Telmisartan Protects the Lipopolysaccharide Intoxicated RAW 264.7 Cell line by Deactivating NFκB Mediated Inflammatory Mechanism

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Abstract

In addition to its AT_1R (angiotensin receptor) blocking properties, telmisartan functions as a partial agonist at peroxisome proliferator-activated receptor-γ (PPARγ) which controls the expression of pro-inflammatory genes. We hypothesized that; in murine RAW 264.7 cell line, the anti-inflammatory effects of telmisartan may be mediated through PPARγ with NFκB deactivation. To test the hypothesis, the anti-inflammatory effect of telmisartan was assessed in lipopolysaccharide (LPS) intoxicated RAW 264.7 cells by MTT cytotoxicity assay. The protective effect was supported by the measuring transcription factor NFκB. The results indicate that telmisartan attenuated pro inflammatory mediator TNFα expression via NFκB deactivation and protected LPS intoxicated RAW 264.7 cell line. In conclusion the study indicates the existence of association between PPARγ and NFκB mediated inflammatory mechanism which was controlled by angiotensin receptor blocker telmisartan in RAW 264.7 cell line via NFκB deactivation effect.

Keywords: Lipopolysaccharide, RAW 264.7, Telmisartan, Cytotoxicity, MTT assay, NFκB, TNFα

1. INTRODUCTION

Angiotensin II (Ang II) type 1 receptor (AT1) stimulation results in hypertension, and AT1 blockers (ARBs) were used to treat high blood pressure. ARBs other beneficial effects include inflammatory and metabolic alterations [1]. Most of the ARBs are biphenyl-tetrazole derivatives with similar but not identical pharmacological profiles [2]. The biphenyl-nontetrazole telmisartan is structurally unique and, in addition to its AT1 blocking properties, it functions as a partial agonist at peroxisome proliferator-activated receptor-γ (PPARγ) [3]. PPARγ full agonists reduce

Pro-inflammatory gene expression [4] and metabolic alterations associated with cardiovascular disease [5]. Anti-inflammatory effects of ARBs, were first established in the peripheral vasculature, later in the cerebral vasculature and in stress-induced gastric ulcerations [6]. These observations suggest that ARBs may exert anti-inflammatory effects beyond those associated with cardiovascular and metabolic disease. Candesartan reduced the peripheral and brain acute inflammation following systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) [7], and in cultured circulating human monocytes expressing few AT1 [8]. The PPARγ activation in the brain suppresses the inflammatory response in neuronal cells, endothelial cells, astrocytes, microglia and also increases Aβ clearance [9] . It has been reported that rosiglitazone, a PPARγ agonist, improves memory and learning in mice over expressed a mutant form of the human amyloid precursor protein [10] and suppresses cognitive function decline in early forms of AD. [11]. Telmisartan is also reported to improve memory impairment in mice that had been injected with Aβ. Therefore, telmisartan is expected to be an option for AD

treatment. Macrophages play essential roles in inflammation and mobilization of the host defense against bacterial infection [12] and they release various inflammatory cytokines which regulate immune function. Further macrophages RAW 264.7 cell line that expresses TLR4 receptor can be an ideal cell line to study the effect because, LPS can trigger excessive and uncontrolled production of inflammatory mediators, through TLR4 receptors,which can lead to potentially lethal systemic disorders such as septic shock. Hence in the present study the protective effect of telmisartan in LPS intoxicated RAW 264.7 cells was assessed through MTT cytotoxicity assay. To support the anti-inflammatory effects of telmisartan, NFκB deactivation along with TNFα expression were measured.

2. METHODS AND MATERIALS

2.1 Essential Chemicals and Reagents

MTT (Sigma, USA), Minimum Essential Medium (MEM) (Invitrogen, USA), Fetal Bovine Serum (Invitrogen, USA), blueprint TM One Step RT-PCR Reaction kit (TAKARA, USA), PCR primers (Sigma, India), tissue culture grade 96 well plates (Cornings, USA), PCR tubes and desired plastic wares (Tarsons, INDIA), PMSF, aprotinin, leupeptin, pepstatin (Sigma, USA) , ECL plus Western detection kit (Amersham Pharmacia Biotech, USA), Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), antibody phospho- p65(Nfκβ) (Santa Cruz Biotech Inc, USA).

2.2 Cell culture and LPS dose optimization by MTT assay

RAW 264.7 cell line was obtained by National centre for cell science (NCCS) Pune, India. Cell lines were maintained in Minimum Essential Medium (MEM) supplemented with 10%

fetal bovine serum (FBS), amphotericin (3μg/ml), streptomycin (250μg/ml) and penicillin (250U/ml) in a carbon dioxide incubator at 5% CO_2. About 2000 cells/well were added in 96 well tissue culture plates (Corning, USA) from well grown culture medium, the viability was tested using trypan blue dye with help of haemocytometer and 95% of viability was confirmed to carry the assay. The cells were then incubated with LPS at different doses (1 to 400μg/ml) for 24h. Then, the medium was changed again for all the groups and 10μl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 150μl of dimethyl sulfoxide (DMSO). The optical density was measured at 590 nm. The toxicity and maximal inhibitory concentration (LD_{50}) value was calculated using Graphpad Prism 4.03.

2.3 Neuroprotective assessment by MTT assay
The cells were incubated with LPS (LD_{50}= 72μg/ml) and with telmisartan (1, 5 and 10μM) for 24h. The medium was changed after 24h and 10μl of MTT (5 mg/ml stock solution) was added, the plates were incubated for an additional 4h. The medium was discarded and the formazan blue insoluble crystal, which was formed in the cells, was dissolved with 150μl of DMSO. The optical density was measured at 590nm. The protective effect of drug was evaluated using Graphpad Prism 4.03.

2.4 Cell morphology observation
About 10,000 cells were plated in six well plate (Corning, USA), incubated with LPS (72μg/ml) and telmisartan (1, 5 and 10μM) for 24 h, the morphological changes in the RAW cells were observed by using phase contrast microscopy (Nikon, Japan).

2.5 Preparation of cell lysates and Immunoblotting
About 2×10^6 cells were plated in tissue culture grade petri plate (Corning, USA), incubated with LPS (72μg/ml) and telmisartan (1, 5 and 10μM) for 24h, the medium was separated and cells were washed with ice-cold phosphate buffer saline, scraped, and centrifuged at 5000 rpm for 5 min at 4^0C. The cell pellet was resuspended in 2000μl of lysis buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM PMSF, 1mM aprotinin, 1mM leupeptin, 1mM pepstatin, 1mM sodium orthovanadate, and 1mM NaF) and incubated on ice for 30min. Cell lysates were obtained by centrifugation at 12,000 rpm for 20min at 4^0C. The aliquot was separated. Cell lysates, medium and pellets were separated and stored at -80^0C. Protein content in the cell lysates were estimated using Lowry's method. Lysates from cells were separated by gel electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then probed with antibody of anti phospho- p65(NFκB) and anti TNFα (Santa Cruz Biotech Inc.) following the instructions provided by the manufacturers. β- actin (Santa Cruz Biotech Inc.) was used for housekeeping gene expression control. Analysis was

performed using the ECL plus Western detection kit (Amersham Pharmacia Biotech) and Gene Tools analysis software (Syngene, Cambridge, United Kingdom)

3. RESULTS

3.1 The morphological changes in RAW 264.7 cell line with LPS treatment
The untreated control cells were appeared cuboidal like shape with thin extensions. As the culture becomes denser, there may be another layer of rounded cells attached to the original monolayer. Morphological changes were observed in the cell lines incubated with 72μg/ml of LPS, compared to the untreated control group. LPS treated cells were bigger in size, irregular shape comparison to the control group. The morphology of the cells was restored with the treatment of telmisartan (5 and 10μm) in the presence of 72μg/ml of LPS. Figure: 1
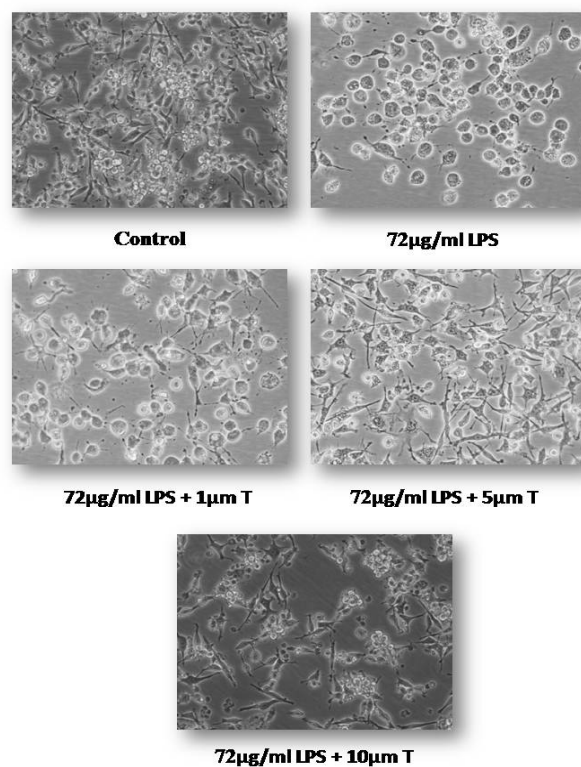

Figure 1: Phase contrast image of RAW 264.7 cell line at 20X magnification.T10- Telmisartan 10μm; T5- Telmiartan 5μm; T1- Telmisartan 1μm;LPS- LPS (72μg/ml) treated. Control- Untreated cells.

3.2 Determination of LD_{50} of Lipopolysaccharide in RAW 264.7 macrophage cell line in MTT assay
LPS-induced cytotoxicity was expressed as cell viability and presented in Figure: 2A. The cell viability of normal control group was designated as 100%, indicating no cytotoxicity. LPS showed the decrease in cell viability in a dose dependent
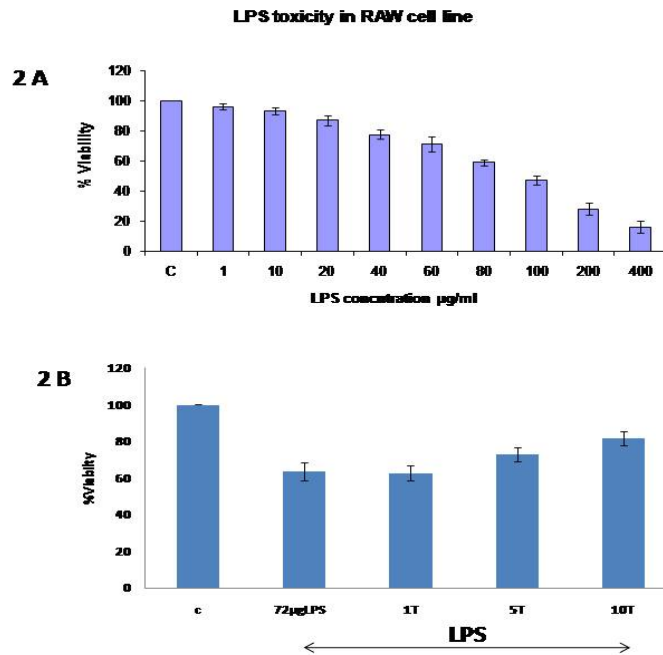
LPS toxicity in RAW cell line

Figure 2:(A) LPS dose optimization in RAW 264.7 cell lines, Data are expressed as mean ± SEM. (n=9). **2(B)** Telmisartan protective effect in RAW 264.7 cell line intoxicated with LD_{50} of LPS. C- Control cells; T10- Telmisartan 10μm; T5- Telmiartan 5μm; T1- Telmisartan 1μm; L- LPS (72μg/ml). (n=9).

manner, indicating cytotoxicity. The LD_{50} of LPS was found to be 72μg/ml. In the present study 72μg/ml was used as effective concentration of LPS to produce 50% cell death and inflammation in RAW cell lines and to test the protective mechanism of telmisartan.

3.3 The protective effect of telmisartan in LD_{50} of LPS in RAW 264.7 macrophage cell line

The protective effect of telmisartan against LPS cytotoxicity was presented in Figure: 2B. Telmisartan (1-10μM) showed protective effect in dose-dependent manner, cells treated with higher dose of telmisartan have shown maximum cell viability. Telmisartan is non-toxic at the highest concentration studied (10μM), data were not presented. The percentage protection of the RAW cells with 10μM telmisartan treatment is approximately 40% compared to LPS treated group.

3.4 Effect of telmisartan on NFκB activation in LPS intoxicated RAW 264.7 cell line

The NFκB activation was significantly increased in the LPS treated cells in comparison to the controls and there was a 75% increase in the activation of NFκB in LPS treated cells compared with control. Telmisartan attenuated the NFκB activation dose dependently. Telmisartan 10μM treatment decreased the activation of NFκB to 30% compared to LPS treated group. Figure: 3
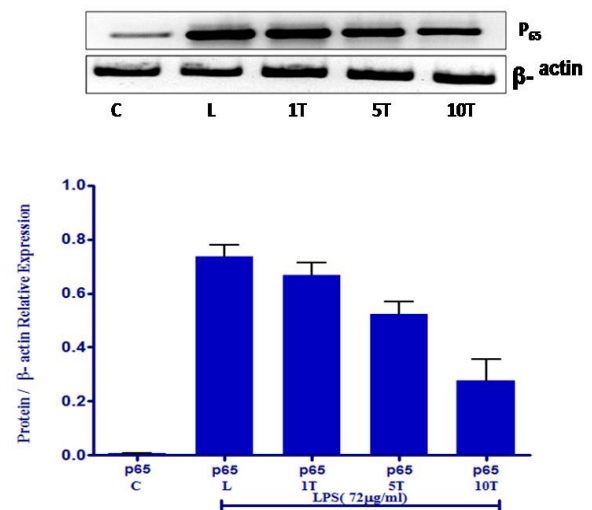

Figure 3: Telmisartan effect on P65(NFκB) activation in LPS intoxicated RAW 264.7 cell line by immune blotting. C- Control cells; T10- Telmisartan 10μm; T5- Telmiartan 5μm; T1- Telmisartan 1μm;L- LPS (72μg/ml) (a) Western blotting for p65 – Phospo-p65 Nfkβ, β- Actin – Beta actin proteins in RAW 264.7 cell line.(b) Relative quantification for protein expression. Data are expressed as mean ± SEM (n=3).

3.5 Effect of telmisartan on TNFα expression in LPS intoxicated RAW 264.7 cell line

The TNFα expression was significantly increased in LPS treated cells compared to the control and there was an 80% increase in relative expression of TNFα in LPS treated cells in comparison to control. Telmisartan 1, 5, 10μM treatment attenuated the TNFα protein expression to 79%, 62%, and 22% respectively in comparison to LPS treated cells. Figure: 4
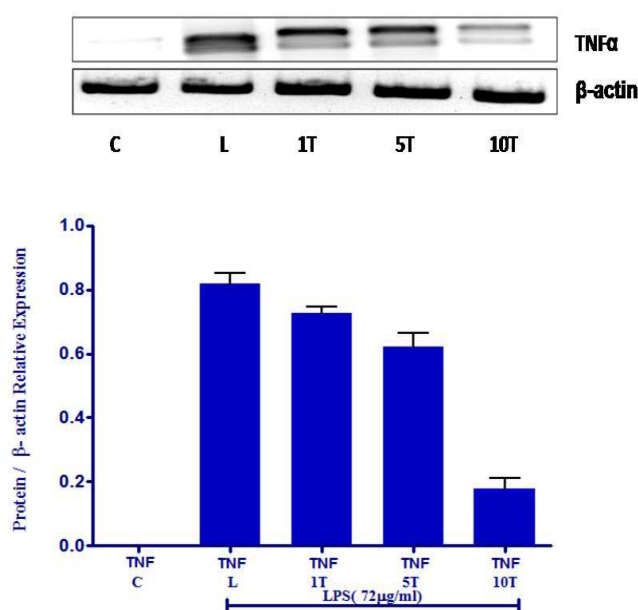

Figure 4: Telmisartan effect on TNFα expression in LPS intoxicated RAW 264.7 cell line by immune blotting.
C- Control cells; T10- Telmisartan 10μm; T5- Telmiartan 5μm; T1- Telmisartan 1μm;L- LPS (72μg/ml) (a) Western blotting for TNF – Phospo-p65 TNF, β- Actin – Beta actin proteins in RAW 264.7 cell line.(b) Relative quantification for protein expression. Data are expressed as mean ± SEM (n=3)

4. DISCUSSION

In this study we demonstrated the telmisartan's protection over the LPS induced inflammatory degeneration in RAW 264.7 cell line through NFκB deactivation. Telmisartan significantly reduced the LPS induced inflammatory response through activation of PPARγ [13]. All ARBs inhibited LPS-induced pro-inflammatory gene expression in THP-1 cells, which is consistent with the recent report of anti-inflammatory effects of losartan through a PPARγ-dependent mechanism [14]. However studies suggest that telmisartan is capable of inducing the activation of PPARγ independently. Further, contrast report was also available stating that telmisartan, oxidase pathways in SK-N-SH neuronal cell line, and had no effect on NFκB activation associated with ERK and p38 MAPK [15]. This result suggests that telmisartan is capable of attenuating inflammation without involvement of PPARγ activation in SK-N-SH cell line that expresses AT$_1$R. So under these circumstances we should not totally exclude the participation of AT$_1$R in the anti inflammatory effects of ARBs independent or associated with PPARγ activation.

LPS activates NFκB via TLR4 receptors and stimulates various inflammatory mediators to induce the inflammatory mediated cytotoxicity. In our study LPS activated the NFκB transcriptional establishment and aggregated the TNFα pro inflammatory mediator protein expression and results in 50% decrease in cell viability. LPS stimulates various transcription factors essentially NFκB via MyD88 mediated activation of p38, ERK and JNK causes a chain of mechanism results in the production of pro inflammatory cytokine TNFα, IL1β, IL6 and inflammatory mediators iNOS, COX-2 etc [16]. In differentiated macrophages, angiotensin II promotes inflammation stimulation and mechanisms similar to the LPS induced inflammation mechanism. [17] Earlier candesartan attenuated angiotensin II mediated cell death in mesangial cells via LPS/TLR4 pathway [18]. NFκB is a main transcription factor that controls the expression of pro inflammatory cytokines and inflammatory mediators. Telmisartan attenuated the LPS mediated activation of NFκB in our demonstration. We interpreted in our study that the activation of p65 subunit, as a consequence of LPS induced NFκB activation, resulting in the elevation of pro inflammatory mediator TNF α. TNFα itself a potent inflammatory mediator that activates the various inflammatory cascades related to disease pathogenesis. TLR4 activates predominantly NFkB, compared to AP1 and IRF3 via MyD88 activation. [19]. Previously, telmisartan also reduced TNF*α* mRNA expression in the hippocampal region of rats with impaired spatial memory in Alzheimer's *in vivo* model. Diminished TNF α production within the brain correlated with decreased local activation of microglia. Microglia are myeloid lineage CNS specific immune cells that are among the first responders to brain injury and function to secrete pro-inflammatory cytokines, enhance antigen presentation, destroy pathogens before they injure neurons, and clear dying cells [20]. Though several factors contribute to microglial stimulation, TNFα directly regulates activation. Mice lacking TNFα receptors showed decreased microglia activation and correspondingly higher levels of neuronal injury in the hippocampus after treatment with a dopaminergic neurotoxin [21, 22]. By our results and previous reports we strongly suggest that telmisartan protected over LPS intoxicated murine RAW 264.7 cell line by attenuating the NFκB activation thereby controlling the expression pro inflammatory mediator TNFα and also we bear in mind that telmisartan a functional modulator of PPARγ. The link between PPARγ and NFκB mediated inflammatory mechanism need to be explored.

5.   CONCLUSION

Angiotensin II receptor type I (AT$_1$) blocker telmisartan protected LPS intoxicated murine RAW 264.7 cell line by attenuating the NFκB activation thereby controlling the expression of pro inflammatory mediator TNFα, which might be an independent mechanism. Telmisartan a functional modulator of PPARγ hence, the link between PPARγ and NFκB mediated inflammatory mechanism need to be explored to further ascertain the telmisartan protective activity.

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