Biochemistry

Rapid Report

pubs.acs.org/biochemistry

Olive Component Oleuropein Promotes β-Cell Insulin Secretion and Protects β-Cells from Amylin Amyloid-Induced Cytotoxicity

Ling Wu,[†] Paul Velander,[†] Dongmin Liu,[‡,§,⊥] and Bin Xu*[,†,‡,§,∥]

[†]Department of Biochemistry, [‡]Center for Drug Discovery, [§]Translational Obesity Research Center, [∥]School of Neuroscience, and [⊥]Department of Human Nutrition, Foods, and Exercise, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: Oleuropein, a natural product derived from olive leaves, has reported anti-diabetic functions. However, detailed molecular mechanisms for how it affects β-cell functions remain poorly understood. Here, we present evidence that oleuropein promotes glucose-stimulated insulin secretion (GSIS) in β-cells. The effect is dose-dependent and stimulates the ERK/MAPK signaling pathway. We further demonstrated that oleuropein inhibits the cytotoxicity induced by amylin amyloids, a hallmark feature of type 2 diabetes. We demonstrated that these dual functions are structure-specific: we identified the 3-hydroxytyrosol moiety of oleuropein as the main functional entity responsible for amyloid inhibition, but the novel GSIS function requires the entire structure scaffold of the molecule.

Natural products derived from olive fruits, olive oil, and olive leaves have received widespread attention because of their potential benefits in preventing several currently prevalent chronic human diseases, including type 2 diabetes (T2D).[1] Oleuropein is a phenolic compound that is mainly found in olive leaves and fruits. This compound, as well as other olive-derived compounds such as ligstroside, is a tyrosol ester of elenolic acid that is further hydroxylated and glycosylated (Figure 1A). Oleuropein has been reported to have beneficial anti-diabetic functions such as reducing the frequency of glycemia and enhanced glucose tolerance in diabetic animal models.[2,3] Oleuropein-containing olive leaf extract has been shown to promote insulin sensitivity and glucose tolerance in overweight humans.[4] However, the mechanisms by which oleuropein contributes to these anti-diabetic functions and whether any structural moiety of oleuropein is responsible for such beneficial effects are not well understood. Oleuropein has also been reported to prevent cytotoxic amyloid aggregation of human amylin, Aβ42, and transthyretin, which is linked to T2D, neurodegeneration, or cardiovascular diseases.[5−7] However, it is not known if oleuropein's amyloid inhibition properties are structure-specific, as very few studies have examined the structure−function relationship. A recent study reported that polyphenolic glycosides and aglycones may utilize different pathways to selectively remodel and inactivate toxic Aβ oligomers.[8]

We identified oleuropein by screening a library of natural compounds (mostly flavonoids and polyphenols) that are known to have anti-diabetic functions in complementary medicine based on a thioflavin T (ThT) fluorescence assay.[9] Several strong inhibitors (including the EGCG control) were identified and confirmed. Among these hits, one that has been described to have multiple health benefits was oleuropein, an olive component (refs 2, 3, and 10 and Figure 1A).

Because oleuropein has the potential to activate G-protein-coupled estrogen receptor GPER/GPR30,[11,12] we hypothesized that oleuropein may have biological functions similar to those of genistein because phytoestrogen genistein is a known GPER/GPR30 agonist and can induce significant anti-diabetic effects.[13] One prominent biological function of genistein is anti-diabetic glucose-stimulated insulin secretion (GSIS). We therefore investigated oleuropein's insulin secretion function in INS-1 β-cells and further validated it with specific signaling pathway analyses.

Oleuropein has also been reported to prevent cytotoxic amyloid aggregation of human amylin,[5] but a detailed structure−activity dissection of the molecule has not been performed. Oleuropein has three parts to its structure: 3-hydroxyhyrosol (3-HT), elenolic acid (EA), and glucose (Figure 1A). To pinpoint which part(s) of the molecule is responsible for its anti-aggregation effects, we took an analytical approach using structural analogues of oleuropein.

Health benefits of olives and its associated natural products have long been recognized, as seen in the Mediterranean diet, but compound-specific effects and mechanisms related to their biomedical and nutritional values are just beginning to come to light.[1,4−6,14,15] Our mechanistic studies will therefore contribute in a timely manner to the improved understanding of those potential "nutraceuticals" for prevention of epidemic aging and metabolic syndromes.

To investigate oleuropein and its analogues' potential GSIS function, we tested them in INS-1 β-cells followed by enzyme-linked immunosorbent assay measurements of secreted insulin concentrations. We observed modest yet significant increases in the level of insulin secretion with increasing doses of oleuropein in the treatment starting in the low micromolar range. Increases of 10−20% in the level of insulin secretion were observed at 30 μM (Figure 1B). The potency of oleuropein is comparable to that of the natural compound genistein as determined by the increase in the level of insulin secretion.[13] In this assay, glucagon-

Received: March 5, 2017
Revised: July 9, 2017
Published: August 22, 2017

DOI: 10.1021/acs.biochem.7b00199
Biochemistry 2017, 56, 5035−5039

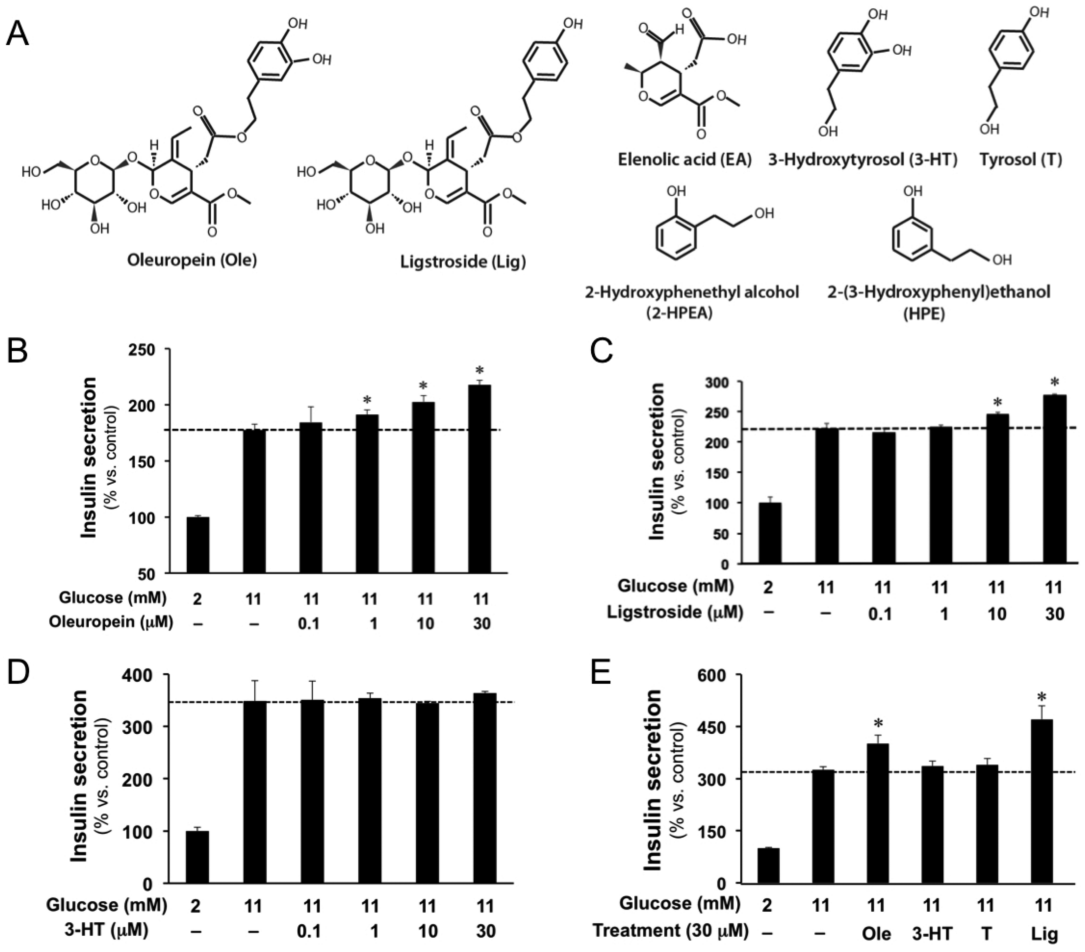

Figure 1. Oleuropein and its analogue, ligstroside, but not 3-hydroxytyrosol or tyrosol, promote glucose-stimulated insulin secretion in INS-1 β-cells. (A) Chemical structures of oleuropein and its analogues. (B–E) Dose-dependent GSIS effects of oleuropein and its analogue, ligstroside, in promoting GSIS in INS-1 β-cells, where 3-hydroxytyrosol serves as a negative control. Effects that are statistically significant with respect to 11 mM glucose controls are indicated with asterisks. Comparative analyses of GSIS effects in INS-1 β-cells with the different compounds are shown in panel E.

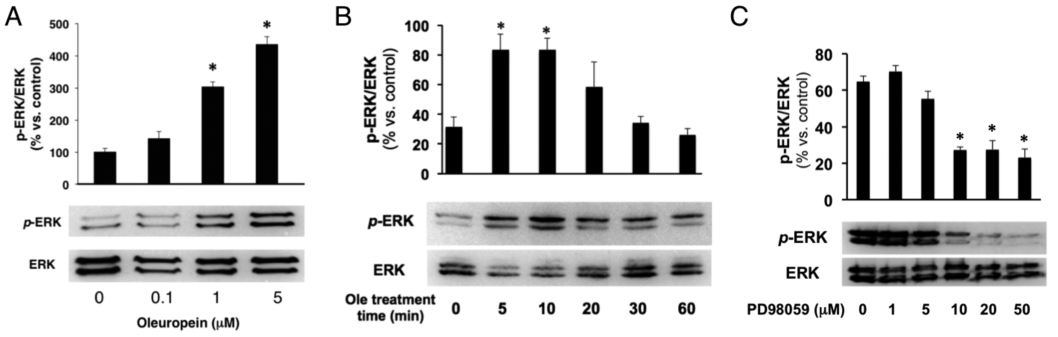

Figure 2. Oleuropein activates ERK/MAPK signaling pathways in INS-1 β-cells. Cells were treated with oleuropein at the indicated concentrations or for the specified durations, and protein extracts were probed by Western blotting. Levels of phosphorylated p-ERK were quantified by densitometry, normalized against total ERK. Effects that are statistically significant with respect to vehicle control or 0 min treatments are indicated with asterisks. (A) Dose-dependent ERK phosphorylation by oleuropein. The treatment time was 10 min. (B) Time dependence of ERK phosphorylation. The concentration of oleuropein in the treatment was 5 μM for both panels B and C. (C) Oleuropein-induced ERK activation is specifically blocked by ERK signaling pathway inhibitor PD98059 in a dose-dependent manner.

like peptide 1 (GLP-1), a potent GSIS agonist, was used as a positive control (Figure S1). We further tested structural analogues of oleuropein. We found that oleuropein's close analogue, ligstroside, retains GSIS function with a potency similar to that of oleuropein (Figure 1C,E), but 3-HT, a component of oleuropein's structure, and its related analogue, tyrosol, have no effects on stimulating insulin secretion (Figure 1D,E). Elenolic acid, another structural component of oleuropein, showed no GSIS activity (data not shown). These results suggest that the main structure scaffold of oleuropein is required for this novel metabolic effect and the 3-hydroxyl group on the 3-HT component is not critical for oleuropein's GSIS function.

DOI: 10.1021/acs.biochem.7b00199
Biochemistry 2017, 56, 5035–5039

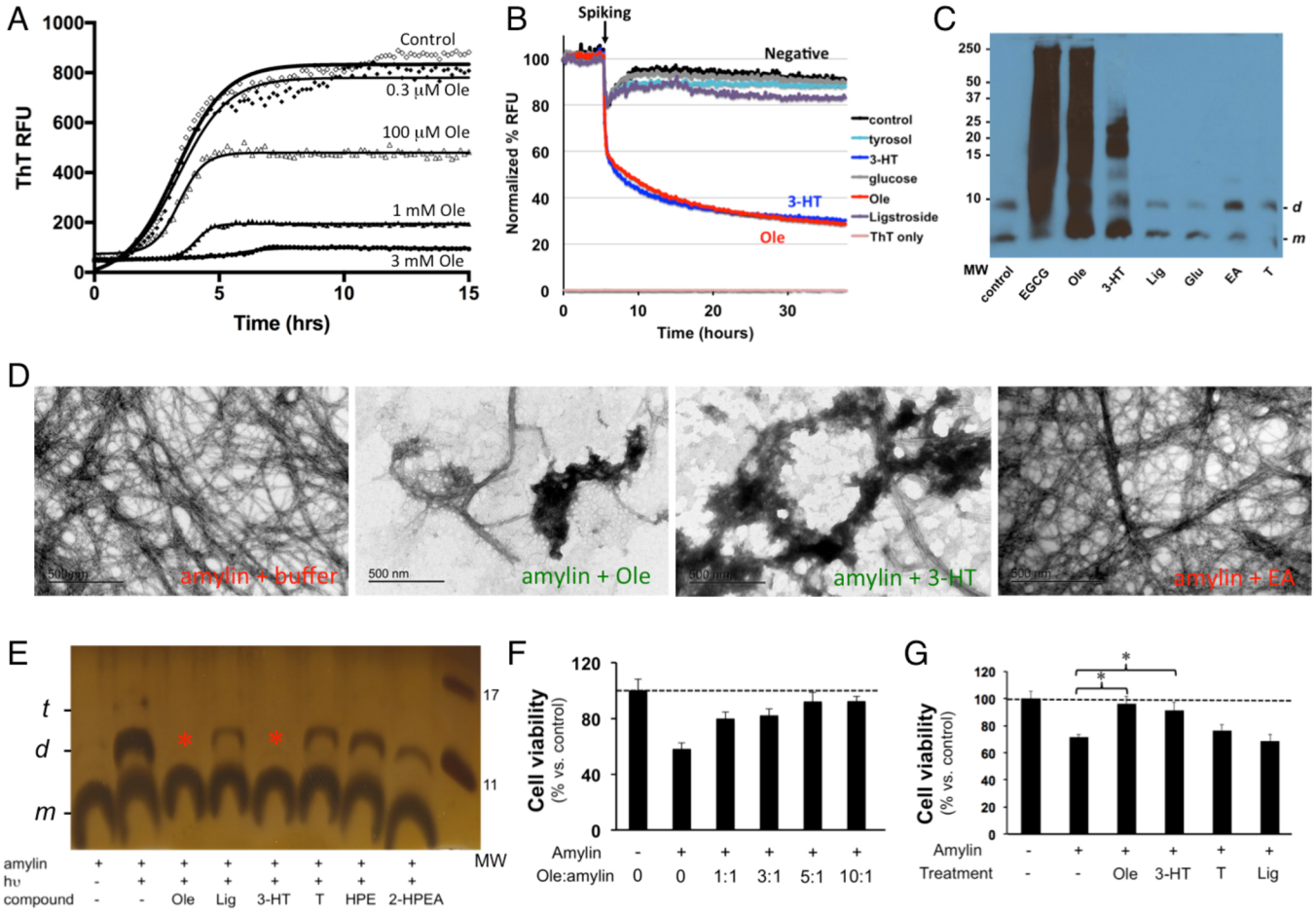

Figure 3. Oleuropein inhibits the formation and cytotoxicity of amylin amyloid primarily through its component 3-hydroxytyrosol moiety. (A) ThT fluorescence-based assay showing dose-dependent amyloid inhibition by oleuropein. (B and C) ThT fluorescence-based and gel-based amyloid remodeling assays showed oleuropein and 3-HT, but not other analogues, remodeled amylin amyloid. In panel B, the vehicle control or specified compounds were spiked into preaggregated amyloid samples (arrowed) that already reached plateaus (equivalent to the 10 h point in panel A). Fluorescence signals after spiking were recorded continuously. The compound:amylin molar ratio was 10:1. In panel C, 3 days after spiking, samples were vacuum-dried, redissolved in 6.5 M urea, and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blotting with amylin-specific antibody T-4157. EGCG served as a remodeling positive control.[17,18] Monomer (m) and dimer (d) sizes are marked. The compound:amylin molar ratio was 20:1. (D) Representative transmission electron microscopy images of amylin amyloid and its treatment with vehicle, oleuropein, 3-HT, or EA. The amylin concentration was 15 μM, and the drug:amylin molar ratio was 20:1. (E) Photoinduced cross-linking of unmodified protein analysis of amylin oligomer formation with various oleuropein analogue treatments. The absence of the cross-linked dimers is marked with the red asterisks. The amylin concentration was 10 μM, and the drug:amylin molar ratio was 10:1. (F and G) Neutralization of amylin-induced cytotoxicity by oleuropein analogues in INS-1 β-cells. The amylin concentration was 5 μM, and the Ole:amylin molar ratios are indicated (F). Treatment of Ole and 3-HT, but not T and Lig, has significant effects that protect INS-1 cell viability (asterisks in panel G).

To further validate and gain mechanistic insights into oleuropein's GSIS effect, we performed cell signaling analyses in INS-1 β-cells. On the basis of the cell signaling activation used by other ligands (such as genistein and GLP-1) that induce GSIS effects in β-cells,[13,16] we used a standard pharmacological inhibitor approach. We tested the involvement of major kinase pathways that are related to metabolism: protein kinase A (PKA), protein kinase C (PKC), ERK/MAPK, PI3 kinase, and the AMP-activated kinase (AMPK).[13,16,19] We used the inhibitors KT-5702 (PKA), H-89 (PKA), Ro-318220 (PKC), LY-294002 (PI3K), Compound C (AMPK), and PD98059 (ERK/MAPK). Except for the ERK/MAPK pathway showing strong activation (Figure 2), we did not observe significant activation by other pathways (data not shown). We identified the ERK/MAPK pathway: we not only observed dose-dependent ERK activation by oleuropein (Figure 2A) but also found that oleuropein rapidly stimulated insulin secretion with peaks at 5−10 min upon treatment (Figure 2B). Consistently, the level of inhibition of ERK phosphorylation decreased with an increased dose of ERK/MAPK-specific inhibitor PD98059 (Figure 2C). Oleuropein was shown to induce AMPK phosphorylation (and mTOR inhibition)-related autophagy in SHSY-5Y neuroblastoma cells.[19] When we performed tests in INS-1 cells on autophagy induction upon oleuropein treatment, we found no significant induction of autophagic markers beclin-1 or LC3 proteins (Figure S2). The differences between our results and the literature could arise from the different cell models used.

Oleuropein's anti-diabetic effects have been in part caused by its neutralizing effects against the cytotoxic amyloids of a peptide hormone, amylin.[5] This 37-residue peptide is co-secreted with insulin from the β-cells. Insulin resistance-associated hyperamylinemia can lead to toxic amylin amyloid deposits in the pancreas, which occurs in up to 90% of T2D patients.[20] To validate oleuropein as an effective amylin amyloid inhibitor

DOI: 10.1021/acs.biochem.7b00199
Biochemistry 2017, 56, 5035−5039

identified from our initial natural product library screens,[9] we performed multiple secondary assays. These orthogonal assays are necessary, partly because of the reported limitations of ThT fluorescence screen assays in defining amyloidogenicity in some cases.[21] We used transmission electron microscopy (TEM) to validate that oleuropein significantly blocked fibrillation under experimental conditions (Figure 3D). We showed in two orthogonal remodeling assays that oleuropein significantly remodeled preformed amylin amyloids such that the intensities of ThT fluorescence signals were significantly reduced (Figure 3B) or led amyloid into nontoxic, presumably off-pathway aggregates that have broad molecular weight distributions as exemplified by EGCG (Figure 3C).[17,18] During the early phase of amyloid formation, we found oleuropein disrupted oligomer (dimer and trimer) formation in an *in vitro* photoinduced cross-linking of unmodified protein assay (Figure 3E). Expectedly, oleuropein inhibits amylin amyloid in a dose-dependent manner (Figure 3A) with an estimated IC_{50} of 100 μM (Figure S3B). Oleuropein also induced a kinetic delay (longer $t_{1/2}$) in the lag phase of amyloid formation at high concentrations (Figure 3A and Figure S3A). Lastly and importantly, oleuropein neutralized amylin amyloid-induced cytotoxicity against β-cells in a dose-dependent manner (Figure 3F,G). Oleuropein itself has no significant effects on cell viability (Figure S5).

To further pinpoint which functional groups in oleuropein are important for its amyloid inhibitory effects, we performed an analogue-based structure−activity relationship analysis. Our assays compared and contrasted oleuropein with each of its component and related analogues (Figures 1A and 3). In both ThT fluorescence and gel-based amyloid remodeling assays, we found oleuropein and 3-HT showed similar remodeling activities whereas all other compounds, including oleuropein analogue ligstroside, 3-HT analogue tyrosol, and oleuropein components EA and glucose, showed no such activities. TEM results validated that both oleuropein and 3-HT, but not EA, significantly reduced the level of fibrillation in comparison to the vehicle control (Figure 3D and Figure S6). Consistently, oleuropein and 3-HT have similar IC_{50} inhibition potencies (Figure S3B). Oligomer formation is the intermediate step in forming mature fibrils, and oligomers are thought to be more cytotoxic than fibrils.[20] Our data suggested that oleuropein and 3-HT inhibit oligomer formation (Figure 3E). Only at a relatively higher concentration did tyrosol, ligstroside, 2-HPEA, and HPE show moderate dimer breakup functions [at this ratio, EA and glucose remained negative (Figure S4)]. Importantly, cell-based assays demonstrated significant neutralization functions of oleuropein and 3-HT, but no activities for ligstroside and tyrosol at comparable concentrations (Figure 3G). All compounds alone have no effects on cell viability (Figure S5B). Ligstroside, a close analogue of oleuropein that is active in stimulating the GSIS effect, has little amyloid inhibitory functions (Figure 3B,C,E,G). Loss of inhibition activities of tyrosol and ligstroside demonstrated that vicinal dihydroxyl groups of the catechol moieties of 3-HT and oleuropein are important for amyloid inhibition functions, as we showed with other catechol-containing compounds.[9] Together, multiple independent lines of data demonstrated that the 3-HT moiety of oleuropein is the main functional entity responsible for its amyloid inhibition.

In summary, here we report, for the first time to the best of our knowledge, that oleuropein is a novel natural compound inducing anti-diabetic GSIS function. Our data suggested that its GSIS function requires the entire structural scaffold of oleuropein. In contrast, the 3-HT moiety of oleuropein is largely responsible for its amyloid inhibition function, which is relevant to its second anti-diabetic function. Our work thus provided insights into the dual anti-diabetic functions of oleuropein. Anti-amyloid effects of oleuropein have been shown in an animal study to effectively counteract the toxicity of a well-studied amyloidogenic peptide, Aβ42.[4] It will be interesting to test the specific effects of oleuropein and its components in counteracting amylin amyloid deposition in the pancreas as well as in positively regulating hyperglycemia in diabetic animal models in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00199.

Experimental procedures and supplemental figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: binxu@vt.edu.

ORCID

Bin Xu: 0000-0002-0490-9540

Funding

This work is in part supported by the U.S. Department of Agriculture (HATCH Funds for Project VA-135992), Commonwealth Health Research Board (CHRB) Grant 208-01-16, the Diabetes Action Research and Education Foundation (DAREF), and Grants 16-1 and 18-4 from the Commonwealth of Virginia's Alzheimer's and Related Diseases Research Award Fund, administered by the Virginia Center on Aging, School of Allied Health Professions, Virginia Commonwealth University.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Kathy Lowe at Virginia-Maryland Regional College of Veterinary Medicine for her excellent technical assistance in using TEM.

■ REFERENCES

(1) Rigacci, S., and Stefani, M. (2016) *Int. J. Mol. Sci. 17*, 843.

(2) Jemai, H., El Feki, A., and Sayadi, S. (2009) *J. Agric. Food Chem. 57*, 8798−8804.

(3) Murotomi, K., Umeno, A., Yasunaga, M., Shichiri, M., Ishida, N., Koike, T., Matsuo, T., Abe, H., Yoshida, Y., and Nakajima, Y. (2015) *J. Agric. Food Chem. 63*, 6715−6722.

(4) de Bock, M., Derraik, J. G. B., Brennan, C. M., Biggs, J. B., Morgan, P. E., Hodgkinson, S. C., Hofman, P. L., and Cutfield, W. S. (2013) *PLoS One 8*, e57622.

(5) Rigacci, S., Guidotti, V., Bucciantini, M., Parri, M., Nediani, C., Cerbai, E., Stefani, M., and Berti, A. (2010) *J. Nutr. Biochem. 21*, 726−735.

(6) Luccarini, I., Ed Dami, T., Grossi, C., Rigacci, S., Stefani, M., and Casamenti, F. (2014) *Neurosci. Lett. 558*, 67−72.

(7) Leri, M., Nosi, D., Natalello, A., Porcari, R., Ramazzotti, M., Chiti, F., Bellotti, V., Doglia, S. M., Stefani, M., and Bucciantini, M. (2016) *J. Nutr. Biochem. 30*, 153−166.

(8) Ladiwala, A. R. A., Mora-Pale, M., Lin, J. C., Bale, S. S., Fishman, Z. S., Dordick, J. S., and Tessier, P. M. (2011) *ChemBioChem 12*, 1749−1758.

(9) Velander, P., Wu, L., Ray, W. K., Helm, R. F., and Xu, B. (2016) *Biochemistry 55*, 4255−4258.

DOI: 10.1021/acs.biochem.7b00199
Biochemistry 2017, 56, 5035−5039

(10) Umeno, A., Horie, M., Murotomi, K., Nakajima, Y., and Yoshida, Y. (2016) *Molecules 21*, 708.
(11) Chimento, A., Casaburi, I., Rosano, C., Avena, P., De Luca, A., Campana, C., Martire, E., Santolla, M. F., Maggiolini, M., Pezzi, V., and Sirianni, R. (2014) *Mol. Nutr. Food Res. 58*, 478−489.
(12) Wang, A., Luo, J., Moore, W., Alkhalidy, H., Wu, L., Zhang, J., Zhen, W., Wang, Y., Clegg, D. J., Xu, B., Cheng, Z., McMillan, R. P., Hulver, M. W., and Liu, D. (2016) *Sci. Rep. 6*, 34302.
(13) Liu, D., Zhen, W., Yang, Z., Carter, J. D., Si, H., and Reynolds, K. A. (2006) *Diabetes 55*, 1043−1050.
(14) Watkins, R., Wu, L., Zhang, C., Davis, R. M., and Xu, B. (2015) *Int. J. Nanomed. 10*, 6055−6074.
(15) Velander, P., Wu, L., Henderson, F., Zhang, S., Bevan, D. R., and Xu, B. (2017) *Biochem. Pharmacol. 139*, 40−55.
(16) Drucker, D. J. (2006) *Cell Metab. 3*, 153−165.
(17) Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) *Nat. Struct. Mol. Biol. 15*, 558−566.
(18) Palhano, F. L., Lee, J., Grimster, N. P., and Kelly, J. W. (2013) *J. Am. Chem. Soc. 135*, 7503−7510.
(19) Rigacci, S., Miceli, C., Nediani, C., Berti, A., Cascella, R., Pantano, D., Nardiello, P., Luccarini, I., Casamenti, F., and Stefani, M. (2015) *Oncotarget 6*, 35344−35357.
(20) Westermark, P., Andersson, A., and Westermark, G. T. (2011) *Physiol. Rev. 91*, 795−826.
(21) Wong, A. G., Wu, C., Hannaberry, E., Watson, M. D., Shea, J.-E., and Raleigh, D. P. (2016) *Biochemistry 55*, 510−518.

DOI: 10.1021/acs.biochem.7b00199
Biochemistry 2017, 56, 5035−5039