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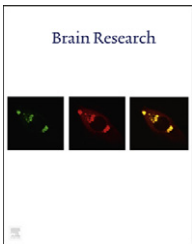

Research Report

Insulin inhibits lipopolysaccharide-induced nitric oxide synthase expression in rat primary astrocytes

Hui Li[a,1], Baoyi Liu[b,1], Jingyang Huang[a], Haili Chen[a], Xiaosun Guo[a], Zhongrui Yuan[a,*]

[a]Department of Pathophysiology, School of Medicine, Shandong University, Jinan 250012, PR China
[b]Department of Respiratory, Qilu Hospital, Shandong University, Jinan, Shandong 250012, PR China

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ABSTRACT

Excessive production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) from reactive astrocytes and microglia may contribute to the development of many types of neurological diseases. Insulin has been shown to inhibit the expression of iNOS, in several organs and cell types. Although insulin and its receptors are present in the central nervous system, the effects of insulin on the iNOS pathway in the brain have not been determined. In this study, using lipopolysaccharide (LPS)-stimulated astrocytes as a model of reactive astrocytes, we investigated the effects of insulin on iNOS expression in activated astrocytes and the mechanism involved. The expression of iNOS was significantly upregulated by LPS in astrocytes. Insulin applied prior to LPS, dose-dependently inhibited LPS-induced iNOS gene expression and iNOS protein levels. In agreement with the suppressive effects of insulin on iNOS expression, insulin also inhibited LPS-induced iNOS activity and NO production. Moreover, insulin was found to significantly inhibit LPS-induced IκB-α phosphorylation and degradation, which led to a decrease in levels of the p65 subunit of NF-κB in the nuclear fraction. Therefore, insulin inhibited LPS-induced iNOS expression via suppressing NF-κB pathway in astrocytes. In addition, treatment with insulin had no effect on LPS-induced PKB phosphorylation. Based on our results, it is plausible to speculate that insulin in the brain may play a neuroprotective role in neurological disorders by controlling the release of NO via the regulation of iNOS expression in astrocytes.

1. Introduction

The activation of glial cells, including astrocytes and microglial cells, has been implicated in the inflammatory responses in brain injury and in neurological diseases such as Alzheimer's disease, Parkinson's disease and stroke (Chen and Swanson, 2003; Diedrich et al., 1987; Miller et al., 2004; Van Eldik et al., 2007). Reactive glial cells are capable of producing a variety of pro-inflammatory mediators, such as tumor necrosis factor-α, interleukin-6, interleukin-1β, neurotrophic factors (Dong and

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; AD, Alzheimer's disease; CNS, central nervous system; ESR, electron spin resonance; PKB, protein kinase B; RT-PCR, reverse transcriptase polymerase chain reaction

*Correspondence to: Wenhua Xi Road No. 44, Jinan, Shandong 250012, PR China. Fax: +86 531 8838 2502.
E-mail addresses: zhongruiyuan@sdu.edu.cn, zhongruiyuan@hotmail.com (Z. Yuan).
[1]These authors contributed equally to this work.

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Benveniste, 2001), as well as potentially neurotoxic compounds, like nitric oxide (NO). NO, a short-lived and diffusible free radical, has been shown to be an important factor in a wide range of physiological processes including the modulation of neurotransmission, blood pressure, memory and gene regulation (Bredt and Snyder, 1994). However, excessive production of NO is neurotoxic (Calabrese et al., 2007). NO is produced as a byproduct of the conversion of L-arginine to L-citrulline by three distinct isoforms of nitric oxide synthase (NOS) (Bredt and Snyder, 1990). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and calcium dependent, while inducible NOS (iNOS) is calcium-independent and generally has limited expression. However, iNOS expression is highly and rapidly induced by exposure to immunological and inflammatory stimuli in a variety of cells (Kleinert et al., 2004), including glial cells (Sheng et al., 2011). Once induced, iNOS leads to continuous NO production, which is terminated by enzyme degradation, depletion of substrates, or cell death (MacMicking et al., 1997). NO derived from iNOS may have antimicrobial, anti-atherogenic, or apoptotic actions (Bogdan et al., 2000). However, aberrant iNOS induction exerts detrimental effects and seems to be a key mediator of glial-induced neuronal death (Bal-Price and Brown, 2001). In fact, there is considerable evidence for the transcriptional induction of iNOS (the high-output isoform of NOS) in the central nervous system (CNS) that is associated with several CNS disorders, including inflammatory, infectious, traumatic, and degenerative diseases (Brosnan et al., 1994; Luth et al., 2001). Therefore, the characterization of signaling pathways controlling the induction of iNOS is an active area of investigation since compounds capable of antagonizing such signaling steps may have therapeutic effects in neurodegenerative disorders.

Brain insulin signaling has been the focus of many studies of the central regulation of energy balance for decades (Schwartz et al., 1992). Moreover, recent studies have demonstrated that insulin levels and/or signaling in the brain is associated with impaired learning, memory, and age-related neurodegenerative diseases (Correia et al., 2011; Talbot et al., 2012). Insulin and its receptors are present in the CNS. Insulin receptors are widely expressed in the CNS (Havrankova et al., 1978;Unger et al., 1991); they are expressed by both astrocytes and neurons (Unger et al., 1991). Insulin enters the CNS through the blood-brain barrier by a saturated insulin receptor-mediated transport process (Baura et al., 1993) and may also be synthesized by neurons in the brain (Devaskar et al., 1994). Accumulating evidence has indicated that brain insulin regulates both peripheral and central glucose metabolism, neurotransmission, learning and memory, and is neuroprotective (Plum et al., 2005;Zhao and Alkon, 2001). However, the exact role of insulin in the brain requires further investigation.

In many organs, insulin not only regulates the expression and activity of eNOS (Ding et al., 2000; Kuboki et al., 2000;Yu et al., 2011) in vascular endothelial cells, but also inhibits the expression and activity of iNOS in macrophages and hepatocytes (Harbrecht et al., 2012; Martins et al., 2008; Stevens et al., 1997). Moreover, we recently found that insulin also induces the expression and activity of nNOS in both neurons and astrocytes (Yuan et al., 2004). However, based on our review of the literature, we found no unequivocal evidence pointing to the direct effect of insulin on the iNOS pathway in the brain or astrocytes. The aim of this study is to test the hypothesis that insulin also inhibits the expression of iNOS in astrocytes. Lipopolysaccharide (LPS), the major component of the outer wall membrane of Gram-negative bacteria, has been widely used as an experimental tool to induce reactive changes including inflammatory stimulation in astrocytes (Wu et al., 2010). We stimulated rat primary astrocytes with LPS to investigate the effects of insulin on iNOS expression in activated astrocytes and the mechanism involved.

2. Results

2.1. *Effects of insulin on LPS-induced iNOS mRNA expression*

We first assessed the effects of insulin on LPS-induced expression of iNOS mRNA by semi-quantitative RT-PCR with specific primers designed to recognize iNOS mRNA. As shown in Fig. 1, the level of iNOS mRNA was upregulated in response to LPS (1 μg/ml for 4 h) and insulin pretreatment (0.03 nM for 1 h) markedly inhibited the LPS-induced expression of iNOS mRNA by 49.4±1.2%. Importantly, insulin had no effect on the mRNA expression of β–actin, the housekeeping gene.

2.2. *Effects of insulin on LPS-induced iNOS protein expression*

To investigate the effects of insulin on LPS-induced iNOS protein expression in astrocytes, flow cytometry analysis was

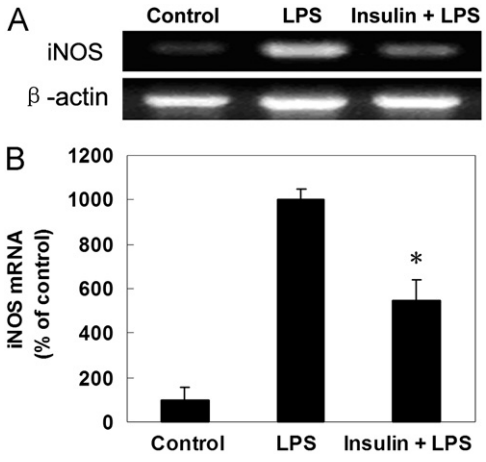

Fig. 1 – Effects of insulin on LPS-induced iNOS mRNA expression in astrocytes. (A) Total RNA was prepared for RT-PCR from astrocytes treated with or without insulin (0.03 nM) for 1 h followed by LPS (1 μg/ml) for 4 h. iNOS-specific sequences (807 bp) were detected by agarose gel electrophoresis, as described in the Experimental Procedure. PCR of β-actin was performed to verify that the initial cDNA contents of samples were similar. Experiments were repeated five times, and similar results were obtained. (B) Relative iNOS mRNA level quantified as compared with control group (normalized to 100%). Data were represented as mean±SD of five independent experiments. There were significant differences between the groups (ANOVA, $p<0.01$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p<0.05$).

performed. Flow cytometry analysis of intracellular antigens is simple and accurate (Sindermann et al., 1997). Samples incubated with secondary antibody alone were used as negative controls (background). As shown in Fig. 2, the fluorescence intensity of negative control samples was very low (mean fluorescence intensity: 12.2±1.6), which confirmed the specificity of this technique. LPS (1 μg/ml for 16 h) increased the expression of iNOS protein by approximately 10 fold. But when cells were pretreated with insulin at 0.003, 0.03, 0.3 and 3 nM for 1 h before LPS treatment, iNOS protein expression was found to have decreased by 24.8±2.2%, 50.8±2.6%, 64.5±6.4% and 64.1±4.9%, respectively.

As shown in Fig. 3, iNOS-immunostaining was mainly distributed in the cytoplasm and the nucleus was faintly

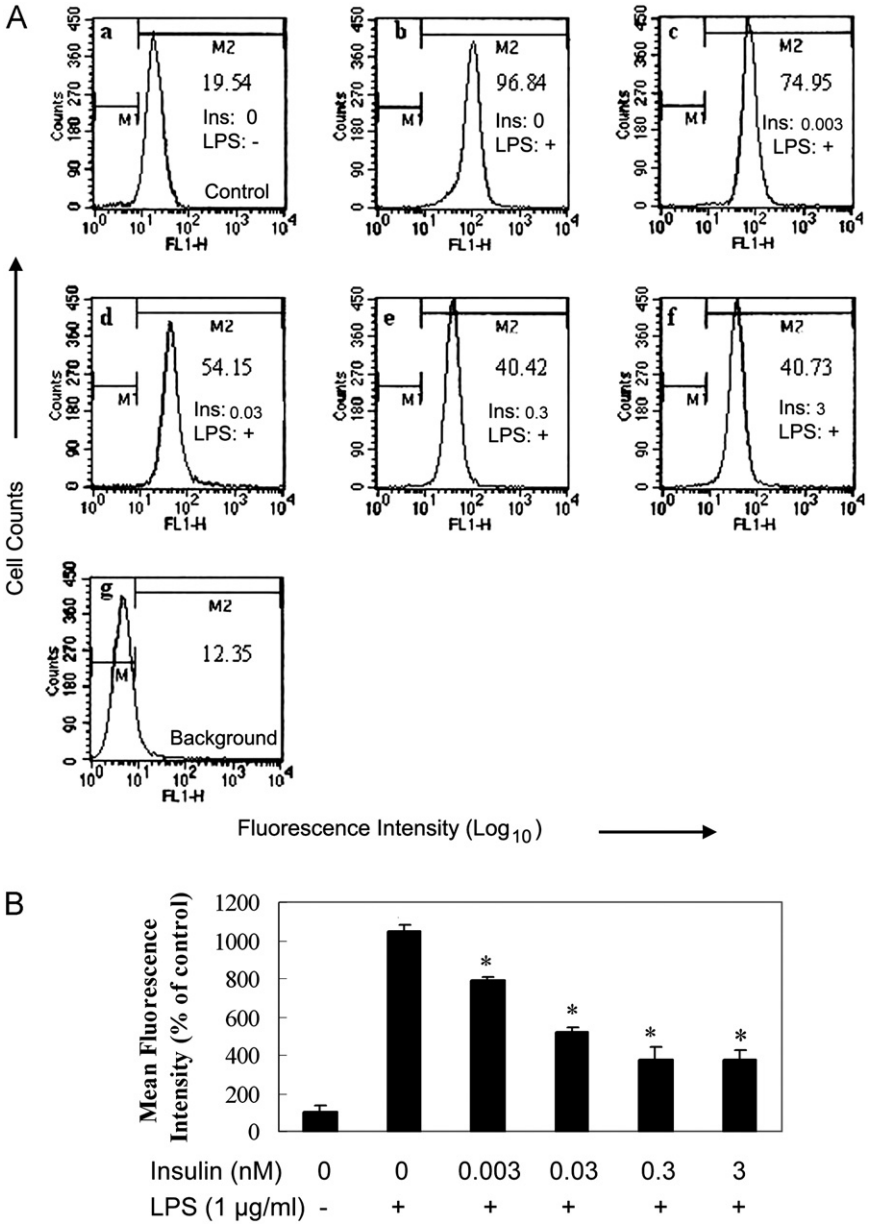

Fig. 2 – Flow cytometry analysis results of iNOS protein in astrocytes after treatment with insulin and LPS. Indirect immunofluorescence and flow cytometry analysis were performed as described in Experimental Procedure. (A) Representative flow cytometric analysis results of iNOS protein in astrocytes. M1 and M2 fractions consisted of mostly iNOS immunoreactive negative and positive cells, respectively. The numerical values in the M2 quadrants are the mean fluorescence intensities of iNOS immunoreactive cells. Cells were pretreated for 1 h with various concentrations of insulin (Ins, nM) as indicated in the M2 quadrants on the plots, and then incubated for an additional 16 h with LPS (1 μg/ml). Secondary antibody alone was used for background fluorescence intensity. Experiments were repeated five times with similar results. (B) Histogram of the level of iNOS immunoreactivity. The relative mean fluorescence intensity of iNOS immunoreactive positive cells was expressed as a percentage of that of control cells. The data were expressed as mean±SD of five independent experiments. There were significant differences between the groups (ANOVA, $p<0.01$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p<0.05$).

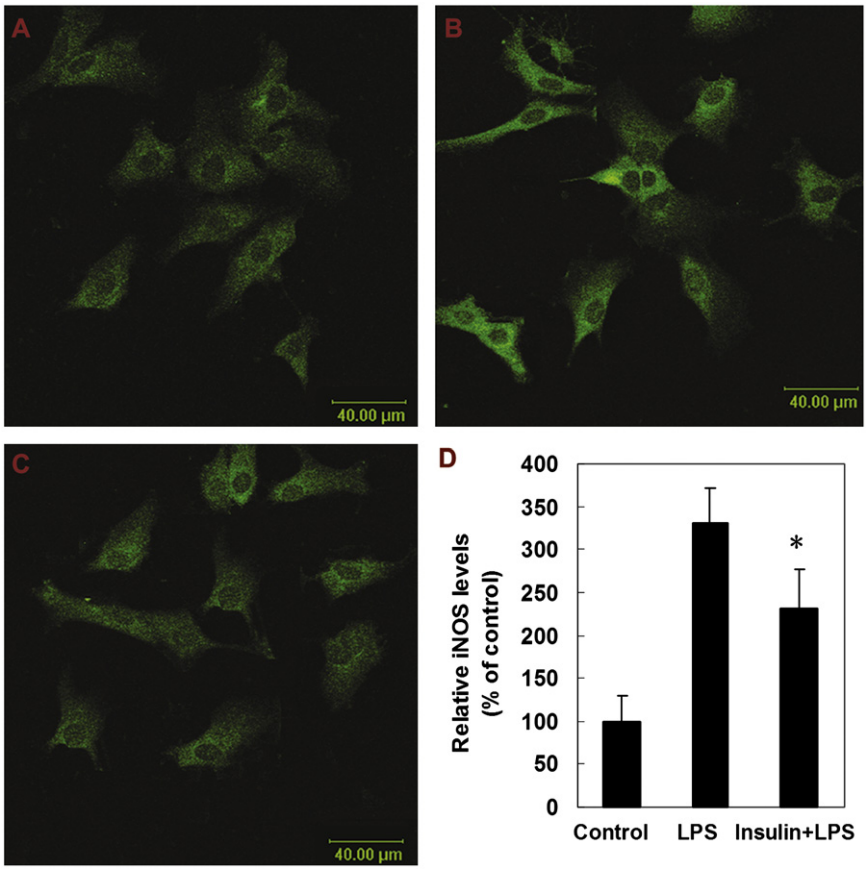

Fig. 3 – Effects of insulin on LPS-induced iNOS expression in astrocytes. (A, B, C) Representative confocal microscopy images of iNOS immunostaining. (A) Control; (B) LPS treated cells (1 μg/ml, 16 h); (C) pretreated with insulin (0.03 nM, 1 h) before exposure to LPS (1 μg/ml, 16 h). Scale bar=40 μm. Immunostaining for iNOS (green) was mainly seen in the cytoplasm. Experiments were repeated five times with similar results. (D) Immunostaining for iNOS was quantified. The data were expressed as mean±SD of five independent experiments. There were significant differences between the groups (ANOVA, $p<0.01$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p<0.05$).

stained. LPS (1 μg/ml for 16 h) significantly increased iNOS-immunoreactivity compared with the control group. Insulin pretreatment (0.03 nM for 1 h) significantly reduced the LPS-induced expression of iNOS in astrocytes.

2.3. *Effects of insulin on LPS-induced iNOS activity*

To investigate the effects of insulin on iNOS activity in astrocytes, electron spin resonance (ESR) technique was used to detect the NO free radical. ESR is a direct and specific method to detect free radicals. Only molecules with unpaired electrons have characteristic ESR spectra that are generated by the coupling of the unpaired electrons, with both the nuclear spin of the molecule and the magnetic fields. For NO, the coupling interaction results in the hyperfine splitting of signals into three peaks at $\alpha_N=12.5$ G and $g=2.035$. Due to its specificity and sensitivity, this method has also been used to assay NOS activity (Zhang et al., 2002) by specifically detecting NO production. Unlike nNOS and eNOS, iNOS is fully active in the absence of either added Ca2+ or calmodulin (Xie et al., 1992). Therefore, under these experimental conditions, the NO was mainly generated from iNOS. Based on an earlier report (Zhang et al., 2002), the height of the first peak of NO signal was taken as the relative activity of NOS. The height was measured and expressed as an arbitrary unit (a.u.) per 2×106 cells. As shown in Fig. 4, iNOS activity in control astrocytes was relatively low. LPS (1 μg/ml for 16 h) increased iNOS activity by approximately six fold. But, when cells were pretreated with insulin at 0.003, 0.03, 0.3 and 3 nM l for 1 h before LPS, iNOS activity was found to have decreased by 20.0±4.4%, 48.7±5.4%, 70.5±1.3% and 70.6±1.9%, respectively.

2.4. *Effects of insulin on LPS-induced nitrite production*

To investigate the effects of insulin on LPS-induced NO production in astrocytes, the generation of NO in cell culture supernatants was determined by measuring nitrite accumulation in the medium using the Griess reagent (Green et al., 1982). As shown in Fig. 5, LPS (1 μg/ml for 16 h) significantly increased nitrite production, but when cells were pretreated with insulin (0.03 nM) for 1 h before LPS was added, nitrite production was found to have decreased by 46.0±8.6%.

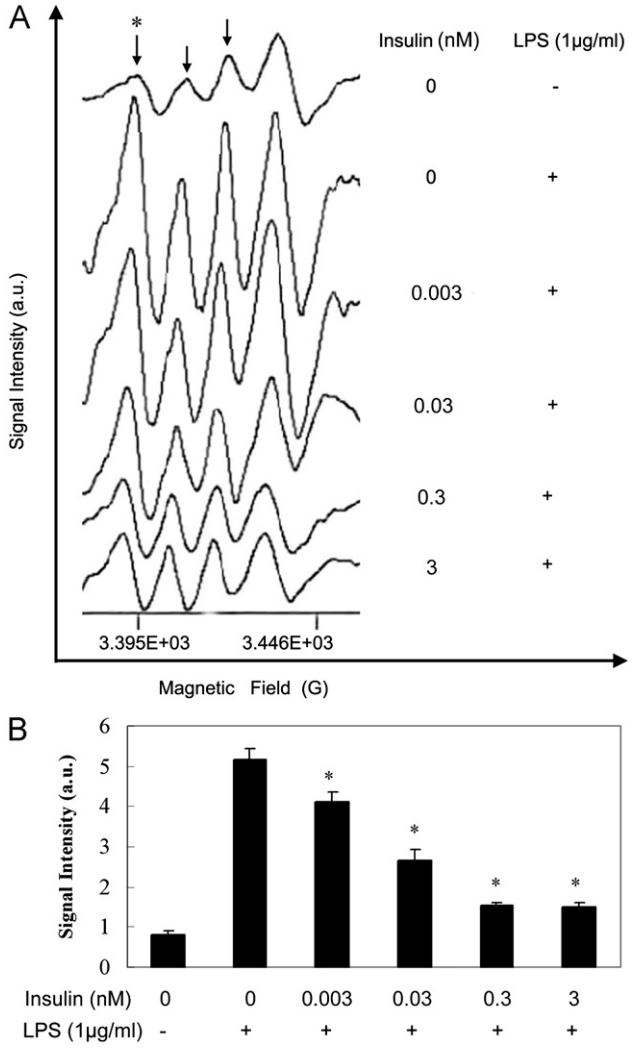

Fig. 4 – Effects of insulin on LPS-induced iNOS activity in astrocytes. (A) Representative ESR spectra of NO produced from astrocytes, trapped by (DETC)2-Fe(II) and extracted by ethyl acetate. The triplet peaks (indicated by arrows) at $a_N = 12.5$ G and $g = 2.035$ were shown. The height of the first peak (indicated by asterisk) was taken for the relative intensity of the NO free radical. Cells were pretreated for 1 h with various concentrations of insulin as indicated above before the addition of LPS (1 μg/ml, 16 h). Spectra were recorded with frequency 9.6 GHz, modulation frequency 100 kHz with 3.2 G modulation amplitude, microwave power 20 mW and central magnetic field 3380 G with scan 400 G. Experiments were repeated five times, and similar results were obtained. (B) Histogram of the level of iNOS activity. The data is expressed as mean ± SD of five independent experiments. There were significant differences between the groups (ANOVA, $p < 0.01$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p < 0.05$).

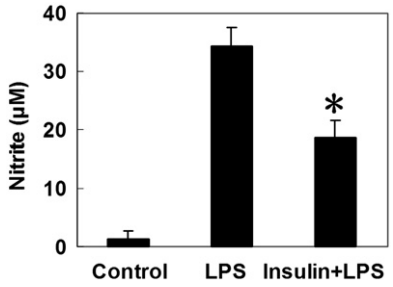

Fig. 5 – Effects of insulin on LPS-Induced NO production in astrocytes. Cells were incubated with or without insulin (0.03 nM) for 1 h and then incubated with or without LPS (1 μg/ml) for 16 h. Control values were obtained in the absence of LPS and insulin. Nitrite levels in culture media were determined using Griess assays and were presumed to reflect NO levels. The data is expressed as mean ± SD of five independent experiments. There were significant differences between the groups (ANOVA, $p < 0.001$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p < 0.05$).

2.5. *Effects of insulin on the LPS-induced phosphorylation of IκB-α, PKB and the nuclear translocation of p65*

There are two NF-κB DNA consensus sequences within the iNOS promoter (Kim et al., 1997), which are responsible for NF-κB DNA-binding activity. We hypothesized that insulin may regulate iNOS RNA levels by regulating the NF-κB pathway. In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor IκB. Upon LPS stimulation, IκB is phosphorylated by its inhibitor IκB kinases, ubiquitinated, and rapidly degraded via the 26S proteosome, thus releasing NF-κB. We examined the effects of insulin on the LPS-induced phosphorylation and degradation of IκB-α using Western blot assay. Insulin was found to significantly inhibit LPS-induced IκB-α phosphorylation and degradation by 37.5 ± 5.2% and 28.5 ± 5.7% respectively (Fig. 6). In addition, we investigated whether insulin prevented the translocation of the p65 subunit of NF-κB from the cytosol to the nucleus after its release from IκB-α. In the case of pre-treatment with insulin, a decrease (40.2 ± 8.0%) in the level of p65 in the nuclear fraction was detected by Western blot analysis (Fig. 6). β-actin and PARP were used as internal controls.

It has previously been suggested that protein kinase B (PKB/Akt) regulates the NF-κB pathway in response to LPS in macrophages (Laird et al., 2009). Therefore, we examined the effect of insulin on LPS-induced activation of PKB in astrocytes. As evident in Fig. 6, treatment with insulin (0.03 nM) had no significant effect on LPS-induced PKB phosphorylation.

3. Discussion

NO has been shown to be an important factor in a wide range of physiological processes including the modulation of neurotransmission, blood pressure, memory and gene regulation (Bredt and Snyder, 1994). However, in pathophysiological conditions, such as brain ischemia or neurodegenerative disorders, NO is formed excessively by NMDA receptor overactivation in neurons, or by iNOS from neighboring glial cells (astrocytes and microglial cells). Excessive production of NO is neurotoxic (Calabrese et al., 2007).

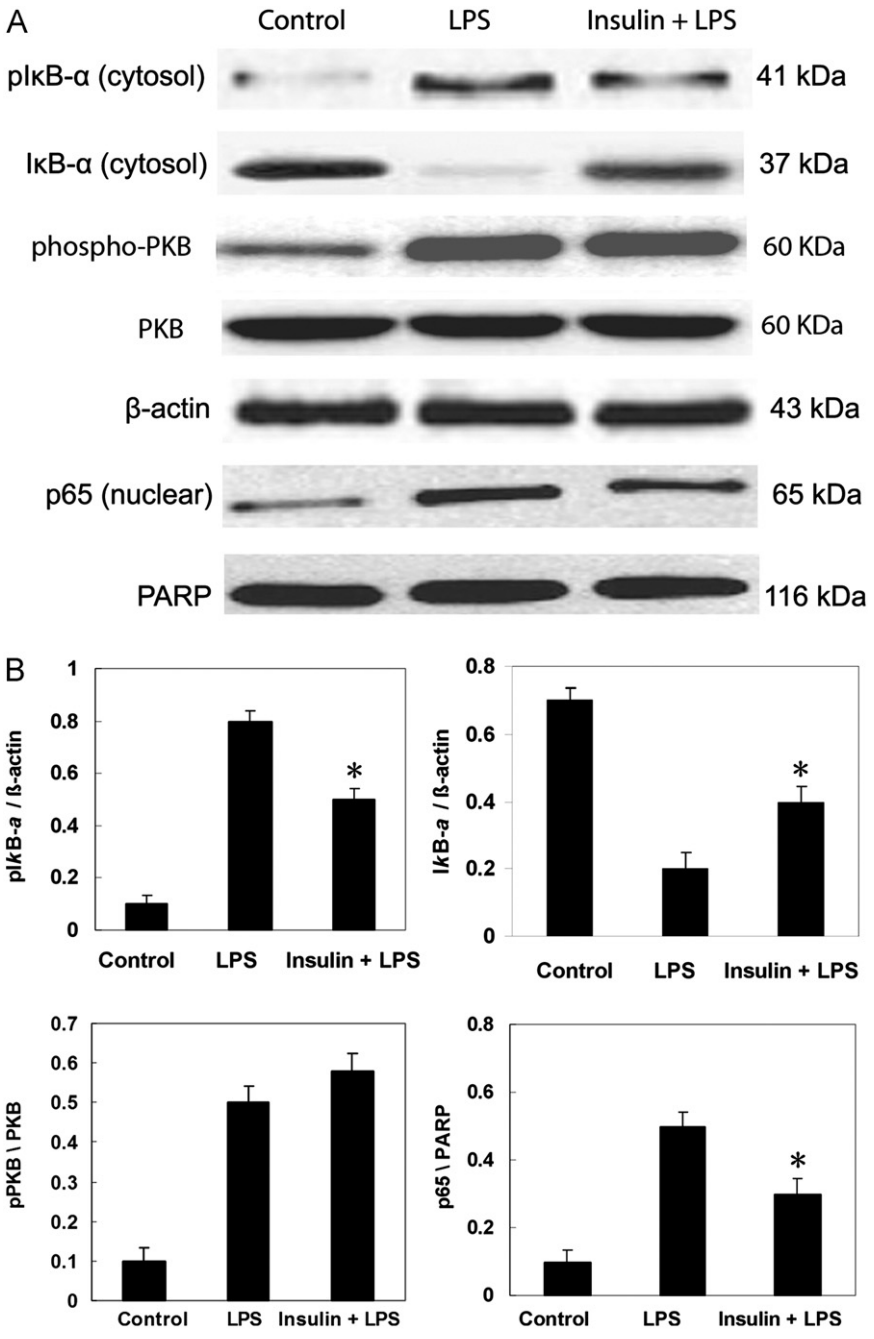

Fig. 6 – Effects of insulin on the phosphorylation of IκB-α and PKB, and the nuclear translocation of NF-κB in astrocytes. (A) Representative immunoblot of phopho-IκB-α, IκB-α, phopho-PKB, PKB and p65 NF-κB in astrocytes. Cells were incubated with or without insulin (0.03 nM) for 1 h and then incubated with or without LPS (1 μg/ml) for 1 h. Cytoplasmic and nuclear extracts were analyzed by Western blotting for phopho-IκB-α, IκB-α, phopho-PKB and total PKB in cytosolic fraction, and p65 NF-kB in nuclear fraction, as described in the experimental procedure. β-actin and PARP were used as internal controls. A representative immunoblot of the five separate experiments is shown. (B) Immunoblot of phopho-IκB-α, IκB-α, phopho-PKB, PKB and p65 NF-κB was quantified by densitometry. The data is expressed as mean ± SD of five independent experiments. There were significant differences between the groups (ANOVA, $p < 0.01$). * indicates significant difference from LPS group (Tukey Kramer post hoc test $p < 0.05$).

Resident glial cells in the brain express iNOS and produce high levels of NO in response to a wide variety of pro-inflammatory and degenerative stimuli (Moncada and Bolanos, 2006; Saha and Pahan, 2006). NO derived in excessive amount from reactive astrocytes and microglial cells is assumed to contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during ischemia, trauma, Alzheimer's disease, Parkinson's disease,

HIV dementia, and amyotrophic lateral sclerosis (Brosnan et al., 1994; Dawson and Dawson, 1998; Mitrovic et al., 1994). Because of its independence of elevated intracellular calcium, iNOS catalyzes a high-output pathway of NO production (Xie et al., 1992). Elevated NO can interact with superoxide anion, generated by the mitochondria or by other mechanisms, leading to the formation of peroxynitrite, a potent oxidant and the primary component of nitro-oxidative stress (Moncada and Bolanos, 2006). NO has been implicated in most neurodegenerative diseases (Murphy, 2000). For example, a hallmark of AD astrogliosis is a strong increase in iNOS levels (Luth et al., 2001). AD lesions display biochemical and histochemical hallmarks of oxidative and nitro-oxidative injury, including nitration of protein tyrosine residues (Castegna et al., 2003;Smith et al., 1997). Thus, the inhibition of iNOS is thought to be a promising therapeutic target in these CNS disorders.

In vitro, primary astrocyte cultures express iNOS in response to cytokines such as interferon-γ, tumor necrosis factor-α and/or the bacterial endotoxin, LPS (Feinstein et al., 1994; Simmons and Murphy, 1994). In this study, in order to investigate the effects of insulin on iNOS expression in activated astrocytes, we stimulated rat primary astrocytes with LPS (Wu et al., 2010). In the present work, we showed that activation of astrocytes with LPS in vitro induced the expression of iNOS and the product of this enzyme, NO. Pretreatment with insulin suppressed the LPS-induced expression of iNOS in a dose-dependent manner. By detecting the production of NO free radical using ESR, we further showed that insulin also inhibited LPS-induced iNOS activity. Consistently, insulin inhibited LPS-induced NO release. Our results are consistent with the report of Martins et al. (Martins et al., 2008). They showed that insulin downregulated LPS-induced expression of iNOS in alveolar macrophages. Similarly, Harbrecht et al. also found that insulin inhibited cytokine-induced iNOS expression in primary rat hepatocytes (Harbrecht et al., 2012). Taken together, these results suggest that insulin not only regulates the expression of iNOS in peripheral tissues but also regulates the expression of iNOS in CNS cells.

There are two NF-κB DNA consensus sequences within the iNOS promoter (Kim et al., 1997), which are responsible for NF-κB DNA-binding activity. Kim YM et al. showed that NF-κB mediated the LPS–induced expression of iNOS in mouse macrophage cell line RAW 264.7 (Kim et al., 1997). Similarly, we also found that LPS induced a significant increase in the nuclear translocation of NF-κB p65 in astrocytes. However, the treatment of astrocytes with insulin before LPS stimulation, significantly reduced the nuclear translocation of this transcription factor possibly by blocking IκB-α degradation, as the level of pIκB-α was decreased compared with the LPS treated group. Consistent with our findings, Martins et al. have documented that administration of insulin before LPS suppressed the expression of iNOS by preventing the nuclear translocation of NF-κB in alveolar macrophages (Martins et al., 2008). In addition, treatment with insulin had no effect on LPS-induced PKB phosphorylation. However, Harbrecht BG et al. showed that insulin inhibited cytokine-induced iNOS expression through PKB-mediated signaling, but had no effect on cytokine-mediated IκB-α levels or NF-κB p65 translocation in primary rat hepatocytes (Harbrecht et al., 2012). These data indicate that insulin regulates the expression of iNOS in different cell types by different mechanisms.

There is a general agreement that insulin is normally present in the brain, however, there is no consensus about actual CNS insulin concentrations in vivo. For example, Craft et al. (Craft et al., 1998) have published 0.003–0.004 nM insulin in the CSF. On the other hand, various laboratories have published markedly different values for immunoreactive insulin (IRI) concentrations in mammalian brain extracts. For example, Bakin et al. have reported extractable rat brain IRI levels of less than 1 ng/g (amount to 0.147 nM) (Baskin et al., 1983). LeRoith et al. have published values of 3–9 ng/g (amount to 0.441–1.323 nM) in rat brain (LeRoith, 1983). In contrast, employing a sensitive ELISA, Schechter et al. have observed higher insulin concentrations in late gestational fetal rabbit brain (approximately 80–90 ng/g, amount to 11.76–13.23 nM) and early neonatal brain (approximately 195 ng/g, amount to 28.665 nM) in comparison to the adult brain (approximately 32 ng/g, amount to 4.7 nM) (Schechter et al., 1992). These disparities have been attributed to different antibodies and insulin standards used in the experiments (Schechter et al., 1992). Nevertheless, the ranges of insulin concentration used in our experiments were within the ranges reported by some laboratories. This suggests that insulin may regulate the expression of iNOS in the brain, under physiological and pathological conditions.

Data of this study are entirely in vitro, further studies in vivo are needed. However, several in vivo studies have given clues on the relationship between the iNOS level and insulin function in the brain. For example, in Alzheimer's disease (AD), a strong increase in iNOS level is associated with astrogliosis (Luth et al., 2001). Recent studies have demonstrated abnormalities in insulin function and insulin signal transduction in brains of patients with AD (Correia et al., 2011;Talbot et al., 2012). Talbot et al. showed that hippocampal brain slices in AD were less responsive to insulin than controls because of increased phosphorylation of IRS-1 that attenuated downstream Akt and ERK signaling (Talbot et al., 2012). It is obvious that insulin resistance in AD occurs not only in peripheral tissues, but also in the brain (Talbot et al., 2012). It still is a matter of debate, whether the abnormal expression of iNOS has some primary importance in the pathogenetic chain and might thus be a potential therapeutic target or only reflects a secondary effect that occurs at more advanced stages of the disease process. However, the functional deficiency of brain insulin might be directly responsible for the elevated iNOS expression and NO production in AD brains, though direct in vivo evidence is needed.

Taken together, our results demonstrate that insulin significantly suppresses the LPS-induced NO production and expression of iNOS, by inhibiting the degradation of IκB-α, which blocks the nuclear translocation of NF-κB in astrocytes, thus preventing iNOS mRNA transcription. It is plausible therefore, that insulin in the brain may play a neuroprotective role in neurological disorders by controlling the release of NO by regulating the expression of iNOS in astrocytes. Clearly, further studies are needed to investigate the effects of insulin on iNOS expression in the CNS in vivo.

4. Experimental procedure

4.1. Cell culture and sample treatment

Primary cultures of astrocytes were prepared from the cerebral cortices of 2–4-day-old Sprague–Dawley rats (Experimental Animal Center, Shandong University, Jinan, China) as previously described (Yuan et al., 2004). Cerebral cortices were removed, dissected free of meninges, digested with 0.06% trypsin and dispersed to single cell suspensions. The cells were plated onto 75-cm^2 culture flasks (Corning Incorporated) at a density of 2×106 cells/flask. Cells were cultured in DMEM supplemented with 20% newborn calf serum plus bicarbonate and antibiotics. Culture medium was replaced with medium containing 10% newborn calf serum twice a week. After 6–7 days, the cultures were mechanically shaken to dislodge microglia and oligodendrocytes. The remaining cells were disassociated with 0.125% trypsin and subcultured. Studies using astrocytes were carried out at 6–7 days after reseeding, when they reached 90% confluence. These cultures contained over 95% astrocytes, as determined by immunostaining for glial fibrillary acidic protein (GFAP).

Cultured cells were pre-treated with vehicle (control) and various concentrations of insulin (from bovine pancreas, Sigma-Aldrich Co.) for 1 h, and then stimulated with or without 1μg/ml of LPS (from Escherichia coli 055:B5, Sigma-Aldrich Co.).

4.2. Determination of iNOS mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Astrocytes cultured in 60-mm dishes were pre-treated with or without insulin (0.03 nM) for 1 h, then stimulated with or without LPS (1 μg/ml) for an additional 4 h. The total cellular RNA was extracted using TRIzol Reagent (Life Technologies Co.). The RNA concentration was measured using the Quant-iT^{TM} RiboGreen® RNA Assay Kit (Life Technologies Co.). From each sample, 1 μg of RNA was reverse transcribed using MuLV RT, 1 mM dNTP and oligo ($dT_{12–18}$) 0.5 μg/μl. PCR was performed on aliquots of the cDNA preparations to detect iNOS and β-actin (the internal standard) gene expressions using a thermal cycler (Perkin-Elmer Cetus, Foster City, CA, U.S.A.). Reactions were carried out in a volume of 25 μl containing; 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10 × reaction buffer, and 100 pmol of 5′- and 3′- primers. After an initial denaturation for 5 min at 95 °C, 35 amplification cycles were performed for iNOS (denaturation at 95 °C for t iNOS, as 30 s, annealing at 60.0 °C for 30 s, and extension at 72.0 °C for 1 min) and β-actin (denaturation at 94 °C for t iNOS, as 1 min, annealing at 60.0 °C for 1 min, and extension at 72.0 °C for 1 min). PCR primers used in this study are listed below and were purchased from Bioneer: sense strand iNOS, 5′-AATGGCAACATC-AGGTCGGCCATCACT-3′; antisense strand iNOS, 5′-GCTGTGTGTCACAGAAGTCTCGAA-CTC-3′; and sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; antisense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCAC-GATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel, and visualized under UV after ethidium bromide staining. Densitometric analysis was performed using NIH image 1.61 software, and iNOS mRNA levels were standardized in proportion to β-actin mRNA. Data are expressed as percentage of LPS-treated group.

4.3. Flow cytometry analysis for the expression of iNOS protein

Flow cytometry analysis was carried out as described by Sindermann et al. with minor modifications (Sindermann et al., 1997). In brief, astrocytes cultured in 75-cm^2 culture flasks were pre-treated with various concentrations of insulin (0, 0.003, 0.03, 0.3, 3 nM) for 1 h and then stimulated with or without LPS 1 μg/ml for 16 h. Aliquots of cell suspension containing 5×10^5 cells were washed with cold PBS, then fixed in 70% ethanol for 30 min. Subsequently cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 45 min on ice. Cells were further blocked with 10% normal goat serum in PBS for 30 min. Then the cells were incubated for 2 h with polyclonal antibody against iNOS (1:200, Santa Cruz Biotechnology, Inc.) and incubated with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, Inc.) for 30 min at 37°c in darkness. Following several washes in PBS, cells were resuspended in 0.5 ml PBS and then filtered through a 100-μm nylon mesh. Finally the cells were analyzed using a FAC Scan flow cytometer (Becton-Dickinson, San Jose, CA) and Cell Quest Software. A gate was set on the FSC-H histogram to select only the cell population, excluding any debris. A total of 1×104 cells were acquired per sample. Samples incubated with secondary antibody alone were used as negative controls (background).

4.4. Immunocytochemistry

Astrocytes cultured in six-well plates containing 12-mm poly-L-lysine-coated glass coverslips were pre-treated with or without insulin (0.03 nM) for 1 h, then stimulated with or without LPS (1 μg/ml) for an additional 16 h. Thereafter the cells were washed in PBS and fixed with 4% paraformaldehyde for 30 min at room temperature followed by permeabilization with 0.3% Triton X-100. After washing with PBS (three times), the cells were treated with anti-iNOS (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200 in 2% BSA for 1 h at 37 °C or overnight at 4 °C. The cells were washed three times and incubated for 30 min with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (1:200 in 5% BSA; Jackson ImmunoResearch Laboratories, Inc.). Subsequently the cells were washed three times and mounted on slides and analyzed by a TCS NT laser confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). The iNOS-immunofluorescence intensity was calculated at 100 × power microscopic fields measuring more than 40 cells per image taken from six areas chosen randomly from each coverslip. Five independent experiments were performed. All data were expressed as mean ± SD.

4.5. NOS enzymatic activity assay

The cells were prepared and assayed as previously described (Yuan et al., 2004). In brief, astrocytes cultured in 75-cm^2 culture flasks were pre-treated with various concentrations of insulin (0, 0.003, 0.03, 0.3, 3 nM) for 1 h then stimulated

with or without LPS (1 μg/ml) for an additional 16 h. The cells were then harvested and counted. The cell numbers of all samples were adjusted to 2 × 106 in 2 ml PBS containing 20 mM glucose. Then L-arginine, $CaCl_2$, NADPH, $FeSO_4$, $Na_2S_2O_4$, and diethyldithiocarbamate (DETC) were added to the cell suspensions to reach the final concentration of 50 μM L-arginine, 500 nM Ca^{2+}, 800 μM NADPH, 2 mM Fe^{2+}, 10 mM $Na_2S_2O_4$, and 10 mM DETC and incubated in the dark for 30 min at 37 °C. The (DETC)-2-Fe(II)–NO complex was extracted with 1:5 (v/v) ethyl acetate for 4 h (Zhang et al., 2002). Finally, ESR samples were measured in a quartz tube at room temperature with an ESP-300 spectrometer (Bruker, Germany) using normal measurement parameters: frequency 9.6 GHz, modulation frequency 100 kHz with 3.2 G modulation amplitude, microwave power 20 mW and central magnetic field 3380 G with scan 400 G. The height of the first peak was taken for the relative intensity of NO free radical (Mulsch et al., 1995; Sato et al., 1994).

4.6. *Measurement of nitrite production*

The nitrite concentration in the culture supernatant was used as a measure of NO production. Astrocytes were pre-treated with or without insulin (0.03 nM) for 1 h, then stimulated with or without LPS (1 μg/ml) for an additional 16 h. Then the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H3PO4, Sigma, Germany). One hundred microliters of culture supernatant and 100 μl Griess reagent were mixed and incubated for 5 min. The absorbance was then measured at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.). Fresh culture media were used as blanks in all experiments. Sodium nitrite (Sigma, St. Louis, MO, USA) was used to generate a standard curve for quantification.

4.7. *Western blot analysis*

Astrocytes were pre-treated with or without insulin (0.03 nM) for 1 h, then stimulated with or without LPS (1 μg/ml) for an additional 1 h. Then cells were washed and collected with ice-cold PBS (1 ml). Cell pellets were resuspended in 300 μl of hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), and incubated on ice for 10 min. Homogenates were separated to supernatant (cytoplasmic compartments) and pellets (nuclear components) by centrifugation at 12,000 g for 5 min. The pellet was gently resuspended in 30 μl of complete lysis buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF, pH 7.9), and supernatant was used as nuclear extract after centrifugation at 13,000 g for 20 min at 4 °C. An aliquot of protein extracts were used to determine protein concentration by the Bradford method. 50 μg of cytoplasmic extracts or 30 μg of nuclear extract protein per lane was separated by SDS-polyacrylamide gels and followed by transferring to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk, and then incubated with the corresponding antibody. Antibodies for phosphor (Ser32)-specific IκB-α, IκB-α, p65, PARP and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for total PKB and phospho-PKB (Thr308) were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence using SuperSignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Densitometric analysis was performed using NIH image 1.61 software, and levels of phosphor-IκB-α, IκB-α and phospho-PKB were standardized in proportion to β-actin, and p65 in proportion to PARP.

4.8. *Statistical analysis*

All data were expressed as mean ± SD. Differences between multiple groups were tested by ANOVA. Tukey–Kramer test was used as a post-hoc test. *P* values of less than 0.05 were considered as significant differences.

Authors' contributions

HL carried out cell culture, treatment with insulin and LPS, iNOS immunocytochemistry, RT-PCR for mRNA assay; BL carried out flow cytometric analysis for the expression of iNOS protein, NOS enzymatic activity assay and statistical analysis; JH helped with measurement of nitrite and flow cytometry analysis; HC helped with electron spin resonance; XG helped with Western blot analysis and data analysis; and ZY was involved in design of the project, interpretation of results, finalizing the manuscript and provided financial support. All authors read and approved the final version of the manuscript. All authors have no competing interests.

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