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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administered. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization. | | | | | |
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1. INTRODUCTION

A physiologically functional insulin molecule is the result of the correct folding of proinsulin, facilitated by the formation of disulfide bridges between its A- and B-peptides and the detachment of the C-peptide. Insulin and C-peptide are then stored in the secretory granules of the pancreatic beta-cells and eventually released together, in equimolar amounts, once the increase of glycemia demands it.

Biological effects of C-peptide has long been hypothesized considering that subjects with type 1 diabetes, who retain low plasma concentrations of C-peptide, seem less prone to develop micro-vascular complications than those in whom beta-cell function has ceased completely. In addition, whole pancreas or beta-cell transplantation, which results in restoration of endogenous insulin and C-peptide concentrations, is accompanied by significant amelioration of diabetes-induced abnormalities, both structural and functional, of the blood vessels, the peripheral nerves and the kidneys. Administration of recombinant insulin alone seems to not be sufficient to avoid the eventual presentation of serious complications associated with the disease.

To substantiate the believe that C-peptide might have therapeutic potential for the treatment of diabetes complications, the physiological significance of C-peptide in normal physiology should be proven by the identification of the C-peptide receptor or receptor complex. To this aim experiments must include binding studies, co-immunoprecipitation, and pull-down assays combined with mass spectrometry analysis. Once the receptor is identified, the C-peptide signaling mechanism(s) can also be determined in order to recognize the C-peptide as a *bona fide* endogenous peptide hormone. (*Am J Physiol Endocrinol Metab* 307: E955–E968, 2014).

2. KEY WORDS

Diabetes, Insulin, C-peptide, C-peptide Receptor.

3. OVERALL PROJECT SUMMARY

In order to more directly identify molecules that interact with C-peptide, the human C-peptide sequence was modified to include biotin and a hemagglutinin epitope (HA) at the amino terminus and an internal photo-leucine. C-peptides containing modified leucines can be cross-linked to nearby molecules after photo-activation. An N-terminal tag allowed the cross-linked molecules to be captured and analyzed by western blotting and mass spectroscopy.

For the purpose of revealing potential C-peptide receptor molecules, we first tested the ability of our modified C-peptide to increase the intracellular calcium concentration relative to native C-peptide in Raw264.7 cells. Both the modified C-peptide and native C-peptide triggered a transient calcium increase and that BAPTA-AM treatment reduced the response in both cases.

HEK293 cells were treated with the modified C-peptide and exposed to UV light to enable the cross-linking of adjacent molecules. In western blots of biotin-captured

molecules from cell lysates, it was difficult to reliably detect a biotin signal indicative of cross-linking, in part because of an abundance of endogenously biotinylated proteins. However, using anti-HA-sepharose beads for capture, we easily found evidence of a cross-linked molecule in the less complex extracellular supernatant obtained after 1,000 x g centrifugation of the intact cells. Western blots revealed a band of about 75,000 M_r when developed for biotin (Fig. 1). A similar result was obtained using antibodies to HA or to C-peptide to analyze the western blots (Fig. 1). The signal intensity was reduced in samples prepared in the presence of an excess of native C-peptide and was not reduced or only slightly reduced by competition with the C-peptide terminal pentapeptide or a mutated pentapeptide (Fig. 2)

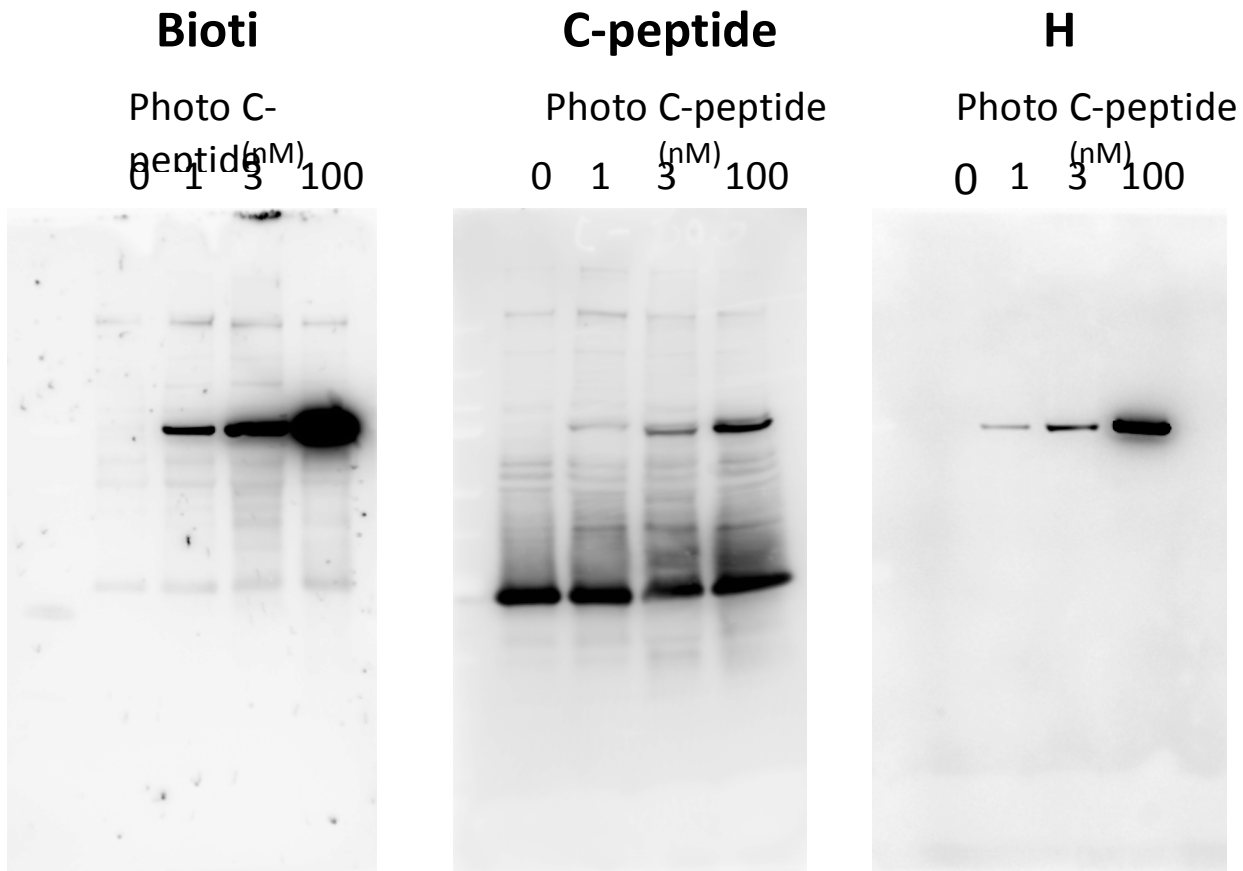


Fig. 1 Capture of an unknown protein by crosslinking with a modified C-peptide. HEK293 cells were treated with the indicated concentrations of BHACPL21. After 40 minutes at 4°C the cells were UV irradiated for 20 minutes. Then cells were centrifuged and the supernatant was filtered. The C-peptide interacting products were affinity purified from the supernatants with anti-HA agarose beads. After SDS gel separation, western blots were prepared and analyzed with anti-biotin antibody, C-peptide antibody, or with anti-HA antibody. The lower molecular weight bands seen with the C-peptide antibody appear to be artifacts since they are present in the sample which does not contain BHACPL21 and the intensity of the bands does not change with BHACPL21 concentration.

Sufficient amounts of this cross-linked molecule were prepared for analysis by mass spectrometry. We initially captured the cross-linked products with anti-HA antibody on beads followed by HA elution and recapture with streptavidin beads. Gel electrophoresis and coomassie blue staining resolved two faint bands at 70,000 M_r and 75,000 M_r . Mass spectrometry identified both bands as the products of either the HSPA1A gene or the HSPA1B gene which encode identical protein sequences of HSP70 having a molecular mass of 70,000 M_r . The size of the 75,000 M_r band fits well with the expected size of the modified C-peptide cross-linked with the HSP70 molecule.

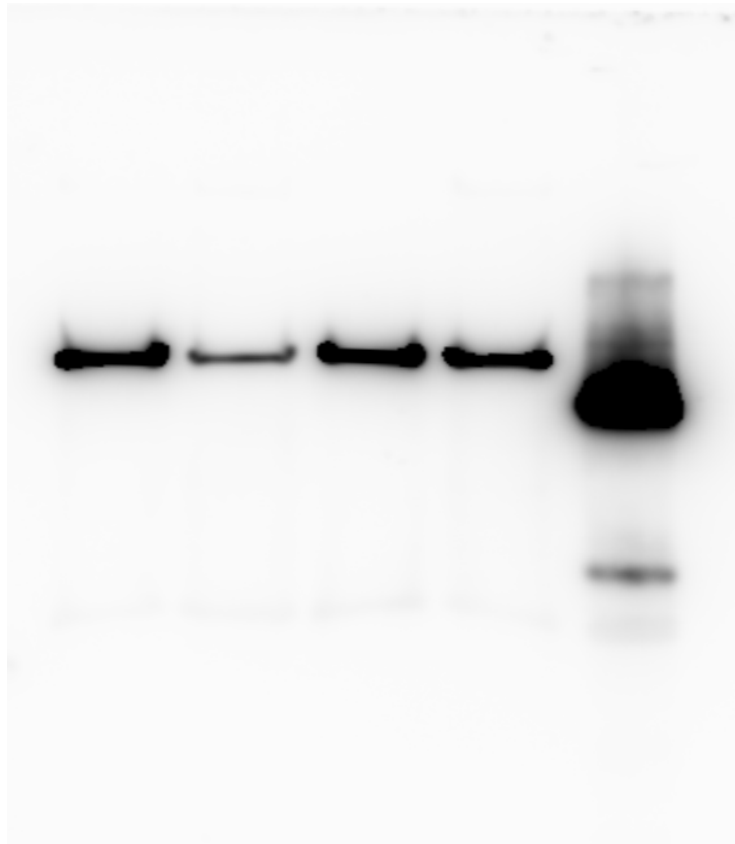


Fig 2 Blocking the cross-linking of modified C-peptide with the unknown protein using C-peptide and pentapeptides. HEK293 cells were treated and processed using BHACPL21 in the presence of a 20 fold excess of native C-peptide (CP) as in Fig. 1. EGSLQ is the C-peptide native sequence in single letter amino acid code. AGSLQ is an altered pentapeptide sequence. Lane 2 shows that native C-peptide reduces the amount of cross-linked product. The pentapeptides appear to have no effect, lanes 3 and 4. Lane 5 shows the cross-linked products of a UV treated mixture of BHACPL21 and C-peptide antibody. Most of the cross-linking is to the heavy chain with a small amount to the light chain.

The HSP70 identification was confirmed by western blot analysis with an HSP70 antibody (Fig. 3). Two bands at 70,000 M_r and 75,000 M_r were observed that corresponded in size to the native HSP70 and the expected size of modified C-peptide cross-linked to HSP70, respectively.

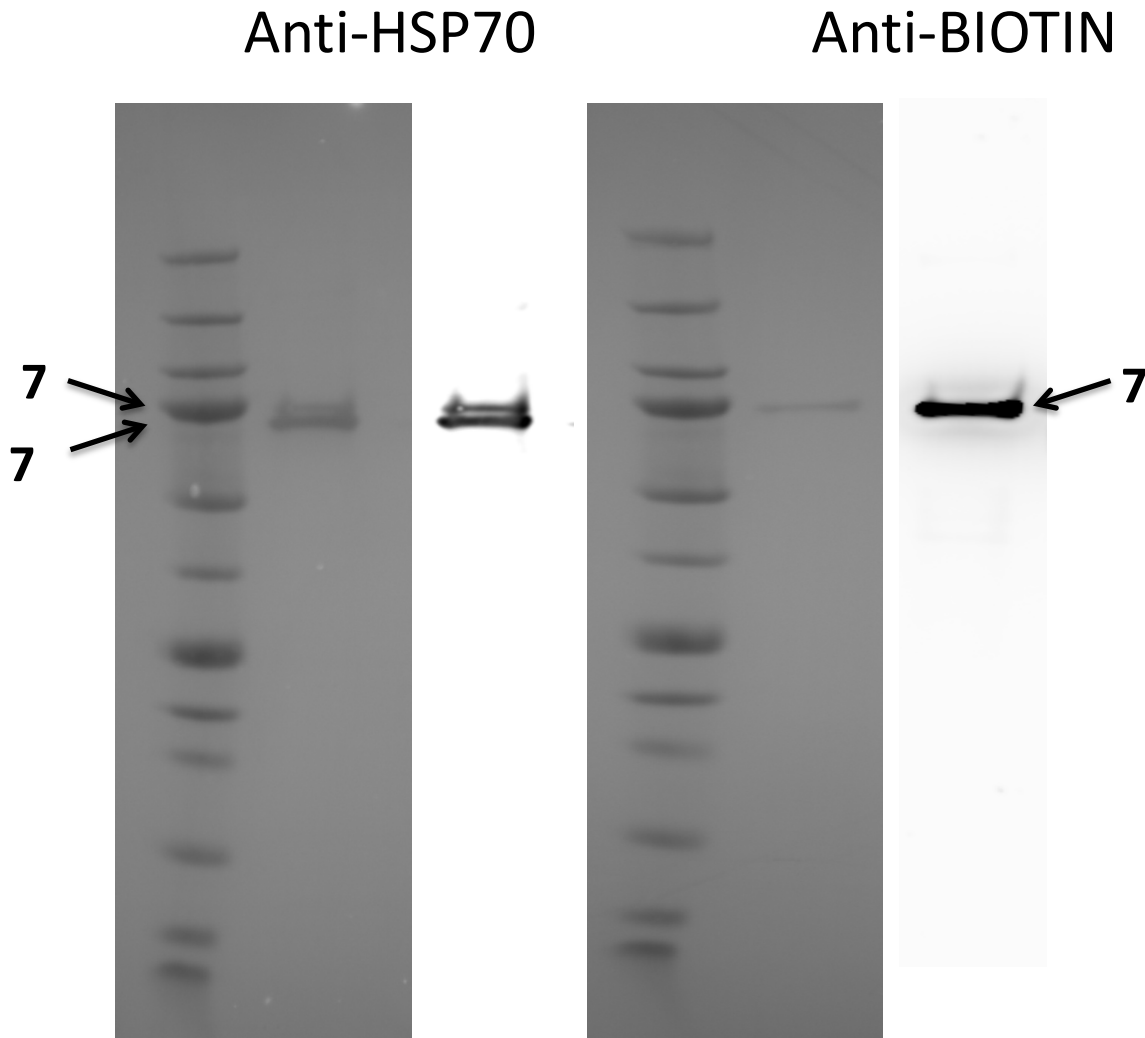


Fig. 3 Confirmation of mass spectroscopy results with HSP70 antibody. HEK293 cells were treated with 50 nM BHACPL21. After 40 minutes at 4°C the cells were UV irradiated for 20 minutes. Then cells were centrifuged and the supernatant was filtered. The C-peptide interacting products were affinity purified with anti-HA agarose beads. After SDS gel separation, western blots were prepared and analyzed with HSP70 antibody (SPA-8131), or with anti-biotin antibody. The whiter lanes show the chemiluminescent detection of the antibodies and the darker lanes are photos of the membranes showing the size of the visible reaction by-products compared with the colored size markers at the left side of the images. The arrows identify the position of the 75,000 M_r marker protein and the expected location of a 70,000 M_r protein. In latter experiments the gels were run longer to increase the separation of the bands seen here.

To test if the C-peptide cross-linked to HSP70 was present in exosomes, we ultracentrifuged the extracellular solution as prepared in Fig. 1 before capturing the C-peptide with anti HA beads. Figure 4 (upper part) shows the western blot analysis of captured and cross-linked HSP70 from the extracellular solution prior to ultracentrifugation compared to the supernatant after centrifugation. The nearly equal signals from the cross-linked C-peptide before and after centrifugation indicate that the cross-linked molecules exist primarily in the exosome-free solution. When the exosomal pellet was dissolved to the original solution volume, we found an abundance of HSP70 Fig. 4 (bottom part). We found no discernable biotin signal that would identify HSP70 cross-linked to C-peptide in the exosome fraction. In addition, the non-UV treated C-peptide captured HSP70 to an extent equal to or slightly greater than the cross-linked C-peptide. Thus, of the total amount of HSP70 that was found in the extracellular fluid, under our experimental conditions, only a small fraction was non-exosomal but included nearly all of the extracellular HSP70 that became cross-linked to C-peptide.

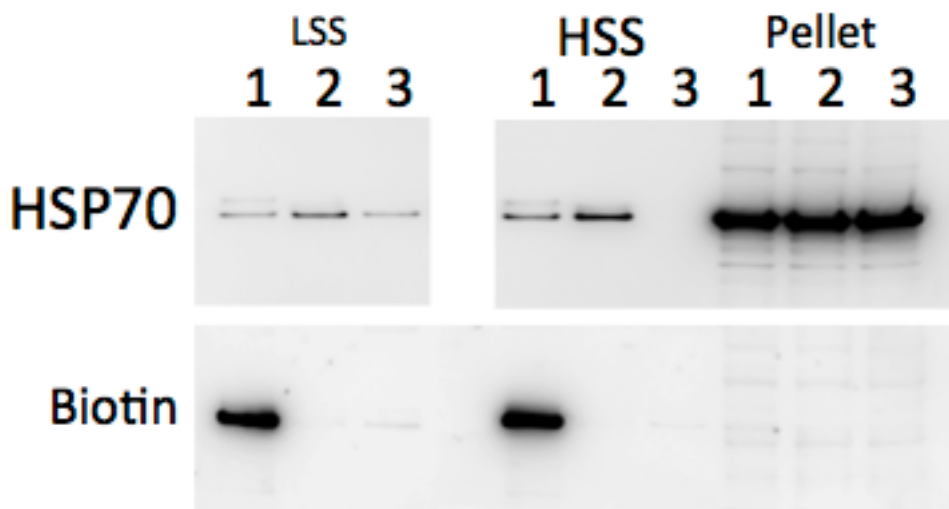


Fig 4 C-Peptide cross-linked to HSP70 is not abundant in exosomes. Sample supernatants were prepared as in Fig. 1. The samples were divided and one half was further centrifuged at 45,000 x g for 2 h to pellet exosomes. Lanes 1, 2, and 3 are BHACPL24 and UV treated, BHACPL24 no UV treatment, and media alone, respectively. Avidin beads were used for capture. LSS is the low speed supernatant equivalent to Fig. 1. HSS is the exosome-free high speed supernatant. The pellets were dissolved to the original volume and analyzed without bead capture. A break was introduced in the image of HSP70 western blot to adjust for a marker lane in the gel of the biotin western blot. The biotin positive bands align in size with the faint bands of lane 1 of the LSS and HSS UV treated samples in the HSP70 blot. The band seen in lane 3 of the LSS samples is unexplained, since there was no BHACPL24 in that sample.

The properties of the substrate-binding site of HSP70 make this region a likely candidate for the site of interaction of C-peptide and HSP70. Because the status of the adenine nucleotide binding domain of HSP70 modulates the functional characteristics of the substrate-binding site, we have used apyrase to deplete ATP at various points during the C-peptide mediated capture of HSP70. In Fig. 5 we show that there is only a minimal if any loss of HSP70 when beads with the captured complex are treated with apyrase. When lysates were treated with apyrase before C-peptide addition or after C-peptide addition but before capture we found an obvious increase in the capture of HSP70 by biotin tagged C-peptide compared to our standard protocol. However, there was not a parallel increase of C-peptide cross-linked to HSP70.

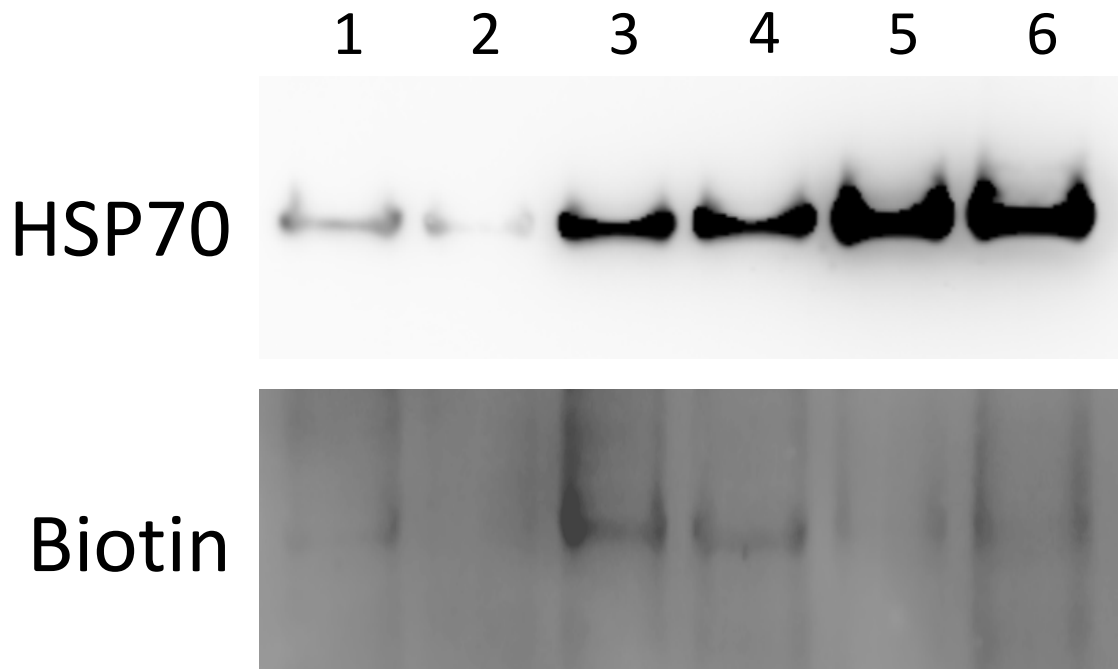


Fig. 5 Apyrase treatment that enhances capture of HSP70 does not facilitate cross-linking. HEK293 cells were first lysed with 0.5% CHAPS. For lanes 1 and 2 media alone was added to the lysate and for lanes 3 and 4 BHACPL21 was added to the lysate. Following UV irradiation and capture with Neutravidin beads, the preparations for lanes 2 and 4 were subsequently treated with apyrase (10U/ml x 20 minutes at room temperature) and washed before preparation for electrophoresis. In lane 5, the cell lysate was treated with apyrase before BHACPL21 addition and in lane 6 the apyrase treatment was after the BHACPL21 addition. This was followed by UV irradiation and bead capture. The upper western blot was developed with HSP70 antibody SPI-8131. The lower western was developed with anti-Biotin antibody to reveal cross-linked, biotin-HA-tagged C-peptide. Lane 4 compared with lane 3 shows there is no loss of captured HSP70 after apyrase treatment. Lanes 5 and 6 show that the sequence of BHACPL21 and apyrase additions did not affect the capture of HSP70. However, the lower panel shows that with the greater capture of HSP70 in both lanes 5 and 6 there is less cross-linking than in lanes 3 and 4.

To test if the C-peptide-HSP70 interaction is involved in cellular responses to C-peptide, we investigated C-peptide induced calcium flux in the presence of HSP70 antibody. The rise in intracellular calcium mediated by C-peptide treatment of RAW246.7 cells was reduced by pretreatment with HSP70 antibody and not with a control antibody Fig. 6. Likewise HSP70 antibody also reduced the uptake of fluorescent labelled C-peptide by HAEC Fig. 7.

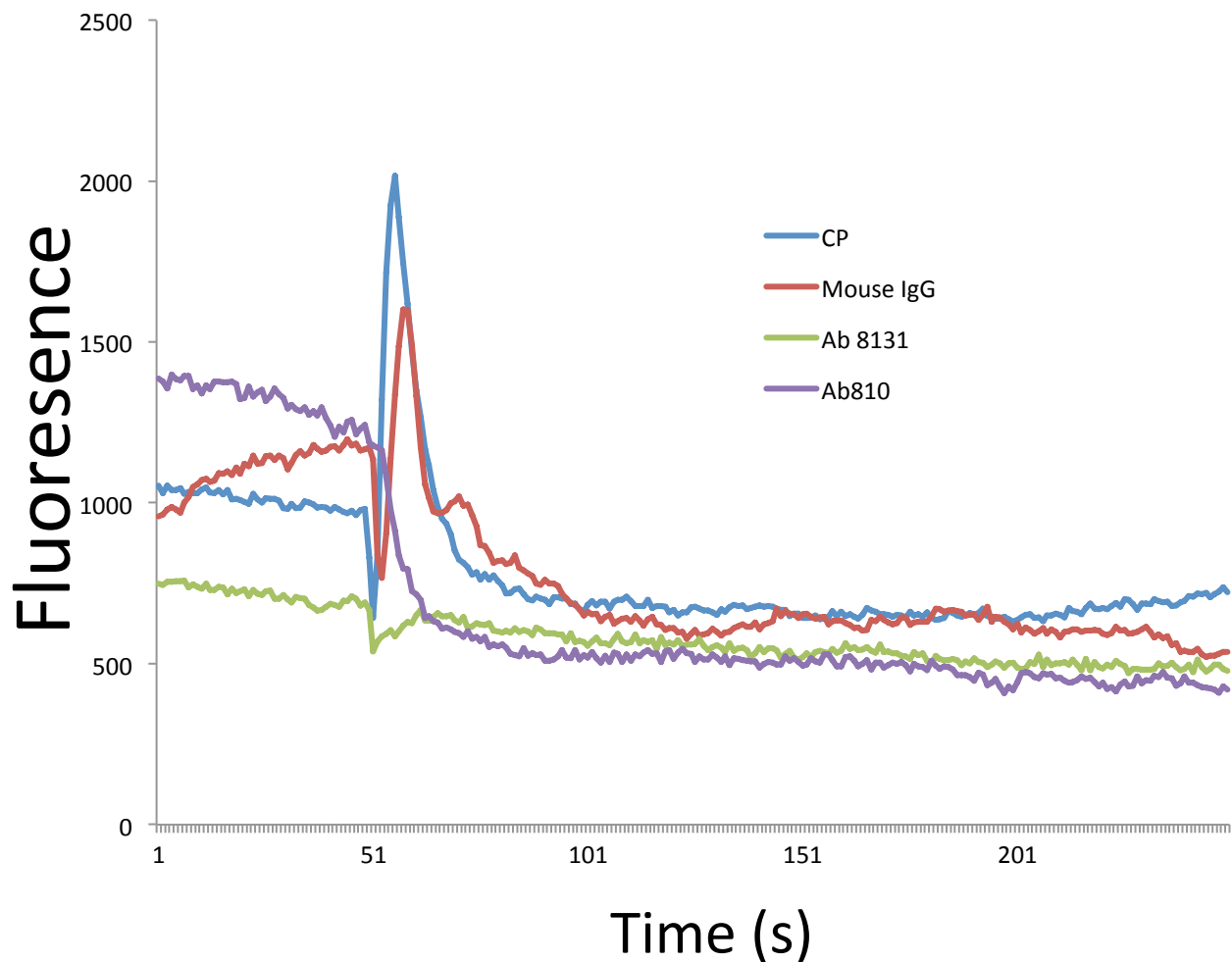


Fig. 6 Anti-HSP70 antibodies block human C-peptide induced intracellular calcium in RAW246 cells. RAW246 cells were grown on glass bottom culture dishes. Culture media was replaced with serum free media overnight before an experiment. Cells were incubated with 1 μ M Fluo-4, 2.5mM probenidic and 20mM HEPES pH7.5 in serum free media, 30mins at 37°C. HSP70 antibodies (SPA-8131 and SPA 810) or a isotype-matched mouse antibody (0.2 μ g/ml) were added. Cells were washed and imaged by live confocal microscopy for 250s. The arrow shows the time of C-peptide (100nM) addition. Cytosolic Ca²⁺ was assessed by the fluorescent intensity of Fluo-4. Data are the mean change in Fluo-4 intensity \pm S.E.M. for 3 experiments.

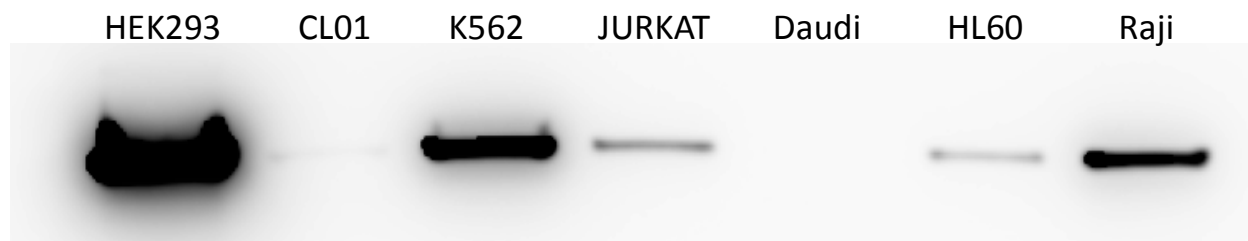


Fig. 7. HSP70 antibody blocks internalization of C-peptide. Human aortic endothelial cells (HAEC) were treated with (a,b) media alone; (c,d) HSP70 antibody (SPA-8131); or (e,f) an isotype-matched control antibody. Then Alexa Fluor 488 labelled C-peptide was added to the cells. Fluorescent images (a,c,e) of representative fields are shown on the left and again merged with a visible light image (b,c,f) on the right.

4. KEY RESEARCH ACCOMPLISHMENTS

The C-peptide-HSP70 interaction was validated by the close interaction requisite for cross-linking, by identification by mass spectroscopy, and with HSP70 antibody.

5. CONCLUSION

Physiologically, the effects of C-peptide may involve HSP70 and may be modulated by particular conformations of HSP70. As both molecules may have roles in inflammation, the consequences of their interaction need to be further investigated. Thus, antagonistic or beneficial effects of the interaction of C-peptide with HSP70 should be considered in the design of experiments to study potential C-peptide receptor molecules and in models of autoimmunity and potential C-peptide therapeutic interactions.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

YEAR 1

Cifarelli V, Trucco M, Luppi P: Anti-inflammatory effects of C-peptide prevent endothelial dysfunction in type 1 diabetes. *Immun Endoc & Metab Agents in Med Chem* 11:59, 2011.

Cifarelli V, Geng X, Styche A, Lakomy R, Trucco M, Luppi P: C-peptide reduces high glucose-induced apoptosis of endothelial cells and decreases NAD(P)H-oxidase reactive oxygen species generation. *Diabetologia* 54:2702, 2011.

Luppi P, Cifarelli V, Wahren J. C-peptide and long-term complications of diabetes. *Pediatric Diabetes* 2011; 12: 276–292.)

Haidet J, Cifarelli V, Trucco M, Luppi P: C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimulated U937 monocytes in condition of hyperglycemia. *Inflammation Research*, In press, 2011

YEAR 2

Luppi P, Cifarelli V, Wahren J: C-peptide and long-term complications of diabetes. *Pediatric Diabetes* 12:276, 2011.

Cifarelli V, Trucco M, Luppi P: Anti-inflammatory effects of C-peptide prevent endothelial dysfunction in type 1 diabetes. *Immun Endoc & Metab Agents in Med Chem.* 11:59, 2011.

Haidet J, Cifarelli V, Trucco M, Luppi P: C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimulated U937 monocytes in condition of hyperglycemia. *Inflammation Research* 6:27, 2012.

Cifarelli V, Lee S, Kim DH, Zhang T, Kamagate A, Slusher S, Bertera S, Luppi P, Trucco M, Dong HH: FOXO1 mediates the autocrine effect of endothelin-1 on endothelial cell survival. *Mol Endocrinol.* 26:1213, 2012.

YEAR 3

Haidet J, Cifarelli V, Trucco M, Luppi P: C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimulated U937 monocytes in condition of hyperglycemia. *Inflammation Research* 6:27, 2012.

Cifarelli V, Lee S, Kim DH, Zhang T, Kamagate A, Slusher S, Bertera S, Luppi P, Trucco M, Dong HH: FOXO1 mediates the autocrine effect of endothelin-1 on endothelial cell survival. *Mol Endocrinol.* 26:1213, 2012.

Haidet J, Cifarelli V, Geng X, Trucco M, Luppi P: C-peptide: A new molecule with anti-inflammatory properties. In: Sima A.A.F (Editor), *Diabetes & C-Peptide: Scientific and Clinical Aspects, Contemporary Diabetes*, Springer, pp. 111-127, 2012.

YEAR 4 / NCE

Rudert W A, Geng X, Fan Y, Trucco M, Human proinsulin C-peptide interacts with HSP70 (manuscript submitted).

Rudert W A, "A Binding Partner for C-Peptide: Implications for Autoimmunity and

Diabetic Complications”, Institute of Cellular Therapeutics, Work-in-Progress Seminar, Allegheny General Hospital, Pittsburgh, PA, October 2, 2014

7. INVENTIONS, PATENTS AND LICENSES

NONE

8. REPORTABLE OUTCOMES

C-peptide may have a role in affecting the function of non-exosomal HSP70. If there is an effect of C-peptide, it would more likely occur where the C-peptide concentration is highest, at the point of insulin release in the interstitial space and the adjacent endothelial cells and capillaries of the pancreatic islets. A protective mechanism involving C-peptide that could naturally modulate an HSP70-mediated-localized-inflammatory response would be significant for the understanding of and potential prevention of islet autoimmunity and its debilitating complications.

9. OTHER ACHIEVEMENTS

NONE

10. REFERENCES

NONE

11. APPENDICES

NONE