Peer-Review Record

Protein homeostasis in the aged and diseased heart

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by Nirjal Mainali, Srinivas Ayyadevara, Akshatha Ganne, Robert J. Shmookler Reis, Jawahar L. Mehta

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Academic Editor: Junichi Sadoshima

Reviewer 1: Anonymous

Reviewer 2: Anonymous

Round 1

Reviewer 1 Report

Overall comment:
This mini-review manuscript highlighted some aspects of recent advances under the broad title “Protein homeostasis in the aged and diseased heart”, which is certainly an important topic appropriate for this journal.

Unfortunately, a good part of the manuscript is not well written and needs substantial revision to be publishable. The main weaknesses include: (1) missing a large proportion of relevant literature on impaired proteostasis in diseased hearts; (2) many statements are conceptually misleading; (3) for the most part, reviews rather than original research articles are cited and, making it even worse, the cited reviews are often quite old, missing newer state-of-the-art reviews that are written by scientists who have made significant original contributions to the topic; (4) in general, this manuscript lacks depth or mechanistic delineation.

Specific points:
1. Line 30-31, The first half of the first sentence of the Abstract: “Protein homeostasis, the balance between protein synthesis and turnover, requires the clearance of misfolded and aggregated proteins, ...” has several issues. First, “turnover” should be replaced by “degradation” as here “turnover” implies a dynamic process that includes both protein synthesis and degradation; second, it is too simplistic to define “protein homeostasis” or “proteostasis” as “the balance between protein synthesis and turnover” because protein quality control (as stated by the subsequent phrase) and even protein trafficking are also part of the proteostasis that is not covered by the definition.

2. Line 49, it is at least quite debatable to list heredity and genetics in parallel as they overlap a lot if not completely superimpose.

3. It is quite abrupt and puzzling why among hundreds of CVD-associated genetic variants, only APOE variants are highlighted in the Introduction.

4. Line 73-74, The statement that "In addition to assisting in refolding, the ERAD pathway helps to limit misfolding by halting or reducing protein synthesis [10]" is apparently incorrect. ERAD is only responsible for degradation of the misfolded proteins that are retro-translocated from ER lumen to the cytosolic side. ERAD here should be replaced with UPR.

5. Line 73-76, the statement that "When both the UPR and ERAD pathways cannot control the accumulation of misfolded proteins, toxic aggregates must be degraded by either or both of the main clearance mechanisms: the ubiquitin-proteosome system (UPS) and autophagy" is also confusing and illogical. Because the UPS and autophagy are part of the ERAD.

6. In many places throughout the manuscript, "proteasome" is misspelled as "proteosome".

7. Line 79. "UPS is a highly regulated process that degrades misfolded, oxidized, or disordered proteins." This is an incomplete statement and can be very misleading because the UPS also degrades perfectly normal but no longer needed proteins in the cell to regulate virtually all cellular functions/processes.

8. Line 79-80, in the sentence that "UPS recognizes proteins in all domains of the cell including membranes, nucleus, cytoplasm, and ER lumen". The inclusion of "ER lumen" here is wrong unless the UPS were recently identified in the ER lumen. If so, please cite the original reference for that. As far as this reviewer knows, the UPS does not exist inside the ER, which is why misfolded ER proteins must be retrotranslocated to the cytosolic side for degradation. There are ubiquitin E3s associated with ER membranes but not in the lumen and proteasomes are outside of the ER as well.

9. Line 85-86, "There are >1000 substrate-specific E3 ligases, ...". This is not accurate, the human genome encodes approximately 600 ubiquitin ligases based on the known molecular features of ligases (HECT, RING, U-box). Indeed, some papers claimed "Over 1000 E3s", which is not vigorous because they counted every component of a E3 complex as a E3. For example, Cullin is only a scaffold protein in Cullin-RING ligases, not a E3 but some people count them as E3's, which apparently is incorrect and reflects a misunderstanding of how ubiquitination works.

10. Not all poly-ubiquitinated proteins are destined for degradation. Ubiquitination has many non-proteolytic fates.

11. Line 91-93, “Cardiac-specific E3 ligases such as atrogin-1, the muscle ring finger (MuRF) family, and C-terminal HSP70-interacting protein (CHIP), assist in the conjugation of ubiquitin to substrates ....” None of the E3s listed here are cardiac-specific; atrogin-1 and MuRF are also expressed in at least skeletal muscle whereas CHIP is expressed in virtually all cell types in the body.

12. Line 94, “Proteasome structure also differs between cardiac and other tissues.” In general, this is a very bold statement without citing much supporting evidence. If you consider differences that are as minute as a post-translational modification (e.g., phosphorylation, acetylation) on individual proteasome subunits, this statement may arguably be true, but proteasome structure in eukaryotes or at least in mammals is highly conserved, just as you stated in the subsequent description.

13. Line 119, “Cardiomyocytes, the contractile muscle cells of the heart ...” Are there any normal muscle cells that are not contractile?

14. Line 122-123, The sentence that “Cardiomyocytes in tissues from animal models predisposed to heart failure contain autophagic vacuoles and degraded mitochondria” is meaningless because the stated features can be found in normal cardiomyocytes as well.

15. Line 124-126, the claims made here should be supported by References.

16. In the context of the passage (Line 131-133), the references (Ref #18-22) cited for the statement that “Several studies have shown that a cascade of cellular-stress events can alter the machinery of UPS and/or autophagy, resulting in their disrupted clearance functions and the intracellular accumulation of misfolded proteins [18-22]” are, for the most part, irrelevant or even inappropriate. This is because you are talking about “myocardial ischemia and heart failure”, but Ref #18 is apparently about colon cancer cells, #19 is a general review article and unlikely emphasizes CVDs, #20 is about ophthalmic diseases, #21 appears to be a general review article and not CV specific, and only #22 appears to be centered in CVDs. In fact, there are a lot of elegant original reports on dysregulation of the UPS and autophagy as well as accumulation of misfolded proteins in ischemic heart disease or heart failure that could and should be cited to support the points here. For example, you may search the publications from Dr. Xuejun Wang (South Dakota) and Dr. Junichi Sadoshima (New Jersey) groups for both original research and high-quality review articles regarding respectively the UPS and autophagy in CVDs including myocardial ischemia and heart failure.

17. When talking about proteostasis, especially protein quality control and degradation, aberrant protein aggregation, and proteotoxicity in diseased hearts, it is simply strange that not even a single original research article or even a review article from some of the pioneers and/or current leaders of this field (e.g., Xuejun Wang, Junichi Sadoshima, ...) was cited.

18. Line 218, myocardial infarction is a type of ischemic injury; so, the two terms should not be listed equally in parallel.

19. Line 219-220, cardiac remodeling also encompasses changes in the non-cardiomyocyte compartment of myocardium; hence, the description of cardiac remodeling here needs to be revised to be more inclusive and accurate.

20. In Figure 2, which meant to illustrate "Compromised protein homeostasis associated with cardiovascular disease and aging can be improved by targeting aggregate proteins". Here for terminally misfolded proteins, a main and least harmful fate is to be degraded by the UPS before they have a chance to form aberrant aggregates; only to the latter, autophagy becomes relevant. The illustration completely misses the role of UPS in dealing with misfolded proteins. This should be added.

Author Response

We would like to thank the reviewers for their exceptionally detailed and helpful critiques. Most of the comments have resulted in improvements to the paper. Our detailed response to critiques is as follows.

Overall comment: This mini-review manuscript highlighted some aspects of recent advances under the broad title "Protein homeostasis in the aged and diseased heart", which is certainly an important topic appropriate for this journal. Unfortunately, a good part of the manuscript is not well written and needs substantial revision to be publishable. The main weaknesses include:

(1)missing a large proportion of relevant literature on impaired proteostasis in diseased hearts;

We apologize for the lack of depth in citing the literature and we have taken your advice and revised the paper to make it more comprehensive in covering the relevant literature.

(2)many statements are conceptually misleading;

The reviewer's point is well taken and the statements and sentences that are misleading are now removed or modified.

(3)for the most part, reviews rather than original research articles are cited and, making it even worse, the cited reviews are often quite old, missing newer state-of-the-art reviews that are written by scientists who have made significant original contributions to the topic;

We have added the relevant references as suggested by the reviewer, and added additional details to the text also for clarity.

(4)in general, this manuscript lacks depth or mechanistic delineation.

The latest version includes these details.

Specific points:
1. Line 30-31, The first half of the first sentence of the Abstract: “Protein homeostasis, the balance between protein synthesis and turnover, requires the clearance of misfolded and aggregated proteins, ...” has several issues. First, “turnover” should be replaced by “degradation” as here “turnover” implies a dynamic process that includes both protein synthesis and degradation; second, it is too simplistic to define “protein homeostasis” or “proteostasis” as “the balance between protein synthesis and turnover” because protein quality control (as stated by the subsequent phrase) and even protein trafficking are also part of the proteostasis that is not covered by the definition.

We regret that we were not clear in our statements, we now replace turnover with degradation as suggested by the reviewer and also discussed protein trafficking in the protein homeostasis.

2. Line 49, it is at least quite debatable to list heredity and genetics in parallel as they overlap a lot if not completely superimpose.

We agree with the reviewer and have now deleted heredity from the sentence.

3. It is quite abrupt and puzzling why among hundreds of CVD-associated genetic variants, only APOE variants are highlighted in the Introduction.

We wanted to highlight the APOE genetic variants as it is implicated in protein aggregation and is enriched and contributes to aggregation in several neurodegenerative diseases as mentioned in previous research as seen in past publications.

4. Line 73-74, The statement that “In addition to assisting in refolding, the ERAD pathway helps to limit misfolding by halting or reducing protein synthesis [10]” is apparently incorrect. ERAD is only responsible for degradation of the misfolded proteins that are retro-translocated from ER lumen to the cytosolic side. ERAD here should be replaced with UPR.

We agree with Reviewer and now deleted ERAD and mentioned UPR and gave the references also. We wanted to make a statement based on the earlier research on both UPR and ERAD are how closely coordinated.

5. Line 73-76, the statement that “When both the UPR and ERAD pathways cannot control the accumulation of misfolded proteins, toxic aggregates must be degraded by either or both of the main clearance mechanisms: the ubiquitin-proteosome system (UPS) and autophagy” is also confusing and illogical. Because the UPS and autophagy are part of the ERAD.

Sorry for not making our point clear, we now rephrased the sentence.

6. In many places throughout the manuscript, “proteasome” is misspelled as “proteosome”.

We are sorry and corrected the misspelled word.

7. Line 79. “UPS is a highly regulated process that degrades misfolded, oxidized, or disordered proteins.” This is an incomplete statement and can be very misleading because the UPS also degrades perfectly normal but no longer needed proteins in the cell to regulate virtually all cellular functions/processes.

Thanks for your valuable comments. We have amended the sentence accordingly.

8. Line 79-80, in the sentence that “UPS recognizes proteins in all domains of the cell including membranes, nucleus, cytoplasm, and ER lumen”. The inclusion of “ER lumen” here is wrong unless the UPS were recently identified in the ER lumen. If so, please cite the original reference for that. As far as this reviewer knows, the UPS does not exist inside the ER, which is why misfolded ER proteins must be retrotranslocated to the cytosolic side for degradation. There are ubiquitin E3s associated with ER membranes but not in the lumen and proteasomes are outside of the ER as well.

We apologize for the error, we have corrected and revised the phrase.

9. Line 85-86, “There are >1000 substrate-specific E3 ligases, ...”. This is not accurate, the human genome encodes approximately 600 ubiquitin ligases based on the known molecular features of ligases (HECT, RING, U-box). Indeed, some papers claimed “Over 1000 E3s”, which is not vigorous because they counted every component of a E3 complex as a E3. For example, Cullin is only a scaffold protein in Cullin-RING ligases, not a E3 but some people count them as E3’s, which apparently is incorrect and reflects a misunderstanding of how ubiquitination works.

We are thankful for the comprehensive information and now edited the sentence to represent the correct substrate-specific E3 ligases.

10. Not all poly-ubiquitinated proteins are destined for degradation. Ubiquitination has many non-proteolytic fates.

We agree with the reviewer that the poly ubiquitination not always leads to degradation and especially in cancer cells it is useful for stabilizing the proteins. Our intention is to just emphasize the fact that polyubiquitin tag is critical in protein degradation.

11. Line 91-93, “Cardiac-specific E3 ligases such as atrogin-1, the muscle ring finger (MuRF) family, and C-terminal HSP70-interacting protein (CHIP), assist in the conjugation of ubiquitin to substrates ....” None of the E3s listed here are cardiac-specific; atrogin-1 and MuRF are also expressed in at least skeletal muscle whereas CHIP is expressed in virtually all cell types in the body.

We have edited the sentence and deleted cardiac from the sentence.

12. Line 94, “Proteasome structure also differs between cardiac and other tissues.” In general, this is a very bold statement without citing much supporting evidence. If you consider differences that are as minute as a post-translational modification (e.g., phosphorylation, acetylation) on individual proteasome subunits, this statement may arguably be true, but proteasome structure in eukaryotes or at least in mammals is highly conserved, just as you stated in the subsequent description.

The sentence Proteasome structure also differs between cardiac and other tissues has now been deleted.

13. Line 119, “Cardiomyocytes, the contractile muscle cells of the heart ...” Are there any normal muscle cells that are not contractile?

We agree and have deleted the word contractile from the sentence.

14. Line 122-123, The sentence that “Cardiomyocytes in tissues from animal models predisposed to heart failure contain autophagic vacuoles and degraded mitochondria” is meaningless because the stated features can be found in normal cardiomyocytes as well.

Previous phrase has been revised to new phrase.

15. Line 124-126, the claims made here should be supported by References.

We have added the reference.

16. In the context of the passage (Line 131-133), the references (Ref #18-22) cited for the statement that “Several studies have shown that a cascade of cellular-stress events can alter the machinery of UPS and/or autophagy, resulting in their disrupted clearance functions and the intracellular accumulation of misfolded proteins [18-22]” are, for the most part, irrelevant or even inappropriate. This is because you are talking about “myocardial ischemia and heart failure”, but Ref #18 is apparently about colon cancer cells, #19 is a general review article and unlikely emphasizes CVDs, #20 is about ophthalmic diseases, #21 appears to be a general review article and not CV specific, and only #22 appears to be centered in CVDs. In fact, there are a lot of elegant original reports on dysregulation of the UPS and autophagy as well as accumulation of misfolded proteins in ischemic heart disease or heart failure that could and should be cited to support the points here. For example, you may search the publications from Dr. Xuejun Wang (South Dakota) and Dr. Junichi Sadoshima (New Jersey) groups for both original research and high-quality review articles regarding respectively the UPS and autophagy in CVDs including myocardial ischemia and heart failure.

We are sorry and the original and key contributing articles to the field are now cited.

17. When talking about proteostasis, especially protein quality control and degradation, aberrant protein aggregation, and proteotoxicity in diseased hearts, it is simply strange that not even a single original research article or even a review article from some of the

pioneers and/or current leaders of this field (e.g., Xuejun Wang, Junichi Sadoshima, ...) was cited.

We apologize again for the omission of some of the seminal papers which have now been added in this section.

18. Line 218, myocardial infarction is a type of ischemic injury; so, the two terms should not be listed equally in parallel.

We agree and replaced Myocardial infarction with ischemic injury.

19. Line 219-220, cardiac remodeling also encompasses changes in the non-cardiomyocyte compartment of myocardium; hence, the description of cardiac remodeling here needs to be revised to be more inclusive and accurate.

We now added non-cardiomyocyte details in the cardiac remodeling section.

20. In Figure 2, which meant to illustrate "Compromised protein homeostasis associated with cardiovascular disease and aging can be improved by targeting aggregate proteins". Here for terminally misfolded proteins, a main and least harmful fate is to be degraded by the UPS before they have a chance to form aberrant aggregates; only to the latter, autophagy becomes relevant. The illustration completely misses the role of UPS in dealing with misfolded proteins. This should be added.

We would like to thank the reviewer for their insightful and detailed critique and now the Figure 2 is modified to include the UPS mediated degradation before the aggregation process.

Reviewer 2 Report

This review article from Mehta and colleagues addresses an important topic in cardiac aging, i.e. protein quality control and its relation to cardiac dysfunction. The paper provides a general overview of the most salient areas of protein homeostasis. Unfortunately, however, the manuscript lacks sufficient depth and detailed mechanistic descriptions that can provide new insights and pose next questions for the audience and future investigation, which is a major limitation.

Author Response

This review article from Mehta and colleagues addresses an important topic in cardiac aging, i.e. protein quality control and its relation to cardiac dysfunction. The paper provides a general overview of the most salient areas of protein homeostasis. Unfortunately, however, the manuscript lacks sufficient depth and detailed mechanistic descriptions that can provide new insights and pose next questions for the audience and future investigation, which is a major limitation.

We have added to the text a more comprehensive description addressing the mechanistic details and added more details and cited relevant publications.

Round 2

Reviewer 1 Report

The revision is responsive and has significantly improved the manuscript, but there are still some minor but important issues that need to be addressed (see below).

1. Line 70-71, The statement that "The ERAD pathway helps to limit misfolding by halting or reducing protein synthesis" remains misleading. ERAD is only responsible for degradation of the misfolded proteins that are retro-translocated from ER lumen to the cytosolic side. The word "ERAD" here should be replaced with "UPR".

2. Line 101 -103: "The 19S regulatory particle may also be replaced by an 11S regulatory particle in mouse heart, complexed with the 19S catalytic core particle (17). The resulting 20S-11S complex appears to improve the catabolism of many cardiac substrates (17)." First, in the above two sentences, the second "19S" should be replaced with "20S". Second, here the cited reference (Reference 17) is again a review article written by some someone who did nothing on the stated discoveries. The main original research articles that reported the discoveries here are Li J et al. FASEB J. 2011 Mar (PMID: 21098724) and Li J et al. J Clin Invest. 2011 Sep (PMID: 21841311), and they should be cited, besides Ref. #17.

3. Line 127-130: "Mice with myocardial infarction (MI) were found to have reduced autophagic flux and mitochondrial respiration, relative to sham-operated mice. This decrease in flux results in an accumulation of damaged organelles, poluubiquitinated proteins, and spheroidal (more rounded) mitochondria (39)." First, "poluubiquitinated" misspelled. More importantly, the first study that rigorously measured myocardial autophagic flux in mice post-MI is reported by Wu P, et al. (PMID: 28694354), which shows myocardial autophagic flux is increased post-MI and this increase is protective because blocking it via CtsD haploinsufficiency is detrimental. The study (Ref. #39) cited by the author actually investigated a reversible heart failure model, wherein pressure overload by transaortic constriction superimposed on acute coronary artery (MI), not just a simple MI model.

4. Reference # 61 cited in the revised manuscript actually delineates a molecular pathway (Calcineurin-TFEB-p62) mediating the activation of autophagy by proteasome malfunction in mouse hearts, which belongs to the added Section "Crosstalk and cooperation between UPS and autophagy" (Line 134-154). Indeed, on one hand, p62 hinders the degradation of ubiquitinated proteins by the proteasome when autophagy is impaired (Refs #53 and 54); but on the other hand, p62 mediates the activation of autophagy by the proteasome malfunction/inhibition (Ref. #61).

Author Response

We would like to thank the reviewers for their detailed and helpful critiques. Most of the comments have resulted in improvements to the manuscript. Our detailed response to critiques are highlighted in the manuscript, and follows below.

The revision is responsive and has significantly improved the manuscript, but there are still some minor but important issues that need to be addressed (see below).

We thank the reviewer for the positive comments.

1. Line 70-71, The statement that "The ERAD pathway helps to limit misfolding by halting or reducing protein synthesis" remains misleading. ERAD is only responsible for degradation of the misfolded proteins that are retro-translocated from ER lumen to the cytosolic side. The word "ERAD" here should be replaced with "UPR".

ERAD is now replaced by UPR in the manuscript.

2. Line 101 -103: "The 19S regulatory particle may also be replaced by an 11S regulatory particle in mouse heart, complexed with the 19S catalytic core particle (17). The resulting 20S-11S complex appears to improve the catabolism of many cardiac substrates (17)." First, in the above two sentences, the second "19S" should be replaced with "20S". Second, here the cited reference (Reference 17) is again a review article written by some someone who did nothing on the stated discoveries. The main original research articles that reported the discoveries here are Li J et al. FASEB J. 2011 Mar (PMID: 21098724) and Li J et al. J Clin Invest. 2011 Sep (PMID: 21841311), and they should be cited, besides Ref. #17.

We are sorry for the omission of these seminal papers, these are now included in the manuscript.

3. Line 127-130: "Mice with myocardial infarction (MI) were found to have reduced autophagic flux and mitochondrial respiration, relative to sham-operated mice. This decrease in flux results in an accumulation of damaged organelles, poluubiquitinated proteins, and spheroidal (more rounded) mitochondria (39)." First, "poluubiquitinated" misspelled. More importantly, the first study that rigorously measured myocardial autophagic flux in mice post-MI is reported by Wu P, et al. (PMID: 28694354), which shows myocardial autophagic flux is increased post-MI and this increase is protective because blocking it via CtsD haploinsufficiency is detrimental. The study (Ref. #39) cited by the author actually investigated a reversible heart failure model, wherein pressure overload by transaortic constriction superimposed on acute coronary artery (MI), not just a simple MI model.

We thank the reviewer for these suggestions which are now included in the manuscript.

4. Reference # 61 cited in the revised manuscript actually delineates a molecular pathway (Calcineurin-TFEB-p62) mediating the activation of autophagy by proteasome malfunction in mouse hearts, which belongs to the added Section "Crosstalk and cooperation between UPS and autophagy" (Line 134-154). Indeed, on one hand, p62 hinders the degradation of

ubiquitinated proteins by the proteasome when autophagy is impaired (Refs #53 and 54); but on the other hand, p62 mediates the activation of autophagy by the proteasome malfunction/inhibition (Ref. #61).

We now moved the reference to the cross talk between UPS and autophagy.

Reviewer 2 Report

Comments:
1. The revised review is improved but still lacks depth of discussion, specifically related to how these protein homeostatic processes contribute to cardiac homeostasis, and heart disease and failure. For example, the authors provide a one-page explanation about how UPS works, and another half-page describing crosstalk between UPS and autophagy, but do not discuss how perturbation of UPS impacts baseline heart function or cardiac stress responses and heart failure. This seems to be important considering the topic of the review.

2. Similarly, hypoxia and oxidative stress and their impact on cardiac injury and dysfunction are discussed, yet the connection between mechanisms that regulate protein homeostasis and hypoxia or oxidative stress is not addressed. This detracts from the cohesion of the review and somewhat distracts from the main focus of the paper.

3. The same is true for the ER stress and mitochondrial stress sections - the discussion of relevant cardiac studies and findings is very limited (1-2 sentences), and no conclusions or critiques are presented by the authors. Consequently, the review could be strengthened by adding discussion and citation of more primary studies to provide a clearer and more inclusive assessment of the field that the audience will appreciate.

4. No figure legends are provided for either Figure 1 or 2.

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Comments:
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We agree with the reviewer and incorporated a discussion on how these pathways impact heart disease.

2. Similarly, hypoxia and oxidative stress and their impact on cardiac injury and dysfunction are discussed, yet the connection between mechanisms that regulate protein

homeostasis and hypoxia or oxidative stress is not addressed. This detracts from the cohesion of the review and somewhat distracts from the main focus of the paper.

We now included discussion of how these impact heart function.

3. The same is true for the ER stress and mitochondrial stress sections - the discussion of relevant cardiac studies and findings is very limited (1-2 sentences), and no conclusions or critiques are presented by the authors. Consequently, the review could be strengthened by adding discussion and citation of more primary studies to provide a clearer and more inclusive assessment of the field that the audience will appreciate.

Thanks for the valuable suggestion, we now expanded the discussion on ER and mitochondrial stress.

4. No figure legends are provided for either Figure 1 or 2.

The figure legend is now added and highlighted in the manuscript.