Author’s response to reviews

Title: Effects of β1-adrenergic receptor blockade on the cerebral microcirculation in the normal state and during global brain ischemia/reperfusion injury in rabbits

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Effects of β1-adrenergic receptor blockade on the cerebral microcirculation in the normal state and during global brain ischemia/reperfusion injury in rabbits

Akila Sridhar

Editor, BMC Pharmacology and Toxicology

Dear Editor:

We are pleased to submit the revised version of our manuscript to be considered for publication in your journal.

Thank you very much for your supportive and constructive comments and for giving us the opportunity to revise and resubmit this manuscript.

In response to the reviewers’ comments, we have conducted several additional experiments and extensively revised the manuscript. In the revised manuscript, all changes are highlighted yellow.
We hope that our paper will be accepted for publication in your journal and look forward to hearing from you.

Sincerely,

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Response to the reviewer’s comments:

Reviewer reports:

Nahid Aboutaleb (Reviewer 1): the work is good it is better to authors show histopathological changes in the brain of rabbits. How did you confirm that model of ischemia was established?

We thank the reviewer for the supportive and constructive comments.

The aim of the present study was to investigate the pharmacological effects of β1-blockade on cerebral microvasculature via a physiological approach, and we did not conduct histological evaluation.

In the present study, the establishment of ischemia was clear and confirmed by microscopic observation in all animals. The pial vessels constricted and the brain surface became pale after clamping the major branches of aorta, indicating the establishment of brain ischemia. Based on the rabbit anatomy and earlier studies, the present method must have induced severe global brain ischemia (Am J Emerg Med 2000;18:31-35, J Neurosurg Anesthesiol 2010;22:207-213). However, we strongly agree that the verification and quantitative assessment of the severity of the brain ischemia in the present study is necessary, and it would support the findings of the study. Therefore, we have conducted an additional experiment using a laser Doppler flowmeter (FLO-C1; Omegaflo, Tokyo, Japan) to measure the cerebral blood flow, in which we confirmed that the clamping of the brachiocephalic, left common carotid, and left subclavian arteries significantly decreases the cerebral blood flow by 89.3 ± 9.2%. We have revised the Methods section as follows:

“To verify brain ischemia/reperfusion in this model, another set of three rabbits were subjected to cerebral blood flow (CBF) measurement. A 1-mm burr hole was made on the parietal bone using a micro drill, and a laser Doppler flowmeter (FLO-C1; Omegaflo, Tokyo, Japan) was inserted perpendicularly to the bone surface. After the Doppler flowmeter was installed, the
rabbits were subjected to the 15-min artery clamping. The CBF decreased by 89.3 ± 9.2% (mean ± standard deviation) after clamping, and recovered after unclamping, confirming that the three-vessel clamping induces brain severe brain ischemia and reperfusion.” (Methods, Line 134) some related works (2019) 9:6044 | https://doi.org/10.1038/s41598-019-42633-9) should be added to enrich text.

> We have cited the reference in the revised manuscript as follows:

“Ischemic brain injury is a major cause of death and morbidity worldwide. Although numerous neuroprotective agents have been investigated, few of them have been shown to impact the clinical outcomes of ischemic brain injury [1].” (Background, Line 73)

Yanqin Gao (Reviewer 2): This manuscript's aim to address the direct effects of β1-blockade on the cerebral microvasculature both in the normal state and ischemia/reperfusion state. A huge literatures report that β1-blocker has the vasodilation function. Author simply used cranial window method as showing the changes of the pial arteriole diameter in the normal and ischemia/reperfusion state.

Authors need to demonstrated what the neuroprotective effect of landiolol via inducing significant vasodilation of the pial arterioles during ischemia/reperfusion.

> Although this study demonstrated the direct local vasodilatory effects of β1-blockade, the results of our study do not directly indicate the neuroprotective effect of β1-blockers, since vasodilation during brain ischemia can be both neuroprotective and detrimental depending on the clinical/experimental settings.

Our intension of the present study was to investigate the direct pharmacological effects of β1-blockade on cerebral microvessels via a physiological approach. Exploratory studies that assess the effects of β1-blockade on neurons and neurological function and the condition in which the vasodilation provide neuroprotection would be beyond the scope of this study.

However, we strongly agree that the lack of neurological evaluation remains a limitation of the present study. Therefore, we have added this limitation to the revised manuscript as follows:

“Third, although we demonstrated the direct local vasodilatory effects of β1-blockade, we did not examine the neuroprotective effect of β1-blockers. The vasodilation of cerebral vessels and subsequent increase in CBF during ischemic brain injuries can be both neuroprotective and harmful [24]. Further exploratory studies are required to assess the effects of β1-blockade on neurons and neurological functions, as well as to determine the conditions in which vasodilation provides neuroprotection, in order to elucidate the neurological outcomes and mechanisms
underlying the neuroprotective effects of β1-blockers reported in the literature.” (Discussion, Line 290)

This study is no novelty and the data is not enough data to support the conclusion.

> Despite its potential clinical and pharmacological impact on the outcomes of ischemic brain injuries, the direct local effects of β1-blockade on cerebral microvasculature have not been investigated. Systemic administration of β-blockers generally affects hemodynamic parameters, such as systemic blood pressure and cardiac output, making it difficult to assess the direct local effects of β1-blockade on the cerebral microvasculature. We therefore used the clinical window method and topical application to evaluate the direct pharmacological effects of β1-blockade on the cerebral microvasculature independently from the systemic conditions and hemodynamic parameters. The present study first demonstrated that the local blockade of β1-adrenergic receptors induces significant vasodilation of the pial arterioles during ischemia/reperfusion injury via the in vivo approach. We believe that findings of the present study would provide new insight into the roles of β1-adrenergic receptors in the ischemic cerebral microvasculature.

To emphasize the strength and novelty of the present study, we have revised the manuscript as follows:

“Despite the potential clinical and pharmacological impact of β1-blockade on the outcomes of ischemic brain injuries, its direct local effects on cerebral microvasculature have not been investigated.” (Background, Line 85)

“In the present study, we first demonstrated that the local blockade of β1-adrenergic receptors leads to vasodilation of pial arterioles especially during ischemia/reperfusion injury.” (Discussion, Line 209)

“we focused on the direct pharmacological effect of β1-blockade on ischemic cerebral microvessels, and we therefore used topical administration instead of systemic administration to evaluate these effects on cerebral microvasculature independently from the systemic conditions and hemodynamic parameters.” (Discussion, Line 285)

Regarding the lack of data that supports the conclusion, we agree that the present results did not directly demonstrate that constrictive microvessels are more susceptible to the vasodilation than intact vessels. We have therefore revised the conclusion as follows:

“By contrast, only a slight dilation of the arterioles was observed in the normal state, indicating that ischemic cerebral microvessels are more susceptible to the vasodilatory effect of the selective blockade of β1-adrenergic receptors compared to normal microvessels.” (Conclusion, Line 301)
Xiaodi Chen, Ph.D., M.D. (Reviewer 3): Comments

In the present study, the authors showed the effect of direct administration of β1 blocker (landiolol) to brain. The brain microvessels were more dilated by landiolol in the ischemic/reperfusion injury group than control group. They concluded that constrictive cerebral microvessels are more susceptible to the vasodilatory effect induced by landiolol. This finding is interesting, but several points need to be addressed to improve this article.

We thank the reviewer for the supportive comments and constructive suggestions. In response to the reviewer’s comments, we have conducted additional experiments and have extensively revised the manuscript.

The authors conducted the direct administration of β1 blocker to brain. Why did you choose this method instead of administrating it from vein systemically? To avoid blood-brain barrier (BBB)? What clinical situation did you assume?

As we discussed in the response to the reviewer 2, systemic administration of β-blockers generally affects hemodynamic parameters, such as systemic blood pressure and cardiac output, making it difficult to assess the direct local effects of β1-blockade on the cerebral microvasculature. We therefore used the clinical topical administration to evaluate the pharmacological effects of β1-blockade on the cerebral microvasculature independently from the systemic conditions and hemodynamic parameters.

We have added this discussion in the revised manuscript as follows:

“We focused on the direct pharmacological effect of β1-blockade on ischemic cerebral microvessels, and we therefore used topical administration instead of systemic administration to evaluate these effects on cerebral microvasculature independently from the systemic conditions and hemodynamic parameters.” (Discussion, Line 285)

Our intention was to investigate the direct pharmacological effects of β1-blockade on the cerebral microvasculature, and we agree that the findings and the methodology of the present study may not be directly applicable to clinical practice at this moment. However, future technological development may enable us to induce β1-blockade selectively in the ischemic brain vessels.

Although clinical application is beyond the scope of the present study, we agree that this remains a limitation of this study. Therefore, we have added this limitation in the revised manuscript as follows:
“Therefore, our findings may not be directly applicable to clinical practice.” (Discussion, Line 289)

Why did the concentration of 10⁻⁶ mol/L produce a peak vasodilatory effect? Isn't landiolol concentration dependent medicine?

Landiolol is certainly a concentration-dependent drug. Based on the molecular weight of landiolol (546), 10⁻⁶ mol/L is equivalent to 0.546 µg/mL. According to an earlier study, the human plasma concentration of landiolol reaches approximately 1 µg/ml when the drug is administered intravenously at a rate of 40 µg/kg/min, the maximum dose for maintenance in clinical settings, for 1 h (Drug Metab. Pharmacokinet., 20:337, 2005). Hence, 10⁻⁶ mol/L (0.546 µg/ml) represents a clinically relevant and high enough concentration, and 10⁻⁴ mol/L represents a very high concentration in the experimental setting. Accordingly, it would be reasonable to attribute the peak vasodilatory effect observed with 10⁻⁶ mol/L to the ceiling effect of this drug at least in the present experimental setting.

We have added this discussion to the revised manuscript as follows:

“In the normal state, landiolol produced the peak vasodilatory effect at a concentration of 10⁻⁶ mol/L. Based on the molecular weight of landiolol (546 g/mol), 10⁻⁶ mol/L is equivalent to 0.546 µg/mL. According to an earlier study, the human plasma concentration of landiolol reaches approximately 1 µg/ml when the drug is administered intravenously at a rate of 40 µg/kg/min, the maximum dose for maintenance in clinical settings, for 1 h [16]. Hence, the concentration of 10⁻⁶ mol/L (0.546 µg/mL) represents a clinically relevant and high enough concentration, while 10⁻⁴ mol/L represents a very high concentration. Accordingly, it would be reasonable to attribute the peak vasodilatory effect observed with 10⁻⁶ mol/L to the ceiling effect of this drug at least in the present experimental setting.” (Discussion, Line 247)

You showed the pial arterioles dilation only in macro image. I think you should do immunostaining for microvessles and show the histological changes in landiolol group (normal brain), landiolol group (I/R brain), and sham group.

How severe is this ischemic/reperfusion injury in your model?

Our intention of the present study is to investigate the pharmacological effects of β1-blockade on cerebral microvessels via a physiological approach. For the assessment of vasodilation, we believe the direct measurement of the vascular diameter on the microscopic view as conducted in the present study would provide enough and better information than histological approaches.
However, as we strongly agree that the confirmation of the structural changes of the vessels and severity of the ischemia/reperfusion is necessary, we have conducted additional experiments.

First, we assessed the vascular permeability using the Evans blue extravasation method. As shown in the revised figure 1, we confirmed the Evans blue leakage from the pial microvessels after ischemia/reperfusion, which indicate the blood-brain barrier disruption after the ischemia/reperfusion in our model.

Revised Figure 1

“Blood–brain barrier disruption after ischemic/reperfusion

Representative images of pial microvessels before and after ischemia/reperfusion. Leakage of Evans blue dye indicates the increased vascular permeability after ischemic/reperfusion.” (Figure legend, Line 417)

“In addition, vascular permeability after ischemia/reperfusion was assessed by Evans blue extravasation [10]. Briefly, 1 mL/kg of 2% Evans blue (Sigma-Aldrich, St. Louis, MO, USA) in normal saline was intravenously administered immediately after the ischemia/reperfusion. Figure 1 shows a representative picture of Evans blue extravasation, confirming the increased vascular permeability caused by the ischemia/reperfusion in this model.” (Methods, Line 141)

We also performed another additional experiment using a laser Doppler flowmeter to measure the cerebral blood flow (Please refer to our response to Reviewer 1). We confirmed that the clamping of the brachiocephalic, left common carotid, and left subclavian arteries significantly decreases the cerebral blood flow by 89.3%.

The results of the additional experiment were included in the revised manuscript as follows:

“To verify brain ischemia/reperfusion in this model, another set of three rabbits were subjected to cerebral blood flow (CBF) measurement. A 1-mm burr hole was made on the parietal bone using a micro drill, and a laser Doppler flowmeter (FLO-C1; Omegaflo, Tokyo, Japan) was inserted perpendicularly to the bone surface. After the Doppler flowmeter was installed, the rabbits were subjected to the 15-min artery clamping. The CBF decreased by 89.3 ± 9.2% (mean ± standard deviation) after clamping, and recovered after unclamping, confirming that the three-vessel clamping induces brain severe brain ischemia and reperfusion.” (Methods, Line 134)

You said systemic hemodynamic parameters were not affected by the topical administration of landiolol. Landiolol can pass the BBB and should affect systemically. Why didn't they affect systemic parameters?
Based on the structural design of the cranial window, we assumed that most of the drug solution infused into the window was drained from the outlet catheter and not absorbed into the systemic circulation. Even if all the solution was absorbed, the average infusion rate used in the present study was 3.3 µg/kg/min (10-4 mM), which is considered equivalent to the adult human dose of 1 µg/kg/min, based on the body surface area (2005 US FDA Guidance, FACEB J. 2008;22:659-61). The infusion rate is smaller than that used in clinical settings (1–125 µg/kg/min), especially for young healthy animals that have no cardiac dysfunction. Because systemic hemodynamic parameters were not affected by the topical administration of landiolol, it appears that landiolol did not affect the systemic condition.

We have added this discussion in the revised manuscript as follows:

“Based on the structural design of the cranial window, we assumed that most of the drug solution infused into the window was drained from the outlet catheter and not absorbed into the systemic circulation. Even if all the solution was absorbed, the average infusion rate used in the present study was 3.3 µg/kg/min (10-4 mM), which is considered equivalent to the adult human dose of 1 µg/kg/min, based on the body surface area [11]. The infusion rate is smaller than that used in clinical settings (1–125 µg/kg/min), especially for young healthy animals that have no cardiac dysfunction. Because systemic hemodynamic parameters were not affected by the topical administration of landiolol, it appears that landiolol did not affect the systemic condition, and the pial vasodilation observed in this study reflects the direct local effects of selective β1-blockade on cerebral microvessels.” (Discussion, Line 212)

Can you show the changes of norepinephrine concentration in blood?

We are afraid we did not measure the blood concentration of norepinephrine. Because landiolol was infused into the cranial window and affected the microvessels extraluminally in the present experimental setting, what affected the vasodilation would be the synaptic concentration of norepinephrine rather than the systemic blood concentration.

Based on the results that showed no significant changes in hemodynamic parameters between the landiolol and control groups, the blood concentration of norepinephrine was not likely to be changed by landiolol.

Can you show the increased vascular permeability of ischemic arteriosus in the present model?

As we described above, the increased vascular permeability after ischemia/reperfusion was confirmed by the Evans blue extravasation.
You said the I/R injury rose susceptibility of landiolol to cerebral microvessles, however, I/R injury may cause change to the β1 receptor. Landiolol is a β1 blocker and you should evaluate the β1 receptor changes in landiolol group (normal brain), landiolol group (I/R brain), and sham group.

We are afraid we did not evaluate β1 receptor changes in the present study. Even if the underlying mechanism is the change in the β1 receptors rather than that in the vascular structures, we believe it is still reasonable to state “ischemic microvessels are more susceptible to the vasodilatory effect of landiolol”, because our results demonstrated that the ischemic microvessels dilate in a greater degree than the intact microvessels after topical administration of landiolol.

As we confirm the increased vascular permeability of the ischemic microvessels via Evans blue extravasation, the structural changes seem to be responsible, at least partly, for the susceptibility of the vessels to the vasodilatory effect of landiolol. However, we agree that there is a possibility that β1 receptor changes due to ischemia/reperfusion are responsible for the susceptibility. Therefore, we have added this discussion to the revised manuscript as follows:

“although the increased vascular permeability of the ischemic microvessels appears to be the responsible mechanism for the increased susceptibility to the vasodilatory effects of landiolol, we could not exclude possible changes in β1 receptors caused by ischemia/reperfusion.”

(Discussion, Line 282)