CO-EXPRESSION OF INOS AND HSP70 IN DIABETES TYPE 1 MAKES A RATIONAL HYPOTHESIS TO EXPLAIN THE DIABETIC NEUROPATHY

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Abstract

The present study explores the co-expression of iNOS and HSP70 in brains of diabetic rats. Methodology involved induction of diabetes type 1 in rats and carrying out immunohistochemical analysis for both iNOS and Hsp70 in the brain tissues. Results revealed that the levels of HSP70 decreased in diabetes type 1 significantly compared with baseline levels for control groups, while the level of iNOS increased dramatically in brain tissue of diabetic rats. Furthermore, the data of the present study showed similar levels of HSP70 and iNOS at baseline level. The expression and localization of iNOS have both shifted in brain of control group from gray matter to white matter in rat diabetic brain.

Conclusions: the imbalance of molecular expression of iNOS and HSP70 offers better molecular environment to facilitate pathogenesis of diabetes and explain partly that the overexpression of iNOS downregulates the expression of HSP70.

Keywords: HSP70, iNOS, diabetes type 1

Introduction

Diabetes is a disease characterized by having high glucose level in blood. Glucose is utilized within cells by insulin; a hormone that helps the glucose gets into cells to provide them with energy. Diabetic symptoms include frequent urination, lethargy, excessive, thirst and hunger. Diabetes can be treated by changes in diet, medications, and in some cases, daily injections of insulin. Diabetes may occur as a result of either a lack of insulin or because of the present of factors that oppose the action in blood glucose, the result of insufficient action of insulin is an increase in blood glucose concentration (Tierney, McGhee, Papadakos, 2002).

Diabetes mellitus is not viewed as a single disease but it is rather a group of metabolic disorders include alterations in the carbohydrate, fat, and protein metabolism associated with absolute and/or relative deficiencies in insulin secretion (West, Ahuja, Bennett, 1983). High blood glucose levels were reported to be toxic, causing serious microvascular and macrovascular damages. Therefore, diabetes is a leading cause of blindness, end-stage renal disease, atherosclerotic macrovascular disease, and a variety of debilitating neuropathies, reducing patients' quality of life and life expectancy (Eizirik, 1995; Kelly et al., 2003).

Diabetic neuropathy is a descriptive term and includes a spectrum of clinical and subclinical syndromes with differing anatomical distributions, clinical courses, and possibly differing underlying pathogenetic mechanisms. Diabetes mellitus leads to diffuse or focal damage to peripheral somatic or autonomic nerve fibers, although indistinguishable syndromes may occur idiopathically or in association with other disorders in nondiabetic individuals (James et al., 2008).

Nitric oxide (NO) is known as a highly reactive signaling molecule that is made in a wide variety of cells, most prominently neurons, skeletal muscle, endothelial cells, and certain immune system cells. In these cells, NO is synthesized by one or more of three highly related NOS isoforms, neuronal NOS (nNOS, or NOS-1), endothelial NOS (eNOS, or NOS-3), and macrophage or inducible NOS (iNOS, or NOS-2), each of which is encoded by a separate gene. Although each NOS isoform is named for the cell type in which it was first isolated, none is confined to any one cell type and most cells express more than one isoform (Marletta, 1993). NO has been associated with numerous critical roles in the body, including maintaining blood pressure, acting as a neurotransmitter in the brain, and as a key component of the immune system's repertoire of antiviral and antibacterial agents. However, it was shown that misregulation and overproduction of NO can occur, and overproduction contributes to the pathogenesis of a range of diseases (Marletta, 1993). iNOS has been shown to play roles in the pathogenesis of hypotension and impaired organ perfusion, as well as inflammatory disorders, such as rheumatoid arthritis, asthma, and inflammatory bowel disease, which are associated with the generation of inflammatory mediators that cause tissue damage. In the case of these inflammatory disorders, NO, generated by iNOS, and prostaglandins, synthesized by the inducible cyclooxygenase COX-2, are key inflammatory mediators (Weinberg, 2000).

One function of NO is to induce prostaglandin production (Salvemini et al., 1993). In one study,a macrophage cell line was treated with inflammatory signals such as lipopolysaccharide to mimic physiological inflammatory diseases; iNOS and COX-2 were

induced. In the same study, it was shown that the application of iNOS inhibitors blocked release of prostaglandins (Kim, Huri, Snyder, 2005).

The NO generated by iNOS directly modulates the blood supply to nerves and participates in microvascular changes following injury (Levy, Zochodne, 2004). NO has direct roles in axon and myelin breakdown following an injury and also contributes to the development of neuropathic pain (McDonald et al., 2007). However, excessive local levels of NO during inflammation may damage axons and growth cones (Zochodne, Levy, 2005).

Heat shock proteins (HSPs) are referred to stress proteins because various forms of stress enhance their transcriptional activation and biosynthesis. HSPs have been shown to play important roles in cell proliferation and the control of cellular functions (Lindquist, 1986). HSPs comprise a family of proteins named according to their molecular weight among which is HSP70. It is known to act as a chaperone, forming complexes with a diverse array of cellular proteins and peptides (Heike et al, 1996). It has been shown that many bacteria and parasites produce HSP70 which has been postulated to serve as an antigen for bacterial and parasitic pathogens (Dubois et al,. 1984; Shinnick, 1991; Erol, Kumar, Carson, 1999). Furthermore, it has been found that there is an antigenic mimicry between the protein of the host and the pathogen and it is thought to be associated with autoimmune diseases (Minota et al., 1988).

It has been demonstrated that a correlation between diabetic neuropathy and plasma levels of HSP 27 (Gruden et al., 2008). HSP 27 is a required intermediate in the pathway of TNF-α induction of the inflammatory mediators cyclooxygenase-2 (COX-2), IL-6, and IL-8. The production of the initiating inflammatory mediators TNF-α and TGF-β results from several of the glucose-induced pathways already outlined (Vincent, Feldman, 2004; Brownlee, 2005). It has been well demonstrated that HSP has a major role in tissue protection and repair against a number of insults and pathological conditions (Powers, Locke , Demirel, 2001; Welsh et al., 1995).

Methodology

Induction of Diabetes

Twenty female, Albino rats, weighing between 53-100 g at the beginning of the study, were housed in single cages with food pellets and water available *ad libitum.* The room was maintained at a constant temperature and humidity on a 12-h/12-h light/dark cycle. All animal treatments were carried out strictly according to the guideline of the Committee of Animal Research at Jordan University of Science and Technology. A total of 20 rats were randomly assigned into two groups: control group (C, n=10) and diabetic control (D, n=10).

The average weight of the two groups was approximately closed to each other. It was 63.2 g, 63.6 g for control and diabetic groups respectively (table 1)

Table1: the weight of rats in experimental groups

Control group	Diabetic group
65	**66**
53	**48**
73	**52**
60	**60**
63	**38**
59	**66**
65	**82**
61	**70**
59	**77**
74	**77**
Average: 36.2	**Average: 63.4**

P >0.05 at the average level

To prepare the diabetic rat model, 10 rats were injected with 1 dose of alloxan (120 mg/kg) for each. After three days, glucose level was above 250 mg/dl (severe diabetes).
This schedule of alloxan injection has been shown to result in persistent diabetes. To ensure that the animals used in this study were affected by the alloxan treatment, glucose levels were measured weekly, and maintained above 250 mg/dl (average glucose= 342.88 mg/dl for Diabetic group).

Immunohistochemistry Staining For Brain Tissue

Immunohistochemical detections of iNOS and HSP70 were performed using commercially available mouse monoclonal antibodies. Immunohistochemical detections of iNOS and HSP27 were demonstrated by using labeled streptavidin biotin LSAB kit, which consists of secondary biotinylated goat anti-mouse antibody and conjugated streptavidin Horse raddish peroxidase followed by 3',3'-Diaminobenzidine (DAB) chromogen.

Sections were processed for immunohistochemistr using conventional techniques.

Immunohistochemical Assessment of Stained Sections

Slides were assessed using adopy photoshop software. Photos for sections were taken and divided into pixels. The total number of pixels was computed and represented both colours (blue and brown), then the brown colour (the colour of the marker under study) was computed and divided by the total number of pixels.

Statistical Analysis

One way ANOVA was completed on all animals followed by paired and unpaired student t-test analysis to determine statistical significance within each group and to compare two groups.All bar graphs illustrate the mean $\pm$ SE. P value < 0.05 was considered statistically significant.

Results

The Expression Pattern Of HSP70

The expression of HSP70 was compared among the two study groups. As mentioned in the methodology section, there were two groups; diabetic groups and control groups (table 2).

The average expression rate of HSP70 was 0.045% in SC group. This rate was decreased in diabetic group to 0.0029. This decreased rate was statistically significant as shown by T test (p value $\leq$0.05).

Table 2: The expression rate of HSP27 in the brain tissue

Group no	Control group	Diabetic group
1	0.018	0.0025
2	0.142	0.0025
3	0.025	0.002
4	0.027	0.0026
5	0.030	0.003
6	0.035	0.0029
7	0.020	0.0035
8	0.060	0.0034
overall average	0.045	0.0029

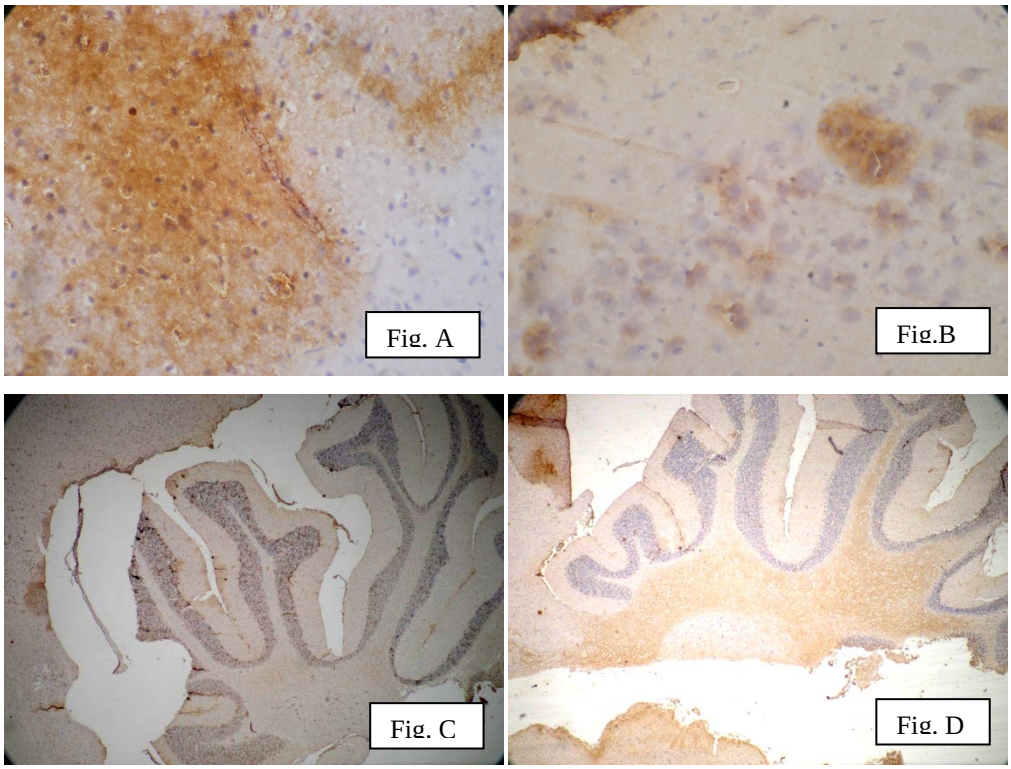
The Expression Pattern Of iNOS

The expression rate of iNOS was evaluated and compared in the brain tissue of the two groups under study. The average of expression rate of iNOS was 0.041% in SC group. The average of expression rate in SD group (0.30%) was increased significantly (p value $\leq$ 0.05).

As it can be shown in figure 4, the expression of both iNOS and HSP70 are approximately similar in the Control group. This may be the balance point before going further appropriate changes according to each biomarker.

Table 3: The expression rate of iNOS in the brain tissue

no	Control group	Diabetic group
1	0.061	0.182
2	0.068	0.153
3	0.015	0.070
4	0.049	0.87
5	0.032	0.090
6	0.050	0.250
7	0.027	0.340
8	0.028	0.450
overall average	0.041	0.300



Figures A-D show the expression of HSP70 and iNOS. Under physiological conditions, HSP70 is expressed to carry out required functions (Figure A), under diabetic conditions, tissue defense mechanisms are impaired and the expression of HSP70 is decreased (Figure B). Slight expression of iNOS is required under physiological condition. The expression of iNOS is mainly confined to gray matter as seen in brain of control group (Figure C), while in diabetic brain, there is a high expression of iNOS with shifting to white matter (Figure D).

Discussion

Diabetes studies on molecular level can help in better understanding of diabetes and its associated neuropathies. The present study was conducted to explore the co-expression of iNOS and HSP70 inbrain of diabetic rats.

The results of our experiments showed that the average expression level of HSP70 was decreased in diabetic group compared with control group significantly (p value $\leq$0.05).

These findings are in line with the results obtained by a study conducted by Mustafa et al (2004). It has also been found that induction of diabetes decreased HSP expression in heart, liver and vastus lateralis (VL) muscles (Hojlund et al., 2003).

The results obtained in the present study can also be explained in the context of other adverse effects of diabetes as impairment of endogenous tissue defense mechanisms and vulnerability of tissues to various types of stress. In this regard, heat shock protein (HSP) family is a major component of the endogenous defense which could protect against tissue

damage by facilitating the re-folding of denatured proteins, maintenance of structural integrity and by acting as molecular chaperones (Yamagishi et al., 2001; Noor et al., 2007).

The data of our results showed that expression level of iNOS was increased significantly in diabetic group compared with control group (p value ≤ 0.05). The obtained results in the present study are consistent with other studies reported in literature. It has been found that iNOS is induced in cells in response to various inflammatory stimuli such as tumor necrosis factor (TNF), interferon-gamma, or lipopolysaccharide (MacMicking et al., 1997). Other studies indicated that the large amounts of NO produced by iNOS contribute to tissue damage even though iNOS induction has key roles in the innate immune system due to the anti-viral and anti-bacterial effects of NO (Vane et al., 1994).

It has been suggested through different studies that hyperglycemia favors, through the activation of NF_B, an increased expression of inducible nitric oxide synthase (iNOS), which is accompanied by increased generation of nitric oxide (Griendling et al., 2003).

Based on the idea that diabetes is a metabolic disease, NO has the ability to react with superoxide to produce the strong oxidant peroxynitrite, which in turn can increase lipid peroxidation, protein nitration, and LDL oxidation, affecting many signal transduction pathways (Stadler et al., 2004). However, there has recently been accumulation of experimental evidence supporting the idea of complex roles for nitric oxide, ROS, and peroxynitrite in the development of early diabetes tissue injury before the evolution of late complications (Gunnett et al., 2002; Baker et al., 1999).

It has been shown by other studies that inducible nitric oxide synthase (iNOS) is not present in normal blood vessels but is expressed in blood vessels during atherosclerosis, diabetes, endotoxemia, and after stroke (Hoque et al., 1998; Forster et al., 1999; Detmers et al., 2000; Hollenberg et al., 2000; Pulido et al., 2000; Zoppo et al., 2000; Bardell et al., 2001). It has also been argued that although expression of iNOS in blood vessels is associated with vascular dysfunction in vascular disease, it is not clear if iNOS is directly involved with mechanisms of impairment. Accordingly, several studies have used gene targeted mice deficient in expression of iNOS to implicate iNOS in mechanisms of vascular dysfunction (Koglin et al., 1998; Kibbe, Billiar, Tzeng, 1999; Zhao et al., 2000). While other studies, however, have used iNOS-deficient mice and gene transfer of iNOS and suggest that iNOS protects vascular function and inhibits hyperplasia and restenosis (Benjamin et al., 1998; Shears et al., 1998; Kanno et al, 2000; Mustafa et al., 2004).

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As previously mentioned, diabetes mellitus is one of the most common endocrine metabolic disorders. Trends in research have emphasized that oxidative stress is a mechanism underlying insulin resistance, type I and type II diabetes, and diabetic complications (Brownlee, 2001). Hyperglycemia leads to oxidative stress because of increased mitochondrial production of the superoxide anion, nonenzymatic glycation of proteins, and glucose autoxidation (Wolff, Dean, 1987; Wolff, Jiang, Hunt, 1991; Wojtczak, Schonfeld, 1993; Baynes, 2003). It has been shown that FFA, which is elevated in diabetes and insulin resistance, may also contribute to the increased production of reactive oxygen species due to increased mitochondrial uncoupling and oxidation (Evans et al., 2002; King, Loeken, 2004).

Conclusion

The data of the present study showed that diabetes is complicated by lowering the expression of HSP70 and increasing the expression of iNOS. Furthermore, we showed for the first time that iNOS changes its localization and expression from gray matter in rat brain of control group to white matter in brain of diabetic rats.

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