European Journal of Pharmacology 740 (2014) 641–644

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

The influence of vitamin-rich diet on the extent of lipoperoxidation in brain of mice during an acute post-insulin hypoglycaemia

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ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form
11 June 2014
Accepted 17 June 2014
Available online 24 June 2014

Chemical compounds studied in this article:
Malondialdehyde (PubChem CID 10964)

Keywords:
Lipoperoxidation
Vitamin C
Vitamin E
Hypoglycaemia
Malondialdehyde
Superoxide dismutase
Glutathione peroxidase

ABSTRACT

Antioxidatives are widely used and recommended in common clinical praxis, even though they may have negative impact on our health under some circumstances (i.e. N-acetylcysteine, vitamin E, risk of lung cancer etc.). Our aim was to evaluate the role of exogenous scavengers in prevention of induced oxidative stress in rodents. Male ICR mice were used and acute hypoglycaemia was induced with insulin. The mice were randomized into eight experimental groups, either pretreated by vitamin C or vitamin E or combinations with respective vehicles. Total malondialdehyde (MDA), superoxide dismutase (SOD), and selenium-dependent glutathione peroxidase (GSHPx) activity were measured in brain tissue samples. ANOVA with a post-hoc Duncan or Turkey's tests were used for statistical evaluation. A statistically significant increase in brain MDA was observed after insulin-induced severe hypoglycaemia relative to normoglycaemia. Animals pretreated with vitamins, both in monotherapy and in combination (both $P<0.05$), had significantly lower MDA values compared with animals without pretreatment. Importantly, significant differences were also observed after combination of vitamin C and E in GSHPx and SOD (both $P<0.05$).

1. Introduction

Diabetes mellitus is chronic disease that places a significant burden on health care systems as well as the social and economic lives of patients. The prevalence of the disease has been steadily rising over the past decades. If not treated adequately, it significantly shortens life expectancy and negatively affects the quality of life (QoL) in diabetic patients (Clarke et al., 2002). Although among children and young adolescents with type 1 diabetes, generic QoL is not impaired compared to healthy peers, a negative impact of diabetes on daily functioning and diabetes-related worries was present in this population (Nieuwesteeg et al., 2012). Despite the use of modern pharmacotherapy, there is a steady accumulation of debilitating effects that cannot be reversed. Therefore, new therapeutic approaches are still urgently needed.

Diabetic patients are at risk of not only hyperglycaemia but also of hypoglycaemia induced by antidiabetic drugs. An episode of severe hypoglycaemia is reported in almost half of all diabetic patients (Amiel et al., 2008; Garber, 2012). Treatment for most diabetic patients involves sulfonylurea derivatives (i.e. glibenclamide etc.) (Greco and Angileri, 2004; Vexiau et al., 2008) or insulin (Chiarelli et al., 1999; Airey et al., 2000).

The extent of brain lipoperoxidation (LPO) during hypoglycaemia and the related changes have not been extensively studied. Nevertheless, there are many studies that have evaluated the relationship between LPO and other pathologic conditions, i.e. hyperglycaemia, hypoxia, ischaemia, lactate acidosis, etc. (Lantos et al., 1994; McGowan et al., 1995b; Ress et al., 1995). However, diabetic patients remain at risk from hypoglycaemia, and more research is needed on how it affects the functions of the central nervous system (McGowan et al., 1995a; Benzi et al., 1987; Haces et al., 2010).

Hypoglycaemia and oxidative stress are usually studied independently, resulting in a lack of studies that evaluated their relative causality. Many studies have been performed in tissue cultures or brain slices (Rego et al., 1999; Saransaari and Oja, 1999). One of the most extensively studied topics regarding LPO and the CNS are the related events of hypoxia and ischaemia. Previously, these conditions were thought to be related and that they were associated with increased reactive oxygen species formation in the central nervous system (Agardh et al., 1991; Horakova et al., 1990).

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http://dx.doi.org/10.1016/j.ejphar.2014.06.015
0014-2999/

The brain lacks its own energy storage mechanism; thus it is dependent on a continuous uptake of both oxygen and glucose. Based on this, a parallel between hypoxia and hypoglycaemia, in terms of risk of lipoperoxidation, was postulated. Moreover, EEG findings show an analogy between hypoglycaemic and hypoxic models (Ugawa and Kanazawa, 1993).

In this experiment, we wanted to determine if administration of exogenous scavengers (ascorbic acid, vitamin E, administered either alone or in combination) could prevent oxidative stress.

2. Materials and methods

2.1. Experimental animals

Male ICR mice, weighing 24–30 g (Velaz, Czech Republic), were used. The animals were adapted to the laboratory environment for at least 1 h before being used. University guidelines for the ethical treatment of animals were followed throughout the experiment. Food was withheld for 16–36 h before intraperitoneal administration of either saline or insulin; access to water was ad libitum. The duration of acute insulin-induced hypoglycaemia lasted from 1 to 3 h and was characterized by decreased motor activity, atonia or occasional seizures. The duration of the experiments was as short as possible, and the number of animals involved was kept to a minimum ($n=8$ per each group and there were 8 groups). The animals were euthanized, with an anaesthetic overdose, immediately after the experiment.

All studies and procedures were approved by the Committee for Protection of Laboratory Animals, 3rd Faculty of Medicine, Charles University.

2.2. Experimental drugs

Each animal was administered either saline or insulin at a dose 24 IU/kg. The animals were randomized into eight experimental groups as summarized in Table 1. Vitamin E (α-tocopherol, olive oil as the vehicle) was given at a dose of 100.0 mg/kg/day, vitamin C (ascorbic acid, distilled water as the vehicle) was given at dose 1000.0 mg/kg/day.

2.3. Blood and tissue sampling

Blood samples were obtained from carotid arteries following decapitation. Samples of brain tissue (right hemisphere) for subsequent biochemical analyses (quantification of MDA, SOD, and GSHPx) were taken post mortem.

2.4. Blood and tissue sample analyses

2.4.1. Measurement of blood parameters

A standard glucometer (EasyGluco set, INFOPIA Co., Ltd.) was used for measurement of plasma glucose concentrations.

2.4.2. Measurement of total malondialdehyde (MDA)

Measurement of MDA in brain tissue was performed using a modified method previously described by Zima et al. (1995).

Chemicals: methanol (gradient grade HPLC, J.T. BAKER, Netherland), 2-thiobarbituric acid (TBA, Sigma-Aldrich, Germany), 2,6-di-tert-butyl-p-kresol (BHT, Fluka, Germany), perchloric acid (Lachema, Czech Republic), sodium hydroxide (Penta, Czech Republic), phosphoric acid (Fluka, SRN), and 1,1,3,3-tetraethoxypropane (TEP, Fluka, Germany). Water was deionized using a Millipore system.

Sampling, processing and storage of tissues and preparation of the MDA–TBA complex: brain tissue was frozen immediately after removal (initially at −20 °C and then at −70 °C). Chemical analysis required tissue homogenization in ice-cold physiological solution. The tissue concentration in the homogenate was 15%. The MDA–TBA complex was prepared using 300 μl of homogenate. The first step was hydrolyzation with sodium hydroxide (3.4 mol/l) to release MDA bound to membrane phospholipids. Deproteination was performed using perchloric acid (3.4 mol/l). Supernatant, for measurement of total MDA, was obtained by centrifugation (3000 rpm). Thiobarbituric acid (0.4%) was added to supernatant and the reaction was run for 40 min at 95 °C. All samples were filtered (0.45 μm, Tessek, Czech Republic) before HPLC injection. The mobile phase was also filtered (0.45 μm, Gelman, Michigan, USA) (Fukunaga et al., 1997; Suttnar et al., 1997). HPLC analyses were performed using a Shimadzu chromatograph (Shimadzu Corporation, Kyoto, JP) consisting of a degasser (DGU-12A), LC solvent delivery unit, auto-injector (SIL-10ADVp), column oven (CTO-10AD), and data station (CLASS VP 4.3). A Supelcosil LC-18 (5 μm, 50 × 4.6 mm i.d.) column was used. An isocratic elution of the MDA–TBA complex, with mobile phase, consisting of methanol/20 mM phosphate buffer, pH 6 (40/60; /v/v), was used in the analysis. The UV–vis detector was set at 532 nm and the flow rate was set at 0.4 ml/min at 25 °C.

The concentration of MDA was then calculated from the calibration curve using tetraethoxypropane as the external standard – the curve was linear from 0.5 to 12.5 μmol/l ($r^2=0.997$).

2.4.3. Measurement of superoxide dismutase (Cu, Zn-SOD) activity

The Cu–Zn-SOD activity was measured using a slightly modified method previously presented by Sun et al. (1988). O_2^- was generated with a xanthine/xanthine oxidase reaction in the presence of tetrazolium salt (nitrotetrazolic blue, NTB). 0.4 ml of chloroform/ethanol mixture (6:10 v/v) was added to 0.5 ml of tissue homogenate, mixed and centrifuged at 20 rpm for 20 min. The supernatant (extract SOD) was diluted in water. In our modification, 1 ml

Table 1
Overview of drugs administered to animals in each group.

Group ($n=8$ per each group) **Route of administration**	**Distilled water (vehicle)** Oral	**Vitamin C** Oral	**Olive oil (vehicle)** Oral	**Vitamin E** Oral	**Saline** Intraperitoneal	**Insulin** Intraperitoneal
Group						
I	+		+		+	
II	+		+			+
III		+	+		+	
IV		+	+			+
V	+			+	+	
VI	+			+		+
VII		+		+	+	
VIII		+		+		+