

2.6.2. 17β-Hydroxysteroid dehydrogenase (17β-HSD)

The activity of 17β-HSD was determined by the method as described previously (Bergmeyer, 1974). 17β-HSD catalyzes the reversible reaction of androstenedione into testosterone using NADPH as a coenzyme. The activity is determined by the optical measurement of the rate of conversion of NADPH to NADP. In brief, the reaction mixture contained 100 μl of testicular supernatant, 200 μl of 0.5 μM NADPH, and 100 μl of 0.8 μM androsten-3,17-dione in a final volume of 3 ml 100 μM phosphate buffer solution (pH 7.4). The reaction was initiated by the addition of the substrate and the decrease in absorbance of NADPH was followed at 340 nm for 5 min at 20 s interval. The enzyme activity was expressed as nmol of NADPH oxidized/min/mg protein.

2.7. Estimation of blood glucose

Blood glucose levels were measured with OneTouch Select blood glucose monitoring system (LifeScan, Inc., USA).

2.8. Analysis of the gene expression by reverse transcriptase polymerase chain reaction (RT-PCR)

Briefly, total RNA from whole brain was isolated according to the RNA isolation kit instructions (One step RNA Trizol Reagent; Biobasic Inc., Canada). RT-PCR was performed with 4 μg of total RNA isolated from tissue by using AMV-one step RT-PCR kit (GeNei, India). The specific sets of primers for target genes are shown in Table 1. PCR amplification was carried out according to a protocol for the initial denaturing step at 95 °C for 10 min; then 30 cycles at 95 °C for 1 min (denaturing), at 55 °C for 1 min (annealing) and 72 °C for 1.5 min (extension); and a further extension at 72 °C for 10 min. To compare the amount of steady state mRNA, 5 μl of each PCR product was resolved onto 1.4% agarose gel using TBE buffer. After electrophoresis, the gels were viewed under UV light and digital images were captured on Gelstan gel documentation system. The densitometric analyses were carried out with lab image platform ver 2.1 software by Kapelan Bio-Imaging GmbH. The expression of each target gene was normalized with internal control and represented as a ratio.

Table 1
Primer sequences for PCR for each target gene.

Gene	Primer
Steroidogenic acute regulatory (StAR) protein	F: 5′-AGCCAGCAGGAGAATGGAGAT-3′ R: 5′-CACCTCCAGTCGGAACACCTT-3′
3β-HSD	F: 5′-AACTGGTCTTCAGGTCACCAGAA-3′ R: 5′-GTCCCCTGCACCTTGTTCA-3′
17β-HSD	F: 5′-CCTTTGGCTTTGCCATGAGA-3′ R: 5′-CAATCCATCCTGCTCCAACCT-3′
Aromatase	F: 5′-TCCTCCTGATTCGGAATTGTG-3′ R: 5′-GGCCCGATTCCCAGACA-3′
CREB-binding protein/E1A binding protein (CBP/p300)	F: 5′-TGCAGTCTGCTACTACTGCG-3′ R: 5′-AAGAGCCGTATGCCAAGGTC-3′
PEPCK	F: 5′-TCCCATTGGCTACGTCCCT-3′ R: 5′-CAGCATTGTGCTTGCTGGTT-3′
Tumor necrosis factor alpha (TNF-α)	F: 5′-TTCCTTACGGAACCCCCTCT-3′ R: 5′-CCCGTAGGGCGATTACAGTC-3′
Interleukin-6 (IL-6)	F: 5′-CACTTCACAAGTCGGAGGCT-3′ R: 5′-AGAGCATTGGAAGTTGGGGT-3′
β-Actin	F: 5′-TTCAACACCCCAGCCATGT-3′ R: 5′-TGGTACGACCAGAGGCATACAG-3′