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Involvement of Nitric Oxide in Insulin-Induced Changes in Memory, Oxidative Stress and Brain Histology in Mice

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ABSTRACT

Insulin has been reported to increase nitric oxide (NO) level in the brain but the biological significance of such effects is not well understood. The present study examined the involvement of NO on insulin-induced effects on brain oxidative stress, learning, memory and histology. Mice were grouped (n=6) and treated intraperitoneally, thus: Control group- distilled water; Insulin group- insulin (10 I.U./kg/day); Insulin+L-NAME group- insulin (10 I.U./kg/day) and L-NAME (50 mg/kg); L-NAME group- L-NAME (50 mg/kg). Learning and memory were assessed using novel object recognition test at the end of the experiment. Concentrations of NO, malonedialdehyde (MDA), as well as glutathione peroxidase (GPx) activity, were determined in brain homogenates using assay kits. Histological examination of brain slides was also conducted. Insulin treatment resulted in higher levels of NO compared to controls ($p<0.005$); and this effect was reversed by L-NAME treatment. With the increased NO level, there was concurrent increase in MDA concentration, decreased GPx activity (increased oxidative stress) and impaired memory in the treated animals. The increased oxidative stress was reversed by L-NAME treatment. Brain slides appeared normal and showed no indication of histopathological changes. These data led to the conclusion that sub-acute insulin treatment caused an NO-dependent increase in oxidative stress in the brain; insulin impaired non-spatial working memory but did not affect brain histology in the treated mice. Insulin treatment may have negative consequences on the brain through increased NO levels.

Key words: *Nitric oxide, Oxidative stress, Learning and memory, Brain histology, Malonedialdehyde, Glutathione peroxidase*

INTRODUCTION

Insulin was discovered as a hypoglycaemic agent several decades ago, and has also been reported to increase NO level in the brain (Montagnani et al. 2001; Vincent et al. 2003), platelets (Trovati et al. 1997) and endothelium (Steinberg et al. 1994), but the biological significance of these effects are not well understood. There are conflicting reports on insulin effects on the brain: improvement in learning and memory (Choopani et al. 2008) and potential harm due to induction of oxidative stress (Monnier et al. 2011); this calls for more studies to better understand these phenomena.

Based on the findings that insulin increases brain NO level (Montagnani et al. 2001; Vincent et al. 2003), and that NO (a free radical) is a known inducer of

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oxidative stress (Knowles and Moncada 1994), it is conceivable that the administration of insulin may increase the level of oxidative stress in the brain. Studies that examined the involvement of NO in the effect of insulin on the brain are lacking. Therefore, we hypothesized that the increase in NO level by insulin could result in increased oxidative stress in the brain, with possible negative consequences. Furthermore, it is not known how the effect of insulin treatment will manifest in brain histology. The present study examined the involvement of NO on insulin-induced effects on brain oxidative stress, learning, memory and histology.

MATERIALS AND METHODS

Experimental Animals and Treatments

There were 24 Swiss albino mice of both sexes, weighing between 19 - 21 g, and kept in spacious cages. They were allowed free access to feed and drinking water and maintained in well ventilated animal rooms under light/dark environment and time. The study was conducted with the approval of, and in accordance with the guidelines of The Animal Use and Care Committee of Ahmadu Bello University, Zaria, Nigeria.

The animals were randomly assigned to four groups (n = 6) and treated 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.) (Sharma et al. 2007); Insulin+L-NAME 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.) (Paul et al. 2003); L-NAME group received L-NAME (50 mg/kg). L-NAME hydrochloride is an nNOS blocker (Griffith and Kilbourn 1996) and was used in this study to examine the involvement of NO in the insulin-induced effects. The treatment was once daily between the hours of 8:00 and 9:00 a.m. and lasted for 7 days (Francis et al. 2008).

Assessment of Short-Term Non-Spatial Working Memory Using Novel Object Recognition Task

Novel object recognition task (NORT) was conducted on the 7th day of the experiment, 30 minutes after the injections, when L-NAME (Matsumoto et al. 2006) and insulin (Francis et al. 2008) have reached adequate blood concentrations. The test was carried out according to Gaskin et al. (2010). The NORT capitalizes on the findings of Berlyne (1950), who showed that rats prefer to explore objects that they have not previously encountered over objects that are familiar to them. A tendency to explore the novel object over the familiar one is interpreted as evidence of memory for the training exposure (Ennaceur and Delacour 1988; Gaskin et al. 2010).

Object recognition experiments were performed in a white wooden box (length 60 cm, width 40 cm, and height 30 cm). Objects to be discriminated were about the same size, made of plastic, deferring in shape and colour. Two days before the test, mice were allowed to explore the box twice for 5 minutes, in order to acclimatize. On the test day, each mouse was placed in the box for 2 sessions of 4 minutes each, separated by an interval of 15 minutes and left to explore objects freely. During the first session (S1) (acquisition trial), two copies of the same object were presented for the mouse to explore. In the second session (S2) (retention trial), mice were presented with one of the objects presented previously in S1 plus a novel object. Exploration was defined as the mice sniffing, gnawing or touching the object with the nose, or head orientation within 1.0 cm of the object. Sitting and/or turning around the object was not considered as exploratory behaviour.

The performance of the mice was recorded using a video camera (HANDYCAM, SONY, Japan) for subsequent evaluation of the following parameters: (a) Time spent by the mice exploring the objects during either S1 or S2; (b) Novel object recognition, which was calculated as the percentage of time spent in exploring the new object with respect to the total amount of time spent in exploring the two objects during S2; and (c) Discrimination ratio- the duration of exploration of the novel object, divided by the total exploration duration of both objects during the test phase. A discrimination ratio equal to 0.5 indicates chance behaviour, while ratios above 0.5 indicate preference for the novel object and, therefore, memory of the familiar object.

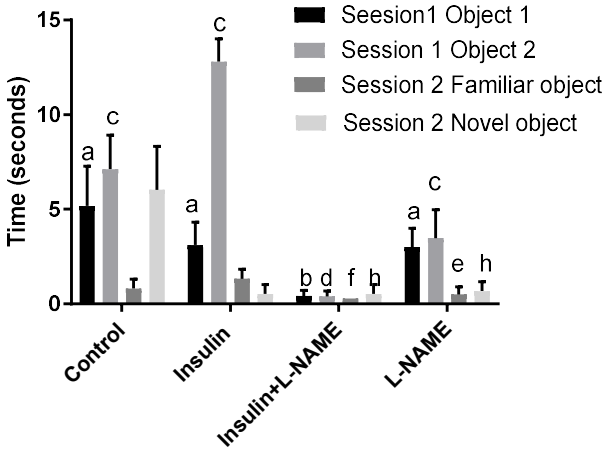

Figure 1: Time (seconds) spent by control and treated mice exploring familiar and novel objects during novel object recognition task. a,b,c,d,e,f,g,h = Bars with different superscripts are significantly different ($p < 0.05$). (Mean ± S.E.M, n = 6)

Biochemical Analysis of Brain Tissue

On the 7th day of the experiment after neurobehavioural studies, the mice were sacrificed by cervical transection under anaesthesia by ketamine/xylazine 65/4 mg/kg, i.p. (Buitrago et al. 2008). Brain tissue was collected and prepared as described previously by Shen et al. (2011). The skulls were opened and the brains quickly harvested and divided into two halves by sagittal section. Each left half of the brain was weighed, and a milligram of it removed and homogenized in 10 mL of ice-cold phosphate-buffered solution (pH 7.0), centrifuged at 3,000 g at 4°C for 5 minutes. The supernatant was transferred into fresh Eppendoff tubes and kept at -20°C till analysis to determine NO concentration, MDA concentration and GPx activity.

Nitric oxide concentration was determined in brain homogenates using commercially available kit (Biovision Inc., Milpitas, CA 95035, U.S.A., Catalog #262-200) and 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.

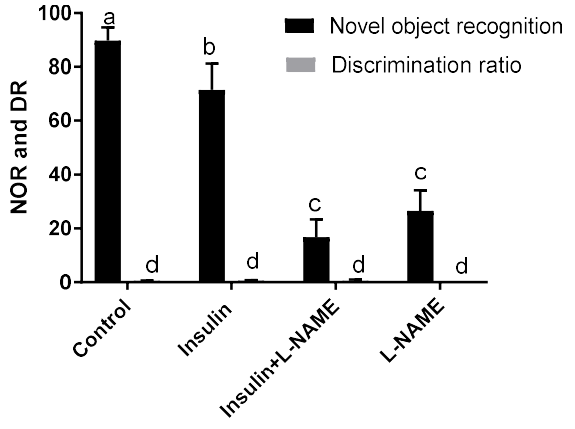

Figure 2: Novel object recognition and discrimination ratio of control and treated mice during novel object recognition task. a,b,c,d = Bars with different letters (between groups) are significantly different ($p < 0.05$). (Mean ± S.E.M, n = 6)

Histological Evaluation of Brain Tissue

Brain tissues were prepared for histological examination as described by Igado et al. (2012). Briefly, the right half of the brain from each mouse was fixed in 10% formaldehyde, dehydrated, embedded in paraffin and cut into 5 µm thick sections using a rotary microtome (Leica RM2125 RTS). Six slides from each brain were prepared and stained using haematoxylin and eosin (H&E). All the tissue slides were examined under light microscope (Olympus CH, Japan) (x200 magnification) for histologic changes in form or shape, loss of cellular integrity, presence of vacuolations and other micro-anatomical alterations. Photomicrographs were prepared using a Sony DSC-W 30 digital camera (Japan) from selected sections to demonstrate the findings.

Statistical Analyses

All data were collated and analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0. Values were expressed as mean ± standard error of mean. General linear model-repeated measures analysis of variance was used to compare mean time spent exploring the objects. One-way ANOVA was used to compare mean values of novel object recognition. Bonferroni test was employed for post-hoc multiple comparisons. Values of $p < 0.05$ were considered significant.

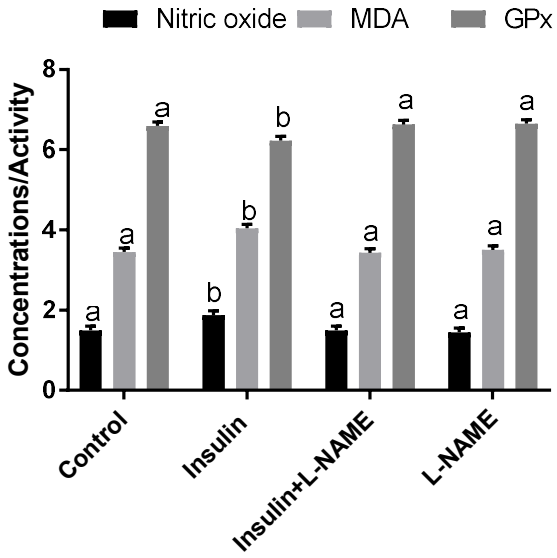

Figure 3: Concentrations of nitric oxide (nmol/µL), malonedialdehyde (µM) and glutathione peroxidase activity (nM GHS/min/mg protein) in brain homogenates of control and treated mice during novel object recognition task. a,b = Bars with different superscripts (between groups) are significantly different ($P < 0.05$). (Mean ± S.E.M, n = 6)

RESULTS

Effects of the Treatments on Short-Term Non-Spatial Working Memory

Time Spent Exploring the Objects

There was a significant overall difference between groups in the amount of time (seconds) spent exploring the different objects ($F(3.21) = 3.134$, $P = 0.048$, multivariate partial Eta2 = 0.320) (Figure 1).

There was also a significant difference within groups in the amount of time spent exploring the familiar and novel objects (Wilk's lambda = 0.414, F(3,21) = 8.480, P = 0.001, multivariate partial Eta2 = 0.586). The control animals spent significantly more time on the novel object compared with the insulin-treated (P = 0.024), insulin+L-NAME-treated (P = 0.044) and L-NAME-treated (P = 0.040). This means that the control mice showed preference for the novel object; hence have intact memory, while the treated animals had impaired memory. The result also shows that treatment with L-NAME did not modify the insulin-induced memory impairment.

Novel Object Recognition and Discrimination Ratio

There was a significant overall difference in novel object recognition compared between the groups (F = 6.988, P = 0.002) (Figure 2). Novel object recognition for the insulin group was significantly lower than that of the control (P = 0.029), indicating impaired memory of the former. In this parameter, the values of the insulin + L-NAME and L-NAME groups were significantly lower compared to those of the control (P = 0.005 and P = 0.015, respectively). Novel object recognition was significantly lower in the insulin+L-NAME group (P = 0.039) and L-NAME group compared to insulin group, showing that L-NAME did not reverse the insulin-induced effects. Discrimination ratio was > 0.5 for all the groups, indicating that the shorter time spent on the novel object was not a chance behaviour, but rather a true lack of preference for the novel object due to poorer memory of the familiar object. There was no significant difference in discrimination ratios between any of the groups (F = 0.866, P = 0.475).

Effects of the Treatments on Nitric Oxide Concentration, Malondialdehyde Concentration and Glutathione Peroxidase Activity in Brain of Mice

There was an overall significant difference in NO levels (nmol/µL) in brain homogenates of mice compared between the groups (P = 0. 013, F(3,16) = 5.533, df = 3, n = 6) (Figure 3). The NO values in the insulin group were significantly higher than those of the controls (P = 0.049). NO levels of the insulin+L-NAME (P = 1.000) and L-NAME (P = 1.000) groups were not significantly different compared with the levels in the control group. Insulin treatment resulted in higher levels of NO compared to controls. This effect was reversed by L-NAME treatment.

There was a significant overall difference between groups in the level of MDA (µM) in brain homogenates (F(3,16) = 18.816, P = 0.001, df = 3, n = 6) (Figure 4). Malondialdehyde values in the insulin group was significantly higher (P = 0.001) than that of the control. The values of MDA in the insulin+L-NAME (P = 1.000) and L-NAME (P = 0.001) groups were not significantly different compared with the control group. This result shows that insulin increased lipid peroxidation and that the effect was reversed by L-NAME, indicating the dependence of the effect on NO concentration.

There was a significant overall difference between groups in GPx activities (nM GHS/min/mg protein) in brain homogenates (P = 0.002, F(3, 16) = 9.463, df = 3, n = 6) (Figure 4). The activity of GPx in the brain homogenate of insulin group was significantly lower compared with that of the control (p= 0.010), indicating increased oxidative stress. Glutathione peroxidase activity in the insulin+L-NAME (P = 1.000) and L-NAME (P = 1.000) groups did not significantly differ from the control, showing that L-NAME treatment has reversed the effect.

Effects of the Treatments on Cerebellar Histology

Figure 4 (A) showed a photomicrograph of cerebellar cortex of mouse treated for seven days with distilled water (Control Group). Normal histology of the cortex and absence of histopathological lesions were observed. Neuronal cells (cross sections of axons) and glial cells (which appear with distinct cell membrane, cytoplasm and nucleus) can be observed. Similarly, normal histology was observed on the entire slides from all the treatment groups as illustrated on Figure 4(A-D).

Taken together, insulin treatment resulted in higher levels of NO compared to controls. The increased NO level was associated with increased oxidative stress and impaired memory. The increased oxidative stress was reversed by L-NAME, suggesting the role of NO in oxidative stress caused by insulin. However, the

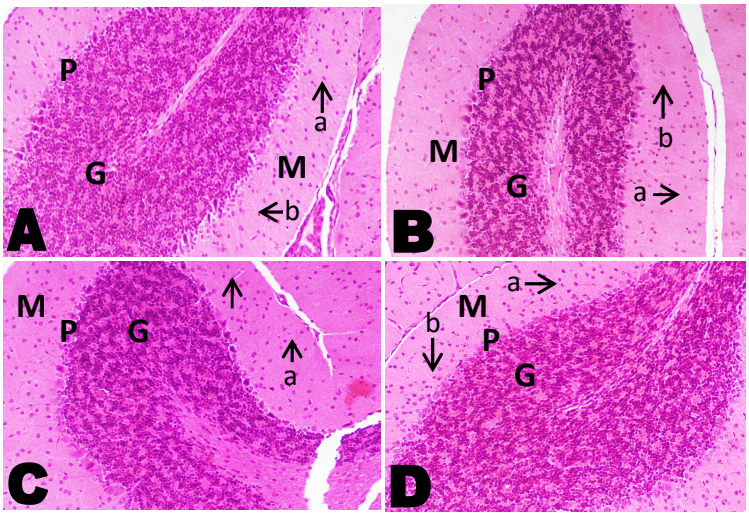

Figure 4: Photomicrograph of Cerebellar Cortex of Mice treated with A: distilled water; B: Insulin; C: Insulin+L-NAME; D: L-NAME. Note the normal appearance and distribution of neuronal and glial cells, with absence of histopathological lesions. a = neuron (cross section); b = neuroglia; P = Purkinje cell layer, M = Molecular layer, G = Granular layer. H & E, ×200.

insulin-induced memory impairment was not reversed by L-NAME treatment. Brain slides gave no indication of histopathological changes.

DISCUSSION

The insulin-induced increase in NO level observed in this study was associated with memory impairment which corroborates the findings of other studies that demonstrated impairment in spatial learning and memory using Morris water maze (Kamal et al. 2012; Yarube et al. 2016). As shown by previous studies, the effect of exogenous insulin on memory varies according to the duration of the treatment, with improvement observed during acute exposure, and impairment reported during chronic exposure (Watson et al. 2003; Craft and Watson 2004; Luchsinger et al. 2004). This study has demonstrated memory impairment even following a sub-acute exposure.

The findings of this study show that insulin may potentially damage the brain by inducing oxidative stress. As reported previously, exposure of the brain to high doses of insulin may negatively affect cognitive signalling pathways (Chiu et al. 2008). NO-dependent oxidative stress may be proposed as the mechanism of insulin-induced memory impairment. This study reports an increased brain levels of NO due to insulin treatment in support of previous studies (Facchini et al. 2000; Choopani et al. 2008, Yarube et al. 2019), even though some other studies have reported to the contrary that insulin inhibited NO production in the rat brain (Li et al. 2013; Liu et al. 2013). The increase in NO levels was reversed by L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor that causes irreversible inhibition on nNOS isoform for a potent effect *in vivo* (Víteček et al. 2012), which was used in this study to create NO deficiency. With the increase in NO concentration, there was also an increase in oxidative stress in the brain of the animals as evidenced by depletion of MDA and reduced activity of GPx.

Even though previous studies have reported insulin-induced increase in brain MDA levels and decreased GPx activity in animals (Patočková et al. 2003; Agrawal et al. 2009), the present study has provided evidence, in addition to the findings in previous studies (above), that there was a concurrent insulin-induced increase in the brain NO in the same animals. Furthermore, the increase in NO concentration and oxidative stress was not observed during NO deficiency (reversed by L-NAME) leading to the deduction that insulin increases brain lipid peroxidation and oxidative stress through an NO-dependent mechanism. This is the novel finding of this study and has provided evidence in support of our hypothesis.

Brain slides of control mice revealed normal histology of the cortex, which was consistent with the normal performance of the control animals during the neurobehavioural experiments, conducted before sacrifice. Similarly, normal histology of the cerebellar cortex was observed in all treated animals. This finding indicates that sub-acute treatment with insulin, L-NAME separately or in combination did not cause significant damage to the cortex detectable by histological examination. The histological findings obtained in the present study disagree with the significant changes reported in this study at the molecular (NO, MDA and GPx) as well as the systemic (learning and memory) levels induced by administration of these substances. The histology findings however, do not exclude changes at the cellular levels such as apoptosis, which could be detectable using the appropriate laboratory methods and which was not investigated in the present study. Further studies are needed to investigate this. The absence of changes reported here may be explained by the relatively short duration of treatment, which did not allow enough time for histological changes to manifest. It is conceivable, that, chronic studies could reveal changes at the histological level detectable by simple microscopy.

Conclusion

In conclusion, the results of this study has demonstrated that sub-acute insulin treatment causes an NO-dependent increase in oxidative stress in the brain as measured by increased MDA concentration and reduced GPx activity; insulin impaired non-spatial working memory but did not affect brain histology in the treated mice. Insulin treatment may have negative consequences on the brain through increased NO levels.

Conflict of Interest

None declared.

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