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Original research article

Neuroprotective effects of pioglitazone against transient cerebral ischemic reperfusion injury in diabetic rats: Modulation of antioxidant, anti-inflammatory, and anti-apoptotic biomarkers

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ABSTRACT

Background: Recent growing consensus introduced thiazolidinediones, agonists of the nuclear receptor peroxisome proliferator-activated receptor gamma as promising candidates in the management of ischemia in various organs. Thereby, interest was raised to investigate the neuroprotective effects of pioglitazone against transient ischemia/reperfusion (I/R) injury in diabetic rats targeting mainly the oxidative-inflammatory-apoptotic cascades which are involved in this insult.

Methods: Forebrain ischemia was induced in streptozotocin-diabetic rats by occlusion of the bilateral common carotid arteries for 15 min followed by 1 h reperfusion. Pioglitazone (10 mg/kg; *po*) was administered daily for 2 weeks prior to I/R.

Results: The drug alleviated hippocampal injury inflicted by diabetes and/or I/R injury where it suppressed nuclear factor kappa (NFκB), and consequently the downstream inflammatory cytokines tumor necrosis factor-α and interleukin-6. In parallel, the anti-inflammatory cytokine interleukin-10 was elevated. Antioxidant potential of pioglitazone was depicted, where it reduced neutrophil infiltration, lipid peroxides, nitric oxide associated with replenished reduced glutathione. Decline of excitatory amino acid glutamate content is a main finding which is probably mediated by the NFκB signaling pathway as well as improved oxidant status. Pioglitazone exerted an anti-apoptotic effect as reflected by the reduction of the cytosolic cytochrome c and the key downstream executioner caspase-3.

Conclusions: Pioglitazone is endowed with neuroprotective properties which are probably mediated by its antioxidant, anti-inflammatory, and anti-apoptotic mechanisms hence may provide a successful agent for the management of ischemic stroke.

Introduction

Thiazolidinediones (TZDs), ligands for the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ), are insulin sensitizers which improve insulin-mediated glucose uptake into skeletal muscles without increasing endogenous insulin release [1,2]. Growing evidence verifies the protective effects of PPAR-γ agonists against ischemic injury in various organs including peripheral nerves [3], liver [4], and heart [5]. Ischemic brain injury causes marked neurological dysfunctions and is a predisposing factor to stroke [6,7]. Diabetes mellitus is also a major risk factor for ischemic cerebrovascular injuries and is considered one of the global etiological risk factors of stroke. Inevitably, diabetic patients are at least 2-fold more liable to have a stroke as compared to non-diabetics. Besides, they reveal poor functional outcomes and prognoses, and exhibit high rates of morbidity and mortality after stroke [8].

Oxidative stress, inflammation, and extensive programmed cell death are common culprits in the pathophysiology of both diabetes and ischemic brain injuries [9–11], accordingly modulation of these detrimental pathways may offer promising opportunities for the management of ischemic injuries in diabetics. In fact, in its early stages cerebral ischemia activates the synthesis and release of inflammatory mediators, such as interleukin IL-1β, IL-6 and TNF-α, which promote the transformation of quiescent microglia into reactive microglia and the migration of granulocytes and macrophages into the ischemic brain parenchyma. Inflammation further precipitates the neuronal death after stroke [12].

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Transcription factors like nuclear factor kappa B (NFκB) are known to be induced during the acute phase after ischemia and promote postischemic inflammation, generation of reactive oxygen species (ROS), and neuronal damage [13].

This study investigated the protective effects of pioglitazone, a potent synthetic PPAR-γ agonist, against transient I/R injury in diabetic rats. Interestingly, the drug was formerly shown to exert eminent neuroprotective effects in neurodegenerative disorders including Parkinson's [14,15], and Alzheimer's [16] diseases, as well as focal cerebral ischemia [17] in preclinical studies.

Materials and methods

Chemicals

Streptozotocin (STZ), pioglitazone and thiopental sodium were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity and analytical grade.

Animals

Male Wistar albino rats weighing 200–250 g were used in the present study. They were allowed an acclimatization period for at least one week prior to testing. Animals were kept under controlled environmental conditions; room temperature (24–27 °C), constant humidity (60 ± 10%), with alternating 12 h light and dark cycles. Standard pellet diet and water were allowed *ad libitum.* All animals' procedures were performed in accordance with the ethical procedures and policies approved by the Ethics Committee of Faculty of Pharmacy, Cairo University and complies with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of STZ (40 mg/kg), freshly prepared in 0.1 M citrate buffer, pH 4.5 [18]. Rats were allowed to drink 5% glucose solution during the first 24 h of diabetes induction to overcome the drug-induced hypoglycemia [19]. Two days later, blood samples were collected from rats' tails and hyperglycemia was confirmed by a blood glucose level reaching 300 mg/dl. Glucose was measured using an analyzer (Roche Diagnostic Accu-Check test strips, Berlin, Germany).

Induction of transient cerebral ischemia

Rats were anaesthetized with thiopental (50 mg/kg, *ip*) and a midline ventral incision was made in the neck. Bilateral carotid artery occlusion using small artery clips was used to induce global cerebral ischemia for 15 min followed by 60 min reperfusion period [10].

Experimental design

Hundred rats were randomly allocated into five groups, the first two groups received single injection of citrate buffer; one group served as normal sham operated while the other was subjected 6 weeks later to transient I/R and served as I/R control group and received 1% Tween 80 (10 ml/kg; *po*) daily during the last 2 weeks before ischemia. Diabetes was induced in the rest of animals which were divided into three groups and kept for 6 weeks; the first one served as diabetic sham operated group; the second was subjected to I/R by the end of the 6th week, the last group pioglitazone (10 mg/kg; *po*) daily [20] for 2 weeks before induction of I/R. Immediately after reperfusion, animals in each group were further subdivided into three sets then sacrificed by cervical dislocation and the both hippocampi of each rat were isolated. Those of the first set ($n = 7$) were used for ELISA estimations; the second one ($n = 6$) was employed for assessment of glutamate content by HPLC; the last set ($n = 7$) served to determine the rest of biochemical parameters.

Two additional groups (8 rats each) were further included in the study to elucidate the effects of pioglitazone on either diabetes or ischemia. In the first one, non-diabetic animals were kept for 4 weeks and then animals were treated with pioglitazone for 2 weeks before I/R induction. While, in the second one diabetes was induced, rats were kept for 4 weeks and were treated with pioglitazone for the last 2 weeks (5th and 6th week) without I/R induction (sham-operated). In these two groups, only apoptotic biomarkers (cytochrome c and caspases-3) as well as glutamate contents were estimated.

Biochemical determinations in hippocampus

Estimation of oxidative stress biomarkers

Lipid peroxides formation was determined in rat hippocampus homogenate (10% w/v normal saline) by estimating the content of thiobarbituric acid reactive substances (TBARS) using malondialdehyde (MDA) as a standard according to the method described by Mihara and Uchiyama [21]. Reduced glutathione (GSH) content was measured using Ellman's reagent as described by Beutler et al. [22]. Total nitrite and nitrate content was assessed according to the method of Miranda et al. [23] based on the Griess reaction and employing vanadium trichloride as reducing agent.

Estimation of inflammatory and apoptotic mediators

The kinetic method described by Bradley et al. [24] was employed to determine myeloperoxidase (MPO) activity. Since the enzyme is located within the primary granules of neutrophils, its extraction necessitates the disruption of the granules to render MPO soluble in aqueous solution. This was achieved by sonication in potassium phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyl-trimethyl ammonium bromide (HTAB), a detergent that releases MPO from the primary granules of the neutrophil [25].

Hippocampal contents of caspase-3, interleukin-6 (IL-6), interleukin-10 (IL-10), as well as tumor necrosis factor alpha (TNF-α) were assessed using enzyme-linked immunosorbent assay (ELISA) kits supplied by R&D Systems, Inc., Minneapolis, USA. Likewise, the contents of NFκB and cytochrome-c were measured using ELISA kits supplied by EIAab Science Co., Wuhan, China.

Estimation of the excitatory amino acid glutamate

Glutamate content was estimated using a fully automated high-pressure liquid chromatography system (HPLC; Perkin-Elmer, Billerica, MA, USA) according to the precolumn phenylisothiocyanate derivatization technique described by Heinrikson and Meredith [26]. Brain residues were reconstituted in 2:2:1 mixture (v) of methanol: 1 M sodium acetate trihydrate:triethylamine then re-dried under vacuum. The reaction of derivatization was performed for 20 min at room temperature using a 7:1:1:1 mixture (v) of methanol:triethylamine:double-distilled deionized water:phenylisothiocyanate, then subjected again to vacuum until dryness. Derivatized amino acids were reconstituted with sample diluent consisting of 5:95 mixture (v) of acetonitrile: 5 mM phosphate buffer (pH 7.2). After sonication, samples were filtered (0.45 μm; Millipore, Billerica, MA, USA). A Pico-Tag physiological free amino acid analysis C18 (300 mm × 3.9 mm i.d.) column from Waters (Milford, MA, USA) and a binary gradient of eluents 1 and 2 (Waters, Milford, USA) were used. The column temperature was set