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Amelioration of neurological and biochemical deficits by peroxynitrite decomposition catalysts in experimental diabetic neuropathy

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ABSTRACT

Diabetic neuropathy, a major complication of diabetes, affects more than 60% of diabetic patients. Recently, involvement of peroxynitrite has been postulated in diabetic neuropathy. In the present study, we have studied the effects of peroxynitrite decomposition catalysts (PDC's)—5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) [FeTPPS] and 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphyrinato iron(III) [FeTMPyP]—in experimental diabetic neuropathy. Male Sprague–Dawley rats, with six weeks of untreated diabetes were treated for two weeks with peroxynitrite decomposition catalysts. Diabetic animals showed a significant decrease in motor nerve conduction velocity and nerve blood flow, nociception as evident from decreased tail flick latency (hyperalgesia) and increased paw withdrawal pressure (mechanical allodynia) along with elevation in peroxynitrite and reduction in nerve glutathione levels. Two weeks treatment with PDC's significantly improved all the above stated functional and biochemical deficits. Aftermath of this study advocates the beneficial effects of peroxynitrite decomposition catalysts in experimental diabetic neuropathy.

1. Introduction

Diabetic neuropathy is a heterogeneous disorder whose enigmatic etiology is characterized by a number of interwoven pathways subsuming increased flux of glucose to polyol pathway, increased hexosamine shunt, aldose reductase activation, decrease in nerve myoinositol content, formation of advanced glycation end products (AGE), impaired neurotrophic support, activation of protein kinase C (PKC), activation of poly (ADP-ribose) polymerase (PARP), impaired insulin/C peptide action etc (Evans et al., 2002; Obrosova et al., 2005a). All these factors result in the formation of a variety of reactive oxygen species (ROS) which are the ultimate malefactor involved in most of the diabetic complications (Pop-Busui et al., 2002; Sima, 2006). Amongst this class of species, the notorious ones are superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydroxyl radical (OH^-). Superoxide though performs some of the house keeping functions and is an audacious defense against microbial invasion, but under pathological conditions, unfettered production of nitric oxide (Fujimoto et al., 2004) coupled with deficient body's antioxidant defense results in reaction of nitric oxide with superoxide to form peroxynitrite which is several times multiple of its parents (superoxide and nitric oxide) in terms of tissue toxicity (Patel et al., 1999). Peroxynitrite can cause hydroxylation and nitration of the aromatic residues of proteins and nucleotides (Stamler, 1994). Peroxynitrite is also responsible for the abrasion of the body's antioxidant defense by inactivation of enzyme superoxide dismutase (Bartosz, 1996; Szabo, 2003). Generation of superoxide and peroxynitrite impairs vascular function in diabetic rats, which precedes the slowing of nerve conduction velocity (Coppey et al., 2002). In addition to these effects, peroxynitrite induced DNA damage leads to over-activation of PARP, a nuclear enzyme involved in DNA repair (Szabo, 2005). Ramifications of PARP over-activation include energy depletion, failure of membrane associated ATP dependent ion pumps and disruption of membrane potential. The deleterious effects of peroxynitrite have been reported in a number of inflammatory conditions as well as in cardiovascular and central nervous system disorders (Cuzzocrea et al., 2006; Mabley et al., 2002). Peroxynitrite is also involved in the pathogenesis of complications associated with diabetes which includes diabetic neuropathy, vasculopathy, retinopathy, and nephropathy (Obrosova et al., 2005b).

Peroxynitrite decomposition catalysts act by promoting the cleavage of peroxynitrite to nitrate thereby reducing the levels of notorious reactive intermediates such as nitrogen dioxide and hydroxyl radicals (Bartosz, 1996; Patel et al., 1999). Neuroprotective potential of peroxynitrite decomposition catalysts in cerebral ischemic reperfusion injury has been demonstrated earlier (Sharma et al., 2004; Thiyagarajan et al., 2004). In the present study we have investigated the neuroprotective potential of peroxynitrite decomposition

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catalysts (FeTPPS and FeTMPyP) in experimental diabetic neuropathy by assessing various functional, behavioral, biochemical and immunohistological parameters.

2. Materials and methods

2.1. Reagents

FeTPPS and FeTMPyP were purchased from Calbiochem, Germany and dissolved in saline. Streptozotocin (STZ) was procured from Sigma-Aldrich, U.S.A and dissolved in citrate buffer (pH 4.4). Glucose oxidase–peroxidase (GOD–POD) kit was purchased from Accurex, India. All other chemicals of analytical grade were purchased locally.

2.2. Animals

Healthy male Sprague–Dawley rats (250–270 g) were obtained from the Central Animal Facility (CAF), National Institute of Pharmaceutical Education and Research (NIPER), Punjab, India. Animals were provided with standard diet and water *ad libitum*. They were housed in plastic cages (two in each) at a controlled temperature 22±2 °C and humidity 55±5%, with 12 hour light and dark cycle. All the experimental protocols were approved by the Institutional Animal Ethics Committee of NIPER.

2.3. Induction of diabetes

Diabetes was induced using a single dose of streptozotocin (55 mg/kg, i.p.). Age matched control rats received an equal volume of vehicle (citrate buffer). Diabetes was confirmed after 48 h of streptozotocin injection by estimating plasma glucose levels using glucose oxidase–peroxidase (GOD–POD) and the rats with plasma glucose level >250 mg/dl were used for further studies.

2.4. Treatment schedule

Diabetic neuropathy was well developed after six weeks of streptozotocin treatment as reported earlier (Sayyed et al., 2006). The two weeks treatment with FeTPPS (1, 3 and 10 mg/kg/day, i.p.) and FeTMPyP (1 and 3 mg/kg/day, i.p.) was started after the sixth week of diabetes induction and continued till the eighth week. All the parameters (motor nerve conduction velocity, nerve blood flow, nociception and biochemical parameters) were measured 24 h after the last dose. The number of animals used for motor nerve conduction velocity, nerve blood flow and thermal hyperalgesia was 6–8 and for biochemical (lipid peroxidation, peroxynitrite and glutathione levels) estimations 3–6.

2.5. Motor nerve conduction velocity

Power Lab 8sp instrument (AD Instruments, Australia) was used for the measurement of motor nerve conduction velocity. Briefly the animals were anesthetized by 4% halothane in a mixture of nitrous oxide and oxygen and anesthesia was maintained with 1% halothane, using gaseous anesthesia system (Harvard apparatus, UK). Motor nerve conduction velocity was measured by stimulating the sciatic (proximal to sciatic notch) and tibial (distally to ankle) nerve using bipolar needle (261/2 gauge) electrodes with 3 volt, single stimulus as described previously (Kumar et al., 2007). Body temperature of rats was maintained at 37 °C using homoeothermic blanket system (Harvard, UK) throughout the experiment. Motor nerve conduction velocity was calculated by following formula:

Motor nerve conduction velocity(m/s)
= (Distance between sciatic and tibial nerve stimulation point)
/(Sciatic M wave latency–*Tibial M wave latency*).

2.6. Composite nerve blood flow

Nerve blood flow was measured using Laser Doppler flowmeter (Perimed, Sweden) as described earlier by Kumar et al. (2007). The Laser Doppler has been compared to other methods and has been reported to be equivalent to other available methods like hydrogen clearance (Takeuchi and Low, 1987) and [^{14}C] iodo antipyrine method (Rundquist et al., 1985) of blood flow measurement. Briefly, the animals were anesthetized by 4% halothane in a mixture of nitrous oxide and oxygen and anesthesia was maintained with 1% halothane, using gaseous anesthesia system (Harvard apparatus, UK). Anesthetized animals were placed on stereotaxic equipment to achieve uniform positioning of Laser Doppler probe each time. Sciatic nerve of the left flank was exposed and laser probe was placed just above the nerve. Nerve blood flow was recorded for 10 min after 15 min of stabilization period. Averages of 10 min continuous recordings were represented as arbitrary perfusion units (PU). During the recording of nerve blood flow, body temperature of the rats was maintained at 37 °C using homoeothermic blanket system (Harvard apparatus, UK).

2.7. Thermal hyperalgesia

Hyperalgesia was assessed using tail immersion test. Rats were trained for three days prior to final measurement. The rat tail was immersed in warm (45 °C) water and the tail flick response latency (withdrawal response of tail) or any signs of struggle were observed as the end point response. Cut-off time was kept at 15 s.

2.8. Mechanical allodynia

To assess the mechanical allodynia, the Electrovonfrey Anaesthesiometer was used (IITC, Woodland Hills CA). The animals were kept on a perforated platform and vonfrey hairs were applied to the underside of their paw. The pressure (g) at which the rat withdraws its right paw was recorded (Sweitzer et al., 2004). Five consecutive readings were taken at an interval of 5 min.

2.9. Peroxynitrite estimation

Peroxynitrite formation was estimated by using a fluorescent dye dihydrorhodamine123 (Szabo et al., 1995; Thiyagarajan et al., 2004). Dihydrorhodamine123 gets oxidized to rhodamine123 in a peroxynitrite dependent manner. Rats were injected dihydrorhodamine123 through the femoral vein (2×10^{-6} M/ml/kg in saline). After 2 h of injection, blood was withdrawn and fluorescence in the plasma was measured using the spectrofluorometer at an excitation wavelength of 500 nm and emission wavelength of 536 nm. The plasma level of rhodamine123 was calculated from the standard curve obtained from authentic rhodamine123 in a concentration of 0–10 nM prepared in plasma obtained from the untreated rat.

2.10. Glutathione estimation

Sciatic nerve homogenate was used for measuring reduced glutathione (GSH) content as described by Moron et al. (1979) with some modification. Nerve was homogenized in phosphate buffer saline (pH 7.4). An aliquot (0.1 ml) of homogenate was mixed with 10% sulphosalicylic acid (0.5 ml) and kept in ice for 20 min. Then the resulting solution was centrifuged at 10,000 g for 5 min at 4 °C, 50 µl of the supernatant was mixed with 450 µl of phosphate buffer and 1.5 ml of 5,5′-dithiobis (2-nitro benzoic acid) in 0.1 M phosphate buffer, (pH 8.0), incubated for 10 min at 37 °C followed by measurement of absorbance at 412 nm spectrophotometrically, using reduced glutathione as an external standard (Moron et al., 1979; Tietze, 1969). Protein content in tissue homogenate supernatant was determined by the Lowry method (Lowry et al., 1951).