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Research Article

Combination of Calcium Channels Inhibitors Prevents LPS – Induced (pro-) Inflammatory Release of the TNF-α and IL-1β, *In Vivo*

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Abstract

Trauma and cardiovascular myocarditis patients, who are exposed to bacterial infections are at risk to be exposed to so-called mortal "LUCOST" (LPS-Unfolded Protein Response (UPR)-Cytokine Overexpression-Signal Transduction). The essential components of LUCOST system are consist of 4 main key players. The aim of this study is to evaluate induction of inflammatory processes and assess the pharmacological inhibitory of tissue cytokines changes, in an innovative prepared model system, *in vivo*.

Methods: The LPS was extracted from patients' burned wounds after *Pseudomonas aeruginosa* isolation (hLPS), with informed consent. The male c57/BL6 mice divided into 6 groups: 1. Two controls, one received nothing another sterile pyrogen-free normal saline, 3. hLPS (3 mg/kg/intraperitoneal), 4. hLPS+ Dantrolene sodium (Dan)-calcium channel inhibitor (CCI1) (40 mg/kg), 5. 2-aminomethyl phenyl borinate (2APB)-CCI2 (1 mg/kg) and 6. hLPS+CCI1/CCI2. Subsequently, mice livers were extracted for evaluating cytokines release after 2, 8 and 24 hrs, LPS inductions.

Results: Compared to the controls, the TNF-α and IL-1β levels in hLPS group significantly increased after 8 and 24 h injection ($P < 0.05$). Treatment with CCIs separately and/or in combination decrease these levels ($P < 0.05$). Compared to all attempts to inhibit calcium overload, hLPS combined with CCI1/ CCI2 showed a remarkable decrease in TNF-α and IL-1β levels after 2, 8 and 24 h ($P < 0.05$).

Our results showed that compared to controls and CCIs separately, using an appropriate combination of both CCIs had better effectiveness to inhibit (pro-)inflammatory processes i.e. the TNF-α and IL-1β release. We expect in the near future such CCIs mixtures being developed as a specific drug to prevent the LUCOST in different heart patients, suffering from infections timely.

Keywords: *Heart; Inflammation; LPS; Gene Splicing; Calcium Channel Inhibitors; LUCOST*

Introduction

Despite recent evidence-based results, which show that damaged tissue calcium overload and cytokine release i.e. TNF-α and IL-1ß are recognized as the most important factors to increase myocardial dysfunction and heart failure, though there are limited pharmacologic drugs development done to prevent their mortal effects [1-4]. On the other hand, still scant studies focused on more details about possible inhibiting pathways via combination of calcium channels inhibitors to manage infected patients, who are suffering from proinflammatory responses and (un-)known side effects resulting to random shut down of organs, however (hypothetical Figure 1).

Figure 1: *Hypothetical model system LUCOST. Infection with microorganisms (Gram-negative bacteria) and their endotoxins might increase mortality and morbidity rate via LUCOST (LPS-UPR-Calcium Overload Signal Transduction) System.*

After traumatic (car) accidents and cardiovascular diseases, different heart patients, who are exposed to bacterial infections are at risk to being exposed to so-called mortal "LUCOST" (LPS-UPR-Cytokine Overexpression Signal Transduction). (hypothetical Figure 2) The essential components of the LUCOST pathways are consist of 4 main key players 1. Initiating stimuli i.e. LPS, LOS endotoxin release from micro-organisms [5-7] 2. Propagating factors and cytokines release i.e. NOX, ROS products, TNF-α, and IL-1ß, [1,3,4,7,9] 3. The UPR activation system [1,9-12] and 4. Terminating factors i.e. calcium overload and disorders, [2,23-26] hyperactivities of cells [17,24,25], and random organs' shut down [1,4,25-31].

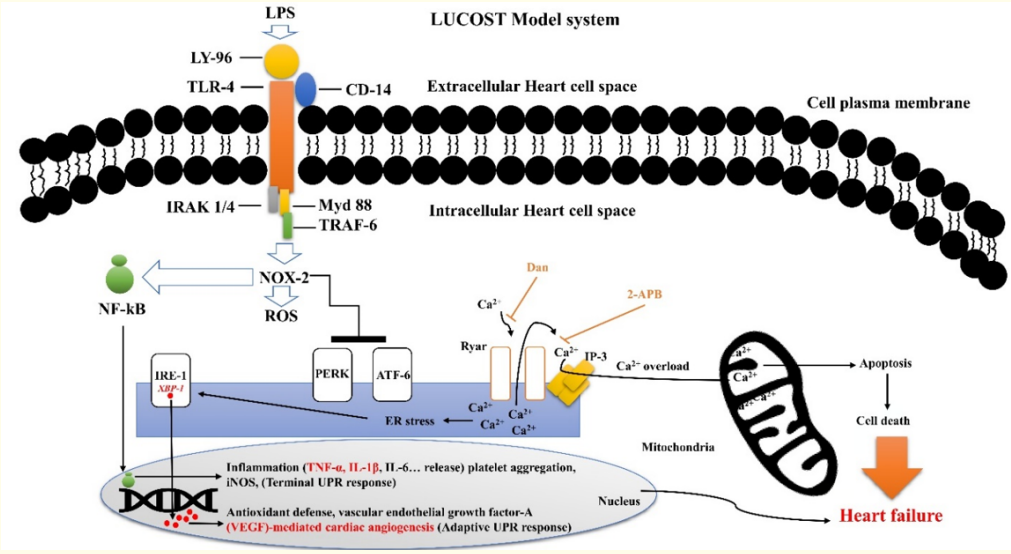

Figure 2: *Study hypothesis the LUCOST. There are different inflammatory and antioxidant mechanism available against exposure to lipopolysaccharide (LPS). Ca+2 released by Inositol three phosphate receptor (IP3R) increases mitochondrial activity during endoplasmic reticulum (ER) stress, in order to revert energy imbalance. When it fails, the cell will die as a result of mitochondrial apoptotic cascade. Ca+2 released from ryanodine receptors on the endoplasmic reticulum could increase Ca+2 overload pool via IP-3 receptors and lead to extra inflammatory response, which might induce heart cell death toward random other organs shut down i.e. heart, brain, lung, kidney. Different molecular pathways of adaptive and terminal UPR system could shift and present Heart failure. LUCOST hypothesis propose applying combination of different Ca+2 Channel inhibitors like Dantrolene Sodium (Dan) and 2-aminomethyl phenyl borinate (2-APB) to prevent terminal UPR activation and (pro)-inflammatory cytokine release in different organs. LY-96: Lymphocyte antigen 96; TLR-4: Toll-like receptor 4; CD-14: Cluster of differentiation 14 marker mad by macrophages and innate immune system; IRAK ¼: N-[1-[2-(4-Morpholinyl)ethyl]-1H-benzimidazol-2-yl]-3-nitrobenzamide; Myd 88: Myeloid differentiation primary response 88; TRAF-6: Tumor necrosis factor receptor (TNFR)-associated factor 6; NOX: (NO and NO2) ROS: Reactive oxygen species, NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, IRE-1: Inositol-requiring enzyme 1; PERK: Protein kinase RNA-like endoplasmic reticulum kinase; ATF-6: Activating Transcription Factor 6; IL-1β: Interleukin 1 beta; VEGF: Vacsular endothelial growth factor-A; UPR: Unfolded protein response; iNOS: Inducible nitrogen oxide synthase.*

One of the most common bacterium that causes infection and inflammatory response is *P. aeruginosa*, which plays a clear role as a pathogen in a certain fatal infection [5]. Primary infections caused by *P. aeruginosa* have been observed in the respiratory tract, bladder, ears, and ulcers from the burns, wounds, and surgical sites as well. This bacterium is one of the main nosocomial infectious microorganisms, which can increase risk of mortality and morbidity rates out- and/or in-Hospitals, eventually.

Different data revealed that bacterial LPS acts as a stimulator of immune system [4-10] and TNFα, which in certain hepatic and heart patients could increase risk of heart failure [1,4].

Given the severity of the side effects of this potent proinflammatory endotoxin on myocardial performance and viability, it is important to characterize the signaling cascades, which is associated as proinflammatory agonists toward cytokine production in certain infected tissue [1-4]. How the LPS's induction mechanism affects liver tissue and hepatocytes, myocardial tissue and cardiomyocytes, resulting in random shut down of organs *in vivo*, is not elucidated completely [1-8].

In the other hand, LPS is the major outer surface membrane components present in almost all Gram-negative bacteria and acts as an extremely strong stimulators of innate or natural immunity, which increases risk of calcium metabolism disorders, septic shock in heart patients toward increased risk of death [1-6,26,27]. In certain patients, after tissue damages and burned-tissue-injuries [1,2], different infection and inflammations, are the main fundamental concerns to treat and/or prevent fatal consequences. Chronic infections increased after LPS concentrations in the systemic blood can prime (pro-)inflammatory responses, and sepsis-related processes (SRPs) [1,6,26-28].

Different Basic researches revealed that might LPS consist of a poly- or oligosaccharide regions, which are anchored in the outer bacterial membrane [7,8]. When LPS released i.e. because of the antibiotics cure and/or attacked –leaking and dying bacteria [6] could induce the migration of Neutrophils from the spleen to the location of T-lymphocytes [8]. The liver is another organ, which also plays a prominent antitoxic role in the production of LPS (anti-) inflammatory responses. Increasing the concentration of liver enzymes is considered as a marker in the acute phase of the inflammation process. Ongoing infections and inflammation might occur in systemic blood circulation after infected tissues damaged harshly, which can be observed in the patients with burns [9], and accidental trauma.

Previous studies have shown that the Calcium ions (Ca^{+2}) intracellular overload could initiate cell death during exposure with LPS [9-24]. There are different inter- and intracellular systems, which are functioning as an homeostatic response mechanism to prevent Ca^{+2} overload for example, the unfolded protein response (UPR), in eukaryotic cells. Ca^{+2} overload might be fatal via the route of the UPR during exposure to the LPS. The UPR-Inositol Requiring Enzyme-1(IRE-1) sensor and its main locomotor product (X-Box binding protein-1 or *XBP-1* transcription factor) may react with certain microbial LPS in direct or indirect pathways via the routes of Fatal (Terminal)-UPR or Survival (Adaptive)-UPR (hypothetical Figure 2) [9-12,23].

The aim of this study is to evaluate induction of inflammatory processes and assess the Ca^{+2} channel Inhibitors (CCIs) administration effects at tissue cytokine changes after LPS injection, in a novel preparation of human-animal inflammation model system, *in vivo*. We assumed that the LPS (side-)effects at systemic blood and the LUCOST system could be managed, *in vivo*.

Here we show that both CCIs could prevent pro-inflammatory role of LPS and cytokine release. This model system could be used as an *in-vivo* model system for the different kinds of research, investigating for a certain anti-inflammatory drugs development, as well.

Materials and Methods

This study was divided in two main research A) isolation of human-tissue-derived bacterial LPS (hLPS) as described [25,26] and B) investigate inflammation and tissue cytokine levels. Our study was carried out in 5 steps: 1) Clinical sampling of patients with burn wounds; 2) Biochemical identification of bacteria *P. aeruginosa* isolated from patients 'damaged burn wound; 3) Extraction of hLPS from isolated bacteria and compare it with Chemical standard LPS available in the market; 4) constructing novel *in-vivo* animal modeling and study purified hLPS versus chemical standard control LPS from Sigma-Aldrich, Germany, without and with the CCIs i.e. Dantrolene sodium (Sigma-Aldrich, Germany)(CCI1) and control groups, 5) Animal tissue released cytokine assay.

Clinical sampling of patients with burn wounds

The test was fully described to the patients and informed consent was obtained for the donation of their sample. Patients with more than 40% burn wound and probability of infection with *P. aeruginosa* infection characteristics was selected. Samples from patients were from the Surgery Department of Velayat Hospital Rasht, Rasht, Iran collected by sterile swab and transferred to the Stewart culture medium (Merck, Germany). Swabs then inoculated to the BHI medium (Merck, Germany), cultured on Cetrimide (Merck, Germany), and blood agar (Millipore, Germany), separately in aseptic condition and finally incubated at 42°C for 48 hours.

Biochemical identification of bacteria isolated from patients

Grown colonies in the Cetrimide and blood agar with specific pigmentation were prepared on the slide for Gram staining kit (Parsian Teb, Tehran, Iran). Additional biochemical and complementary experiments were carried out to determine bacterial identities such as Citrate, Oxidase, Indole, Urea and the Triple sugar iron agar (TSI) (Millipore, Germany).

LPS extraction from patient's bacterial isolation kit

After bacterial strain was confirmed, subsequently the extraction of endotoxin from patients specific isolated *P. aeruginosa* bacteria was carried out by using specific LPS intron purification kit based on the manufacturer's instructions. Pure *P. aeruginosa* sub-cultured and undergo endotoxin extraction. The extracted endotoxin was visualized via 12% Polyacrylamide Gel Electrophoresis (PAGE), stained with silver nitrate. The HPLC conformational analysis utilized for isolated hLPS validation with using commercial *P. aeruginosa* LPS (SIGMA-Cat.no: L8643) as standard control. Purified LPS kept at 4°C till the next use.

Modeling and biologic assays

Six to eight weeks old 25 grams male c57/BL6 mice have selected for the study. All animals exposed to 12-12 light-dark circles equally and had unrestricted access to water and food during the experiment. Groups include: 1) Control 1 (received nothing), 2) Control2 (sham) that received sterile pyrogen-free normal saline, 3) hLPS group (3 mg/kg/intraperitoneal (IP)), 4) hLPS+Dantrolene Sodium (Dan = CCI1) (40 mg/kg), 5) 2 -APB (1 mg/kg) (2-APB = CCI2) and 6) hLPS+Dan+2APB (CCI1+CCI2), respectively.

Cytokine assay

Changes in the level of animal liver cytokine profiles measured and monitored at 2, 8, and 24 hours post injections of LPS for IL-1β and TNF-α by using specific enzyme-linked Immunosorbent assay (ELISA) kits (Zell bio GmbH, Germany) for IL-1β and IBL international kits (USA) for TNF-α evaluation (Figure 4A and B).

Statistical analysis

The results of the study were analyzed using the IBM SPSS software version 16 in three different time intervals (2, 8 and 24 hours). One-way analysis variance (ANOVA) and Tukey test were used for comparison between TNF-α and IL-1β levels in different groups. For all tests statistically significance was considered when $P< 0.05$. All data have expressed as mean ± standard deviation (n = 36).

Results

We isolated hLPS and compared it with standard control LPS to approve correctness the purification process; subsequently studied the hLPS effects on the LUCOST systems (Figure 2). Moreover, we measured hLPS effects at the TNF-α and IL1-ß expression without and with the CCIs (Figure 3). In one hand, we reproduced and confirmed the most results of the previous studies have done *in vivo*, but our novel finding was that the hLPS induction mechanism of the tissue TNF-α release could be prevented by using an appropriate combination of the CCIs (Figure 3), which could be used for pharmacologic goals.

Quality control and quality assurance of LPS

The isolated bacteria from human burned wounds were confirmed as *P. aeruginosa* sort, based on culture and biochemical identification observations, and positive oxidase test. Besides, we confirmed our isolated bacterial hLPS from human wound has comparable quality with chemical LPS (Sigma, Germany) assessed by using HPLC analysis and checked it out by 12% PAGE silver staining (data not shown).

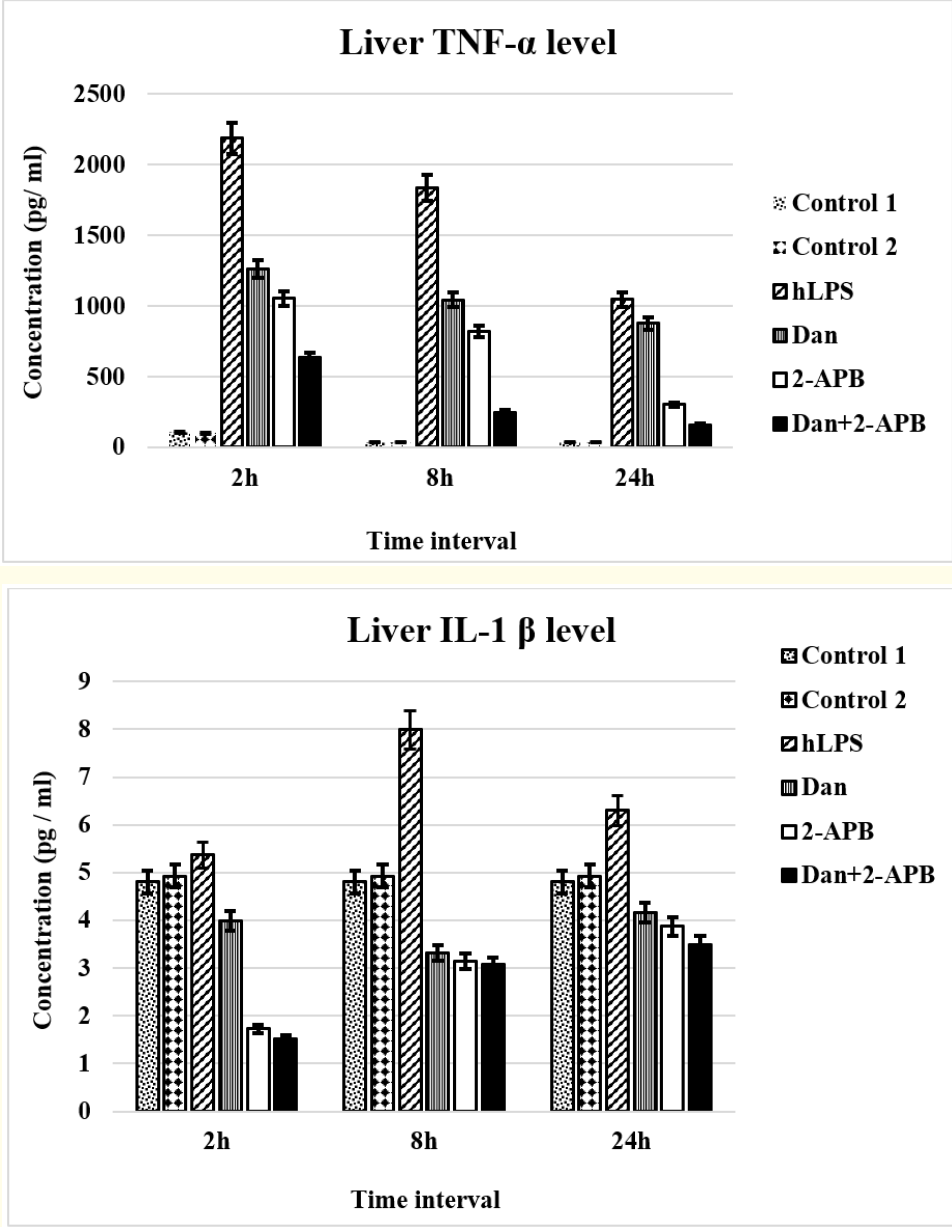

Figure 3: *Cytokines release after IP-hLPS induction. A) Tumor necrosis factor alpha (TNFα) and B) Interleukin 1 beta (IL-1ß). hLPS: The extracted LPS from patients' burned wounds after P. aeruginosa isolation and culture, Dan: Dantrolene sodium; 2-APB: 2-aminomethyl phenyl borinate.*

We used mice as an animal modeling to treat them with purified hLPS, standard chemical LPS, biosimilar hLPS, without and with the CCIs for 2,8, and 24 hrs. As a control group for different subjects undergoing (pro)inflammatory stimuli we injected sterile normal saline (negative controls).

hLPS induced TNF-α levels increased

We measured cytokines release of the TNF-α and IL-1β at 2, 8 and 24 hrs after IP-injection of hLPS to the animals. We observed that compared with both negative and positive controls, after hLPS injections both cytokines levels showed significant increased after 2, 8 and 24 hrs ($P < 0.05$).

After receiving inhibitors separately i.e. CCI1, CCI2 and/or in combination CCI1/2, compared to controls and those mice receiving the hLPS alone, at all test hours, the TNF-α and IL-1β levels were significantly decreased, in isolated tissues ($P < 0.05$). Meaning that our approaches were effective enough to inhibit hLPS induced LUCOST side effects (Figure 3).

It is noteworthy that release of the TNF-α and IL-1β after hLPS to the animals were not the same and TNF-α levels were remarkably higher that IL-1β, however. Meaning that might the TNF-α signaling either are more sensitive than IL-1β, and/or IL-1β amount is less in liver tissue. Compared to controls combined solution of calcium channels inhibitors, showed additive effects against both TNF-α and IL-1β expression, after 2, 8, 24 hrs, respectively (Figure 4). Moreover, we observed that the percentage of inhibition effect was higher at tissue TNF-α than the IL-1β.

Discussion and Conclusion

In this study we used an *in-vivo* described model system and used a novel approach to investigate LPS effects at the LUCOST and the cytokines release *in vivo*. Subsequently, we observed that human LPS can affect pro-inflammatory cytokine release, correlated with lethal effects of the LUCOST, and myocardial tissue damages, as broadly described [1-26].

In our previous study we investigated calcium ions influx and effluxes during calcium paradox in the rodent's heart and how calcium overload could affect UPR system, the SRPs, and myocardial disorders [2,3]. Gaspers LD 2006 has postulated that the second messenger IP3 elicits Ca^{2+} signals that control many important processes in hepatocytes, including mitochondrial metabolism [33-35], glycogen degradation [34] and gene expression [35]. The essential components of this signaling pathway are the formation of IP3 at plasma membrane, binding of IP3to IP3Rs and release of Ca^{2+} from the intracellular to releasing a series of periodic [Ca^{2+}] spikes activating LUCOST system toward tissue damage, myocardial dysfunctions [1,24,33-37].

In the other hand, there has not been released any report about pharmacologic and anti-inflammatory effects of the CCIs against endotoxic shock caused by *P. aeruginosa*'s LPS [26], and how *in-vivo* such LUCOST system could be managed in certain infected patient with cardiovascular diseases. Recall, the *P. aeruginosa*, considered as an important opportunistic pathogen in nosocomial infections of burned wounds [25,26], and traumatic patients.

The use of the CCIs in our study potentially could affect the gene expression of adaptive UPR sensors (like *XBp*-1). When adaptive UPR is affected, this event leads to a beneficial effect on decreasing the amount of the inflammatory mediators, which might have determining roles in dealing with different lethal signal transductions, and subsequently, resulting in an increase/decrease in lethal side effects.

Recent studies shown that the correlation of the UPR- *XBP-1* splicing transcription factor could be vital in heart angiogenesis during cardiac hypertrophy *in vivo*. (40) Both pro/inflammatory cytokines TNF-α and IL-1β are the most important products of *XBP-1* splicing transcription factor, and (fatal) UPR activation during Ca^{2+} overload (Figure 2).

We used CCIs to prevent Ca^{2+} toxic efflux through the cell, affect UPR- *XBP-1* splicing, dawn-regulate the expression and release of pro/inflammatory cytokines like TNF-α and IL-1β by attenuating the endoplasmic reticulum (ER) stress. We approved an appropriate combination of the CCIs could inhibit significantly calcium overload. Moreover, we introduced here a new mixture of CCIs to decrease the fatal levels of TNF-α and IL-1β, pharmacologically.

Taken together our results highlight more about possible management of the LUCOST system *in vivo*, and unravel other aspects of the signal transductions correlated to (un-)known processes toward random shut down of damaged tissues and organs, which might by specific. In the near future we hope that such drugs could be produced to prevent mortal LUCOST in the cardiovascular patients suffering from endotoxemia.

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