Author's response to reviews

Title: Polydatin up-regulates Clara cell secretory protein to suppress acute lung injury induced by LPS in vivo and in vitro

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Author's response to reviews: see over
Dear Editor Miko Galeng and Kazuhisa Iwabuchi

Thank you very much for your letter and advice. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in blue in the revised manuscript. Point by point responses to the reviewers’ comments are listed below this letter.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With best wishes,

Yours sincerely,

Tracy

We would like to express our sincere thanks to the reviewers for the constructive and positive comments.

Replies to Reviewer 1

Specific Comments

Comment 1—“Change the language, even the title, to reflect that this study shows that PD up-regulates CCSP but not make the claim that PD suppresses acute lung injury.”

Answer 1: You mean that we must change the title as “Polydatin up-regulates Clara cell secretory protein of lung induced by LPS in vivo and in vitro”. But we had investigated the protective effects of polydatin on acute lung injury following endotoxic shock in rats seven years ago. We had proved that “In addition to prohibit the dropping of MAP, PD could alleviate the acute lung injury induced by LPS which increased the levels of lung coefficient, lung penetrating index, the protein concentration of the bronchoalveolar lavage fluid (BALF) and the content of NOS in lung tissue. Enough morphological evidence could be found in pathological sections. (Shu
Shiyu, etc. Protective effects of polydatin on acute lung injury following endotoxic shock in rats. China Pharmacy, 2003, 14(3):143-144. All of our subsequent researches were based on these results. Meanwhile, some researchers of China such as professor Zhao Kesen, Jin Chunhua and Jin Lijuan also did sufficient researches to prove that polydatin could suppress lung injury induced by LPS. They had published their results in many domestic journals. (Mo GY, Jin LJ, Jin CH. Polydatin prevents endotoxin-induced acute lung injury in rats. Zhonghua Jie He He Hu Xi Za Zhi. 1993 Jun;16(3):153-4, 187-8. Chinese. etc)

Then we wanted to explore the potential mechanism of polydatin of lung protection. We found that polydatin could inhibit phospholipase A$_2$ (PLA$_2$) activity and the gene expression. PLA$_2$ is one of the key inflammatory mediators which lead to lung injury. But CCSP is an endogenous PLA$_2$ inhibitor. So we did the research on the chain of PD-CCSP-PLA$_2$-lung injury. We had proved that polydatin could protect lung injury induced LPS through up-regulating the expression of CCSP. We will show more explanation about this in answering comment 4. Can we keep the original title or change the title as “Polydatin up-regulates Clara cell secretory protein to suppress phospholipase A2 of lung induced by LPS in vivo and in vitro”?

Comment 2—“Edit manuscript for clarity especially in methods and figures (see below).”

Answer 2: We have done some modification about the methods and figures.

Comment 3—“If the researchers have any data which clearly shows a link between CCSP increase and a decrease in PLA2 inflammatory markers, they should show it.”

Answer 3: You mean that we should show the results about the link between CCSP increase and PLA2 decrease.

First, the fact is truth that CCSP is an endogenous PLA2 inhibitor. It has been clearly identified that CCSP acts as an endogenous inhibitor of PLA$_2$ with CCSP-deficient mice [1-5] (i.e. Si RNA) by binding calcium, a cofactor for secretory PLA$_2$ activation, or phosphatidylcholine, a substrate of PLA$_2$ [6]. And this conclusion has been cited by many researchers.


Second, we had observed the reverse changes of gene expression between CCSP and PLA2.

<table>
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<tr>
<th>Target</th>
<th>Oligonucleotide sequence</th>
<th>Tm (°C)</th>
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</thead>
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</tr>
<tr>
<td></td>
<td>R: 5'-CCATACCCAGGAAGGGCTT-3'</td>
<td></td>
<td></td>
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<tr>
<td>CCSP</td>
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<tr>
<td></td>
<td>R: 5'-ACACAGAGGACTTTGTTAGAT-3'</td>
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<td>R: 5'TTCATAGAGCCTCTCATCACACG 3'</td>
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</table>

Figure 1 Effects of polydatin on the expression of CCSP mRNA in rat lung. (A) Relative expression of CCSP, quantified by real-time PCR. (B) Electrophoresis showing the RT-PCR results. I: sham operation group, injected with normal saline; II: endotoxic shock group, injected with LPS (10 mg/kg body weight); III: PD treatment group, injected with LPS (10 mg/kg body weight), then a 0.5% PD solution (0.2 ml/kg body weight) 1 hour later; IV: PD
pretreatment group, injected with a 0.5% PD solution (0.2 ml/kg body weight), then LPS (10 mg/kg body weight) 0.5 hours later; V: PD control group. *P<0.01 vs. group I; †P<0.05 and ‡P<0.01 vs. group II; ‡P<0.05 vs. group III.

Figure 2 The dose–effect relationship between polydatin and CCSP mRNA expression in rat lung. (A) CCSP mRNA expression in PD treatment groups with different doses by real-time PCR and RT-PCR. (B) CCSP mRNA expression in PD pretreatment groups with different doses by real-time PCR and RT-PCR. bP<0.05 vs. 1 mg·kg⁻¹ PD group, eP<0.05 vs. 5 mg·kg⁻¹ PD group, and jP<0.01 vs. 10 mg·kg⁻¹ PD group.

Figure 3 Effects of polydatin on the expression of sPLA₂/cPLA₂ mRNA in rat lung. A. Relative
expression of sPLA₂, quantified by real-time PCR. B. Relative expression of cPLA₂, quantified by real-time PCR.

I: sham operation group, injected with normal saline; II: endotoxic shock group, injected with LPS (10 mg/kg body weight); III: PD treatment group, injected with LPS (10 mg/kg body weight), then a 0.5% PD solution (0.2 ml/kg body weight) 1 hour later; IV: PD pretreatment group, injected with a 0.5% PD solution (0.2 ml/kg body weight), then LPS (10 mg/kg body weight) 0.5 hours later; V: PD control group. P<0.05 and P<0.01 vs. group I; P<0.01 vs. group II; P<0.05 and P<0.01 vs. group III.

Figure 4 The dose–effect relationship between polydatin and sPLA₂ mRNA expression in rat lung.

A. CCSP mRNA expression in PD treatment groups with different doses by real-time PCR and RT-PCR. B. CCSP mRNA expression in PD pretreatment groups with different doses by real-time PCR and RT-PCR. P<0.01 vs. 1 mg·kg⁻¹ PD group, P<0.05 and P vs. 5 mg·kg⁻¹ PD group, and P<0.05 vs. 10 mg·kg⁻¹ PD group.
Figure 5 The dose–effect relationship between polydatin and cPLA$_2$ mRNA expression in rat lung.
A. cPLA$_2$ mRNA expression in PD treatment groups with different doses by real-time PCR. B. cPLA$_2$ mRNA expression in PD pretreatment groups with different doses by real-time PCR. *P <0.01 vs. 1 mg·kg$^{-1}$ PD group, eP<0.05 and fP<0.01 vs. 5 mg·kg$^{-1}$ PD group, and iP <0.05 vs. 10 mg·kg$^{-1}$ PD group.

Comment 4 –“Is the question posed by the authors well defined? Not really, the authors appear to make the claim that CCSP upregulated by PD can mitigate LPS induced injury through inhibition of PLA2. This is easily seen in their title ‘Polydatin up-regulates Clara cell secretory protein to suppress acute lung injury induced by LPS in vivo and in vitro’. However, the paper does not present any evidence that the CCSP actually suppresses PLA2 or acute lung injury but instead lists references to this fact. The main question of this paper should have been does PD upregulate CCSP? And then use the discussion to expand on the implications of this for prevention of lung injury seen in their previous publications.”

Answer 4: We have not presented clearly enough about the relationship among PD-CCSP-PLA2-Lung injury. All of the questions mentioned above can be answered in union.

First, it has been proved by some other researchers except us that polydatin can suppress the acute lung injury.


**Second,** our previous experimental results indicated that polydatin had prophylactic and therapeutic effects (the former is more distinct than the latter) on acutely injured lungs in rats with endotoxic shock by **inhibiting phospholipase A₂ activity and the gene expression of secretory phospholipase A₂ type II A (sPLA₂-IIA).**


SPLA₂-IIA is a main subtype and the main representative of PLA₂ family to induce inflammation and lung injury.


**Third,** the fact is truth that **CCSP is an endogenous PLA2 inhibitor.** It has been clearly identified that CCSP acts as an endogenous inhibitor of PLA₂ with CCSP-deficient mice [1-5] (i.e. Si RNA) by binding calcium, a cofactor for secretory PLA₂ activation, or phosphatidylcholine, a substrate of PLA₂ [6]. And this conclusion has been cited by many researchers.


⑥ Yoshikawa S, Miyahara T, Reynolds SD, Stripp BR, Anghelescu M, Eyal FG, Parker JC: Clara

**Fourth**, the purpose of this study was to determine the potential mechanism between polydatin and PLA₂. We intended to explore whether polydatin inhibit PLA2 through the endogenous PLA2 inhibitor –CCSP.

**Fifth**, in your opinion, the references were not enough to show the links between CCSP and PLA2. We have done some experiments to verify the facts. The results have been shown on comment 3.

*Comment 5 –“Are the methods appropriate and well described?*

*In general, they are. However, I would suggest describing the types of treatments before discussing the details of treatments and sacrifice.*

*Also this part of the methods was confusing, since it talks about subdiving the treatment groups into 4 subgroups even though a treatment concentration of 0.2ml/kg body weight was already described.*

“For group III and group IV, LPS (10 mg/kg body weight) was injected, and a 0.5% PD solution (0.2 mL/kg body weight) was injected 1 hour later or 0.5 hours before, respectively. The rats in group V received the 0.5% PD solution (0.2 ml/kg body weight) only.

The PD treatment and pretreatment groups were each further divided into four sub-groups (n=6 each) that received 1 mg·kg⁻¹, 5 mg·kg⁻¹, 10 mg·kg⁻¹ or 30 mg·kg⁻¹ PD, respectively.”

Answer 5: You mean that we should describe the types of treatments before discussing the details of treatments and sacrifice. We had changed the sequence of content as your requirement.

Let me show you the animals grouping. First, we concerned about the effects of polydatin on rat lung with different modes of processing.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The animal grouping and drug injection</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>I</td>
<td>NS</td>
</tr>
<tr>
<td>II</td>
<td>LPS</td>
</tr>
<tr>
<td>III</td>
<td>LPS</td>
</tr>
</tbody>
</table>
Then, we concerned about the different doses of polydatin (1 mg·kg\(^{-1}\), 5 mg·kg\(^{-1}\), 10 mg·kg\(^{-1}\) or 30 mg·kg\(^{-1}\)) on rat lung in treatment groups and pretreatment groups respectively. A 0.5% PD solution (0.2 mL/kg body weight) equal to 1 mg PD /kg body weight. 1 mg PD /kg body weight is the lowest effective dose according to our research. So we used this dose to show the dose-effect relationship again. It is not a conflict for us to get the conclusion.

*Comment 6*—*“Are the data sound? Yes, however, it would be a lot better if they looked at an inflammatory marker decreasing with CCSP increasing in expression.”*

Answer 6: You mean that we should add the link between PLA2 and CCSP. We had shown the content of this part on comment 3.

*Comment 7*—*“Does the manuscript adhere to the relevant standards for reporting and data deposition?*

Yes, however, the figure legends need improving so that the reader does not have to always refer to the text to understand them. In particular, each figure should clearly state animal/cells being tested, time of exposure. In addition, for figure 2, it should be made clear that it is PD pretreatment/treatment with the addition of endotoxin similar to what is done in other figures.

Answer 7: Yes, we had done the revision about the figures and the figure legends.

*Comment 8*—*“Are the discussion and conclusions well balanced and adequately supported by the data?*

Sort of. The discussion and results clearly demonstrate that PD upregulates CCSP. The rest is speculative. For example, the conclusion starts off with: “We have demonstrated that PD can modulate CCSP expression to inhibit PLA2.” This is abit misleading since no PLA2 measurements were completed in this study.”

Answer 8: We had added the parts of PLA2 measurements. CCSP is an endogenous
phospholipase A2 inhibitor. It’s a truth which has been identified clearly. It is the main reason why we chose CCSP not others to explore the potential relationship between PD and PLA2. Many scientific discovery is based on the speculation.

Comment 9—"Are limitations of the work clearly stated? No"

To our knowledge, this is the first study to demonstrate the effect and mechanism of polydatin on CCSP. The potential mechanism between PD and CCSP requires further investigation.

Comment 10—“Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes”

Comment 11—“Do the title and abstract accurately convey what has been found?

No, for the reasons stated above. ”

Answer 11: Please see the answer of comment1.

Comment 12—“Is the writing acceptable? In general yes, there are a few small grammatical errors and a global issue with respect to lack of spaces between references numbers and text in some cases. ”

Yes, I had tried my best to modify them.

Replies to Reviewer 2
Specific Comments
Comment 1 -- “Was lethal effect of LPS alleviated by PD treatment in vivo? If all rats were dead even treatment with PD in this model, what is the benefit of PD in the LPS-induced shock?”

Answer 1: This question is difficult to answer. You mean to assess the effect of PD when the rats are dying of LPS-induced shock.

We used LPS (10 mg/kg body weight) which was not lethal immediately. Of course, there were some individual differences among the rats. Most rats could be alive over six hours. But over the dose, the rats were inclined to die quickly. So we used the dose of 10 mg/kg body weight LPS
to copy the animal model. We had explored the effects of polydatin on the rats’ lung with LPS-induced shock several years ago. We had published these contents on the Journal of China Pharmacy (Shu Shiyu, et al. Protective effects of poydatin on acute lung injury following endotoxic shock in rats. China pharmacy, 2003,14(3):143-144). We measured the MAPs (mean artery pressures), the level of lung coefficient, lung penetrating index (LPI), the protein concentration of BALF (bronchoalveolar lavage fluid) and the content of NOS in lung tissue. The histologic changes of the lung were observed under microscope. We found that polydatin could alleviate the acute lung injury induced by LPS by prohibiting the dropping of MAP and the increasing of lung coefficient, LPI, the protein concentration of BALF and the content of NOS in lung tissue. Enough morphological evidence could be found in pathological sections. Especially, the protective effects were more obvious in poydatin pretreatment group.

In our opinion, polydatin can delay the process of shock and lung injury in some extent. When the shock and lung injury is in very serious condition, nothing can change the irreversible consequence. Serious sick, a hopeless case.

Comment 2—“Authors describe that serum CCSP is transferred from lung to serum and its level may be a maker of the bronchoalveolar blood-gas barrier integrity and permeability. Authors should show some reference papers to support this phenomenon.”

Answer 2: “CCSP may act as a marker of the bronchoalveolar blood-gas barrier integrity and permeability” is a conclusion from a literature and I will add the reference.


Comment 3—“It seems to be anti-parallel correlation between the lung tissue CCSP level and serum level. If it is consistent, why the results of Group3 and 4 of Fig4A are not significant each other, while the group3 looks different from group 4 in Fig2C#”
Answer 3: It is a helpful question which gives us a new idea. There is no doubt that PD can up-regulate the CCSP level of lung tissue (PD control group, group 5). LPS can down-regulate the CCSP level of lung tissue (endotoxic shock group, group 2). In group 4 (PD pretreatment group), PD first up-regulates the CCSP level of lung tissue. In group 3 (PD treatment group), LPS was injected first and the level of CCSP was down-regulated by LPS. The level of CCSP in group 4 was higher than that in group 3 in previous time. Although bronchoalveolar blood-gas barrier integrity in group 4 was better than group 3, because the level of CCSP is much higher than that in group 3. The bronchoalveolar blood-gas barrier permeability in group 3 is much higher than that in group 4. So the CCSP level in serum in both groups will be almost the same. We use a very simple graph to show the phenomenon.

Comment 4—“What is the physiological role of decreased serum CCSP for protecting endotoxin shock induced by LPS injection intravenously?”

Answer 4: Clara cell secretory protein (CCSP, CC16, CC10 and uterglobin) is a 16-kDa homodimeric protein that is secreted by non-ciliated bronchiolar (Clara) cells into the mucus lining the bronchial epithelium of the mammalian lung. It is one of the most abundant proteins in the airway mucus of mammals. As a biomarker of Clara cells and lung health, CCSP has been proposed as a useful diagnostic marker of toxicant exposure or airway epithelial damage. Some studies have reported that intratracheal administration of exogenous CCSP such as recombinant human CCSP to the lungs alleviates inflammation. Although evidence points to antioxidant, anti-inflammatory, immunomodulatory, anti-cytokine, pollutant clearance, anti-fibrosis, anti-tumor invasion and anti-protease activity, its endogenous mechanism is not known. Studies on CCSP-deficient mice have indicated that CCSP acts as an endogenous inhibitor of PLA₂ by binding calcium, a cofactor for secretory PLA₂ activation, or phosphatidylcholine, a substrate of...
Various clinical and experimental studies suggest that blood CCSP is a sensitive marker to detect early permeability changes of the lung epithelial barrier and/or to evaluate the integrity of the Clara cells, a privileged target of many pneumotoxicants. The integrity of the tight junctions is regarded as the major factor in providing barrier properties to the airway epithelia. Under circumstances of normal epithelial function and intact tight junctions, only small amounts of intra-tracheally introduced tracers reach the blood. Like other low-molecular size proteins, plasma CC16 is rapidly eliminated by glomerular filtration before being taken up and catabolized by renal tubules. So, the CCSP level in serum is too low to measure in normal animals.

LPS can increase the alveolar capillary membrane permeability. (Fisher P, Millen JE, Glausser FL. Endotoxin-induced increased alveolar capillary membrane permeability. Circ Shock 1977; 4: 387-395. Burrell R, Lantz RC, Hinton DE. Mediators of pulmonary injury induced by inhalation of bacterial endotoxin. Am Rev Respir Dis 1988;137:100-5). In animals, LPS is associated with an elevated serum concentration of CC16 in spite of a marked decrease of secretion and synthesis at the lung level, suggesting a passage of CC16 from the airways to the vascular compartment as a consequence of increased airway permeability. Disruption of the epithelial barrier results in an increased movement across the airway mucosa. This increased permeability is a bi-directional event, i.e. it leads to an increased transport of serum components such as water, solutes and proteins into the air spaces and a concurrent flow of endogenous and exogenous macromolecules from the air spaces into the blood.


Overall, the CCSP level of serum CCSP may act as a marker of the bronchoalveolar blood-gas barrier integrity and permeability. CCSP of serum can not prohibit the lung injury induced by LPS indirectly or directly.

Comment 5--“Basic experimental condition of PD treatment was done using 0.5% PD
(0.2ml/kg). This dose corresponds to 1mg/kg. But in other experiments examining PD dose response, 1mg/kg was less effective (Fig.3, 4BC). Why authors used 1mg/kg in Fig.2, Fig.4A, Fig.5?"

Answer 5: The experiment of Fig 2, Fig 4a was done earlier than the dose-response relationship of PD (Fig 3 ABC). The 1mg/kg PD is effective to increase the CCSP expression to protect lung. The dose less than 1 mg/kg is no effective. The dose of 1mg/kg PD can show the differences among the five groups. But the 1 mg/kg PD is less effective than the different dose groups such as 5 mg/kg, 10 mg/kg and 30 mg/kg. The way of grouping is completely satisfied for our experiment.

Fig 5 is about the cell experiment. We used 0.5 m mol/L PD which is different from the animal experiment.

Comment 6--Page 14 Western blotting

How much protein from the lung homogenate was applied in the western blotting?

Answer 6: The quantity of protein from the lung homogenate depends on the size of lung tissue we use. The concentration of total protein was 10µg/µl. We used 10µg total protein to measure.

Comment 7--“Fig2C What is the unit of Y-axis#(ng/#tissue protein)?”

Answer 7: There is no unit of Y-axis. It is a ratio.

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Answer 9: We had added the missing right parenthesis.

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Answer 10: We have revised mg/L to mg/kg.

Comment 10--“Fig.5A Y-axis, “CCSP protein expression” should be revised as “CCSP mRNA expression”.”
Answer 10: We have revised CCSP protein expression as CCSP mRNA expression.

Thanks for your valuable time and efforts on our article again.

Replies to Reviewer 2
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