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Original Paper

GSK-3β Inhibition Attenuates LPS-Induced Death but Aggravates Radiation-Induced Death via Down-Regulation of IL-6

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Key Words
Glycogen synthase kinase-3 • LPS • Radiation • IL-6

Abstract
Background: Exposure of high dose ionizing radiation is lethal. Signal pathways involved in radiation biology reaction still remain illdefined. Lipopolysaccharides (LPS), the ligands of Toll-like receptor 4(TLR4), could elicit strong immune responses. Glycogen synthase kinase-3β(GSK-3β) promotes the production of inflammatory molecules and cell migration. Inhibition of GSK-3βprovides protection against inflammation in animal models. The aim of the study was to investigate role of GSK-3β in LPS shock and ionizing radiation. ***Methods:*** WT or IL-6$^{-/-}$mice or cells were pretreated with SB216763, a GSK-3β inhibitor, and survival of the mice was determined. Cell viability was assayed by Cell Counting Kit. Apoptosis was assayed by Annexin V-PI double staining. Serum concentrations of IL-6 and TNF-α were determined by ELISA. ***Results:*** SB216763 attenuated LPS induced mice or cell death but aggravated radiation induced mice or cell death. SB216763 reduced IL-6, but not TNF-α levels *in vivo*. IL-6$^{-/-}$ mice were more resistant to LPS-induced death but less resistant to radiation-induced death than wild type mice. ***Conclusions:*** Inhibition of GSK-3β conferred resistance to LPS shock but fostered death induced by ionizing radiation. Inhibition of GSK-3β was effective by reducing IL-6.

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Introduction

Exposure of high dose ionizing radiation is dangerous and lethal [1, 2]. Radiation exposure leads injuries of hematopoietic system (HP) and gastrointestinal tract (GI). Elucidation of the mechanism of radiation injuries can propel the progress in radiation injuries medical aids and bio-defense applications [3-6]. However, signal pathway involved in the radiation biology reaction still remains unclear.

Glycogen synthase kinase-3β (GSK-3β) is now recognized as a key component of a large number of cellular processes and diseases [7, 8]. Several mechanisms play roles in controlling the actions of GSK-3β, including phosphorylation, protein complex formation, and subcellular distribution [9-11]. Dysregulation of GSK-3β is linked to several prevalent pathological conditions, such as diabetes, insulin resistance, and Alzheimer's disease [12]. GSK-3β promotes the production of inflammatory factors and cell migration, which together make GSK-3β a powerful regulator of inflammation, thus GSK-3β inhibition provides protection against inflammation in animal models [13]. The involvement of GSK-3β and inflammation in these diseases are highlighted [14], however the roles of GSK-3β in radiation biology process and biodefense applications have not yet fully investigated.

Toll-like receptors (TLRs) are not only the detector of organisms ranging from bacteria to fungi, protozoa, and viruses, but also play important roles in many biological process [15-18]. LPS triggers sever immunological reactions resulting septic shock and death [19]. The mechanism of LPS action is indirect, mediated primarily through cytokines such as interleukin-6(IL-6), tumor necrosis factor-α (TNF-α) secreted by activated macrophages in spleen, liver and other sites [20].

In this study, we used glycogen synthase kinase inhibitor, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), to investigate the roles of GSK-3β in LPS shock and ionizing radiation, and found that in the two scenarios, GSK-3β may play different roles.

Materials and Methods

Reagents

3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), a GSK-3β inhibitor, was purchased from merck Millipore. LPS from Escherichia coli was purchased from Sigma (St. Louis, Mo), and LPS was re-purified as previous described [21]. Re-purified LPS was dissolved in pyrogen-free PBS (Baisai, Shanghai, China) [5]. DMEM and Fetal Calf Serum were from PAA, Austria [5].

Breeding and treatment of Mice

Adult WT mice and IL-6$^{-/-}$ (C57BL/6 mice, 6weeks, 20g) were obtained from the Model Animal Research Centre of Nanjing University (Nanjing, China) as described previously [4, 5]. Mice were pretreated with or without 10μM SB216763 (Sigma-Aldrich, Taufkirchen, Germany) by intraperitoneal injection [13]. Controls were incubated with 0.2% dimethyl sulfoxide and/or 15mM NaCl. 24 h later, these mice received two treatments: total-body irradiation (5, 7, 9Gy) (TBI) and LPS [5]. LPS was injected at a gradient different (200μg/mice, 500μg/mice, 1000μg/mice). Control mice were treated with pyrogen-free PBS. Here we got four different treated mice mice pretreated with SB216763, then treated with LPS (SB216763, LPS); mice only treated with LPS (PBS, LPS); mice treated with SB216763 and radiation (SB216763, TBI); mice only treated radiation (PBS, TBI). The following experiments were all base on the four types mice. Mice only treated with PBS were used as blank control. Then these mice were routinely cared and observed daily. All mice were housed in a Specific Pathogen-Free (SPF) facility for all experiments [4, 5, 22-24]. All animal experiments were undertaken in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, National Academy Press, Washington, DC, revised1996), with the approval of the Laboratory Animal Center of the Second Military Medical University, Shanghai [5].

Radiation

^{60}Co-gamma rays in radiation center (Faculty of Naval Medicine, Second Military Medical University, China) were used for the radiation purpose [4, 5, 23, 25, 26]. Mice and cells (with or without SB216763 pretreatment) were exposed to different doses of radiation, depending upon the requirement of the present study [4, 6, 23, 24, 27].

Survival assays

After the experiment treatment, mice returned to the animal facility and routinely cared after treatments. Survival was checked daily for 30 days [5, 26].

Cell viability analyses and apoptosis rate detection

L929 cell line was obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 6-well plates at 37 °C in a humidified atmosphere of 5% CO_2 with DMEM and 1640 Medium (PAA, Austria) containing 10% fetal calf serum (PAA, Austria) [6]. Exponentially growing cells were used for experiments. Cells were seeded in 96-well plates and pretreated with or without SB216763, 24h later, were irradiated or added LPS separately. Then, cells were further cultured for 48h. Cell viability was determined by WST assay using a Cell Counting Kit (Dojindo) [3]. Cells were prepared and labeled with Annexin V-FITC and propidium iodide (PI) (provided by BIPEC), following the manufacturer's instructions as previously described [12].

Statistical analysis

Comparisons between experimental groups and relevant controls (but not survival curves) were performed using a Student's t-test [5, 23, 26]. Differences in survival of the various groups of mice were assessed using Kaplan-Meier plus Cox Regression Analysis with the SPSS (Statistical Program for Social Sciences) software [4, 5, 22, 26]. The SPSS software generated a P value and Chi-Square value for each analysis; $P< 0.05$ was considered a statistically significant difference.

Results

SB216763 decreased LPS induced mice mortalities but increased radiation induced mice mortalities

Mice were pretreated with SB216763 then received LPS or total-body irradiation treatment and were monitored daily. After 30 days, we drew the survivorship curves. We found that all survivorship curves showed go-down trend. Higher concentration of injected LPS and dose of radiation leaded higher mortalities. Interestingly, SB216763 pushed the two similar curves to two different directions. In all different LPS dose (1000μg, 500μg, 200μg/mice), mice pretreated with SB216763, in the equal time point, comparing with mice only treated with LPS, showed higher survival rate (Fig. 1A). But on the contrary, mice pretreated with SB216763, exposed to 5, 7 and 9 Gy radiation, have lower survival rate, comparing with mice only treated with total-body irradiation (Fig. 1B).

So, these data above showed that that GSK-3β inhibition regulated LPS and radiation induced death, and attenuated LPS induced death but aggravated radiation induced death.

SB216763 decreased LPS induced cells death but increased radiation induced cells death

To confirm whether SB216763 has different effects on LPS and radiation induced death *in vitro*, L929 cells were pretreated with or without SB216763 for 24h, and then treated with LPS or radiation. Cell viability and apoptosis were assayed. *In vitro* results were similar with *in vivo* results. SB216763 reduced the effect of LPS inducing L929 cells to death, and aggravated death induced by radiation (Fig. 2A, B). Next, we tried to confirm whether LPS and radiation can induce cell apoptosis with or without SB216763. Cells apoptosis were analyzed by FACS after exposure to 0.1μg/ml LPS or 5 Gy. We found that both induced cells apoptosis, but SB216763 reduced the effect of LPS, SB216763 together with radiation

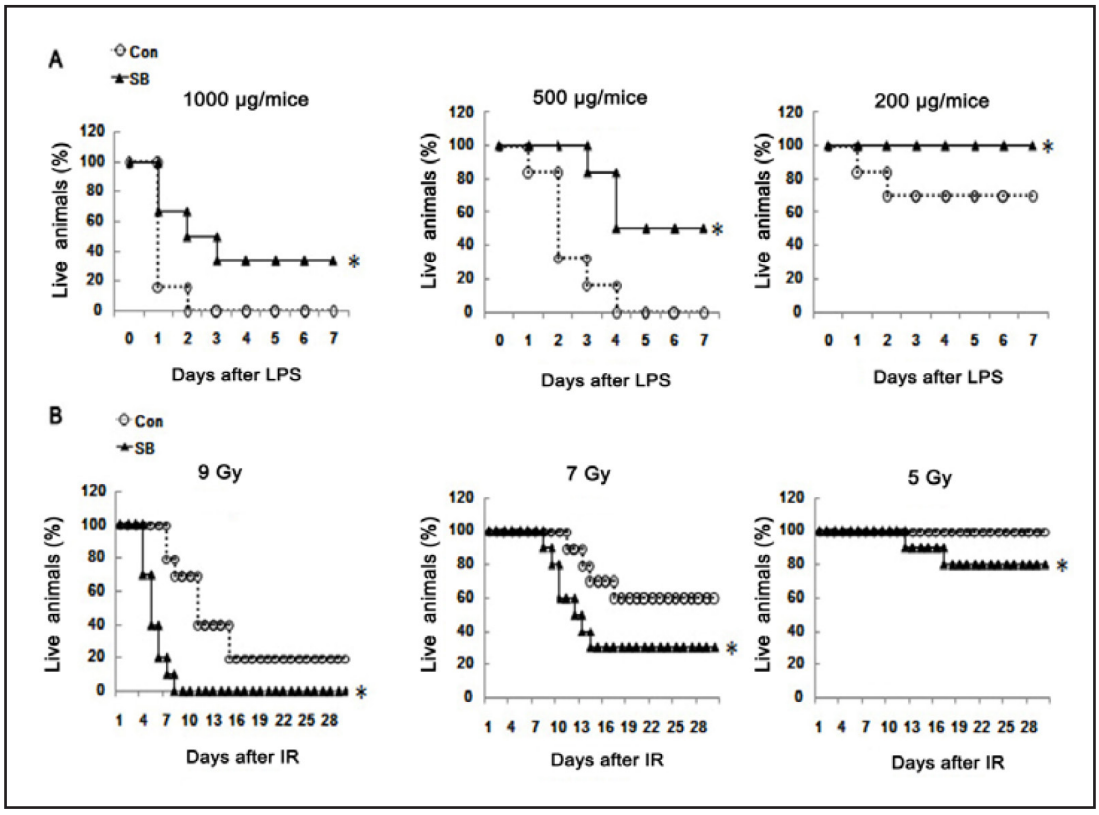

Fig. 1. SB216763 decreased LPS induced mice mortalities but increased radiation induced mice mortalities. (A) SB216763 pretreated mice (N=30) and WT control mice (N=30) were each randomly divided into three groups (SB216763 pretreated mice (N=10) and WT control mice (N=10) in each group), and exposed to LPS (1000μg, 500μg, 200μg/mice). Survival was monitored until day 30 after treatment. (B) SB216763 pretreated mice (N=30) and WT control mice (N=30) were each randomly divided into three groups (SB216763 pretreated mice (N=10) and WT control mice (N=10) in each group), and exposed to 9, 7 or 5 Gy ^{60}Co-γ radiation (dose rate: 1Gy/min). Survival was monitored until day 30 after treatment. *: P<0.05.

resulted more cells apoptosis (Fig. 2C, D). The similar results were obtained by using T cells (data not shown).

So, both *in vivo* and *in vitro* results indicated that GSK-3β played different roles in the death or apoptosis induced by LPS or radiation.

SB216763 reduced IL-6, but not TNF-α levels in vivo

What is the cellular mechanism involved in the process? Certainly, LPS could lead to lethal immunological response, and total-body irradiation is poisonous to cells *in vivo*, especially proliferating cells. However, which pro-inflammatory factors were involved in the death caused by LPS or radiation were still not clearly. 24 h after LPS or radiation treatment, we euthanized the survival mice for measuring IL-6 and TNF-α in their serum and found that LPS and radiation both caused the high level of IL-6 and TNF-α in serum (Fig. 3A, B, C, D). For SB216763 pretreated mice, the alteration of these two cytokine was different. SB216763 suppressed both the up-regulation of IL-6 caused by LPS and radiation (Fig. 3A.C). For mice treated with radiation, SB216763 showed more effect, almost reducing the level of IL-6 down to control level. The TNF-α induced by LPS and radiation seemed having no relationship with GSK-3β, the alteration caused by SB216763 showed no significant statistical difference.

It seemed that IL-6 induced by LPS and radiation could be suppressed by SB216763. So, is IL-6 the factor involved in the process related with GSK-3β?

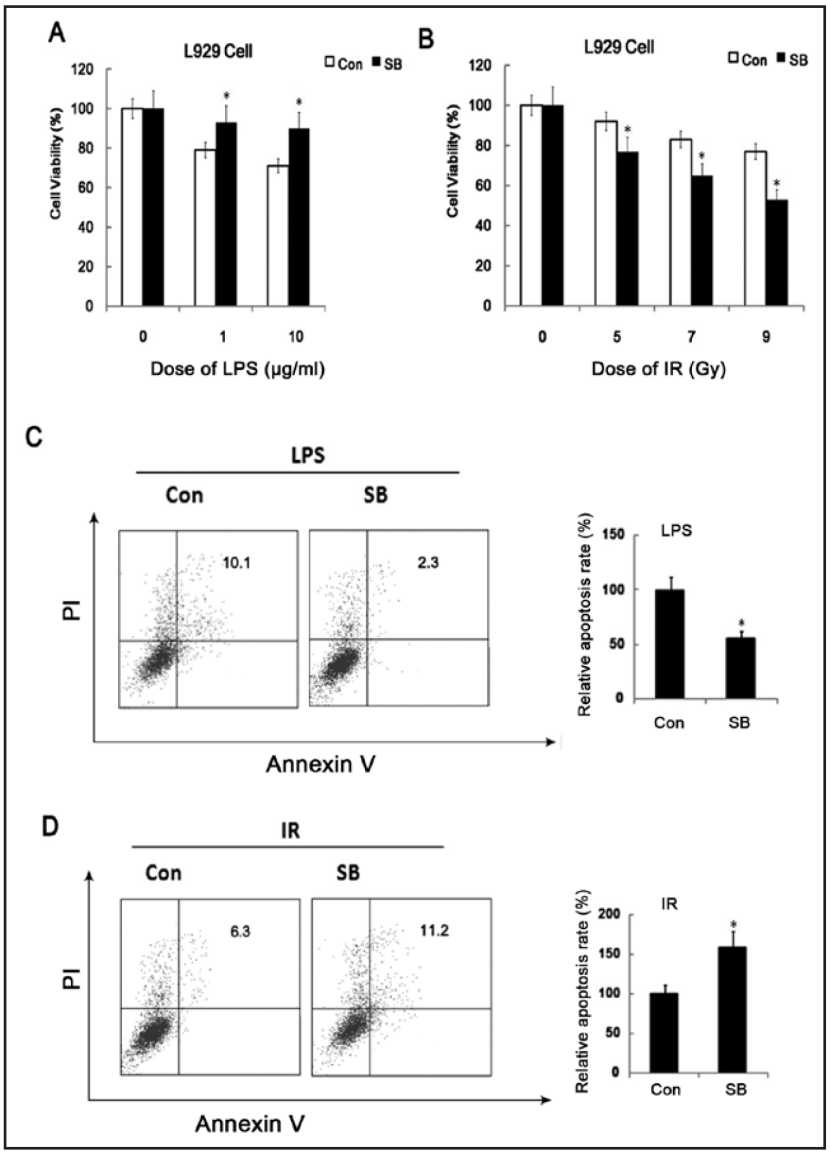

Fig. 2. SB216763 decreased LPS induced cells death but increased radiation induced cells death. L929 were seeded in 96-well plates and pretreated with or without SB216763, 24h later, were added LPS (0.1, 1, 10μg/ml) or irradiated (5, 7, 9Gy)separately. 48h later, cell viability was detected by WST assay using a Cell Counting Kit(Dojindo). Then apoptosis of 0.1μg/mlLPS group and 5Gy irradiated group were assayed. Relative apoptosis rate was calculated (Control group was treated as 100%). Every experiment was independently repeated three times. *: $P<0.05$. (A) Cells viability in LPS treated group. (B) Cells viability in irradiated group. (C) Cells apoptosis in LPS treated group. (D) Cells apoptosis in irradiated group.

IL-6$^{-/-}$ mice resisted to LPS induced death while increased more radiation induced mortalities

We tried to confirm the role of IL-6 involved in LPS and radiation by using IL-6 knockout mice (IL-6$^{-/-}$mice). IL-6$^{-/-}$mice and WT mice were treated with LPS and radiation. All mice were monitored daily. After 30days, we drew the survivorship curves. In Fig. 4A, survival rate curve illustrated a significant increase in response to LPS treatment in IL-6$^{-/-}$ mice. In contrast, survival rate was decreased in IL-6$^{-/-}$mice when they were treated with 7Gy comparing with IL-6$^{+/+}$ WT mice (Fig. 4B). IL-6$^{-/-}$ mice exhibited the similar role of SB216763 in response to LPS or radiation.

So, these results supported our central hypothesis that SB216763 attenuated LPS induced mice or cell death but aggravates radiation induced mice or cell death both via reducing IL-6.

Discussion

To our best knowledge, it is the first time to prove that SB216763, an inhibitor of glycogen synthase kinase-3β, could attenuate LPS-induced death but aggravate radiation-

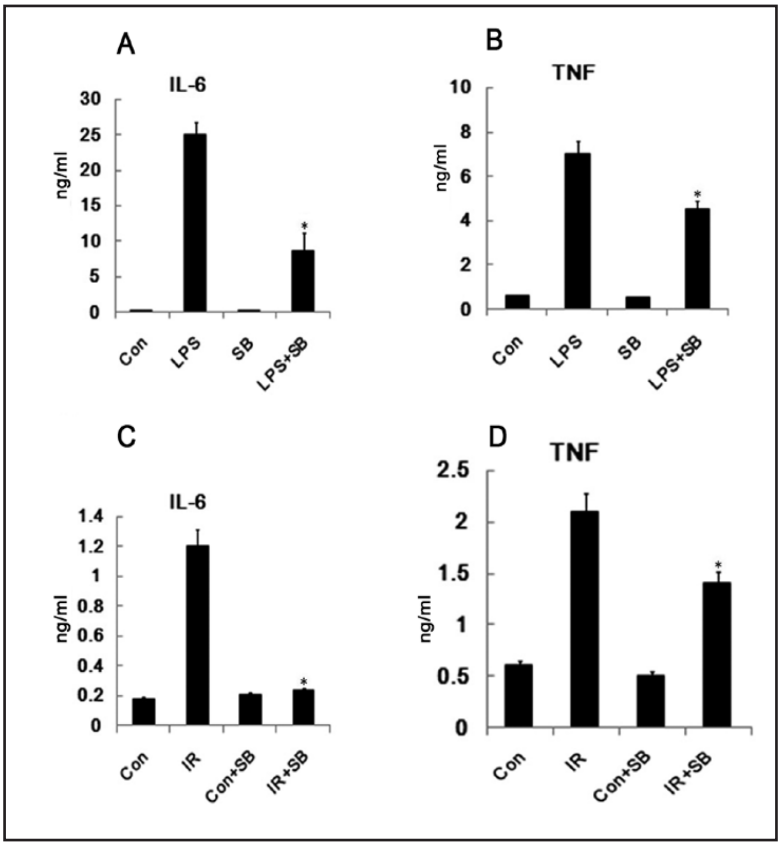

Fig. 3. SB216763 reduced IL-6, but not TNF-α levels *in vivo*. SB216763 pretreated mice (N=20) and WT control mice (N=20) were each randomly divided into two groups (SB216763 pretreated mice (N=10) and WT control mice (N=10) in each group). Each group was exposed to LPS (1000μg/mice)(N=10) or 7Gy radiation (N=10). 24h later, mice were euthanized for their serum. IL-6 and TNF-α were measured by ELISA. Results were the mean±SD. *: P<0.05. (A) The IL-6 serum level in LPS treated group. (B) The TNF-α serum level in LPS treated group. (C) The IL-6 serum level in radiation group. (D) The TNF-α serum level in radiation group.

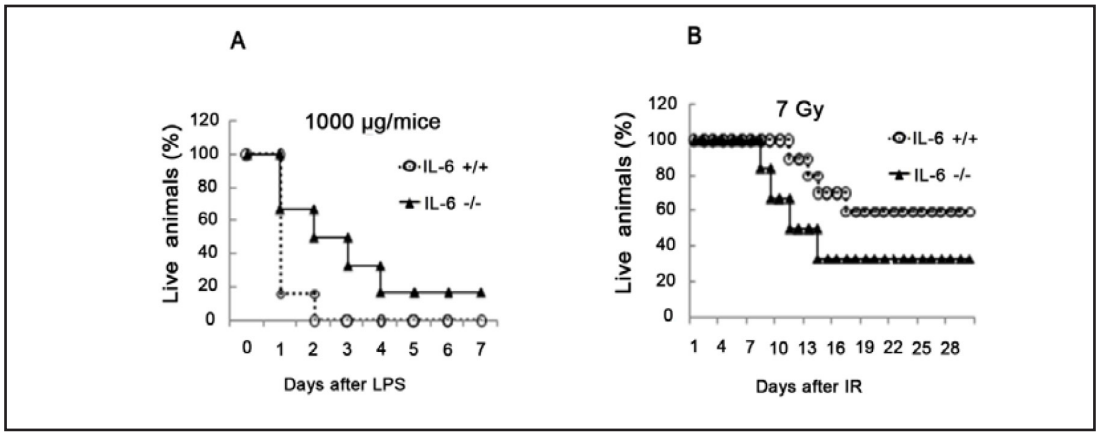

Fig. 4. IL-$6^{-/-}$ mice resisted to LPS-induced death while had more radiation induced mortalities. IL-$6^{-/-}$ mice (N=20) and WT control mice (N=20) were each randomly divided into two groups. Each group was exposed to LPS (1000μg/mice) or radiation (7Gy). (A) IL-$6^{-/-}$ mice survival monitored until day 30 after LPS expose (1000μg/mice). (B) IL-$6^{-/-}$ mice survival monitored until day 30 after radiation (7Gy).

induced death both *in vivo* and *in vitro*. knock out IL-6 also attenuated LPS-induced death but aggravated radiation-induced death. Interestingly, SB216763 suppressed both IL-6 induced by LPS or radiation. Thus, we guessed that inhibition of GSK-3β attenuated LPS-induced death but aggravated radiation-induced death via IL-6 .

It is interesting to found that glycogen synthase kinase-3β played different roles in LPS and radiation biology process. Comparing with radiation stimulation, LPS stimulation is

simple and well-studied [13, 19]. LPS is an extracellular signal, it does not alter or destroy intracellular material directly, but acts as a trigger initializing a cascaded reaction [19, 20]. In this study, It is the overreaction immune responses induced by LPS kill treated mice. Inhibition the expression of IL-6 attenuated LPS-induced death. The activation of TLRs stimulated immune cells and caused the secretion of cytokines and chemokines such as IL-6 and TNF-α to modulate innate and acquired immune response [13-15].The most important signal pathways provoked by TLRs are the transcription factor NF-κB and mitogen-activated protein kinases(MAPKs) pathway, which regulate the expression of many immune and inflammatory genes [28]. We also proved that mice deficient in TLR4 were more susceptible to radiation, and Myd88, not TRIF, may be the critical adaptor in TLR4 induced radiation resistance [5].

GSK-3β plays a key role in numerous cell signaling pathways including inflammation. Many of the GSK-3β targets are components of the transcriptional machinery, especially transcription factors such as CREB [29], NF-κB or c-myc [30]. GSK-3β is a crucial regulator of innate inflammatory processes, it promotes TLR-induced production of pro-inflammatory IL-6 and TNF-α at least in part by promoting NF-κB activity in monocytes [8]. Inhibition of GSK-3β suppressed the expression of NF-κB mediated pro-inflammatory genes in LPS-stimulated macrophages such as NOX, IL-6 [13]. Our results further showed that SB216763 inhibited the activation of GSK-3β, thus attenuated the expression of NF-κB mediated pro-inflammatory cytokines IL-6, not TNF-α.

Recent concerns for the accidental or deliberate exposure of the general population to radiation due to terrorism have resulted in studies of agents to mitigate or treat the symptoms of radiation exposure. Various radioprotective strategies have been explored including compounds that scavenge free radical and modulate the DNA repair process, or growth factors and cytokines that function through receptor mediated mechanisms [6, 31, 32].

The toxicity of ionizing radiation is associated with massive apoptosis in radiosensitive organs [5, 33]. GSK-3β is a key regulator of radiation-induced apoptosis, and small molecule inhibitors of GSK-3β could protect irradiated hippocampal neurons from apoptosis and improve cognitive performances in irradiated mice [34]. Meanwhile, another study reported that the small molecule inhibitors of GSK-3β prevented radiation-induced death in mouse intestine by reducing apoptosis of the epithelial cells of the crypts and dramatically increase animal survival [21]. Since our results have shown that GSK-3β inhibitor elicited protective role in LPS induced death, we explored the possibility of developing small molecule inhibitors of GSK-3β as radiation protectors against tissue injury. In our study, we found that inhibition GSK-3β did not serve as radiation-protectors, which was inconsistent with previous reports. Perhaps this outcome was dependent on cell types and other experimental conditions. On the contrary, we found inhibition of GSK-3β caused higher mortalities in mice treated with radiation and IL-6 was involved in the mechanism. Does IL-6 have a role of radiation protection? What is the relationship between inflammation and radiation exposure? A better understanding of the molecular, cellular and physiological mechanisms related to the effects of GSK-3β on TLR and radiation requires further studies.

Conflict of Interest

The authors have declared that no competing interests exist.

Acknowledgements

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