The Effects of Vitamin E on Lipid Peroxidation, Nitric Oxide Production and Superoxide Dismutase Expression in Hyperglycemic Rats With Cerebral Ischemia-Reperfusion Injury

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ABSTRACT

OBJECTIVES: Recent studies have revealed that nitric oxide (NO) in concert with lipid peroxidation (LP) may play an important role in pathophysiological mechanism(s) of cerebral ischemia. In addition, hyperglycemia exaggerates the pathological changes during cerebral ischemia. The purpose of this study was to investigate the effects of vitamin E (VE) on LP, NO production, and superoxide dismutase (SOD) expression in hyperglycemic rats after cerebral ischemia-reperfusion injury.

MATERIALS and METHODS: Malondialdehyde (MA) (indicator of LP) in plasma and brain tissue, total nitrite/nitrate (metabolites of nitric oxide) in plasma, nitrite in brain tissue, and SOD in red blood cells were detected and the results were compared before and after VE administration in hyperglycemic rats with cerebral ischemia-reperfusion injury induced by two common carotid artery occlusions.

RESULTS: Ischemia-reperfusion injury together with hyperglycemia caused elevation in NO metabolites and MA levels and this elevation was more prominent in hyperglycemic rats. SOD was also increased in ischemia-reperfusion, and VE administration had positive effects on the SOD level. In addition, VE administration caused a significant decrease in NO metabolite levels after ischemia-reperfusion injury.

CONCLUSIONS: These results suggest that prophylaxis with VE may have positive effects on reducing cerebral damage after stroke in patients with diabetes mellitus.

KEY WORDS: Cerebral ischemia, Nitric oxide, Superoxide dismutase, Vitamin E

INTRODUCTION

Cerebral damage due to ischemia results in the production of reactive oxygen species (ROSs), including superoxide anion and hydroxyl radicals (7, 19). After reperfusion, the production of ROSs is notably increased, which may damage the cells by oxidizing proteins such as DNA and by promoting lipid peroxidation (LP) of cellular membranes (6, 8). Production of nitric oxide (NO) in the brain after ischemia has been demonstrated and it reacts with superoxide anion to form peroxynitrite, which appears to be one of the most important mechanisms leading to cell death after ischemia-reperfusion injury (IR) (1, 23). Both human and animal studies have shown

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that coexistence of hyperglycemia with stroke is associated with increased oxidative stress, which contributes to more severe neuronal dysfunction. In addition to ROSs, hyperglycemia may also cause an increase in superoxide dismutase (SOD), one of the antioxidant enzymes involving the brain defense mechanisms (2). The brain has also natural non-enzymatic defense mechanisms, which consist of the vitamins A, C, and E. The effects of vitamin E (VE) on diminishing LP and consequently ischemic brain damage have been proven (10, 12, 16). Furthermore, VE appears to be the first line of defense against peroxidation after cerebral ischemia.

In this experimental study, we tried to investigate the effects of VE treatment on LP, NO production, and SOD expression in hyperglycemic rats following IR injury. Malondialdehyde, one of end-products of LP, was measured in order to evaluate LP. Since NO is a rapidly decaying gas and cannot be detected directly, NO products such as nitrite and nitrate allow us to estimate NO generation. Therefore, nitrite and nitrate were measured in this experiment in order to evaluate NO production.

MATERIALS and METHODS

Animal groups

This experimental study was performed in accordance with an institutionally approved protocol and guidelines provided by the Istanbul University Council for Animal Care. The study group consisted of adult male Wistar Albino rats weighing 200-250 g. Purina diets and tap water were supplied to them ad libitum. During the experimental procedures, the rats were housed at 24 ± 3 °C room temperature and 12-hour light/dark cycles.

At the beginning, the rats were divided into two broad groups: control (n=8) and hyperglycemic (n=10). Hyperglycemia was induced by intravenous (tail vein) injection of 65 mg/kg streptotozocin and the control group received the same amount of physiological saline by the same route. After 48 hours, hyperglycemia was confirmed by the measurement of blood glucose.

The control group was further subdivided into two groups: sham-operated (n=4) and normoglycemic sham + IR (n=4) groups. The hyperglycemic group was also subdivided into two groups as hyperglycemic IR (n=6) and hyperglycemic IR + VE (n=4) groups. Alpha-tocopherol was administered intraperitoneally at a dose of 60 mg/kg 2 days per week for 4 weeks before IR injury.

Surgical procedures

All surgical procedures were performed under general anesthesia induced by intramuscular administration of ketamine hydrochloride (100 mg/kg) and the dose was maintained as needed. Rats were endotracheally intubated and mechanically ventilated with a room air apparatus (Harward rodent ventilator model 683, Harward Apparatus, Inc., South Natick, Massachusetts). Following neck dissection, bilateral common carotid arteries were clipped with surgical microclips for 15 minutes in order to induce forebrain ischemia. Then reperfusion was allowed and the rats were sacrificed under the general anesthesia after 60 minutes of reperfusion. Sham-operated animals underwent the same surgical procedure without vessel occlusion. The thorax was

Table I. Summary of the results obtained in this study.

Groups	RBC SOD (U/g Hb)	Tissue MDA (nmol/g wet tissue)	Plasma MDA (nmol/mL)	Tissue nitrite (μmol/mg protein)	Total plasma nitrite+nitrate (μmol/L)	MAP (mm Hg)
Sham-operated (n=4)	2842 ± 113.76	129.30 ± 3.85	6.27 ± 0.61	0.480 ± 0.014	17.1 ± 0.5	116 ± 1.4
Sham + IR (n=4)	3559. 25 ± 179.59	160.1 ± 5.57	7.12 ± 0.39	0.485 ± 0.026	19.3 ± 0.6	76 ± 1.0
Hyperglycemic + IR (n=6)	2623.67 ± 211.58	215.9 ± 8.19	8.06 ± 0.32	0.508 ± 0.032	27.2 ± 0.6	110 ± 1.0
Hyperglycemic + IR + VE (n=4)	2789.5 ±167.48	180.63 ± 6.88	6.88 ± 0.46	0.550 ± 0.035	19.9 ± 0.4	98 ± 2.3

IR: ischemia-reperfusion; MAP: Mean arterial pressure; MDA: Malondialdehyde; RBC: red blood cell; SOD: Superoxide dismutase; VE: Vitamin E.
All data in the table is expressed as mean ± standard deviation.

opened and an incision made on the right ventricle of the heart and 3 ml of blood was obtained in a sodium heparin tube. As soon as possible, each blood specimen was centrifuged at 2,500 rpm for 10 minutes and the supernatant was stored at –80 °C until assayed.

Brain sample preparation

Forebrains from all animals were taken out quickly, washed with chilled n-saline and homogenized or sonicated immediately to give 10-20% homogenate in tris-HCL 0.1 M pH=7.3 buffer for biochemical estimation. This procedure was performed at 4 °C. The homogenate was then centrifuged for 10 minutes at 15,000 rpm and the supernatant was used for biochemical study. The supernatant was stored at –80 °C for the enzyme and the protein assays.

Assay procedure for protein

The total protein concentration in the cytosols was measured by the method of Lowry et al. (15) using bovine serum albumin as the standard.

Red blood cell SOD determination

SOD activity was measured by the modified method of Sun, et al (24). This assay for extracellular SOD activity is based on inhibition of $\bullet O_2?$ mediated reduction of nitroblue tetrazolium (NBT) to formazan, a photoabsorbant product. One unit SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %. Solutions containing 40 ml of 0.3 mmol/L xanthine, 20 ml of 0.6 mmol/L diethyenetri-aminepentaacetic acid, 20 ml of 150 mmol/L NBT, 12 ml of 400 mmol/L Na2CO3, and 6 ml of bovine serum albumin were incubated with 10 μL of CSF. Superoxide radicals were generated by adding 167 U/L xanthine oxidase and the measurements were recorded at 560 nm and 37 °C for 5 minutes, using the spectrophotometer. Total SOD activity was measured as the effectiveness of the supernatant to inhibit dismutation of $\bullet O_2?$ generated by the xanthine-xanthine oxidase reaction. Total SOD activities were expressed in units per gram hemoglobin (U/g Hg).

Plasma and tissue MDA determinations

Thiobarbituric acid reacting substance (TBARS) levels as the marker of LP were determined with the spectrophotometric method described by Buege and Aust (4). Stock solution included 15 % trichloroacetic acid, 0.375 % thiobarbituric acid, 0.25 N hydrochloric acid. The samples were heated in a water bath for 20 minutes and, after cooling, centrifuged at 2000 rpm for 15 minutes. The formation of a pink color

indicated a reaction between one molecule of TBARS and two molecules of TBAs, and was measured spec-trophometrically at 560 nm. Plasma and tissue MDA levels were expressed as nmol/mL plasma and nmol/g wet tissue, respectively.

Analysis of NO metabolites (nitrite-nitrate)

Nitrite-nitrate concentrations were measured as described previously, using an automated procedure and a NO2-/NO3- kit (Boehringer Mannheim, Mannheim, Germany - NO2-/NO3- kit) based on the Griess reaction. After reaction with the Griess reagent, the NO2-/NO3- concentrations (μmol/g) were derived at 550 nm spectrophotometrically. Data (total nitrite + nitrate) in plasma and in tissue (nitrite) were expressed as μmol/L and μmol/mg protein, respectively.

Statistical analysis

Data were analyzed using the SPSS statistical program (SPSS 11.0, Chicago, Illinois, USA). The results were expressed as mean ± standard deviation (means ± SD). One-way analysis of variance (ANOVA), Kruskal-Wallis, and Mann-Whitney U tests were used in the statistical analysis. "p" values less than 0.05 were considered statistically significant.

RESULTS

Glycemia

Average glycemia prior to ischemia in hyperglycemic and control group was 388.80 ± 35.92 and 126.75 ± 7.45 % mg, respectively. The difference was statistically significant ($p < 0.01$).

Levels of RBC SOD

One of the most intriguing findings related to SOD in this experiment was that RBC SOD activity increased considerably after IR. We found a statistically significant difference between sham-operated and sham + IR groups ($p < 0.05$). The mean value in hyperglycemic + IR group was smaller than sham + IR group ($p < 0.05$). On the other hand, the difference between sham + IR and hyperglycemic + IR + VE group and between hyperglycemic + IR and hyperglycemic + IR + VE group was not statistically significant ($p > 0.05$). These findings suggested that administration of VE has raised SOD levels, an antioxidant enzyme.

Levels of MDA

When the sham-operated group was compared to sham + IR group, we found a statistically significant difference in tissue levels of MDA ($p < 0.05$) but not in plasma levels ($p > 0.05$). In addition to IR, hyperglycemia led to considerable increase in tissue MDA levels. The difference between sham + IR and

hyperglycemic + IR groups was quite significant (p < 0.01). In contrast, there was no difference between sham + IR and hyperglycemic+IR+VE groups and between hyperglycemic+IR and hyperglycemic +IR+VE groups (p>0.05), suggesting that administration of VE, a natural antioxidant has caused to decrease LP.

When regarding the plasma MDA levels, hyperglycemia and IR together caused significant increase in MDA levels when compared to sham + IR group (p < 0.05). However, there was no statistically significant difference between sham + IR and hyperglycemic +IR+VE (p> 0.05), demonstrating VE administration caused a reduction in the level of plasma MDA, a by-product of LP. We also found statistically significant difference between hyperglycemic +IR and hyperglycemic +IR+VE group (p< 0.05).

NO metabolites (nitrite/nitrate)

When the sham-operated group was compared to sham + IR group regarding nitrite levels, we found a statistically significant difference in the plasma (p < 0.05), but not in tissue (p > 0.05). Another important finding demonstrated in this study was that total plasma nitrite/nitrate levels were significantly increased in ischemia and ischemia plus hyperglycemia. Regarding the plasma levels, there was a significant difference between sham + IR and hyperglycemic + IR (p < 0.05). No difference was found when sham + IR and hyperglycemic + IR + VE groups were compared (p > 0.05). Administration of VE to hyperglycemic + IR rats caused a decline in plasma total NO metabolites (hyperglycemic + IR vs. hyperglycemic + IR + VE; p < 0.05). However, we found no significant difference among the groups when regarding cerebral tissue nitrite levels (p> 0.05).

DISCUSSION

Stroke is one of the leading causes of death in developed countries and the risk factor hyperglycemia has been found to be associated with increased mortality and morbidity in patients with diabetes (5, 14). The deleterious effects of hyperglycemia on cerebral ischemia are mainly due to increased superoxide production by, particularly spontaneous oxidation of glucose. It has been demonstrated that superoxide is one of key factors in brain edema formation and apoptotic cell death after IR (11). The formation of free radicals induces LP that results in membrane damage and finally cell death.

NO plays a physiological role in low concentrations but when concentrations are increased beyond a certain critical level, this molecule becomes enormously cytotoxic. The cytotoxicity of increased NO comes mainly from the reaction with superoxide anion, which in turn forms extremely toxic peroxynitrite or it can cause injury to the cell by interacting with proteins, metals, iron-sulfur containing or heme-containing compounds (1, 23). Excessive NO production has been implicated in ischemic neurodegeneration (9, 18).

The brain has enzymatic and non-enzymatic (natural) antioxidant mechanisms. One of the members of enzymatic antioxidant mechanisms is SOD, an endogenous antioxidant enzyme involved in the detoxification of superoxide radicals. Among the various natural antioxidants, lipid soluble nutrient VE scavenges lipid peroxyl radicals and in so doing it inhibits LP (10, 12). However, in pathological conditions, including cerebral ischemia and ischemia associated with hyperglycemia, antioxidants may not overcome free radicals due to over-production. Administration of antioxidants may therefore have a beneficial effect on patients suffering from ischemia or ischemia on diabetes.

SOD production is induced by increased superoxide production during ischemia, particularly in reperfusion injury (2, 22). It has been demonstrated that lower serum SOD levels correlate negatively with infarct volume (22). Neuronal damage is reduced in mice that are treated with SOD, suggesting a protective role (13, 17, 20). Conversely, knockout mice for SOD genes are more susceptible to cerebral ischemia than wild type (21), indicating the contribution of superoxide radicals to ischemia.

Natural antioxidants (vitamins A, E, and C) have been proved to be useful for reducing LP and they also diminish ischemic damage (3, 16, 10, 12). Pre-ischemic administration of VE was found to reduce the volume of ischemic infarct and functional consequences of embolic infarction in rats (10, 16). In addition, it has been demonstrated that VE can prevent apoptosis secondary to ischemia in hippocampal neurons (25) and post-ischemic LP as well (10). Administration of VE alone or in combination with other vitamins, such as vitamin C, increases the activities of antioxidant enzymes such as SOD, catalase, glutathione-S-transferase in rats following cerebral ischemia (12). Therefore, it seems reasonable that patients at high risk of stroke (patients with diabetes mellitus, hypertension, etc.) should be treated with a diet enriched with antioxidant vitamins.

Our findings are consistent with the literature stating a considerable increase in RBC SOD, plasma and tissue MDA and plasma NO metabolites after ischemia induction when compared to control animals. In addition, we found that LP and NO metabolites increased considerably when IR injury was created in hyperglycemic rats, demonstrating the deleterious effect of hyperglycemia on ischemia. There is a decrease in MDA levels and NO metabolites and increase in SOD activities after administration of VE suggesting a protective effect of a natural antioxidant VE.

In conclusion, the administration of VE before cerebral ischemia reduced lipid peroxidation and nitric oxide production in rats. These results suggest that the strategies to scavenge free radicals and to minimize LP and nitric oxide production in hyperglycemic stroke may be neuroprotective and useful. Prophylaxis with VE may be considered in patients with diabetes mellitus for reducing cerebral damage after stroke.

Acknowledgement

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