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Modulation of the oxidative stress by metformin in the cerebrum of rats exposed to global cerebral ischemia and ischemia/reperfusion

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Abstract. – OBJECTIVES: Oxidative stress plays a major role in the pathogenesis of ischemic and reperfusion injury to many organs, including the brain. Chronic metformin treatment is associated with a lower risk of stroke in clinical populations. The aim of the present study was to investigate the effect of metformin on the oxidative stress induced in experimental model of incomplete global cerebral ischemia and ischemia/reperfusion in adult male Wistar rats.

MATERIALS AND METHODS: Metformin was administered to rats orally by gavage 500 mg/kg once daily for one week before induction of cerebral ischemia (rats were subjected to 30 min of ischemia before decapitation) and ischemia/reperfusion (rats were subjected to 30 min of ischemia then 60 minutes of reperfusion before decapitation). The selected parameters for oxidative stress were the activities of the antioxidant enzymes: glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase as well as malondialdehyde (MDA) levels.

RESULTS: Metformin reduced the elevated activites of GSHPx, SOD and catalase as well as MDA levels in cerebrum of rats exposed to ischemia and ischemia/reperfusion injures.

CONCLUSIONS: Metformin improved the oxidative stress induced by ischemia and ischemia/reperfusion injuries. This may be a mechanism that explains the cerebroprotective effect of the drug.

Key Words:
Metformin, Ischemia/reperfusion, AMPK, Oxidative stress.

Abbreviations

GSHPx = Glutathione peroxidase; ROS = Reactive oxygen species; MDA = Malondialdehyde; SOD = Superoxide dismutase.

Introduction

Stroke is a serious and common pathological condition[1]. It is one of the main causes of death and disability worldwide[2]. It is becoming increasingly clear that oxidative stress and excessive inflammatory response are implicated in the pathogenesis of ischemic and reperfusion injury to many organs, including the brain[3]. Reactive oxygen species (ROS) have been indicated as one of the earliest and most important components of tissue injury after reperfusion of ischemic organ and the extent of brain injury appears to depend on the experimental pattern of ischemia/ reperfusion: free radical production is continuous during ischemia, while during reperfusion it is primarily confined to the early stage when fresh oxygen is supplied to the ischemic region[4]. The brain is very susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid (PUFA) contents, relatively low antioxidant capacity and inadequate neuronal cell repair activity[5]. Overproduction of reactive oxygen species (ROS) results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death[6].

Metformin is used for the management of type 2 diabetes mellitus. Its effects are mainly the consequence of reduced hepatic glucose output through inhibition of gluconeogenesis and, to a lesser extent, of increased insulin-stimulated glucose uptake in skeletal muscle and adipocytes[7]. In clinical populations, chronic metformin treatment is associated with a lower risk of stroke, reducing cardiovascular mortality by 26%. This protection is independent of its glucose-lowering effect[8]. Several studies have shown reduced cardiovascular-related mortality rates in metformin users compared with sulfonylurea monotherapy users[9], indicating that met-

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formin might have some additional cardiovascular protective effects beyond its antihyperglycaemic properties. In that way, many studies have demonstrated that metformin possesses antioxidant properties that could participate to its cardiovascular protective effects. Such antioxidant properties could explain some of the pharmacological actions of this drug through a modulation of redox-dependent transduction pathways[10].

In mice, short-term metformin treatment (for 3 days) exacerbated stroke damage; in contrast, relatively long-term treatment (for 3 weeks) with metformin given before stroke was neuroprotective[11].

The present study was designed to investigate the effect of one week treatment with metformin on the neurotoxicity caused by cerebral ischemia and ischemia/reperfusion injury in the rat. The malondialdehyde (MDA) levels as well as the activities of the enzymes; glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase have been estimated in the cerebrum with, and without pretreatment with metformin.

Materials and Methods

Animals

Adult male Wistar rats, weighing 180-200 g, were obtained from National Research Laboratory, Cairo, Egypt. Animals were housed under controlled environmental conditions, fed standard pellet chow (El Nasr Chemical Co., Cairo, Egypt) and permitted free access to tap water. All experimental protocols were approved by the Ethics Committee of Zagazig University.

Drugs and Chemicals

Ethyl carbamate "Urethane" crystals (Prolabo, Paris, France), metformin powder (CID Pharmaceutical Co, El Talbeya, Egypt). Metformin was dissolved in saline (vehicle) and administered 500 mg/kg[12], via adjusted gavage tube, 0.2 ml/rat, daily for 7 days before induction of cerebral ischemia and ischemia/reperfusion.

Experimental Procedures

Study Design

30 rats were randomly allocated into five groups, each group contain six rats: *Sham operated group:* Rats were subjected to the same surgical procedures as described below, except for common carotid artery ligation. *Ischemia-untreated group*: Rats were subjected to 30 min of ischemia before decapitation.

Ischemia metformin-treated group: Rats pretreated with metformin were subjected to 30 minutes of ischemia before decapitation.

Ischemia/reperfusion-untreated group: Rats were subjected to 30 min of ischemia then 60 minutes of reperfusion before decapitation.

Ischemia/reperfusion metformin-treated group: Rats pretreated with metformin were subjected to 30 minutes of ischemia then 60 minutes of reperfusion before decapitation. The animals in the control groups were administered saline 0.2 ml/rat (vehicle for metformin) orally through gavage tube for 7 days before the surgical procedures.

Induction of Ischemia and Ischemia/Reperfusion

Rats were anaesthetized through i.p. injection of 1.25 g/kg urethane. Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternomastoid and the sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained[13]. Ischemia was achieved by clamping the bilateral common carotid arteries for 30 min using non-traumatic artery clamps. For induction of ischemia/reperfusion, recirculation of blood flow was established by releasing the clamps and restoration of blood flow in the carotid arteries was confirmed by careful observation. Reperfusion was allowed for 60 min. Sham-operated rats underwent identical surgical procedures except that no artery clamps were applied. After decapitation, both cerebral hemispheres of each rat were dissected, ice cooled, weighed, homogenized and centrifuged to obtain the cerebrum extract. The activities of the enzymes, glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase were measured in the brain extract.

Preparation of Cerebrum Homogenate

Preparation of the homogenate and measurement of total cerebrum proteins were carried out as previously described by Wong et al[14] with some modifications. Briefly, cerebral hemispheres were weighted and washed twice in phosphate buffered saline (PBS, O°C), then immersed in liquid nitrogen for 10 minutes followed immediately with grounding and the brain tissue powder were resuspended in 9 vol (W/V) of

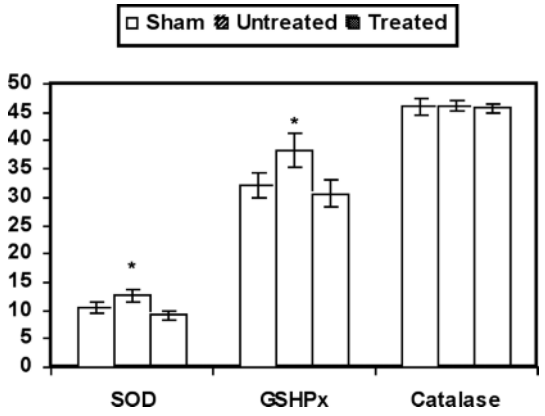

Figure 1. The activities of the antioxidant enzymes SOD, GSHPx and catalase in sham operated, ischemia-untreated and ischemia metformin-treated groups. *Significantly different ($p < 0.05$).

0.1%. Triton X-100 containing 1.0 mM potassium phosphate buffer (pH 7.2). Homogenates were centrifuged at 5000 rpm for 15 minutes at 4°C, the clear supernatant was removed, and aliquots were then taken and stored at –80°C. Protein content of the supernatants determined using protein assay kit. Catalase, SOD and GSHPx activities as well as MDA level were assayed in the prepared homogenate supernatant.

Determination of MDA Level

MDA level was measured according to Polidori et al[15].

Assay of Catalase Activity

Catalase activity was measured according to Abei[16].

Measurement of SOD

SOD activity was measured by the inhibition of pyrogallol autooxidation according to Marklund and Marklund[17].

Assay of GSHPx Activity

GSHPx activity was measured as described by Lawrence and Burk[18].

Statistical Analysis

Results were tabulated as mean ± SEM of six animals in each group. Data were analyzed using one-way analysis of variance (ANOVA test) followed by LSD (Least Significance Difference) test. Statistically significant difference was considered when $p < 0.05$.

Results

Cerebral ischemia resulted in significant ($p < 0.05$) increments in the level of MDA and activities of the antioxidant enzymes SOD and GSHPx in cerebrum extract of the control subjected to cerebral ischemia (vehicle pretreated group) compared to the sham operated group. However, the catalase activity was not significantly affected. In the metformin-pretreated group the level of MDA as well as the activities SOD, GSHPx and catalase activities were insignificantly affected in relation to sham operated group (Figures 1 and 3).

The activities of the antioxidant enzymes SOD, GSHPx, and catalase as well as the MDA level, in cerebrum extract, were significantly ($p < 0.05$) increased in vehicle-pretreated group subjected to

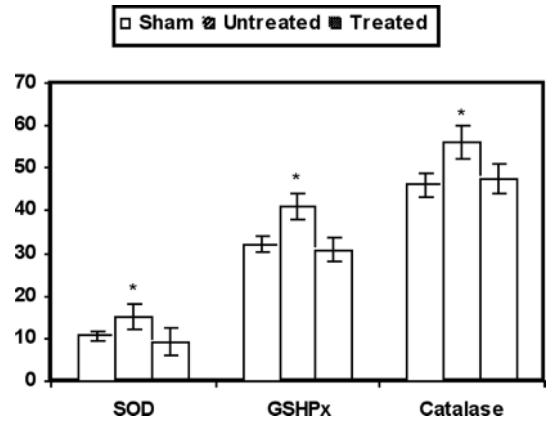

Figure 2. The activities of the antioxidant enzymes SOD, GSHPx and catalase in sham operated, ischemia/reperfusion-untreated and ischemia/reperfusion metformin-treated groups. *Significantly different ($p < 0.05$).

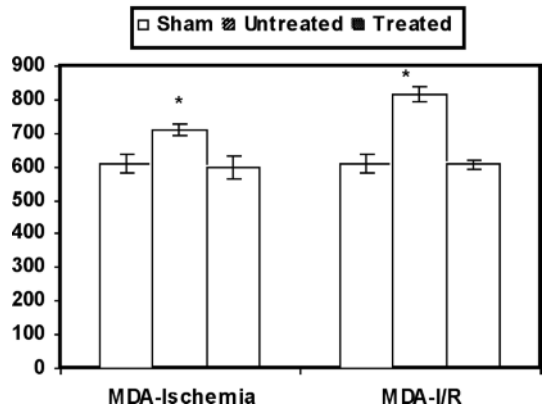

Figure 3. The levels of MDA in sham operated, ischemia-untreated, ischemia metformin-treated, ischemia/reperfusion-untreated and ischemia/reperfusion metformin-treated groups. *Significantly different ($p < 0.05$).

cerebral ischemia/ reperfusion compared to the sham operated group. In metformin-pretreated group, there were no significant change in the MDA level as well as the activities SOD, GSHPx and catalase after cerebral ischemia/reperfusion as compared to the sham operated group (Figures 2 and 3).

Discussion

The majority of *in vivo* models of cerebral ischemia rely on vessel occlusion predominantly affecting the forebrain[19]. Bilateral occlusion of the common carotid arteries in rats is a common model of incomplete global cerebral ischemia[20], and results in a 50% decrease in cerebral blood flow[21]. This induced partial ischemia, without affecting the circle of Willis (collateral circulation), has been suggested to reflect the early events occurring during transitory ischemic attacks more closely[22]. In this study, the MDA level which is a product of lipid peroxidation was increased as a sequence of brain ischemic insult and a further increase in its activity was reported after ischemia/Reperfusion (I/R). These results are in agreement with many works that demonstrate an increase of lipid peroxidation products in experimental studies during cerebral I/R injury as indirect evidence of oxidative stress[23]. It was reported an elevation of free radicals in the first 5 minutes of 1 hour cerebral ischemia; moreover, a second elevation of free radicals gradually occurred on reperfusion[24]. The present study demonstrated that the cytosolic activities of GSHPx and SOD were increased as a result of brain ischemic insult and more increments were detected on I/R. However, the catalase enzyme level was, only, elevated after I/R. This increase may be related to reperfusion-induced free radicals overproduction. In the present study we demonstrated that cerebral ischemia and I/R resulted in an increase in SOD activity indicating that the brain's antioxidant machinery is activated in response to excessive generation of ROS. The enzyme SOD catalyzes the conversion of superoxide anions to molecular oxygen and hydrogen peroxide, which requires to be scavenged further by tissue thiols, such as GSH, and by catalase[25]. Furthermore, apart from its own toxicity, hydrogen peroxide in the presence of iron leads to the generation of toxic hydroxyl radicals[26]. Collectively, the results obtained in the present model confirm the presence of a significant level of oxidative stress resulting from I/R. In this investigation, pre-treatment of rats daily with metformin for one week, caused a substantial reduction of injury induced by cerebral ischemia and ischemia followed by reperfusion. In particular, in the cerebrum of rats that had undergone ischemia or I/R, metformin attenuated the extent of oxidative stress and reduced the elevated levels of GSHPx, SOD, MDA and catalase. These results suggest that the protective effects of metformin against I/R injury may be attributed to its ability to reduce oxidative stress. These results are in agreement with that obtained with Huo et al[27] who suggested that metformin reduces ROS levels by inducing antioxidant thioredoxin (Trx) expression through activation of the AMP-activated protein. Previous papers reported that acute activation of AMPK increased ischemic damage further confirms that acute AMPK (adenosine monophosphate-activated protein kinase) activation is detrimental in stroke, consistent with previous findings from other pharmacological and genetic studies[11]. The detrimental effect of acute AMPK activation may be mediated, at least in part, by enhancement of lactic acidosis. Chronic metformin treatment may lead to sublethal metabolic stress and downregulate AMPK protecting the brain from subsequent injury. Hypoglycemia during biguanide therapy is essentially unknown. These agents are, therefore, more appropriately termed "euglycemic" agents[28]. Impairment of insulin sensitivity was occurred under ischemic stress that results in hyperglycemia. Furthermore, the development of hyperglycemia/glucose intolerance after cerebral ischemic stress, called post-ischemic glucose intolerance, may trigger the aggravation of neuronal damage[29]. Metformin is the only oral antidiabetic medication that has been shown to decrease diabetic cardiovascular complications in large-scale clinical trials[9]. It has been shown to reduce intracellular ROS[30]. However, the precise mechanism of metformin's antioxidant actions is not completely understood. Metformin acts partially through activation of the AMPK, which mediates many of its cardiovascular-protective effects. AMPK is involved in regulating many cellular functions including endothelial nitric oxide synthase (eNOS) activation, angiogenesis and proliferation[31]. Activation of AMPK pathway inhibits vascular inflammation, prevents endothelial injury induced by hyperglycemia and FFAs[32]. The findings of this work are in parallel with Mahrouf et al[10] who demonstrated that metformin possesses antioxidant properties that could participate to its cardiovascular protective effects. Such

antioxidant properties could explain some of the pharmacological actions of this drug through a modulation of redox-dependent transduction pathways. Metformin exerts antioxidant properties at the cellular level, by inhibition of intracellular ROS production in stimulated endothelial aortic cells, through the reduction of PKC membrane translocation and/or activity. Whether such inhibition of the PKC pathway by metformin might be associated to a modulation of the AMPK pathway, the proposed redox-dependent mechanism for the pharmacological effect of the antidiabetic drug remains to be clarified. Also the present findings agree with Arpita et al[33] who concluded that metformin therapy protects against diabetes associated oxidative stress and inflammation which indicates that it may be considered as a preferred oral antidiabetic agent in type 2 diabetes mellitus. Other reports postulated that, metformin protects against myocardial ischemia/reperfusion injury by activating AMP-activated protein kinase[34]. It was demonstrated that the widely used diabetes drug metformin is sufficient to activate the atypical protein kinase C transcription cofactor CBP pathway in neural precursors and, thereby, to enhance neurogenesis and raise the possibility that metformin could provide the basis for a therapeutic strategy for the human nervous system[35].

Conclusions

Treatment with metformin for one week before induction of cerebral ischemia or I/R exerts cerebroprotective effects, possibly by reducing the oxidative stress. Further experimental and clinical studies are required to confirm this effect.

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Conflict of Interest

The Authors declare that they have no conflict of interests..

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