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Lithium attenuates lipopolysaccharide-induced hypothermia in rats

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Abstract. – BACKGROUND: Changes in body temperature are common features among patients with sepsis and septic shock. Similarly, systemic administration of bacterial endotoxin (lipopolysaccharide, LPS) to rats leads to an initial hypothermia followed by elevation in body temperature. These changes in body temperature are accompanied by increased levels of prostaglandin E2 (PGE2) in the hypothalamus.

OBJECTIVE: This study examined the effects of lithium and SB216763 – two different glycogen synthase kinase (GSK)-3 inhibitors – on LPS-induced changes in body temperature and hypothalamic PGE2 levels in endotoxemic rats.

MATERIALS AND METHODS: Endotoxemia was induced by intraperitoneal injection of LPS (10 mg/kg). Lithium (100 mg/kg) and SB216763 (5 mg/kg) were administered at 2 h before LPS. Body temperature and mortality were monitored during 48 h after LPS injection. In another protocol, rats were sacrificed at 2 h post LPS injection and then, blood, liver and hypothalamus were extracted for inflammatory mediators determination.

RESULTS: Lithium but not SB216763 significantly reduced LPS-induced hypothermia, while both compounds did not alter the subsequent elevation in body temperature. Moreover, only lithium significantly reduced hypothalamic PGE_2 levels. On the other hand, both compounds significantly reduced plasma, hepatic and hypothalamic tumor necrosis factor-α and decreased plasma PGE_2 levels. Both compounds did not alter LPS-induced mortality.

CONCLUSIONS: These results suggest that the attenuation of LPS-induced hypothermia by lithium may derive from its reduction of hypothalamic PGE_2 levels.

Key Words:
Cytokines, Inflammation, Lithium, SB216763, Sepsis.

Introduction

Sepsis and septic shock (SS) occur when a systemic inflammation is initiated by circulating bacteria, bacterial components or other pathogens such as fungi[1-3]. The pathophysiology of sepsis and SS is very complex. It is characterized by a profound systemic inflammatory response and robust production of inflammatory mediators, fever and/or hypothermia, lactic acidosis, severe hypotension and decreased organ perfusion – all of which lead to multi-organ dysfunction and death[1-4]. In the United States, severe sepsis and SS account for at least 10% of admissions to intensive care units[3,5]. Despite advancements in treatment strategies, patients with sepsis and SS have a mortality rate of 20 to 40%[3,5,6]. These data underscore the need for novel and more effective therapies.

One of the cellular pathways that have been associated with the pathogenesis of sepsis and SS is the enzyme glycogen synthase kinase (GSK)-3. GSK-3 is a serine/threonine kinase ubiquitously distributed in mammalian tissues including the brain[7,8]. In mammals, GSK-3 exists in two isoforms: GSK-3β and GSK-3β[7,8]. GSK-3β has been studied more than GSK-3β and it was linked to several human illnesses, including bipolar affective disorder and Alzheimer's disease[8]. A relationship between GSK-3β and inflammation was first described by Hoeflich et al[9] who found that GSK-3β is required for activation of the transcription factor nuclear factor (NF)-κB. They showed that GSK-3β facilitates the activity of NF-κB, resulting in an enhanced inflammatory response in mice[9]. Consistently, Dugo et al[10] found that the selective GSK-3β inhibitors SB216763 and TDZD-8 reduced the inflammatory response in endotoxemic rats, which was accompanied by a significant reduction in organ dysfunction[10]. Martin et al[11] showed that administration of selective GSK-3β inhibitors to rodents before treatment with lipopolysaccharide (LPS) significantly reduced NF-κB activity and

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decreased the production of pro-inflammatory mediators. In addition, these authors showed that GSK-3β inhibition significantly reduced LPS-induced mortality in mice[11]. Subsequently, other studies have also shown that selective GSK-3β inhibitors reduce production of pro-inflammatory mediators and decrease mortality in animal models of severe inflammation and sepsis[12-18]. Alternatively, other studies found that inhibition of GSK-3β had no effect[19] or led to stimulation[20-22] of NF-κB activity.

As mentioned, changes in body temperature (BT) are common features among patients with sepsis and SS. Consistently, systemic administration of LPS to rats induces a biphasic febrile response – an initial hypothermia followed by a BT elevation (fever)[23,24]. The precise mechanism underlying the *hypothermic* response to LPS in rodents is not fully understood and it seems that many inflammatory mediators contribute to this complicated process[23-25]. One of the mediators which play a pivotal role in the BT changes occurring after LPS administration is prostaglandin E_2 (PGE_2)[23-25]. Administration of LPS significantly increases PGE_2 levels in the hypothalamus during the hypothermic as well as the hyperthermic phases of the febrile response[23-25].

Despite the evidence linking GSK-3β to the pathophysiology of sepsis, to our knowledge, previous studies have not examined the effect of GSK-3β inhibition on BT changes occurring in septic animals. Therefore, the major aim of the present study was to examine the effect of GSK-3β inhibitors on LPS-induced changes in BT in endotoxemic rats. To this end, we used lithium, a non-selective GSK-3β inhibitor[26,27] widely used as a treatment for bipolar disorder[28], and, SB216763 – a selective GSK-3β inhibitor[29]. Furthermore, we examined the effects of lithium and SB216763 on LPS-induced inflammation and mortality.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 220-250 g were used throughout the studies. The animals were housed 3 per cage and maintained under controlled environmental conditions (ambient temperature 22 ± 1°C, relative humidity 55-58%, photoperiod cycle 12 h light: 12 h dark), fed Purina Lab Chow and water *ad libitum*. Only animals with no overt pathology were included in the studies. The procedures of the study were in accordance with the Guidelines of the Committee for the Use and Care of Laboratory Animals in Ben-Gurion University of the Negev, Israel (authorization #IL-61-11-2010).

Treatment with GSK-3 Inhibitors

Lithium chloride (LiCl) and SB216763 were purchased from Sigma-Aldrich (St. Louis, MO, USA). LiCl was dissolved in NaCl 0.9% and SB216763 in dimethyl sulfoxide (DMSO). The drugs were then filtered to produce sterile solutions for injection. Rats were treated with a single intraperitoneal (ip) injection of LiCl (100 mg/kg) or SB216763 (5 mg/kg) at 2 h before treatment with LPS. Control animals were treated with the vehicles (sterile NaCl 0.9% or DMSO, ip; both bought from Sigma-Aldrich).

Induction of Endotoxemia

LPS from *Escherichia coli* (bought from Sigma-Aldrich) was dissolved in sterile NaCl 0.9%. At 2 h after injection of GSK-3 inhibitors, rats were injected ip with LPS 10 mg/kg to induce endotoxemia. At the same time, control rats were injected with sterile NaCl 0.9%.

Measurement of Body Temperature

BT was measured with a plastic-coated thermocouple probe (HL 600 Thermometer, Anristu Meter Co., Tokyo, Japan) inserted into the rectum. Rats were acclimated to this procedure during one week before the experimental protocol was initiated.

Experimental Designs

The study included 2 experimental designs. The *first* design was conducted to examine the effect of GSK-3 inhibitors on LPS-induced changes in BT and mortality. In these studies rats were treated with LiCl and SB216763 at 2 h before the injection of LPS. BT was measured before (time "zero") and at 1.5, 6 and 24 h post LPS injection. Mortality was monitored during 48 h after LPS injection. The *second* design was conducted to examine the effect of GSK-3 inhibitors on LPS-induced inflammation. Rats were treated with LiCl and SB216763 at 2 h before the injection of LPS. At 2 h post LPS injection rats were sacrificed, blood was collected in heparin-coated tubes and simultaneously, their livers and hypothalami were quickly excised. Hypothalami were cleaned of blood and adipose tissue and together with the livers were immediate-

ly transferred to –80°C. Blood was centrifuged at 4000 rpm, 4°C for 5 min. Plasma samples were separated and kept at -20°C.

Preparation of Hypothalamic and Liver Homogenates

Each hypothalamus was weighed and manually homogenized for 10 seconds in 300 µl of a cold phosphate-buffered saline solution containing protease inhibitors (homogenizing buffer). Thereafter, tissue homogenates were centrifuged at 10,000 rpm, 4°C for10 minutes. Moreover, 0.5 g of each liver was dissected and manually homogenized for 30 seconds in 2 ml of homogenizing buffer. Tissue homogenates were centrifuged twice[30] at 10,000 rpm, 4°C for 10 minutes. After centrifugation, supernatants of hypothalamus and liver samples were collected and immediately transferred to –80°C for further determination.

Determination of TNF-α and PGE_2 Levels

Samples were assayed for TNF-α and PGE_2 content using ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The lower detection limit of the TNF-α and PGE_2 assays were 62.5 and 39 pg/ml, respectively. For samples in which level of the examined mediator was below the lower detection limit of the assay, results were expressed as "undetectable" and analyzed as zero. On the other hand, after an initial screen we found that the levels of TNF-α and PGE_2 in liver samples were extremely high and beyond the higher detection limit of the assays. Therefore, in order to obtain values that are within the detection limit of the assays we diluted all liver samples × 40 times.

Statistical Analysis

Statistical evaluation was carried out using factorial analysis, Student's *t*-test (two-tailed) or chi-square Fisher Exact test, according to the type of the tested parameter. Normally distributed data and continues variables are presented as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. Importantly, all hypothalamus samples were homogenized in 300 µl of homogenizing buffer, regardless of their weight. Similarly, all liver samples (0.5 g) were homogenized in 2 ml buffer. Thus, the content of inflammatory mediators in resultant homogenates was *lower* than its actual content in the original tissue, especially in liver samples which were centrifuged twice. Results in the figures of TNF-α and PGE2 were calculated as follows: *ELISA result in pg/ml divided by sample weight in milligrams*. Results are presented as: pg/ml (pg/mg wet weight).

Results

Effect of GSK-3β Inhibitors on LPS-induced Changes in BT

As seen in Figure 1, treatment with LPS led to a typical biphasic febrile response – a significant hypothermia at 1.5 h post LPS injection followed by a significant elevation in BT at 6 and 24 h post LPS injection. Pretreatment with LiCl significantly reduced the magnitude of LPS-induced hypothermia but did not alter the subsequent hyperthermia. On the other hand, pretreatment with SB216763 did not affect LPS-induced changes in BT at all time points of measurement.

Effect of GSK-3β Inhibitors on LPS-induced Production of TNF-a and PGE_2

As seen in Figure 2A, TNF-α levels were very low in plasma of control rats and in the animals treated only with LiCl or SB216763. Treatment with LPS led to a significant increase in plasma TNF-α levels at 2 h post injection. Pretreatment with LiCl and SB216763 significantly reduced LPS-induced increase in plasma TNF-α (Figure 2A). Moreover, as compared to control, treatment with LPS significantly increased plasma PGE_2 levels (Figure 2B). Pretreatment with LiCl and SB216763 significantly reduced LPS-induced increase in plasma PGE_2 levels.

As seen in Figure 2C, as compared to control, LPS treatment significantly increased hepatic TNF-α levels. Pretreatment with LiCl and SB216763 significantly reduced hepatic TNF-α levels in LPS-treated animals (Figure 2C). On the other hand, hepatic PGE_2 levels were similar in all treatment groups (Figure 2D).

As seen in Figure 2E, LPS did not significantly alter hypothalamic TNF-α levels. Pretreatment with LiCl and SB216763 significantly decreased hypothalamic TNF-α levels both in vehicle- and LPS-treated rats (Figure 2E). Moreover, LPS significantly increased hypothalamic PGE_2 levels (Figure 2F). Pretreatment with LiCl significantly reduced hypothalamic PGE_2 levels both in vehicle- and LPS-treated rats. SB216763 did not alter hypothalamic PGE_2 levels (Figure 2F).

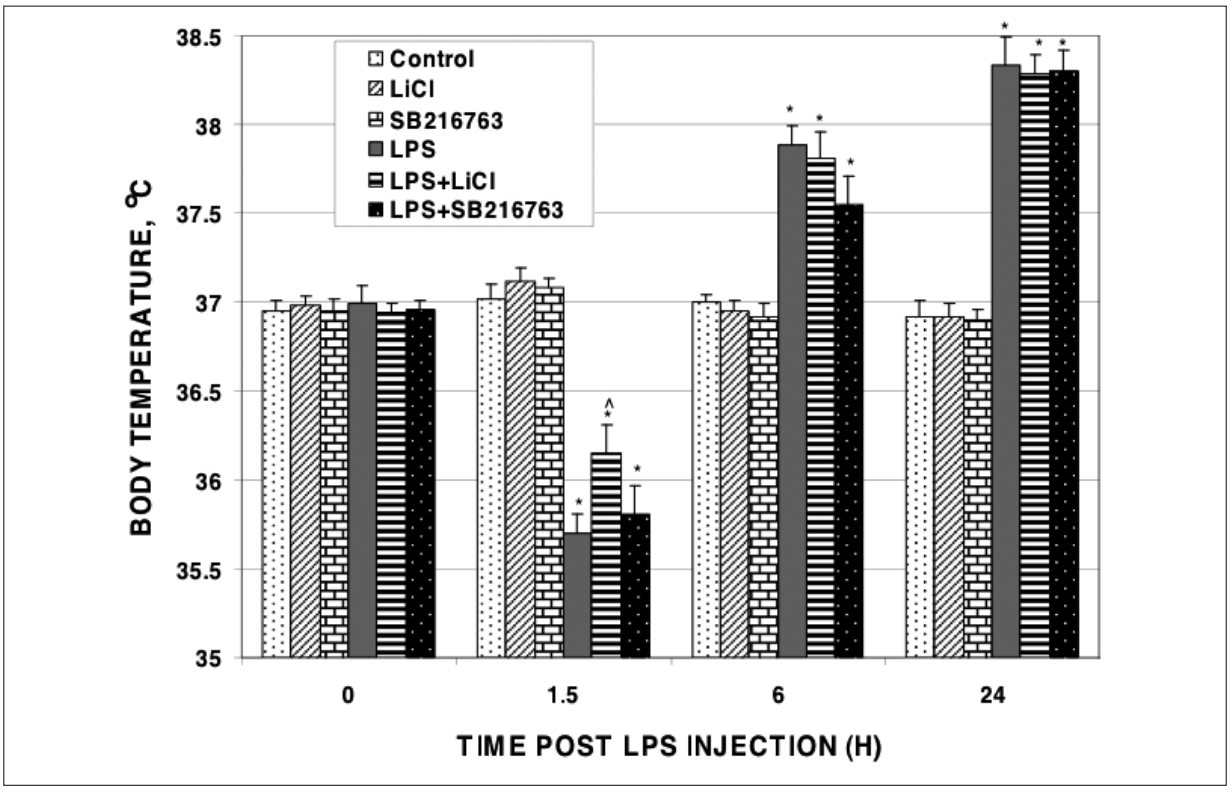

Figure 1. Effect of GSK-3 inhibitors on LPS-induced changes in BT of endotoxemic rats. Rats were treated with LiCl (100 mg/kg) or SB216763 (5 mg/kg) at 2 h before LPS (10 mg/kg) injection. BT was measured before (time "0") and at 1.5, 6 and 24 h post LPS injection. Data express the results of one out of three independent experiments in which identical results were obtained. Each column is the Mean ± SEM of 6 (control, LiCl, and SB216763) or 12 (LPS, LPS + LiCl, and LPS + SB216763) rats per group. *$p < 0.05$ vs. Control, ^$p < 0.05$ vs. LPS.

Effect of GSK-3β inhibitors on LPS-induced mortality

The effect of GSK-3 inhibitors on mortality of endotoxemic rats was examined in three independent experiments in which similar results were obtained. As seen in Table I, treatment with LPS led to a cumulative mortality rate of 50%. Pretreatment with LiCl or SB216763 did not significantly reduce LPS-induced mortality (mortality rates: 44.4% and 47.2%, respectively).

Discussion

This study demonstrated that lithium but not the selective GSK-3β inhibitor SB216763 attenuated LPS-induced hypothermia in rats. LiCl and SB216763 exhibited potent anti-inflammatory properties as they decreased TNF-α and PGE_2 levels. On the other hand, both compounds did not reduce LPS-induced mortality.

Acute pretreatment with LiCl significantly decreased LPS-induced hypothermia (Figure 1). Classical anti-inflammatory drugs attenuate LPS-induced hypothermia by reducing hypothalamic PGE_2 production[24]. In this study, both LiCl and SB216763 significantly reduced TNF-α and PGE_2 in the plasma (Figure 2A & B). In addition, both compounds reduced TNF-α levels in the hypothalamus. This latter finding is not surprising as a number of previous studies have already shown that lithium inhibits TNF-α levels in brain cells[31-36]. However, only LiCl significantly reduced hypothalamic PGE_2 levels (Figure 2F). Thus, the anti-hypothermic effect of LiCl seems to derive from its ability to reduce PGE_2 levels in the hypothalamus. Interestingly, LiCl reduced hypothalamic PGE_2 levels also in LPS-non-treated rats (Figure 2F), attesting for its ability to reduce PGE_2 production *in vivo*[37,38]. Previous studies have shown that GSK-3β inhibitors reduce the expression of cyclooxegenase-2 (the enzyme that produces PGE_2 particularly during inflammation)[14,16]; however, contradicting results have also been reported[39,40]. In the current study, SB216763 decreased PGE_2 levels in the plasma

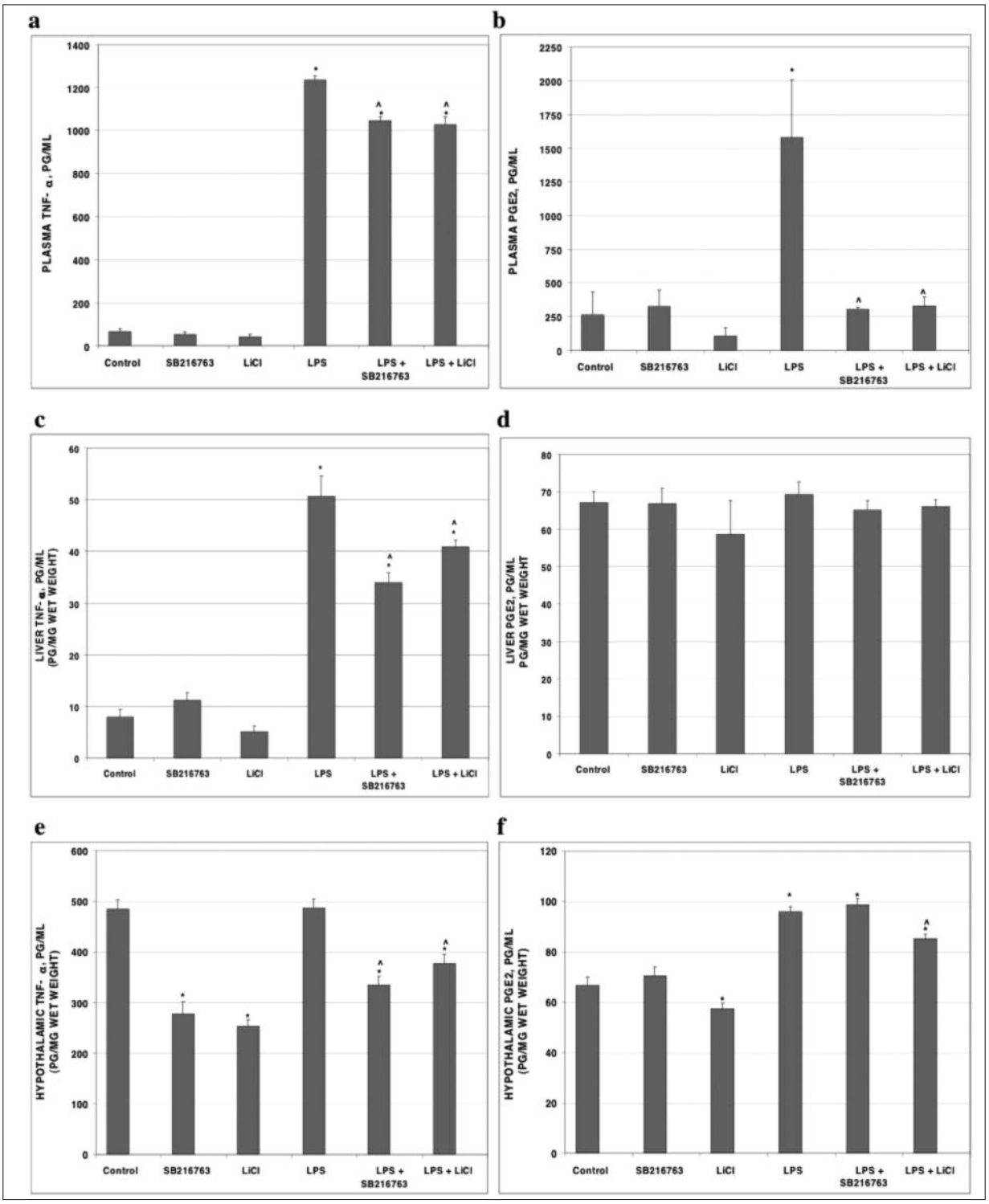

Figure 2. Effect of GSK-3 inhibitors on LPS-induced production of TNF-α and PGE2. Rats were treated with LiCl (100 mg/kg) or SB216763 (5 mg/kg) at 2 h before injection of LPS (10 mg/kg). At 2 h post LPS injection rats were sacrificed and immediately thereafter blood was collected and livers and hypothalami were excised and processed as described in "Materials and Methods". Levels of TNF-α and PGE2 in plasma, liver and hypothalamus (a and b, c and d, e and f; respectively) were measured using ELISA assays. Content of TNF-α and PGE2 in liver and hypothalamic samples was plotted per wet weight of each sample. Data express the results of one out of two independent experiments in which similar results were obtained. Each column is the Mean ± SEM of 6 (control, LiCl, and SB216763) or 12 (LPS, LPS + LiCl, and LPS + SB216763) rats per group. *$p < 0.05$ vs. Control, ^$p < 0.05$ vs. LPS.

Table I. Effect of GSK-3β inhibitors on mortality of endotoxemic rats,

Group	Control	SB216763	LiCl	LPS	LPS + SB216763	LPS + LiCl
n	18	18	18	36	36	36
48-hour survival, number (%)	18 (100)	18 (100)	18 (100)	18 (50)	19 (52.8)	20 (55.6)
p-value*		1	1		0.814	0.637

Mortality was evaluated in three independent experiments. The data presented in this table include the results of all three experiments. Rats were treated with LiCl (100 mg/kg) or SB216763 (5 mg/kg) at 2 h before injection of LPS (10 mg/kg). Mortality was monitored during 48 h after LPS injection. Values represent the number of animals who survived in each group (survival rates are given as percentages in parenthesis). *The statistical significance of difference between the groups was examined according to whether they were or were-not treated with LPS. SB216763 alone and LiCl alone were compared to control; LPS + SB216763 and LPS + LiCl were compared to LPS.

but not in the hypothalamus – this may explain its inability to affect LPS-induced hypothermia (Figure 1). It is worth noting that both LiCl and SB216763 did not alter the hyperthermic phase of the febrile response (Figure 1). These data suggest that the anti-hypothermic effect of LiCl derive from its ability to reduce hypothalamic PGE_2 levels rather than its inhibition of GSK-3β.

Previous studies have shown that selective GSK-3β inhibitors reduce LPS-induced production of pro-inflammatory mediators[10-15] and attenuate mortality in septic animals[20-22]. In the current study we used two GSK-3 inhibitors: LiCl – a non-selective GSK-3β inhibitor, and, SB216763 – a highly selective GSK-3β inhibitor[29]. We found that pretreatment with LiCl and SB216763 was mostly associated with a decrease in TNF-α and PGE_2 production in LPS-treated rats (Figure 2). Liver TNF-α and PGE_2 levels were tested because the liver is an organ highly affected by endotoxemia and bacterial sepsis. It produces large amounts of cytokines[30,41] and is also responsible for metabolism of such cytokines. The liver is a major source of peripherally produced cytokines that signal the brain through vagal afferents, leading to increased hypothalamic PGE_2 production[42-44]. We found that TNF-α and PGE_2 levels in the liver were extremely high even in control animals. As mentioned in "Materials and Methods", liver homogenates were diluted 40 times in order to enable measurement of TNF-α and PGE_2 by ELISA. Consistent with previous studies[30,41], LPS significantly increased hepatic TNF-α levels (Figure 2C). LiCl and SB216763 significantly reduced LPS-induced increase in hepatic TNF-α (Figure 2C). On the other hand, LiCl and SB216763 did not significantly alter hepatic PGE_2 levels (Figure 2D). It is worth noting that the method of hepatic homogenates preparation significantly affected the ELISA results. In a preliminary experiment, after homogenizing liver samples, we performed a single centrifugation cycle and observed a non-significant increase in hepatic TNF-α in LPS-treated rats (as compared to control; data not shown). However, when two centrifugation cycles were performed, hepatic TNF-α levels were found significantly higher in LPS-treated than in control rats (Figure 2C). A single centrifugation cycle (at 10,000 rpm, 10 minutes) yielded cloudy homogenates which appeared to contain large amounts of cell debris.

Furthermore, pretreatment with LiCl and SB216763 was not associated with a significant decrease in LPS-induced mortality (Table I). The reason for the discrepancy between our results and those of previous studies[16-18] is currently not understood. Differences in experimental design (particularly species[45]) may account, at least in part, for this discrepancy. In this regard, despite the encouraging results of previous studies that used selective GSK-3β inhibitors as a treatment against sepsis-induced organ dysfunction and mortality in animals[10,11,17,18], GSK-3β inhibition did not yet become a clinically used treatment for sepsis in humans. To our knowledge, GSK-3β inhibitors have never been tested in a randomized clinical trial in septic patients. Similarly, GSK-3β inhibitors were tested in several animal studies of mood disorders[46-50] in which they were found to reduce mania-and-depressive-like behaviors. However, again, none of these compounds reached the stage of a randomized clinical trial in humans (as a treatment for mood disorders).

GSK-3β is a multi-functional enzyme the inhibition of which is expected to have a powerful influence on multiple cellular functions and signaling pathways. For example, GSK-3β inhibition has been associated with potent antiapoptotic ef-

fects in multiple experimental models. Nonetheless, the therapeutic benefit of this antiapoptotic effect is not indisputable – inhibition of GSK-3β is associated with accumulation of β-catenin which may result in tumor promotion[51]. Moreover, in the pioneer study by Hoeflich et al[9] in which a relationship between GSK-3β and NF-κB was established, sustained inhibition of GSK-3β in mice resulted in extensive hepatocyte *apopotosis* and mortality in-uterus. Similarly, Gomez-Sintes et al[52] showed that *in vivo* GSK-3β inhibition in mice resulted in increased neuronal apoptosis and impaired motor coordination. Thus, the safety of sustained GSK-3β inhibition *in-vivo* remains unclear and necessitates further research.

Conclusions

This study demonstrated that LiCl reduced LPS-induced hypothermia probably due to a reduction in hypothalamic PGE_2 levels. Moreover, LiCl and SB216763, two mechanistically-different GSK-3 inhibitors, reduced LPS-induced production of TNF-α and PGE_2 but failed to decrease mortality in endotoxemic rats.

Funding Interests

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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