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Functional and biochemical evidence indicating beneficial effect of Melatonin and Nicotinamide alone and in combination in experimental diabetic neuropathy

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ABSTRACT

Oxidative stress resulting in excessive generation of ROS is a compelling initiator of DNA damage along with damage to various cellular proteins and other macromolecules. Poly(ADP-ribose) polymerase (PARP) activation in response to DNA damage, stirs an energy-consuming cellular metabolic cycle; culminating into cell death. The present study was designed to determine the effect of combining an antioxidant, Melatonin and a PARP inhibitor, Nicotinamide on the hallmark deficits developing in diabetic neuropathy (DN). Streptozotocin (STZ, 55 mg/kg, i.p.) was administered to induce diabetes. Six weeks post diabetes induction, two week treatment with Melatonin (3 and 10 mg/kg) and Nicotinamide (100 and 300 mg/kg) either alone or in combination was given. Effect of these interventions on the functional, behavioral and biochemical changes caused by hyperglycemia were studied in treated animals. Melatonin and Nicotinamide alone as well as in combination ameliorated the functional deficits along with improvement in pain parameters. The combination also demonstrated an essential reversal of biochemical alterations. Nitrotyrosine and Poly ADP Ribose (PAR) immunopositivity was significantly decreased in sciatic nerve micro-sections of treatment group. The results of this study advocate that simultaneous inhibition of oxidative stress-PARP activation cascade may prove useful for the pharmacotherapy of DN.

1. Introduction

The hyperglycemia is the backbone of the pathophysiology of diabetes leading to the development of complications like diabetic neuropathy (DN) through many intertwined cellular pathways which have been shown to coalesce into a common fate i.e. oxidative stress. Reactive oxygen species (ROS) are notorious for contributing to cell and tissue dysfunction and damage in DN. Hyperglycemia unleash multiple pathways such as enhanced aldose reductase activity (Srivastava et al., 2005), increased advanced glycation end products (Sugimoto et al., 2008) and altered protein kinase C activity (Yamagishi et al., 2008) to induce oxidative stress (Vincent et al., 2008). Prolonged hyperglycemia, through overproduction of ROS, is likely to damage dorsal root ganglion mitochondrial DNA, adding to long-term nerve dysfunction. Many studies have supported this hypothesis, including *in vivo* and *in vitro* measurement of oxidative stress in sensory neurons and dorsal root ganglion (Schmeichel et al., 2003). Exhaustion of natural antioxidant depot in the vascular endothelium and schwann cells of the sciatic nerve may contribute to the neurovascular and metabolic deficits in DN (Ilnytska et al., 2006). Beneficial effects of various antioxidants in experimental DN have been shown by our group (Arora et al., 2008; Kumar et al., 2007; Negi et al., in press; Sayyed et al., 2006; Sharma et al., 2009) and also by other investigators (Coppey et al., 2001a,b).

Melatonin (*N*-acetyl-5-methoxytryptamine), synthesized in the pineal gland during the night, is a well known biological antioxidant and it also stands out as a powerful neutralizer of peroxynitrite and hydroxyl free radical. Melatonin has been shown to condense several complications of diabetes such as altered pain perception (Arreola-Espino et al., 2007), oxidative–antioxidative status (Anwar and Meki, 2003) and the pancreatic (Kanter et al., 2006), renal (Derlacz et al., 2005) and liver (Guven et al., 2006) injury. However, its efficacy in nerve functional parameters and innate antioxidant mechanisms in diabetic rats has not been assessed.

Abbreviations: DN, Diabetic Neuropathy; MNCV, motor nerve conduction velocity; NBF, Nerve blood flow; ND, Non-diabetic; PARP, Poly (ADP–ribose) polymerase; SOD, Superoxide dismutase; STZ, streptozotocin.

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Deriving from various experimental evidence it has been hypothesized that PARP activation is one of the key pathogenetic mechanisms leading to the development of DN. Consistent with this fact is the observation that PARP inhibition or its genetic ablation caused correction of diabetes-associated endoneurial nutritive NBF and vascular conductance deficits in the peripheral nerve (Obrosova et al., 2008). Activation of PARP is now viewed as an important effector of oxidative-nitrosative stress. Under normal conditions, PARP activity is relatively quiescent. However, under conditions of oxidative stress, excessive DNA single-strand breakage is triggered by ROS leading to overactivation of PARP. Activated PARP initiates an energy-consuming cycle resulting in rapid depletion of the intracellular pools of NAD^+ and ATP, hampering glycolysis and mitochondrial respiration, eventually leading to cellular energy crisis and cell death (Obrosova et al., 2005). Oxidative stress followed by PARP activation also stimulates activation of redox-sensitive transcription factors such as NF-*k*B and AP-1, key regulators of inflammatory cytokines and chemokines (Hassa and Hottiger, 2002).

PARP inhibitors that have been tried in diabetic neuropathy include 1,5-Isoquinolinediol (Obrosova et al., 2004) and 4-Amino-1,8-napthalimide (Sharma et al., 2008). Nicotinamide is a water soluble B group vitamin that serves as a precursor of NAD^+, a free radical scavenger, and a weak PARP inhibitor. An array of activities displayed experimentally by Nicotinamide including improvement in energy metabolism in brain following transient focal cerebral ischemia (Yang et al., 2002) and inhibition of apoptosis in neuronal cells (Stevens et al., 2007), makes it a lucrative agent for the treatment of DN.

Even today the pathogenetic mechanisms leading to DN are fathomless mysteries and till date no experimental evidence have been generated which claim to reverse the deficits associated with DN utilizing a solo neuroprotective approach. The present study is centered on two of the premier, correlated pathways that may be considered as the heart of pathophysiology of DN i.e. oxidative stress and PARP overactivation. Considering the role executed by these pathways in the pathophysiology of DN, simultaneously targeting oxidative stress-PARP cascade using combination of antioxidant (Melatonin) and PARP inhibitor (Nicotinamide) can crystallize all the efforts made to ameliorate the epidemic of DN.

2. Materials and methods

Unless otherwise stated, all chemicals were of reagent grade and were purchased from Sigma (St Louis, MO). Poly (ADP Ribose) (PAR) antibody from Alexis Biochemicals, USA. Nicotinamide adenine dinucleotide (NAD) & [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) was obtained from HiMedia laboratories, India. Halothane was obtained from Nicholas Piramal, India. Glucose oxidase-peroxidase glucose kit was purchased from Accurex, India.

3. Induction of diabetes and experimental design

The experiments were performed in accordance with regulations specified by the Institute Animal Ethics Committee (IAEC), NIPER. Male Sprague Dawley rats (250–270 g) were used and were fed on standard rat diet and water *ad libitum*. Diabetes was induced by Streptozotocin (STZ) at a dose of 55 mg/kg (i.p.). Blood samples were collected from tail vein ~48 h after STZ administration. The rats with blood glucose more than 250 mg/dl were considered as diabetics and were further considered for study. The experimental groups were comprised of non-diabetic control group (ND), diabetic control rats (STZ-D), and diabetic rats treated with Melatonin (D + M3 and D + M10 respectively for 3 and 10 mg/kg, p.o.), Nicotinamide (D + N100 and D + N300 respectively for 100 and 300 mg/kg, p.o.) and combination of Melatonin 3 mg/kg and Nicotinamide 100 mg/kg (D + Combi). The treatment was started 6 weeks after diabetes induction and was continued for two weeks (Fig. 1). The behavioral and biochemical experiments were performed 24 h after administration of last dose.

4. Anesthesia, euthanasia and tissue sampling

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). Core temperature was monitored and maintained (37 ± 1 °C) using a rectal probe with help of homeothermic blanket. Animals were sacrificed with high dose of anesthesia. Both nerves were dissected and were collected till trifurcation. The nerves to be used for immunohistochemistry were fixed in formalin. For biochemical estimations nerve was homogenized in phosphate buffer and was used for various estimations.

5. Functional studies

5.1. Motor nerve conduction velocity (MNCV)

MNCV was determined in the sciatic-posterior tibial conducting system using Power Lab 8sp system (ADInstuments, Bellaviata, NSW, Australia) as previously described (Arora et al., 2008). Sciatic nerve was stimulated with 3 V proximally at sciatic notch and distally at ankle via bipolar electrodes. Receiving electrodes were placed on the muscle of foot. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. MNCV was calculated by subtracting the distal latency from the proximal latency, and then dividing by the distance between the stimulating and recording electrode. MNCV was expressed in m/s.

5.2. Sciatic nerve blood flow

Immediately after nerve MNCV determination, NBF was measured using LASER Doppler system (Perimed, Jarfalla, Sweden) (Sharma et al., 2008). Briefly, animals were anesthetized and body temperature was monitored using a rectal probe and maintained with the help of homeothermic blanket throughout the experiment, sciatic nerve was exposed by giving incision on the left flank and the laser Doppler probe (tip diameter 0.85 mm) was applied just in contact with an area of sciatic trunk free from epi or perineurial blood vessels. Flux measurement was obtained from the same part of nerve and for the same time period (over a ten-minute period). The blood flow was reported in arbitrary perfusion units (PU).

6. Behavioral studies

6.1. Thermal hyperalgesia

The thermal hyperalgesia to both hot (45 °C) and cold (10 °C) immersion test was studied. The tail flick latency was taken as end point in the tail immersion test. The flicking of tail or the symptoms

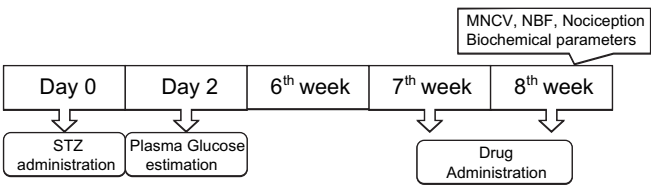

Fig. 1. Schematic representation of experimental design.