Foot-and-mouth disease virus induces PERK-mediated autophagy to suppress the antiviral interferon response

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DOI: 10.1242/jcs.240622

Editor: Derek Walsh

Review timeline
Original submission: 16 October 2019
Editorial decision: 27 November 2019
First revision received: 1 May 2020
Accepted: 19 May 2020

Original submission
First decision letter

MS ID#: JOCES/2019/240622

MS TITLE: Foot-and-mouth disease virus induces PERK mediated autophagy to suppress antiviral interferon response


ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers’ reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the ‘Manuscripts with Decisions’ queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using ‘Tracked changes’ in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the ‘Response to Reviewers’ box. Please attend to all of the reviewers’ comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.
Reviewer 1

Advance summary and potential significance to field

In the manuscript by Ranjitha et al., the authors report a new viral evasion mechanism by foot and mouse disease virus (FMDV) in porcine cells. FMDV infection triggers unfolded protein response (UPR) via ER-stress pathway which is used by the virus to induce autophagy. Virus-induced autophagy is required by FMDV replication, analyzed both chemical and genetic inhibition of autophagy pathway. The virus-induced autophagy is utilized by the virus to dampen host interferon (IFN) response. FMDV infection has earlier been shown to be connected with autophagy pathway and the current study added a new element to the existing literature.

Comments for the author

The study is based on a logical theme and the data are supportive of their conclusion. However, a major concern is that the study is entirely based on a porcine cell line and, therefore, some relevance to human system e.g. in human cells, should be explored to validate the results. The authors have largely depended on the chemical inhibitors, which can be non-specific. The relation of UPR with autophagy and/or IFN responses is not genetically connected.

In addition, here are some minor concerns:
1. Fig 1E should be supplemented with immunoblot
2. The discussion is far too long, should be condensed
3. The authors should summarize the findings in a diagram

Reviewer 2

Advance summary and potential significance to field

Several studies have reached differing conclusions about how UPR induction and autophagy impact FMDV replication. This report seeks to clarify those discrepancies using a series of chemical biology and molecular virology approaches. The authors conclude that FMDV induces the UPR and autophagy which facilitates virus replication by suppressing the type I interferon response. While the data in general are of good quality, several inconsistencies cloud the interpretation and prevent the comparison of complementary data obtained by different assays. In addition, the conclusion that suppressing the induction of interferon is the sole reason activation of the UPR is required for FMDV replication is not supported by the data.

Specific issues to be addressed are noted below.

Comments for the author

1) The multiplicity of infection used and the time points chosen are inconsistent throughout manuscript the manuscript. For example, RT-qPCR is performed at 12 hpi after an infection is performed after an MOI of 0.1 at 12 hpi. However changes in protein expression are measured at earlier times after infection at an MOI of 1.0. The changes in UPR and autophagy markers should be measured at consistent times after infection at the same MOI to allow comparison of data from different assays.

2) The change in PERK protein levels does not necessarily indicate increased PERK activity. Can PERK activity be measured directly? Perhaps by measuring autoactivating PERK phosphorylation? In addition, the phosphorylation of the PERK substrate eIF2a-P should be measured by Western blot over timecourse of infection.

3) The authors should include control to show that the BiP inhibitor and ISRIB are working as expected in these cells at these concentrations. The cellular toxicity of the compounds at the concentrations used should also be provided.

4) In the image provided I cannot clearly see double membrane vesicles.
5) Figure 2 and 5 show no increase in LC3B-I and II at 6 hpi by Western blot as compared to uninfected cells, yet Figure 4 shows a large increase LC3B-I and II protein levels in infected cells. What is the nature of this discrepancy?

6) Figure 6 clearly shows a decrease in interferon levels in cells depleted of LC3B. However LC3B could play multiple roles in FMDV replication, as could autophagy. Thus while the induction of autophagy could enhance replication by preventing interferon expression, UPR induction and autophagy could also enhance FMDV replication independent of its effects on interferon. Data showing the effect of autophagy inhibition on virus replication in interferon deficient cells, or in the absence of interferon (e.g in the presence of interferon neutralizing antibody) would address this concern, and provide more substantial support for the author’s conclusion. In the absence of such evidence, the conclusion should be modified.

7) In general, the manuscript would benefit from more extensive proofreading to correct grammatical errors. The authors should also ensure that all statements of previously published facts are accompanied by appropriate references.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Ranjitha and colleagues examines how ER stress pathways, including associated UPR and autophagy, are activated during foot-and-mouth disease virus (FMDV) and how this affects viral replication. This study systematically determined the particular ER stress pathways that lead to enhanced viral replication through complementary approaches and employed the use of commercially available inhibitors to these pathways as controls. This was a well-designed study that has led to a novel finding in the field that the induction of ER stress and autophagy during FMDV infection may be a mechanism used by the virus to dampen the induction of type I and type III interferons and promote replication.

Comments for the author

Major comments:
1. In general, the grammar in the manuscript needs improvement. Often times, sections and sentences were difficult to read and the word choice didn’t always convey the appropriate meaning.
2. Figure 1E represents phosphorylation of eIF2 (p-eIF2) during FMDV infection using confocal microscopy; however, a western blot depicting the ratio of p-eIF2:eIF2 would be more informative.
3. eIF2 doesn’t transcriptionally induce ATF4, rather ATF4 is preferentially translated during p-eIF2. Therefore, it’s unclear why it would be transcriptionally upregulated in response to p-eIF2, as was stated in lines 142-143 of the manuscript.
4. More cells need to be quantified in Figure 3, which shows only one example of double-membrane vesicles formed following FMDV infection. Does this happen in every infected cell? How many DMVs per cell?
5. In figure 4E, the authors show that FMDV induces LC3B-II puncta. However, this figure is confusing for two reasons. The first is that the mock cell shown in Fig. 4E has no puncta, but the quantification in 4F shows that mock cells have an average of 20 puncta/cells. Therefore, there is a discrepancy between the image shown and the quantified data. The authors should show a more representative cell in Fig. 4E. The second is that the characteristic LC3B-II puncta shown in response to FMDV infection don’t look characteristic. Are these puncta similar to those induced by non-virus mediated autophagy? It would be helpful to include this control.

Minor comments:
1. Overall, this manuscript would benefit from increased text detail in the results section, for example Line 143 refers to transcript level data for ATF4, but no reference to a figure is given, and Line 146 gives the MOI used for RT-qPCR and western blotting experiments, but not the microscopy images.
2. In Figure 5D the authors state the viral titer is significantly increased in the Rapamycin treated condition, as compared to the control. However, the titers appear the same at 6h, and at 12h, I’m
not sure if these two samples were directly compared to each other to be able to state significance. Both the text and the figure should be clarified.

3. Many of the autophagy-related statements throughout the results were not referenced and the manuscript would be strengthened with references to several of the facts stated.

First revision

Author response to reviewers' comments

Dear Editor,

Thank you for giving us an opportunity to submit a revised draft of our manuscript titled ‘Foot- and-mouth disease virus induces PERK mediated autophagy to suppress antiviral interferon response’ (JOCES/2019/240622) to Journal of Cell Science.

We are grateful to the reviewers for their insightful comments on our manuscript. We have been able to incorporate changes to reflect most of the suggestions provided by the reviewers. The reviewers’ comments helped us to overall improve the manuscript. The manuscript is revised in the light of comments of the reviewers.

Please find below point by point response to the reviewers’ comments.

Thanking you.

With warm regards.

Sincerely,

Suresh Basagoudanavar

Reviewer 1:

Advance Summary and Potential Significance to Field:

In the manuscript by Ranjitha et al, the authors report a new viral evasion mechanism by foot and mouse disease virus (FMDV) in porcine cells. FMDV infection triggers unfolded protein response (UPR) via ER-stress pathway, which is used by the virus to induce autophagy. Virus-induced autophagy is required by FMDV replication, analyzed both chemical and genetic inhibition of autophagy pathway. The virus-induced autophagy is utilized by the virus to dampen host interferon (IFN) response. FMDV infection has earlier been shown to be connected with autophagy pathway and the current study added a new element to the existing literature.

Comments for the Author:

1) The study is based on a logical theme and the data are supportive of their conclusion. However, a major concern is that the study is entirely based on a porcine cell line and, therefore, some relevance to human system e.g. in human cells, should be explored to validate the results.

Response: We agree with the reviewer that the study is entirely based on a porcine cell line. In the case of our study, it seems slightly out of scope to study in human cells because the natural host of FMD virus is ruminants. Cattle and swine are most susceptible. Humans are not considered susceptible to infection. And FMD is not readily transmissible to humans and is not a public health risk. Further, LFBK cells are highly sensitive for FMDV infection. Therefore, study of FMDV pathogenesis is more relevant in porcine cell line than in human cell lines.

2) The authors have largely depended on the chemical inhibitors, which can be non-specific. The relation of UPR with autophagy and/or IFN responses is not genetically connected.

Response: We agree with this comment. Therefore, in the revised version, we have carried out
genetic inhibition of PERK mediated UPR pathway by using gene knockdown approach, and studied its effect on the viral multiplication, autophagy and interferon levels during FMDV infection. In line with the chemical inhibition, we found that knockdown of PERK results in reduction of FMDV titer, decreased LC3B-II levels, and increased antiviral interferon response (Figure 3).

3) In addition, here are some minor concerns:
1. Fig 1E should be supplemented with immunoblot
Response: We agree with this suggestion. We accordingly carried out western blotting of p-eIF2α protein expression following FMDV infection (1 MOI). Tunicamycin and Thapsigargin treatment were used as positive control for ER stress induction and subsequent phosphorylation of eIF2a. Unfortunately, by using the antibody (Anti-phospho-eIF2α, Ser51, Cat#3398S from Cell Signaling) we could not see band reactivity even with positive control. However, the same antibody when used for immunofluorescence showed intense staining. Therefore, along with confocal microscopy for phospho(Ser51)-eIF2α (using anti- Phospho-eIF2α (Ser51) (D9G8) Rabbit mAb #3398S), we have included supplementary figure of time course of phosphorylation of the PERK substrate eIF2a assessed by immunofluorescence following FMDV infection. Time dependent virus cytopathic effect associated phospho-eIF2α signal was evident (Fig. S1).

2. The discussion is far too long, should be condensed
Response: As per the suggestion, we have now revised the discussion and tried to keep it condensed.

3. The authors should summarize the findings in a diagram
Response: Thank you for the suggestion. In the revised MS, we have included Figure 8, a diagram representing ER stress/PERK mediated autophagy activation and its effect on interferon production during FMDV infection to emphasize our study.

Reviewer 2:

Advance Summary and Potential Significance to Field:

Several studies have reached differing conclusions about how UPR induction and autophagy impact FMDV replication. This report seeks to clarify those discrepancies using a series of chemical biology and molecular virology approaches. The authors conclude that FMDV induces the UPR and autophagy, which facilitates virus replication by suppressing the type I interferon response. While the data in general are of good quality, several inconsistencies cloud the interpretation and prevent the comparison of complementary data obtained by different assays. In addition, the conclusion that suppressing the induction of interferon is the sole reason activation of the UPR is required for FMDV replication is not supported by the data. Specific issues to be addressed are noted below.

Comments for the Author:

1) The multiplicity of infection used and the time points chosen are inconsistent throughout manuscript the manuscript. For example, RT-qPCR is performed at 12 hpi after an infection is performed after an MOI of 0.1 at 12 hpi. However changes in protein expression are measured at earlier times after infection at an MOI of 1.0. The changes in UPR and autophagy markers should be measured at consistent times after infection at the same MOI to allow comparison of data from different assays.
Response: Thank you for the suggestion. In the revised version, we carried out RT-qPCR experiment with 1 MOI at 6 hpi (Figures 1B and 5A). Also, changes in UPR and autophagy markers are measured at consistent times after infection at the same MOI.

2) The change in PERK protein levels does not necessarily indicate increased PERK activity. Can PERK activity be measured directly? Perhaps by measuring autoactivating PERK phosphorylation? In addition, the phosphorylation of the PERK substrate eIF2a-P should be measured by Western blot over time course of infection.
Response: We agree with the reviewer. As per the suggestion we accordingly carried out western blotting of p-PERK and p-eIF2α protein expression following FMDV infection (1 MOI). Tunicamycin
and Thapsigargin treatment were used as positive controls. As mentioned above, unfortunately, in the western blotting, by using the antibody (Anti- phospho-PERK, Thr980, Cat# 3179S from Cell Signaling; Anti-phospho-eIF2a, Ser51, Cat#3398S from Cell Signaling) we could not see band reactivity even with positive control. Therefore, the data could not be included in the revision. However, of the two, the anti-phospho-eIF2a antibody when used for immunofluorescence showed staining. The data is included as Supplementary Fig 1. The FMDV infection time dependent phosphorylation of eIF2a (PERK substrate), clearly demonstrates the activation of PERK pathway. Further, by knockdown of PERK gene, we show that downstream LC3 lipidation and virus multiplication were affected (Fig.3). These support the conclusion drawn in this study.

3) **The authors should include control to show that the BiP inhibitor and ISRIB are working as expected in these cells at these concentrations. The cellular toxicity of the compounds at the concentrations used should also be provided.**
   Response: We agree with the reviewer and have incorporated the suggestion in supplementary figure S2 which shows in vitro cytotoxicity of drugs- BiP inhibitor (VER 155008) and ISRIB. The drugs were not toxic to the cells at the concentration utilized in the study.

4) **In the image provided I cannot clearly see double membrane vesicles.**
   Response: In the revised MS, we have replaced Figure 4C which is more magnified than the earlier one. We can appreciate the double membranous nature of the vesicles (indicated by arrows).

5) **Figure 2 and 5 show no increase in LC3B-I and II at 6 hpi by Western blot as compared to uninfected cells, yet Figure 4 shows a large increase LC3B-I and II protein levels in infected cells. What is the nature of this discrepancy?**
   Response: There is definitely increase in the level of LC3B-II at 6 hpi, showing the induction of autophagy (in Fig.2 and Fig.5 (Fig. 6 in the revised MS). The discrepancy observed by the reviewer could be due to differences in the amount of loading of samples between the experiments. Nevertheless, the conclusions drawn in the study remain unaffected.

6) **Figure 6 clearly shows a decrease in interferon levels in cells depleted of LC3B. However LC3B could play multiple roles in FMDV replication, as could autophagy. Thus while the induction of autophagy could enhance replication by preventing interferon expression, UPR induction and autophagy could also enhance FMDV replication independent of its effects on interferon. Data showing the effect of autophagy inhibition on virus replication in interferon deficient cells, or in the absence of interferon (e.g in the presence of interferon neutralizing antibody) would address this concern, and provide more substantial support for the author’s conclusion. In the absence of such evidence, the conclusion should be modified.**
   Response: Thank you for this suggestion. It is definitely interesting to explore this aspect. As of now, we do not know the exact mechanism involved in the modulation of innate immunity by the FMDV induced autophagy. We plan to carry out further studies in this direction in future. Our data clearly shown that the inhibition of autophagy enhances the antiviral IFN levels. As discussed in the MS, possibly FMDV induced autophagy may be interfering with the RIG- mediated IFN production, as we show the levels of Atg5-Atg12 increasing with the infection (Fig. 5). It is previously reported that Atg5-12 conjugate associates with the CARD domain of the RIG-I and IFN-β promoter impairing their interaction (Jounai et al., 2007). Further, by PERK (UPR) gene knockdown, we show that autophagy marker LCB lipidation was affected while an enhanced IFN-β and IFN-α3 proteins were observed with reduced viral titre. These data make us believe that UPR induction and autophagy play role in FMDV replication by interfering with interferon production.

7) **In general the manuscript would benefit from more extensive proofreading to correct grammatical errors. The authors should also ensure that all statements of previously published facts are accompanied by appropriate references.**
   Response: Thank you for bringing our attention about grammatical errors. The issue has been addressed in the revised MS. Also we have included additional appropriate references wherever necessary.

**Reviewer 3:**
**Advance Summary and Potential Significance to Field:**
The manuscript by Ranjitha and colleagues examines how ER stress pathways, including associated UPR and autophagy, are activated during foot-and-mouth disease virus (FMDV) and how this
affects viral replication. This study systematically determined the particular ER stress pathways that lead to enhanced viral replication through complementary approaches and employed the use of commercially available inhibitors to these pathways as controls. This was a well-designed study that has led to a novel finding in the field that the induction of ER stress and autophagy during FMDV infection may be a mechanism used by the virus to dampen the induction of type I and type III interferons and promote replication.

Comments for the Author: Major comments:

1. In general, the grammar in the manuscript needs improvement. Often times, sections and sentences were difficult to read and the word choice didn’t always convey the appropriate meaning.

Response: We regret for the poor presentation in the first submission. We have now revised the English grammatical errors and phrases. We hope that the revised manuscript addresses the concerns raised by the reviewer and is now easy for understanding by the readers.

2. Figure 1E represents phosphorylation of eIF2 (p-eIF2) during FMDV infection using confocal microscopy; however, a western blot depicting the ratio of p-eIF2:eIF2 would be more informative.

Response: We agree with the suggestion. However, as mentioned in response to reviewer 1, we had difficulty in getting westernblots for p-eIF2. But using the same antibody we are showing virus cytotoxic effect and time-dependent p-eIF2 expression by using immunofluorescence (Suppl. Fig. S1), along with confocal microscopy for phospho(Ser51)-eIF2α (Fig. 1).

3. eIF2 doesn’t transcriptionally induce ATF4, rather ATF4 is preferentially translated during p-eIF2. Therefore, it’s unclear why it would be transcriptionally upregulated in response to p-eIF2, as was stated in lines 142-143 of the manuscript.

Response: We agree that ATF4 is preferentially translated during p-eIF2, following the repression of global protein. It was reported previously that ATF4 mRNA transcript levels are elevated in response to ER stress (Harding et al., 2000; Lu et al., 2004; Adachi et al., 2008; Dey et al., 2010), as well as in response to amino acid starvation (Siu et al., 2002). Based on this, we assume that ATF4 is regulated at transcriptional level, apart from its translational control. Therefore, we have now modified the sentence in the discussion to read as ‘The transcript level of ATF4 was increased significantly (p<0.01) at 6 hpi, the time point at which enhanced phosphorylation of eIF2α was observed’ at 6 hpi (Fig. 1E).

4. More cells need to be quantified in Figure 3, which shows only one example of double-membrane vesicles formed following FMDV infection. Does this happen in every infected cell? How many DMVs per cell?

Response: The graph of the quantification of the number of autophagosome vesicles per cell in mock and FMDV infected LFBK cells is included in Figure 4D. Counting was obtained from 10 each of mock and FMDV infected cells. 23-33 DMVs in the range of could be observed per cell.

5. In figure 4E, the authors show that FMDV induces LC3B-II puncta. However, this figure is confusing for two reasons. The first is that the mock cell shown in Fig. 4E has no puncta, but the quantification in 4F shows that mock cells have an average of 20 puncta/cells. Therefore, there is a discrepancy between the image shown and the quantified data. The authors should show a more representative cell in Fig. 4E. The second is that the characteristic LC3B-II puncta shown in response to FMDV infection don’t look characteristic. Are these puncta similar to those induced by non-virus mediated autophagy? It would be helpful to include this control.

Response: Thank you for highlighting this. We understand the confusion regarding the LC3B-II punctae. In the revised MS, the number of punctae are recounted and included the modified graph (Figure 5F). The confocal microscopy of FMDV-infected LFBK cells showed an increase in the level of endogenous LC3B. The yellow punctae due to the colocalization /association of FMDV with the LC3B-II autophagosomes and increase in the number of LC3B-II red punctae indicates the induced autophagy is due to FMD virus (Figure 5E). Rapamycin treatment was used as the control for autophagy. It produced similar punctae (Included in the revised MS, as supplementary Fig. S3).

Minor comments:

1. Overall this manuscript would benefit from increased text detail in the results section, for example Line 143 refers to transcript level data for ATF4, but no reference to a figure is given,
and Line 146 gives the MOI used for RT-qPCR and western blotting experiments, but not the microscopy images.
Response: As suggested, the corrections are included in the revised MS. Previously we had used different MOI for RT-qPCR (0.01 MOI) and western blotting and microscopy (1 MOI). However, as per the suggestion of Reviewer 2, we now carried out experiments with uniformly 1 MOI throughout, to allow comparison of data from different assays. The same is now revised.

2. In Figure 5D the authors state the viral titer is significantly increased in the Rapamycin treated condition, as compared to the control. However, the titers appear the same at 6h, and at 12h, I’m not sure if these two samples were directly compared to each other to be able to state significance. Both the text and the figure should be clarified.
Response: In Fig. 5D (which is Fig 6D in the revised MS), at 12 h, rapamycin treated and infected cells showed increase in the viral yield. The rapamycin treated sample was compared to FMDV infected cells at 12 h. The increase was significant with p= 0.0368. The same is now clarified in the text and figure in the revised MS.

3. Many of the autophagy-related statements throughout the results were not referenced and the manuscript would be strengthened with references to several of the facts stated.
Response: As per the suggestion, where necessary additional references are included.

Second decision letter

MS ID#: JOCES/2019/240622

MS TITLE: Foot-and-mouth disease virus induces PERK mediated autophagy to suppress antiviral interferon response

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The study reports a new molecular mechanism by which the FMDV induces autophagy to suppress cellular antiviral response.

Comments for the author

The authors addressed all of my comments and the study is suitable for publication.