Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

Synergistic action of ursolic acid and metformin in experimental model of insulin resistance and related behavioral alterations

Ashish Mourya, Ansab Akhtar, Swati Ahuja, Sangeeta Pilkhwal Sah*, Anil Kumar

Pharmacology Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India

ABSTRACT

Chronic restraint stress (CRS) is known to cause metabolic and neurological complications in a number of ways. Prolonged exposure to stress evident by increased corticosterone level led to impaired altered insulin signaling and oxidative stress in mice, in the present study. Impaired insulin signaling or insulin resistance was characterized by hyperglycemia, hyperinsulinemia, hyperlipidemia, hypoadiponectinemia, increased glycosylated haemoglobin and HOMA-IR. It was also associated with increased proinflammatory cytokine TNF-α levels. CRS also caused significant increase in acetylcholinesterase activity and oxidative stress in brain along with cognitive impairment in behavioral test. Ursolic acid, metformin, gliclazide and their combinations when administered daily for 30 days significantly improved insulin sensitivity apart from behavioral and biochemical alterations in stressed mice. Treatment with drugs also decreased serum corticosterone and TNF-α levels. The findings of our study revealed that improvement in insulin sensitivity, learning and cognitive performance in stressed mice was attributed to attenuation of proinflammatory cytokines and oxidative stress. Moreover, combination of [Metformin (150 mg/kg) + Ursolic acid (10 mg/kg)] produced enhanced improvement in insulin sensitivity and cognitive impairment as compared to their individual effects, suggesting possibly the common mode of anti-inflammatory and antioxidant mechanisms.

1. Introduction

The prevalence of Type-2 diabetes, a chronic metabolic disorder is increasing rapidly and 90% of the cases are characterized by insulin resistance. According to the WHO, 422 million people were Type-2 diabetic in 2014 and this number is estimated to be around 694 million by 2030 (Khodabandehloo et al., 2016). Insulin resistance, a pre-diabetic state occurs when sensitivity of insulin to control its metabolic actions decreases. Thus, IR is characterized by hyperinsulinemia and hyperglycemia in fasting condition, increased glycosylated hae-moglobin (HbA1c), impaired glucose tolerance, impaired insulin tolerance, hyperlipidemia, hypoadiponectinemia, and increased inflammatory markers in plasma (Ye, 2013).

Several factors contribute to the development of insulin resistance, out of which obesity and chronic stress play a major role (Ye, 2013). Hypothalamic-Pituitary-Adrenal (HPA) axis and sympathoadrenal system are involved in the pathophysiology of chronic stress related complications by release of proinflammatory mediators (Chiba et al., 2012). Prolonged stress causes HPA-axis fatigue leading to down-regulation of glucocorticoid receptors. Thus the protective action of cortisol in blocking the translocation of nuclear factor kappa β (NFκβ)

declines, resulting in production of pro inflammatory cytokines (Tian et al., 2014). Inflammatory mediators such as TNF-α, IL-β, IL-6 released during stress and obesity induce insulin resistance by activating JNK/AP-1 and NFkβ pathways which cause phosphorylation of Insulin receptor substrate (IRS) at serine-307 site, negatively altering insulin signaling thus causing insulin resistance (Khodabandehloo et al., 2016).

Insulin also plays an important role in learning and memory in the central nervous system and it was reported that Alzheimer's disease (AD) patients exhibit impaired insulin signaling (Kim and Feldman, 2015). The insulin signaling through protein kinase B/glycogen synthase kinase (Akt/GSK3β) pathway promotes neuronal survival by directly inactivating the proapoptotic machinery. Insulin has been shown to regulate extracellular signal regulated kinase mitogen activated protein kinase (ERK), a kinase which is involved in memory consolidation and this enzyme is compromised in AD patients. The risk of developing AD is 65% in case of type-2 diabetes. Clinical data have also found central insulin resistance in AD patients suggesting the role of altered insulin signaling in learning and memory impairment (Dineley et al., 2014).

Ursolic acid (UA) is a lipophilic pentacyclic triterpenoid present as waxy coat on apples and many herbs (Castro et al., 2015; Kunkel et al.,

2012). It is known to possess a number of biological activities such as anti-inflammatory, antidiabetic, antioxidant, anticancer and hepatoprotective (Ahmad et al., 2015). The anti-inflammatory effect of UA is mediated through the suppression of NFκβ and inhibition of NFκβ regulated genes such as proinflammatory cytokines, cyclooxygenase-2 (COX-2) and lipooxygenase (LOX) (Checker et al., 2012). Some studies have reported improvement in glucose tolerance by UA treatment (Jayaprakasam et al., 2006).

The numbers of patients who are insulin resistant are increasing alarmingly. Thus understanding the relationship between stress and its role in exacerbating insulin resistance and associated complications is essential for the identification of potential therapeutic targets. Therefore, the present study has been designed to investigate the effect of ursolic acid and its mechanism in experimental model of insulin resistance and associated memory impairment.

2. Materials and methods

2.1. Animals

Male albino LACA mice (30–40 g) (n = 60) were procured from the Central Animal House facility of Panjab University, Chandigarh, India. Animals were allowed free access to food and tap water and were kept under standard conditions of temperature (25 ± 2 °C) and relative humidity (45 ± 10%) with alternate 12 h light and dark cycle. The animal care and experiments involved in this study were approved by the Institutional Animal Ethics Committee with approval number (PU/IAEC/S/15/06, 15/09/2015) and experiments were performed according to the National Science Academy Guidelines for the use and care of animals.

2.2. Drugs and treatment schedule

Ursolic acid was purchased from Sigma-Aldrich (St. Louis, Mo., USA). Gliclazide and metformin were obtained from Zing Lifecare, Mumbai. Ursolic acid (5, 10 mg/kg) and Gliclazide (10 mg/kg) were dispersed in 0.25% w/v Na-CMC and administered via oral route using oral gavage. Metformin (150 mg/kg) was dissolved in distilled water and administered orally. All the drugs were administered 45 min prior to the animals being subjected to chronic restraint stress and continued for 30 days. The doses of ursolic acid, metformin and gliclazide were selected on the basis of previous studies (Castro et al., 2015; Kumar et al., 2008; Matsuyama-Yokono et al., 2009; Yoon et al., 2007). Animals were acclimatised to laboratory conditions for one week before starting experiments.

Animals were randomly divided into following ten groups each containing six animals.

Treatment doses and schedule

S. no.	Group	Treatment
1	**Naïve**	Maintained on a normal diet, free access to water
2	**CRS**	Chronic restraint stress (2 h daily for 30 days)
3	**UA (5)**	Ursolic Acid (5 mg/kg) + Restraint Stress (2 h daily for 30 days)
4	**UA (10)**	Ursolic Acid (10 mg/kg) + Restraint Stress (2 h daily for 30 days)
5	**Met (150)**	Metformin (150 mg/kg) + Restraint Stress (2 h daily for 30 days)
6	**Met (150) + UA (5)**	Metformin (150 mg/kg) + Ursolic Acid (5 mg/kg) + Restraint Stress (2 h daily for 30 days)
7	**Met (150) + UA (10)**	Metformin (150 mg/kg) + Ursolic Acid (10 mg/kg) + Restraint Stress (2 h daily for 30 days)
8	**Glic (10)**	Gliclazide (10 mg/kg) + Restraint Stress (2 h daily for 30 days)
9	**Glic (10) + UA (5)**	Gliclazide (10 mg/kg) + Ursolic Acid (5 mg/kg) + Restraint Stress (2 h daily for 30 days)
10	**Glic (10) + UA (10)**	Gliclazide (10 mg/kg) + Ursolic Acid (10 mg/kg) + Restraint Stress (2 h daily for 30 days)

2.3. Chronic restraint stress

Animals were restrained individually for 2 h daily at variable time periods between 9.00 and 17.00 h for 30 days in a modified wooden restrainer with dimensions (7.5 cm length, 3 cm width and 4 cm height) containing wire mesh on one side for proper breathing of the animal and a small opening on another side through which the tail can be protruded out. The movement of the animal was restricted by applying zinc oxide hospital tape. After 2 h, the mouse was released by removing the tape after moistening with acetone in order to minimize pain or discomfort. The study was conducted as per the protocol depicted in Fig. 1.

2.4. Measurement of systolic blood pressure and body weight

Systolic blood pressure was recorded using non-invasive blood pressure recorder (AD instruments, Australia) on day 0, 15th and 30th. This instrument calculates only systolic blood pressure using tail-cuff method in slightly restraint but conscious rats (El-Bassossy et al., 2012). Rats were acclimatized to the warming chamber (35 °C), restrainers, and the tail cuff for 20 min per day for at least 3 days before BP measurements were taken. BP measurements were carried out by the same investigator at a same time in the morning. The BP measurement started with 5–10 min of equilibration in the warming chamber and under restraint. This was followed by 10 repetitions of automated inflation-deflation cycles. The mean of 6 readings within a 5–10 mm Hg range was used as the systolic blood pressure.

Body weights were also measured on day 0, 15th and 30th, and percentage change in weight was calculated.

2.5. Behavioral test for learning and memory

2.5.1. Morris water maze

The Morris water maze (MWM) test was performed to observe the effect of CRS on cognitive function of animals. The experimental apparatus consisted of a circular water tank (105 cm diameter, 30 cm height) filled with water up to a height of 15.5 cm. A platform (9 cm in diameter, 14.5 cm in height) was submerged 1 cm below the water surface and placed at the midpoint of one quadrant. The tank was located in a test room, which contained various prominent visual cues. Each mouse received four training periods per day for 4 consecutive days. For each trial, the mouse was placed in the water facing the wall at one of four starting positions, and the time required for the released mouse to find the hidden platform was recorded. A mouse that found the platform was allowed to remain on the platform for 15 s and then returned to its cage for the inter-trial interval. A mouse that did not find the platform within 90 s was placed on the platform for 15 s at the end of the trial. Latency to escape from the water maze (finding the submerged escape platform) was calculated for each trial. On day 5, the probe test was carried out by removing the platform and allowing each mouse to swim freely for 90 s. For the probe trials, the number of entries and time spent in the target quadrant (where the platform was located during hidden platform training) was measured and calculated. All data were recorded with a computerized video system (software Ethovision 3.1) (Morris, 1984).