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Insulin-induced oxidative stress in the brain is nitric oxide-dependent

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ABSTRACT

Insulin is known to increase brain nitric oxide (NO) level and to cause oxidative stress but the relationship between these phenomena has not been well elucidated. This study aimed to examine the role of NO in the insulin-NO-oxidative stress axis in the brain. Mice were grouped into four (n = 5) and treated for seven days with 0.2 ml deionized water (control); 10 I.U./kg insulin; 10 I.U./kg insulin + 50 mg/kg L-NAME; and 50 mg/kg L-NAME. The mice were anaesthesized using ketamine + xylazine and sacrificed at the end of the study. Forebrain was immediately harvested from which brain homogenates were prepared in order to determine NO and malondialdehyde (MDA) concentrations as well as glutathione peroxidase (GPx) activity using commercially available kits. Data were processed using IBM SPSS Statistics 20.0. Nitric oxide values were higher in the insulin group ($p < 0.05$) but not in the insulin+L-NAME ($p > 0.05$) group when compared with the control. Values of MDA in the insulin and insulin+L-NAME groups were higher ($p < 0.05$) and the same ($p > 0.05$), respectively, than those in the control group. The activity of GPx in the insulin group was lower ($p < 0.05$) than, but that of the insulin+L-NAME was the same ($p > 0.05$) as in the control group. Insulin increased NO concentration and oxidative stress as indicated by increased MDA concentration and decreased GPx activity in the treated mice. This insulin effect was reversed by L-NAME (a non-specific NO inhibitor). These data suggest that insulin increased oxidative stress in the brain through an NO-dependent process. Insulin treatment may be harmful to the brain.

1. Introduction

Since its discovery, insulin has gradually evolved from a peripheral glucose-control hormone to a brain hormone [1] that not only exerts trophic effects but also performs regulatory functions in the brain [2,3]. Due to its numerous beneficial effects on the brain, insulin is considered as a promising therapy for diabetes- and age-related neurodegenerative disorders, including Alzheimer's disease [4].

Insulin, initially used as a replacement therapy in type 1 diabetes mellitus (T1DM) is increasingly being used in the treatment of type 2 diabetes mellitus (T2DM) not controlled by diet, exercise and oral anti-hypoglycaemic agents. Its use leads to better results in terms of decreasing microvascular complications and mortality compared to oral hypoglycaemic agents [5]. Given the high and rising prevalence of diabetes, estimated to stand at about 300 million worldwide by 2025 [6], and with the development of non-invasive insulin delivery methods such as intranasal insulin administration [7,8,9,10], the use of insulin to treat diabetes is expected to increase significantly in the nearest future.

However, amidst the positive effects of insulin, there could be potentially negative ones due to the ability of insulin treatment to induce oxidative stress. Separate studies have reported that insulin increases brain nitric oxide (NO) level [11,12,13] and induces oxidative stress [14] but the relationship between these phenomena has not been well elucidated. In other words, the insulin-NO-oxidative stress axis has been investigated but in separate studies and with incongruent reports. According to Choopani et al. [15], insulin administration induces NO synthesis in the hippocampus with subsequent improvements in learning and memory. Contrary to this, Monnier et al. [14] reported that elevated doses of insulin promoted oxidative stress in humans with T2DM receiving insulin therapy and suggested that the oxidative stress could mediate some deleterious effects in the brain. Indeed, given the fact that NO, a free radical, is an inducer of oxidative stress [16], it is conceivable that brain redox status could worsen due to insulin treatment.

No previous research has investigated the role of NO in the insulin-NO-oxidative stress axis in the brain in a single study, which

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is what this work sought to achieve. The effects of exogenous insulin administration on brain NO concentration and oxidative stress (measured by malonedialdehyde [MDA] concentration and glutathione peroxidase [GPx] activity was investigated in this study. It was hypothesized that insulin administration affects oxidative stress only in the presence of NO.

2. Materials and methods

2.1. Animals and grouping

Twenty four mice of both sexes, weighing between 19–21 g were used. They were kept in spacious cages and allowed free access to feed and drinking water throughout the duration of the experiment. The animals were grouped (n = 5) and treated daily for 7 days thus: Control group received distilled water (0.2 ml) sub-cutaneously (*s.c.*); Insulin group received insulin (Actrapid, Novo Nordisk A/S, Denmark) (10 I.U./kg/day *s.c.*); Insulin + L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) group received insulin (10 I.U./kg/day *s.c.*) and L-NAME (sc-200333, Lot # L1514; Santa Cruz Biotechnology, Dallas, U.S.A.) (50 mg/kg i.p.); L-NAME group received L-NAME (50 mg/kg). At the end of the experiment the animals were sacrificed under anaesthesia by injection of ketamine + xylazine 65/4 mg/kg, i.p. [17].

2.2. Samples and data collection

Mice were sacrificed 30 min after the last injection. Brain tissues were collected and prepared as described previously by Shen et al. [18]. Briefly, brain tissue was immediately harvested and weighed over ice blocks. About 1 mg of the fore-brain tissue was removed and homogenized in 10 mL of ice-cold phosphate-buffered solution (pH 7.0), centrifuged at 4 °C and 3000 *g* for 5 min. The supernatant was transferred into fresh Effendorf tubes and kept at −20 °C till analysis to determine NO concentration, MDA concentration and GPx activity.

Nitric oxide level was determined in brain homogenates using commercially available kit (Biovision Inc., Milpitas, CA 95035, U.S.A., Catalog #262-200) according to the manufacturer's instructions. The concentration of MDA was determined using commercially available colorimetric thiobarbituric acid reactive substances (TBARS) microplate assay kit, obtained from Oxford Biomedical Research, Oxford, MI 48371, U.S.A. (Product number: FR40). The activity of GPx was measured using the GPx cellular activity assay kit (Biovision Inc., Milpitas, CA 95035, U.S.A., Catalog #K762-100) according to manufacturer's instructions. This study was conducted in conformity with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978)

2.3. Statistical analyses

All data were collated and analyzed using IBM SPSS Statistics version 20.0. Values were expressed as mean ± S.E.M. One-way ANOVA was used to compare means; Bonferroni test was employed for *post-hoc* multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Nitric oxide concentration

There was an overall significant difference in NO concentrations (nmol/μL) in brain homogenates of mice, when compared between groups ($P = 0.01$, $F_{(3,16)} = 9.870$, df = 3, n = 5). Nitric oxide values in the insulin group was significantly higher than those of the controls ($P = 0.006$), but the difference between those of the insulin+L-NAME ($P = 1.000$) or L-NAME ($p = 1.000$) groups were not significantly different when compared with the control group (Fig. 1).

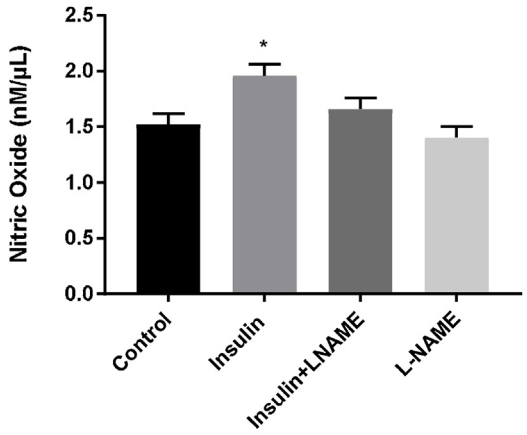

Fig. 1. Nitric oxide level in brain homogenates of control and treated mice. (Mean ± S.E.M, n = 5) *Significantly higher ($P < 0.05$) than in the other groups.

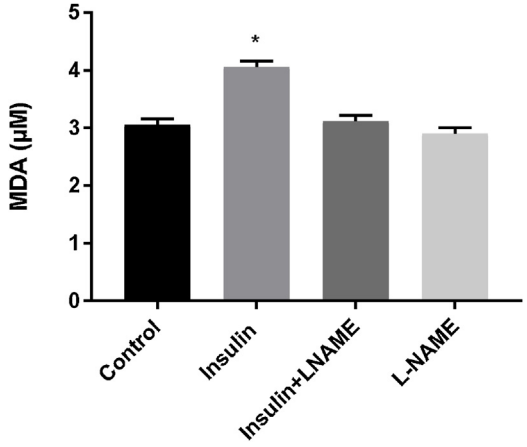

Fig. 2. Malondialdehyde level in brain homogenates of control and treated mice. (Mean ± S.E.M, n = 5) *Significantly higher ($P < 0.05$) than in the other groups.

3.2. Malondialdehyde concentration

There was a significant overall difference between groups in the concentration of MDA (μM) in brain homogenates ($F_{(3,16)} = 53.508$, $P = 0.001$, df = 3, n = 5) (Fig. 2). Malondialdehyde values in the insulin group was significantly higher than that of the controls ($P = 0.001$). The levels of MDA in the insulin+L-NAME ($P = 1.000$) and L-NAME ($P = 0.807$) groups were statistically the same, when compared with those of the control group.

3.3. Glutathione peroxidase activity

There was a significant overall difference between groups in the activity of GPx (nM of GHS oxidized per min per mg protein) in brain homogenates ($P = 0.001$, $F_{(3,16)} = 8.994$, df = 3, n = 5) (Fig. 3). The activities of GPx in the insulin group was significantly lower when compared with that of the control group ($P = 0.002$). Glutathione peroxidase activity in the insulin+L-NAME (p = 1.000) and L-NAME (p = 1.000) groups were not significantly different compared with control.

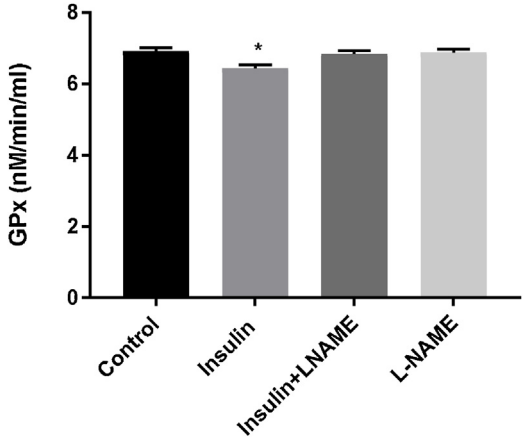

Fig. 3. Glutathione peroxidase activity in brain homogenates of control and treated mice. (Mean ± S.E.M, n = 5) *Significantly lower ($P < 0.05$) than in the other groups.

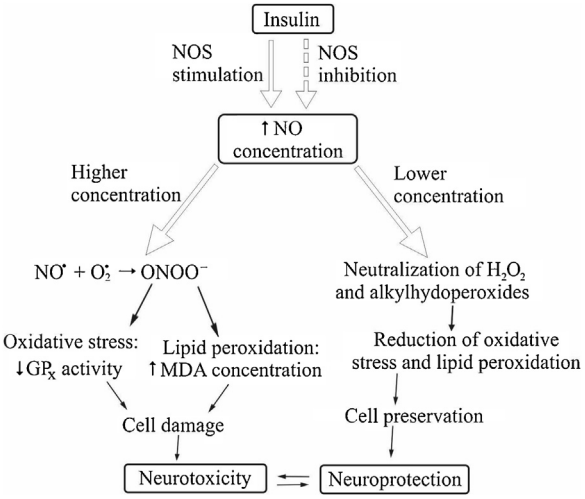

Fig. 4. Mechanism through which insulin increases oxidative stress in the brain. Note that the ability of insulin to induce oxidative stress is NO-dependent (blocked by NOS inhibitors) (dashed arrow) as demonstrated in this study. NO (nitric oxide), MDA (malonedialdehyde), GPx (glutathione peroxidase), $NO^{\bullet}$ (nitrite radical), $ONOO^{-}$ (peroxinitrite), $O_2^{\bullet}$ (superoxide), H_2O_2 (hydrogen peroxide), ↑ and ↓ (increase and decrease in NO, MDA and GPx, respectively).

4. Discussion

In the present study, insulin treatment has significantly increased brain NO level, and the effect which was reversed by L-NAME treatment. This result corroborates earlier reports that insulin activates nitric oxide synthase (NOS) to increase NO synthesis in the brain [15]. L-NAME hydrochloride – a non-selective NOS inhibitor (irreversible inhibition on nNOS isoform), was used as NO inhibitor to create NO deficiency. L-NAME, with a half-life of 12–19 min may act as a weak NOS inhibitor, but it is readily hydrolyzed by ubiquitously present esterases to L-NNA (L-N$^{??}$–Nitroarginine) [19] for its full inhibitory effect *in vivo* [20]. The NO metabolite L-NNA shows a biphasic pharmacokinetic profile with a terminal half-life of 20 h in rats [21].

It is noteworthy that NO production might lead to different consequences in the brain - either neurotoxicity or neuroprotection (Fig. 4) depending on the location and level of NO production, the extent of oxidative stress and type of neurodegenerative process [22]. NO can act directly as an antioxidant to protect against cellular injury caused by H_2O_2, XO and alkylhydroperoxides [23]. At higher concentrations NO can interact with superoxide anion leading to formation of the powerful oxidant peroxynitrite, resulting in cell damage and altered neuronal physiological function [22]. The dual role of NO as an antioxidant on one hand and inducer of oxidative stress on the other explains the contrasting role of insulin as beneficial most of the time and harmful in certain circumstances. Indeed, previous studies have reported contrasting results with regards to the consequences of increased brain NO levels following insulin administration in the form of improvement [24] and impairment [25,26] of functions. This study proposes the mechanism of this contrasting role of insulin. Our results agree with previous reports that insulin induced an increase in brain NO level [27,13,14] but are contrary to other reports that insulin inhibited lipopolysaccharide-induced iNOS activity and NO production in rat brain [28,29].

There was significant increase in lipid peroxidation due to insulin among the mice in this study as demonstrated by the significant increase in MDA levels observed in these animals. Patockova et al. [30] and Agrawal et al. [31] previously reported similar findings that insulin caused lipid peroxidation and increase in MDA levels in the brain. The present study demonstrated, in addition to the above findings, that there was corresponding insulin-induced increase in brain NO in the same animals, which was reversed by L-NAME. The findings indicate that insulin increased brain lipid peroxidation through an NO-dependent mechanism.

The activity of brain GPx was decreased significantly by insulin in the treated mice, in line with previous reports of Agrawal et al. [31]. This effect was reversed by L-NAME, indicating that the effect was NO-dependent. Insulin, thus, increased oxidative stress, which resulted in reduced antioxidant capacity of the brain, expressed as reduced GPx level.

It has been suggested that the consequence of T2DM is oxidative stress due to several mechanisms related to hyperglycaemia [4] mediated via the formation of advanced glycation end-products (AGEs), glucose autoxidation, mitochondrial dysfunction, endoplasmic reticulum stress and impaired antioxidant defenses [32,33]. However, as reported by Craft [34] the hyperglycaemia in T2DM occurs concurrently with chronic peripheral hyperinsulinaemia [34]. In addition, Monnier et al. [14] also observed hyperinsulinaemia in humans with T2DM receiving insulin therapy. This study has provided evidence to demonstrate that the increase in oxidative stress reported in T2DM may partly be caused by hyperinsulinaemia via increase in NO level, more so that insulin increases NO level not only in the brain, but in other tissues such as platelets [35] and endothelium [36]. The findings of this study provide additional insight into how hyperinsulinaemia could promote the development of certain negative consequences of T2DM such as vascular damage, cognitive impairment and dementia, which have hitherto been linked to hyperglycaemia.

5. Conclusion

Our data demonstrate that insulin administration increases oxidative stress and lipid peroxidation in the brain of mice by increasing MDA concentration and reducing GPx activity, through an NO-dependent process. Insulin treatment may be harmful to the brain.

Conflicts of interests

The authors declare no potential conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pathophys.2019.02.003.

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