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Mol Endocrinol. 26:1213, 2012.

INTRODUCTION/ORIGINAL STATEMENT OF WORK

Diabetes mellitus is a life-threatening disease that places children (type 1) and adults (type 2) at risk of complications of blindness, kidney damage and heart disease. Diabetes afflicts 16 million Americans, with more than 800,000 new cases diagnosed each year. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications. Costs associated with diabetes are estimated to reach \$132 billion/year. Significantly, the number of diabetes cases in the United States mirrors national rates. Type 1 diabetes (T1D) patients lack physiological levels of insulin in their bloodstream due to the autoimmune destruction of the insulin producing pancreatic beta cells. Type 2 diabetes (T2D) patients are, instead, afflicted by a heterogeneous set of sub-syndromes characterized by peripheral insulin resistance with or without production insufficiency. Both T1D and T2D patients are at increased risk for damages of both micro- and macro-vascular tissues, which eventually bring to the well known, tragic, diabetic complications.

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.

In preliminary results, we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)- κ B pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). 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 (5). In this project, we will set a number of experiments specifically designed to isolate the CPR and characterize its intracellular signaling activity, with the following specific aims:

1. **To isolate the C-Peptide Receptor (CPR).** We have designed and synthesized a set of biotinylated C-peptides including wild type and two mutants previously shown to not bind cellular surface membrane. These peptides will be allowed to internalize into endothelial and smooth muscle cells. Endosomes containing the biotinylated C-peptides will be isolated by cell fractionation, solubilized, and the C-peptide/CPR complexes isolated. Proteins of the wild type but not the mutant C-peptide/ receptor complexes will be sequenced.

2. **To identify endosomes as the subcellular site of C-peptide/CPR signaling.** Endosomes are likely candidate sites for intracellular signaling by the C-peptide/CPR complex. In this aim, we will block C-peptide internalization at different stages, by using pertussis toxin and endosomal Rab5 trafficking mutants, to determine at which station along its endocytic route C-peptide activates its intracellular signaling pathway.

3. **To investigate the anti-inflammatory effect of C-peptide on high glucose-induced vascular dysfunction *in vivo*.** We have definite proof that C-peptide displays a powerful anti-inflammatory effect on endothelial *cells in vitro*. It is important to investigate whether this anti-inflammatory activity of C-peptide is also observed *in vivo*. To this aim, we will inject C-peptide in a mouse model of diabetes-induced vascular disease and study the effect on adhesion molecule expression and macrophage accumulation particularly in the aortic segment.

Our first quarterly scientific progress report for the second year of our project (09/28/11 – 12/27/11) described the following:

Our efforts were focused on the experiments aimed at isolating the C-Peptide Receptor (CPR) as presented in our original **Aims 1 and 2**. These experiments are ongoing in the laboratory right now.

Three were the considered strategies to be undertaken to perform this difficult task and obtain meaningful results

1. Pull-down experiments: Use a set of biotinylated C-peptides, including a wild-type and a randomized version of C-peptide, in pulled-down experiments in an effort to isolate the specific proteins that will be interacting with C-peptide following its physiological internalization in early endosomes. We expect that the proteins that are pulled-down by the biotinylated wild-type C-peptide be different that the ones from the scrambled C-peptide probe. Since the last Progress Report we have perfected the procedure for endosome isolation from HEK and endothelial cells and performed several pull-down experiments. The interacting proteins have been sent to Sequencing Facilities for protein identification.

We are now improving the experimental procedure of these experiments by cross-linking the C-peptide to the plasma membrane so that the binding with the hypothetical CPR will be stronger and more resistant to the different procedures of isolation.

2. siRNA experiments: It has been suggested that the CPR is a G-protein-coupled receptor from studies in which pertussis toxin inhibits the binding to cell membrane and some intracellular signaling. Based on these published results, we have knocked down gene expression of G proteins, subunits that are susceptible to pertussis toxin. To this aim, we have transduced endothelial cells with several Ga shRNA Lentiviral particles, and measured inflammatory cytokine secretion as a functional read-out under high glucose in the presence or absence of C-peptide. We have generated preliminary results in which we identify some Ga subunits as those involved in the anti-inflammatory effect of C-peptide. As an important control, we are now checking with Western blot whether we were successful in knocking down gene expression of all the G proteins, the subunits that we are studying.

If so, we can certainly conclude that a specific Ga protein is mechanistically responsible for the inhibitory effect of C-peptide on cytokine secretion under high glucose.

3. Microarray experiments: As an additional approach to help identification of the CPR, we have also performed microarray experiments in C-peptide treated and not treated endothelial cells to see whether certain G-protein associated genes are up-regulated or down-regulated by C-peptide.

The Bioinformatics Core of the University of Pittsburgh is now analyzing the results from 5 independent experiments performed.

Specifically, to the goal of attempting to isolate the C-peptide receptor by cross-linking a biotin-labeled C-peptide, nearly 100% of the C-peptide in our experiments will have the capacity to be cross-linked. Some specific position of the cross-link to C-peptide may not be optimal, but we have the option of testing several different positions to maximize the success of this step. We have obtained chemically synthesized C-peptide that contains biotin and has also been modified to include cross-linkable amino acids. Specific amino acids from the sequence of C-peptide were selected for modification with the expectation that the C-peptide function would not be adversely affected. Two modified C-peptides have been obtained and one is being tested. We will be testing the second one when we have perfected the various tests and procedures using the first one. We have shown that stimulation of Ca^{++} flux by C-peptide or by the first of the modified C-peptides are indistinguishable. We have also shown that the modified C-peptide can be cross-linked to C-peptide antibody and not to an unspecific antibody.

In this reporting period we have been focusing of the separation of the biotinylated molecules from cell lysates. During the course of these experiments we found prominent natively biotinylated proteins present in the cells we are studying. They are present in cell lysates, which have not been exposed to the biotinylated C-peptide and their recovery is reduced by the addition of free biotin to the lysate, Figure 1.

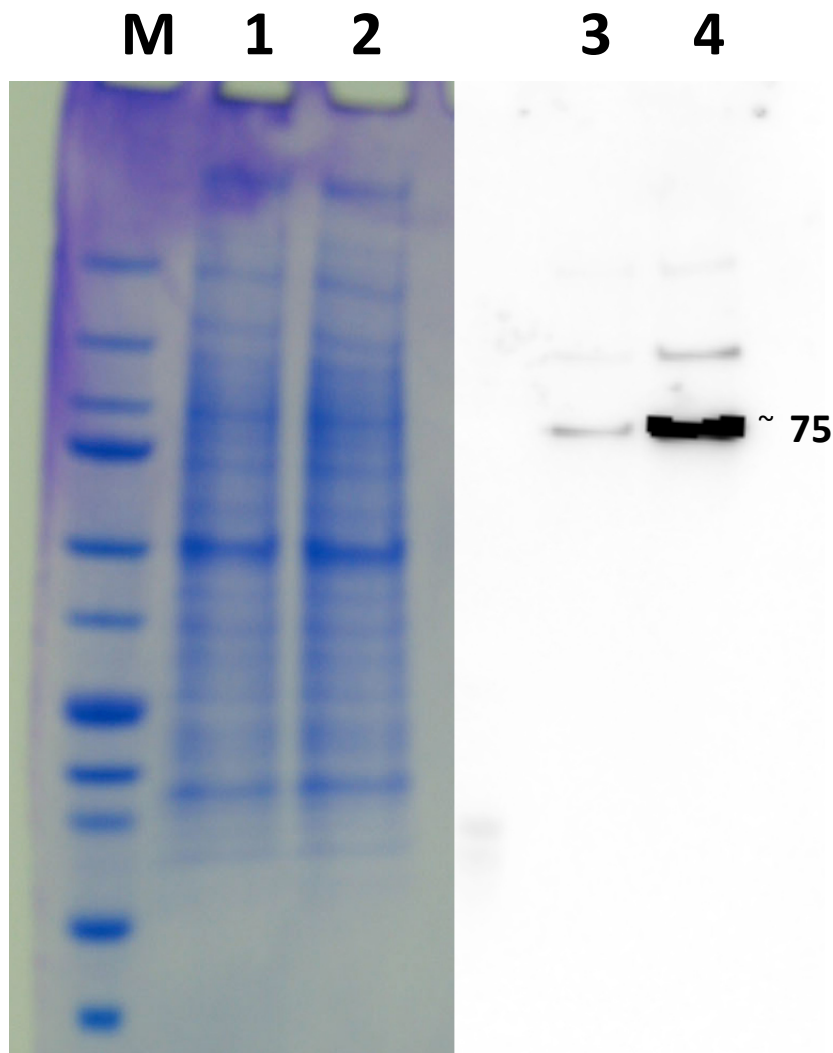


Figure 1. Untreated whole cell lysates were exposed to streptavidin beads in the presence or absence of soluble biotin, lanes 1 and 2, respectively. After washing the beads with phosphate buffered saline, the retained proteins were denatured and separated by SDS gradient-gel electrophoresis. Coomassie blue staining and western analysis for the presence of protein linked biotin (lanes 3 and 4 correspond to lanes 1 and 2) revealed considerable non-specific binding. The reduced band at 75 Kd in lane 3 indicates that free biotin blocked the retention of this band indicating the presence of natively biotinylated protein. (The streptavidin beads had previously been selected for minimal non-specific binding.)

These native proteins may potentially interfere with our experiment in two ways.

First if they are very abundant they may compete with the capture of our cross-linked receptor. We are now evaluating the capacity of the streptavidin beads relative to the abundance of these proteins and will be conducting our experiments under conditions that do saturate the biotin binding capacity of the beads to better recover the cross-linked receptor.

The second possibly is that the molecular weight of the receptor is too similar to these native molecules. In this case, more abundant native proteins can mask, in our gel analysis, the presence of the receptor. If this distinction becomes necessary, we will use C-peptide antibody to separate the C-peptide cross-linked protein from the natively biotinylated proteins. Because the C-peptide antibody that we use works well for detecting C-peptide after exposure to the denaturing conditions of Western blots, we believe it will also be suitable for capturing the receptor protein cross-linked to C-peptide.

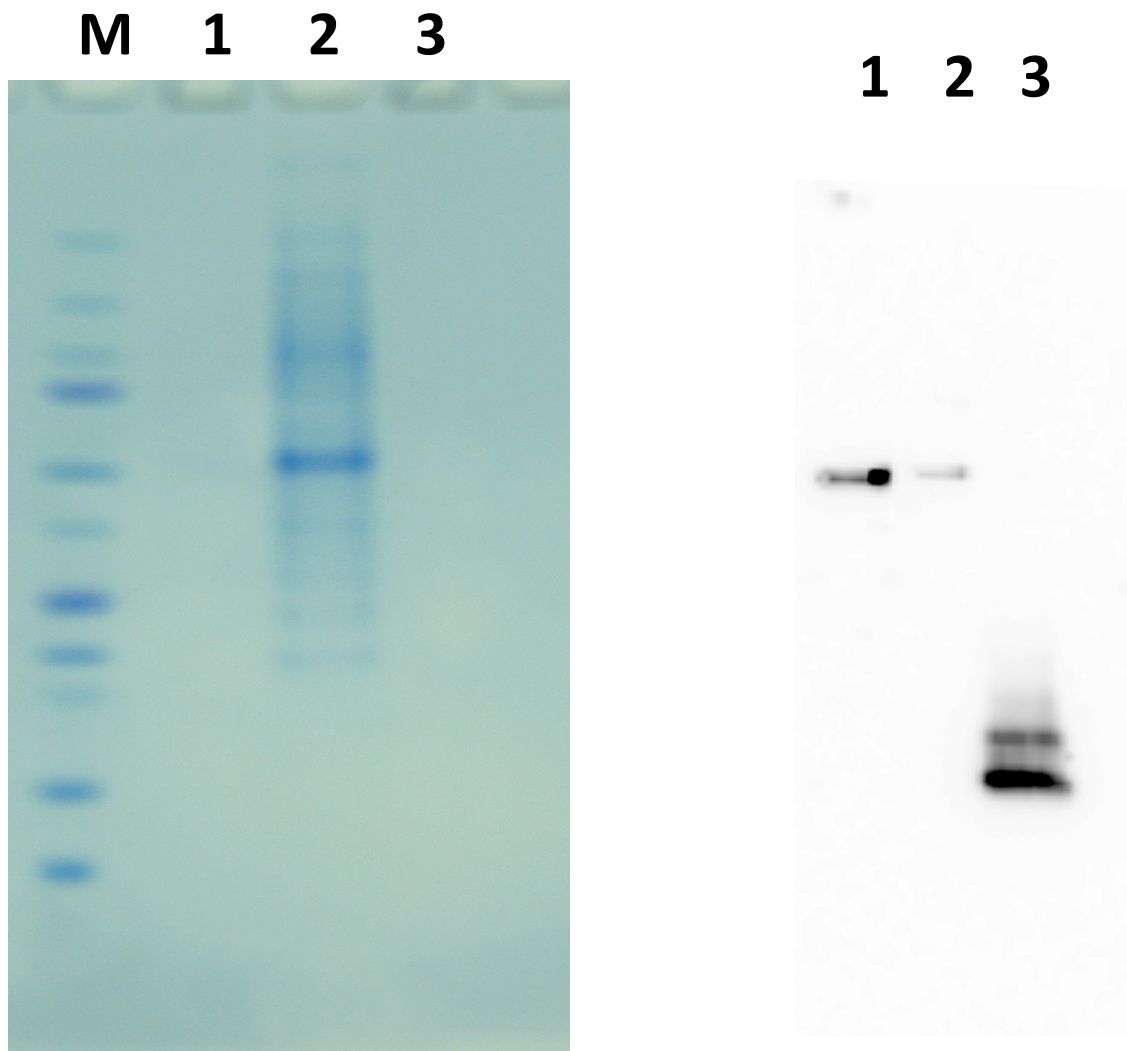


Figure 2. Left Panel. **Reduction of Non-specific Binding.** Cell lysates were combined with streptavidin beads washed before analysis by gel electrophoresis. Lane 1 and 3 were washed with 8M Urea, 10% NP40; Lane 2 with 0.5 M Sodium Acetate pH 4.5. In lane 3 the beads had not been treated with cell lysate.

Right Panel. **Retention of biotinylated protein after stringent washing.** C-peptide antibody and the biotinylated C-peptide containing photoactivatable leucine were combined, treated with UV light and added to Streptavidin beads and washed. The heavy chain of C-peptide antibody, which had been cross-linked to the biotinylated C-peptide, is revealed in western blot analysis with anti-biotin antibody. There is greater retention of the biotinylated protein after an 8M urea 10% NP40 wash (lane 1) than with 0.5 M sodium acetate pH 4.5 (lane 2). Lane 3 is the modified C-peptide run in the gel as a control.

Because of the significant non-specific retention of protein, as seen in Figure1, we have developed stringent washing conditions for the streptavidin coated magnetic beads used to capture the biotinylated proteins. Many conditions were tested with little improvement. However, the biotin-streptavidin binding is sufficiently strong that we were able to use conditions, which are usually too disruptive for protein capture techniques. The use of 8M Urea, 10% NP40 appears to be effective in reducing the retention of non-specific hydrophobic proteins by the magnetic beads without dislodging the biotinylated proteins, Figure 2. With these developments we will soon be conducting the first experiments to reveal a C-peptide receptor protein.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts will be now concentrated on the experiments aimed at isolating the C-Peptide Receptor (CPR) combining these three strategies: **Pull-down** experiments with the **siRNA** experiments and the **Microarray** experiments described before.

Our second quarterly scientific progress report for the second year of our project (12/28/11 – 03/27/12) described the following:

The efforts were focused on the experiments aimed at isolating the C-Peptide Receptor (CPR) to fulfill the goals of the originally proposed **Aim 1**, at testing the anti-inflammatory effect of C-peptide on high glucose-induced vascular dysfunction *in vivo*, to fulfill the goals of the originally proposed **Aim 3**.

For **Aim 1**, following the strategy of “**pull-down the receptor**”, we have obtained the first evidence that we may have a protein band resolved by SDS gel electrophoresis that is associated with C-peptide.

Figure 1.

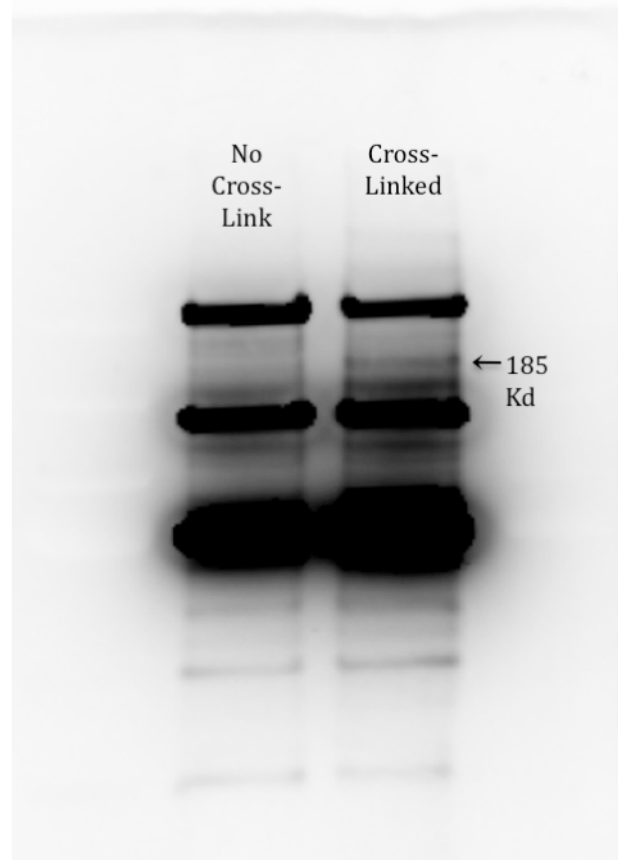


Figure 1. C-peptide associated protein cross-linked to C-peptide. Mouse macrophages (RAW 264) were treated with photo-linkable, biotinylated C-peptide and then half of them were UV irradiated for 20 minutes. The cells were lysed and the soluble fractions (each containing 6.5 mg of protein) were combined with Miltenyi streptavidin beads. The beads were washed with 8M urea-0.5% NP40 and then eluted with SDS gel loading buffer. 13% of the recovered fractions were analyzed on SDS gradient gels. Biotin containing proteins are revealed with an anti-biotin FAB antibody fragment. A band estimated to be 185 Kd is present only in the sample cross-linked with the modified C-peptide (right lane). The prominent bands seen in both lanes are endogenous biotinylated proteins.

It appears to become biotinylated when it is cross-linked to our modified C-peptide, which contains both biotin and a photo-activated cross-linking site. For this experiment, cells were treated with the modified C-peptide and some were exposed to UV light to activate the cross-linking process. Streptavidin beads were used to capture biotinylated proteins from the total protein extracts of the two cell preparations. In the figure, we see an abundance of natively biotinylated proteins that are present in both extracts as well as a band at 185 Kd, which is unique to the C-peptide cross-linked preparation. This western blot was

developed with an anti-biotin antibody. We have not yet been able to obtain similar results with a C-peptide antibody, which is most likely due to a lower avidity of the C-peptide antibody. We have been testing a panel of C-peptide antibodies to select an antibody that will improve the sensitivity of the C-peptide detection in western blots. We expect that, in addition to the normal variation between antibodies, the epitope of C-peptide that is recognized by a specific antibody needs to be far enough from the site of cross-linking that the antibody binding is not diminished. To test for an optimal C-peptide sensitivity, we are cross-linking C-peptide bound to one of the C-peptide antibodies and then comparing the Western blot detection sensitivity of the other antibodies in our panel. We will also be using the antibody cross-linked to C-peptide as a qualitative internal control to estimate the abundance of the C-peptide associated proteins in the western blots.

We have also begun experiments with other modified C-peptides that differ in the amino acid position that is involved in forming the cross-link. These experiments may confirm the above findings and might also improve the efficiency of the cross-linking step.

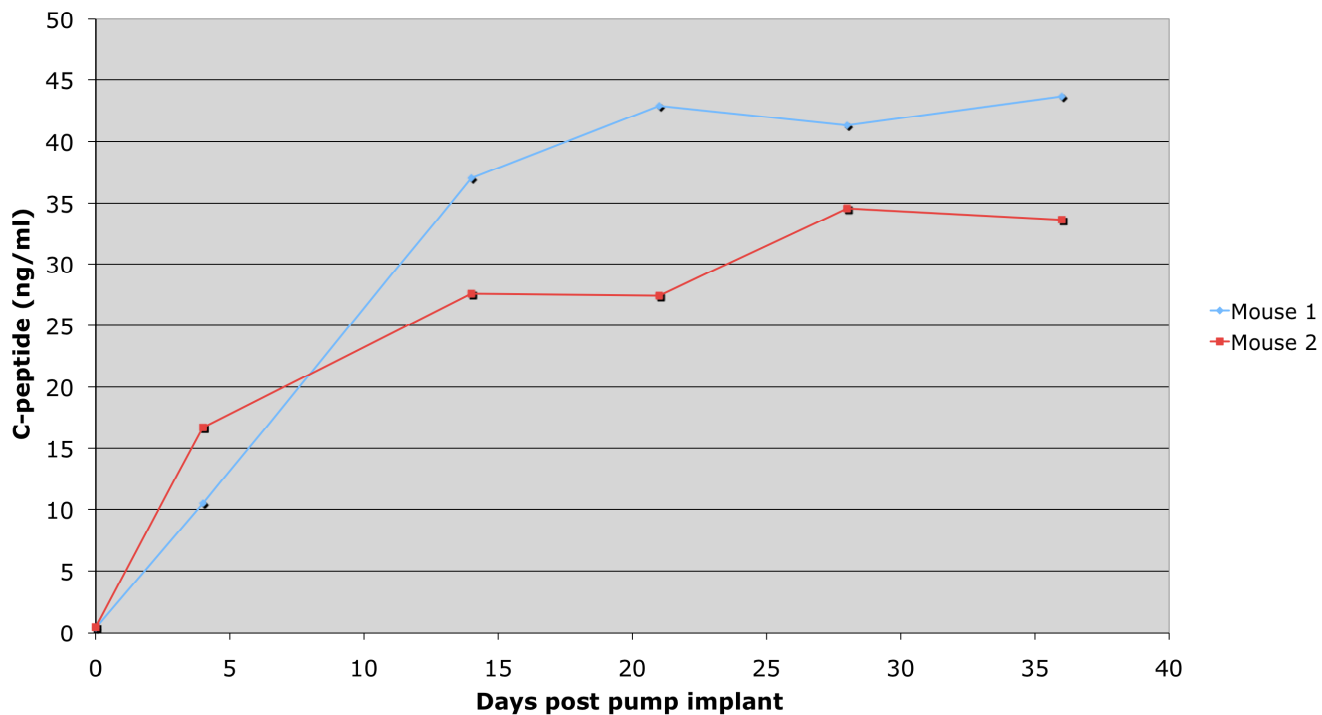
During this period we have also considered the possibility that since the C-peptide is internalized to endosomes, the time that the C-peptide is in contact with surface receptors may be transient and that the cross-linking to these molecules may thus be less frequent. As a possible experimental approach to this problem, we are attempting to first bind the biotinylated C-peptide to beads, which may then interfere with the normal internalization and increase the time that the C-peptide is in contact with the surface receptor. We also plan to saturate these beads with free biotin to block the binding of any natively biotinylated proteins. To begin these experiments we would like to demonstrate that the bead bound C-peptide is able to induce the calcium flux that occurs with soluble C-peptide. Our first attempts at this have not been successful and we have reasoned that the biotinylated C-peptide may need a longer linker between the biotin and C-peptide to allow the bound C-peptide to be more freely available to the receptors on the cells. We are having these modified versions of the C-peptide made for these studies.

For **Aim 3**, we started by determining if the human C-peptide could be delivered in the mice over time using the Alzet mini infusion pumps, without degrading it. The pumps were filled with 200 mg of human C-peptide (Phoenix Pharmaceuticals, Inc), 1 mg/ ml, and placed subcutaneously on the back of diabetic (i.e., streptozotocin treated) mice. The pumps are designed to deliver 6 ml per day. Serum was taken on day 0, 4, 14, 21, 28 and 36. Then, the serum was analyzed for C-peptide content (by ELISA). **Figure 2**.

The results of this preliminary test are that the human C-peptide can be detected up to 36 days after implanting the pump and that the mice either did not have any endogenous C-peptide or the test did not detect any mouse C-peptide as evidenced by the low reading at day 0 (before the pump was implanted)

The levels of human C-peptide detected in this test were found to be, on average, about 4 times the normal concentration for a mouse. We are planning a larger experiment in which we will deliver either a normal concentration or double the normal concentration of C-peptide, in combination with insulin therapy and a high fat, high cholesterol diet. We will then check for atherosclerotic plaques in the aorta of low density lipoprotein receptor deficient (LDLR^{-/-}) mice that have been rendered diabetic, to compare the amount of plaques to control mice receiving the same treatment but without C-peptide.

Figure 2: human C-peptide in mouse serum



12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts will be now concentrated on the experiments aimed at isolating the C-Peptide Receptor (CPR) and at acclimating the LDLR^{-/-} mice to the high fat, high cholesterol diet before making them diabetic chemically with streptozotocin and start the C-peptide infusions again.

Our third quarterly scientific progress report for the second year of our project (03/28/12 – 06/27/12) described the following:

With regards to Aim 1.

In our previous report we had observed a 185 kd band that was cross-linked with our modified C-peptide probe and detected with HRP streptavidin. We have made attempts to further study this protein, knowing that it was a relatively minor component of the cellular lysate of the macrophage lysates we were preparing. Efforts to increase the amount of cell lysate or to increase the sensitivity of C-peptide detection in our western blotting methods have not been successful as far as improving the resolution of this band and have also not been consistent in reproducibly revealing it. We were not able to reveal the same band with C-peptide antibody.

As we continued in this direction we were concerned that we were also not obtaining evidence of a background of non-specific cross-linking of the C-peptide probe. We then investigated the possibility that free amino acids in the culture media were competing with the target proteins in the cross-linking reaction. We tested this possibility by attempting to cross-link our probe to C-peptide antibody as we have done before in amino acid free solutions, but now in the presence of amino acids. Although we observed differences in the efficiency of the cross-linking reaction when using different antibodies, we did not observe a dramatic reduction in the presence of free amino acids verses their absence. However, this may have been related to the inaccessibility of the reactive sites in the antibody-C-peptide complex to the free amino acids and may not exclude the possibility of a negative effect. Nevertheless, when the cross-linking reaction was performed with our cellular lysates we did not see any improvement of the detection of cross-linked C-peptide.

Based on our previous work, we expected that C-peptide might be rapidly taken up by cells and delivered to endosomal compartments. In this process the C-peptide may not be in contact with a receptor for a sufficient length of time to allow the cross-linking of C-peptide and the receptor molecule to occur to any great extent.. To address this possibility, we reasoned that a large micro-bead with bound C-peptide may be able to settle onto attached cells growing in a dish and also resist the internalization process so that the contact period of the C-peptide and the receptor is increased. However, the surface of the micro-beads at the level of molecular dimensions is quite irregular and the C-peptide may need a linker arm for it to project away from the bead surface sufficiently enough to allow free interaction with receptor molecules on the cell surface. With this in mind we have designed a set of three new cross-linkable C-peptide molecules. Each of these molecules each will have a hemagglutinin antigenic epitope inserted between the N-terminal biotin and the C-peptide sequence. This will serve both as the linker extension and also as another marker that can be probed on our western blots. Very sensitive reagents are available for the hemagglutinin epitope, and the epitope will also be useful for us to avoid the signal from the biotinylated endogenous proteins that we observe when using biotin detection reagents. The three new probes differ in the position of the cross-linking site in the C-peptide sequence. The delivery of these new molecules has been delayed, but we expect to receive them before the end of July.

We have also reexamined some published reports of rhodamine labeled C-peptide binding by intact cells (R. Rigler, et al., PNAS, 1999, 96:13318). This report was based on measurements of surface phenomena and seemed to indicate a long-temporal-surface presence of a C-peptide-receptor complex. We had previously set these observations aside because in our hands the C-peptide seemed to be readily internalized. Nevertheless, the findings were that the putative C-peptide receptor was present at 75 receptors/mm² and that receptor binding could be blocked by unlabeled C-peptide. For us, the puzzling part was that 85% of the binding could be displaced by non-labeled C-peptide over the course of three hours. The possibility that a C-peptide-receptor complex remained on the surface for such long times did not seem to match our other observations. However, because this binding was also observed in detergent solubilized cellular fractions (M. Henriksson, et al., Biochem Biophys Res Commun, 2001, 280:423) and that the specified detergent, CHAPS, was also know to maintain binding activity of solubilized G-protein coupled receptors, we chose to test this detergent- solubilized fraction

with our cross-linking method. We also expected that receptors that were this abundant should easily be detected with our methods provided that the cross-linkable sites were favorably positioned or that the CHAPS detergent might better expose cross-linkable sites. Thus, streptavidin beads were loaded with our biotinylated C-peptide, washed, and blocked with an excess of biotin to reduce the capture of endogenous biotinylated proteins. These beads were then mixed with a CHAPS solubilized cell fraction and then cross-linked by UV irradiation. An identical sample was prepared but without the UV treatment. We quickly noticed that the UV treated beads were more slowly eluted from the Miltenyi magnetic capture device. The control sample beads were eluted almost completely with 100 ml of gel loading buffer, while the cross-linked sample required more than 500 ml to recover most of the beads.

Figure 1 shows the results of western analysis detecting biotinylated proteins size separated by SDS gel electrophoresis. The lanes from the cross-linked sample show bands with molecular weights of 16, 21, 29, 35, and 48 kd that are not seen in the non cross-linked fractions. The bands with molecular weights of 83, 150 and >250 kd are in both preparations and may represent proteins that are natively biotinylated and that bind non-specifically to the beads. The increased intensity of these bands in the cross-linked fractions may reflect an additional biotinylation that occurs when the protein non-specifically binds closed enough to one or more of the C-peptide molecules to be cross-linked. The proteins that are cross-linked are expected to be 3,271 daltons larger than their native size because of the C-peptide addition.

Several experiments are underway to confirm and explore the details of this observation. Most important is that we show that native C-peptide can specifically compete with our cross-linking C-peptide. So far, the evidence may only indicate that the molecules that are cross-linked are associated with a C-peptide complex that may involve other molecules in less favorable positions for a cross-link to occur. This can be explored by altering the position of the photo-leucine incorporated into the C-peptide sequence and will be first tested with the C-peptides that are now being prepared. We will also be increasing the scale of our procedure to obtain sufficient material for identification by mass spectrometry. These larger scale experiments will also allow us to examine if there are other proteins that are also enriched in the cross-linked preparation.

With regards to Aim 3:

After the conclusion of the preliminary experiment to determine the feasibility of delivering the human C-peptide using the Alzet mini infusion pumps, we did a larger experiment starting with 9 mice to determine what concentration of C-peptide would be most effective for our purposes. The normal level of C-peptide in a mouse varies with strain and environmental stress so it is difficult to determine normal levels. An article that was published last year (Vasic D, Marx N, Sukhova G, et al.: C-peptide promotes lesion development in a mouse model of arteriosclerosis. *J Cell Mol Med.* 2012) indicates that C-peptide given at 4 fold the “normal” amount was damaging in an arteriosclerosis mouse model. Our mouse model is of a similar strain and the results from our preliminary test showed similar levels of C-peptide as was detected in the article. Using this

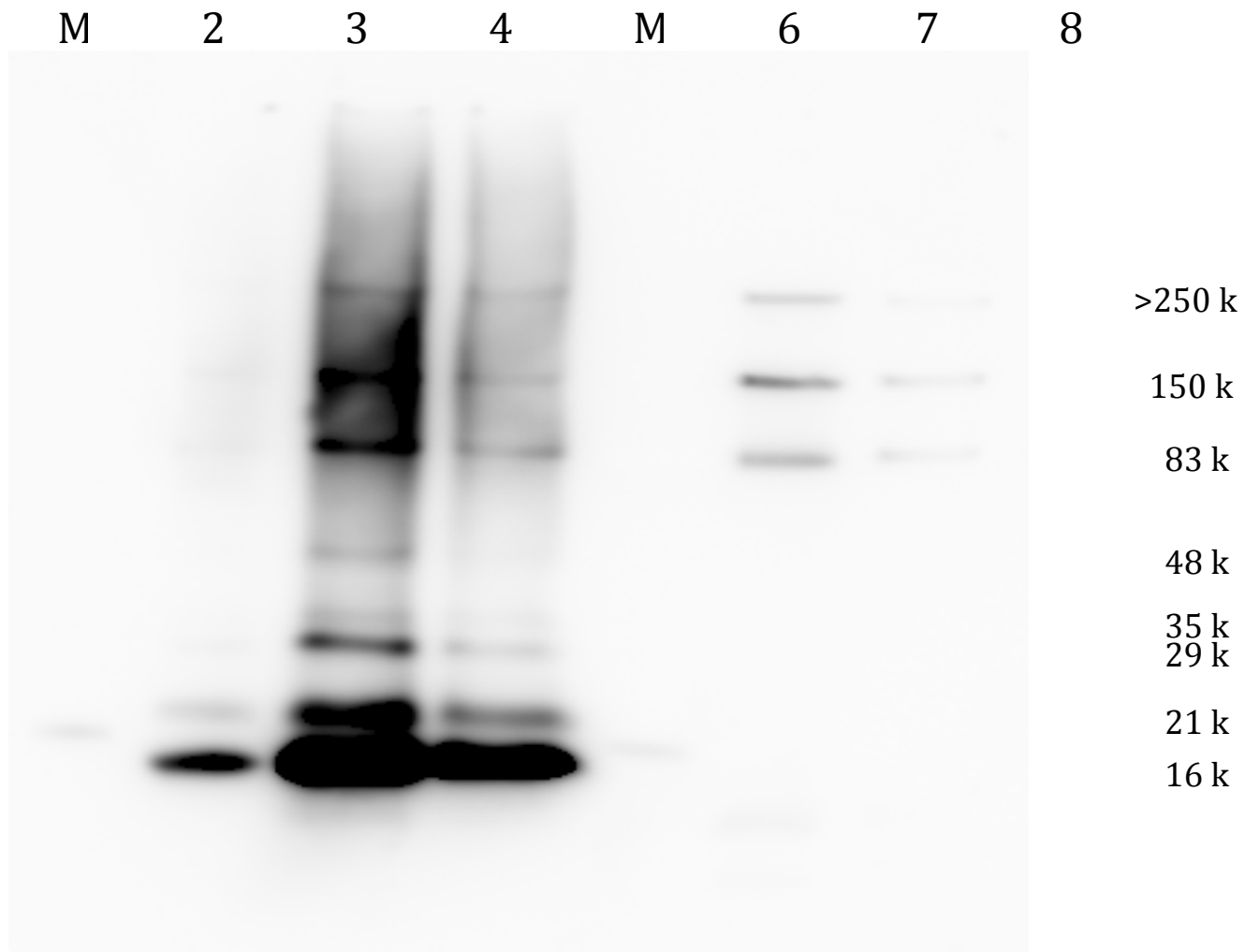


Figure 1. Biotinylated C-peptide cross-linked to proteins from CHAPS solubilized cell fractions and detected with anti-biotin antibody. Lanes 2, 3, and 4 are successive 100 ml fractions eluted from the cross-linked sample. Lanes 4, 5, and 6 are from the non-crossed linked sample. The lanes marked M contained molecular weight standards used to estimate the sizes in the right column. The indicated sizes include the 3,271 kd of the cross-linked C-peptides. The 20 kd marker protein which is visible in this image interacts weakly with the biotin detection reagents.

as a basis, we decided to try a normal level (1 fold) of C-peptide and 2 fold of normal. We made the mice diabetic, placed insulin pellets and the pumps with C-peptide or saline under the skin, and measured human C-peptide in the mouse serum. There were 3 mice each receiving either 1 fold or 2 fold C-peptide and 3 controls receiving saline.

Unfortunately, 2 mice died after being made diabetic. In the end, only 3 mice survived for the full follow-up (~45 days) and it was believed they died because they were not eating the special diet and they lost too much weight as shown in the chart of their weight over time (**Figure 2**). Of the 3 surviving mice, there was one from each category and the human C-peptide levels are shown in our next chart (**Figure 3**). The 2 fold pump appeared to be defective and delivered the C-peptide at a higher rate than expected.

After this unfortunate outcome, we changed the source of the special diet and found the mice liked it better and would eat it. We also decided to use one concentration of C-peptide (1.5 fold of normal) to eliminate one of the variables in this already difficult experiment. With these new parameters, we have started a larger experiment but there are still problems with mortality due to diabetes. Tight regulation of blood glucose in mice over time is difficult whether maintaining them with insulin injections or with the insulin pellet implants. We are a little over a month into this 3 month experiment.

Figure 2

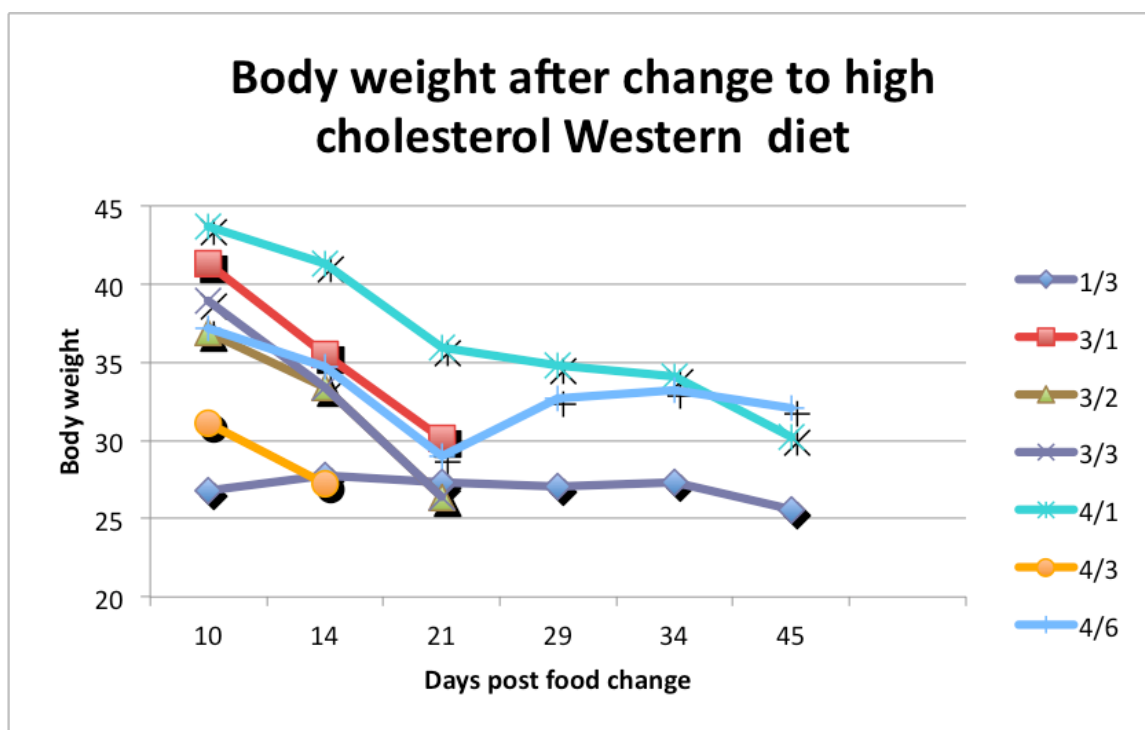
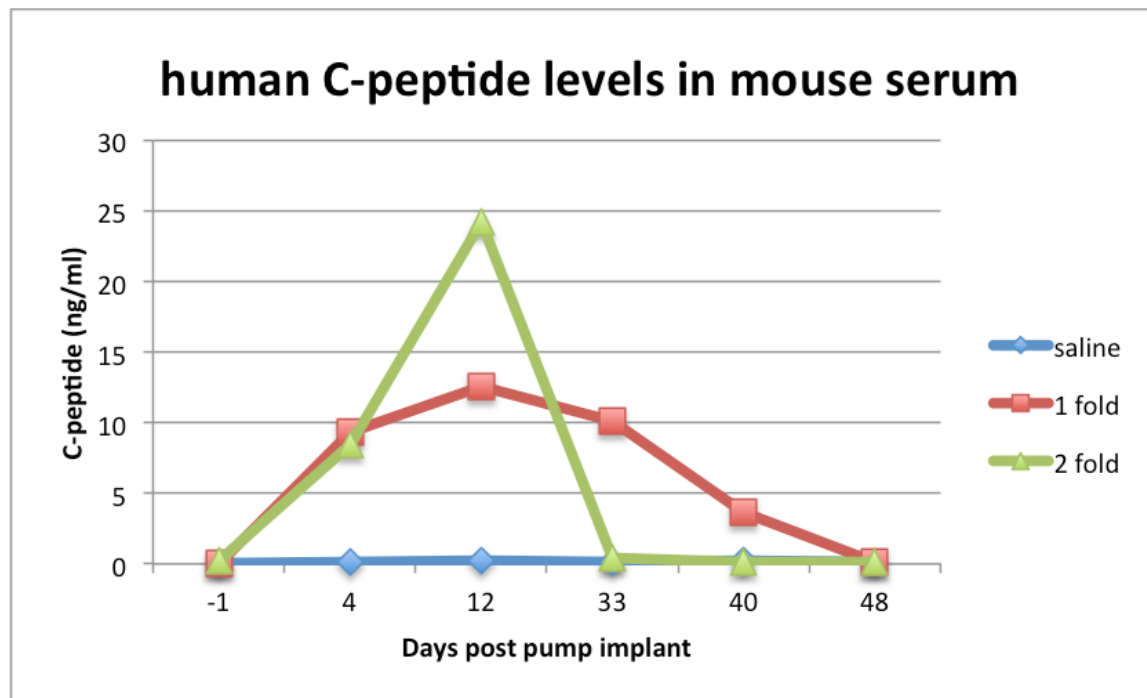


Figure 3



12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts still continue to perform experiments aimed at isolating the C-Peptide Receptor (CPR) and at acclimating the LDLR^{-/-} mice to the high fat, high cholesterol diet before making them diabetic chemically with streptozotocin and start the C-peptide infusions again.

In the fourth quarterly scientific progress report (06/28/12 - 09/27/12) of year 02, we now report on our new and year 02 cumulative results.

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.

In previous experiments we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)- κ B pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). 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 (5).

During this quarter we have continued our efforts to obtain a molecule that binds C-peptide. Our earlier attempts used biotin tagged synthetic C-peptides that contained modified leucines which could be cross-linked to an interacting molecule after activation by UV light. In these experiments we first showed that calcium flux could be induced by our modified C-peptides just as effectively as with unmodified C-peptide. When we attempted to isolate cross-linked molecules by capturing biotin, we discovered that there was an abundance of endogenous biotin labeled molecules. An excess of biotin blocked the capture of these molecules. Thus, it appeared to be specific binding of biotin rather than non-specific binding to the capturing beads. Although we adjusted the biotin capture capacity to accommodate the excess of endogenous molecules we did not observe stronger bands on western blots relative to control samples. In this case, a relatively weaker signal could have been easily obscured by the signal from a nearby endogenous biotinylated protein in our western blots.

During the course of these studies we used one of two different controls to confirm any potentially positive results. One in which the modified, cross-linkable C-peptide was present but the sample was not cross-linked with UV light, and the other when an excess of unmodified C-peptide was added during the binding step to specifically block the binding of the modified C-peptide and then both the experimental sample and the control were treated with UV light. In some cases, we noted small differences, but they either were not reproducible or they did not stand up against both controls. We then altered our approach by first capturing the modified C-peptide on streptavidin beads and blocking the remaining streptavidin with an excess of biotin. We then attempted to capture a C-peptide interacting molecule from cell lysates followed by activation of the cross-linking with UV light. Although we observed an intense cross-linked band in western blots, we discovered that not all subunits of the bead-bound streptavidin tetramer were anchored to the beads and the intense band was C-peptide cross-linked to unanchored streptavidin monomers that were released during the denaturing gel electrophoresis.

While these experiments were ongoing, we recognized that we should consider another capture method. We now have three additional modified C-peptides (BHACPL21, BHACPL24, & BHACPL26, Table 1) that still have biotin but also include an 11 amino acid, commonly used,

Table 1. Photo-cross-linkable C-peptides

BCPL21	Biotin- EAEDLQVELGGGPGAGSLQPLALEGSLQ
BCPL21+26	Biotin- EAEDLQVELGGGPGAGSLQPLALEGSLQ
BHACPL21	Biotin- GYPYDVPDYSLGE AEDLQVELGGGPGAGSLQPLALEGSLQ
BHACPL24	Biotin- GYPYDVPDYSLGE AEDLQVELGGGPGAGSLQPLALEGSLQ
BHACPL26	Biotin- GYPYDVPDYSLGE AEDLQVELGGGPGAGSLQPLALEGSLQ

Human C-peptide sequence in bold letters.
[Red **L** are leucines modified to enable photo activated cross-linking]

antigenic epitope of hemagglutinin at the amino terminal end of C-peptide. We chose to retain the biotin, because it is a very sensitive tag for our western blots since the sensitivity of the available C-peptide antibodies on western blots is significantly lower than with biotin.

There were difficulties in the preparation of one of the peptides, BHACPL21, and its purity is only 70%, so we are first conducting experiments with BHACPL24 and BHACPL26. By cross-linking these peptides to C-peptide antibody we have validated the capture protocol when using the hemagglutinin antigen and have shown that free hemagglutinin peptide present during the capture step will block the recovery of the antibody cross-linked to C-peptide.

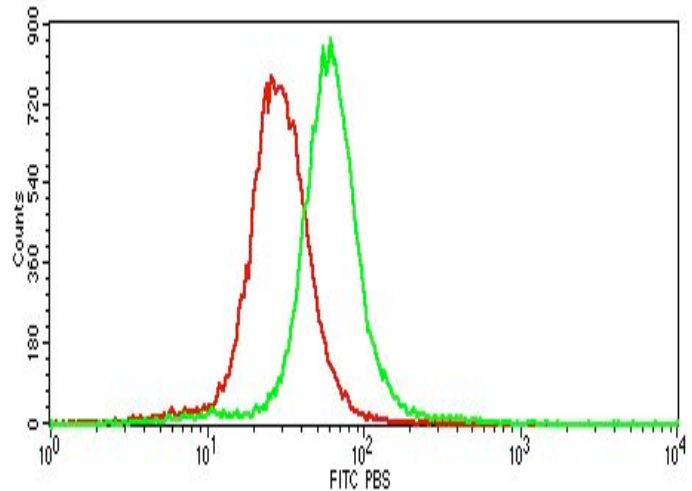
We have also expanded our studies to include HEK293 cells, as well as HEK293 cells grown in serum-free suspension cultures. Many of the successful reports of C-peptide binding use a short period of serum-free incubation and we would like to avoid the interference of any C-peptide which may be present in the serum or possibly other serum factors that may compromise the C-peptide binding. These suspension cultures will also be easily scaled up to be able to pursue a positive result.

We are also examining a recent report that alpha enolase binds c-peptide (Ishi et al., J. Biochem. 2012, 152:53). While alpha enolase has long been known for its involvement in glycolysis, it is now recognized that it has multiple functions that include cell surface binding of plasminogen. This recent report showed that c-peptide activated ERK phosphorylation similar to plasminogen, and that the c-terminal lysine residue of alpha enolase was necessary for both the c-peptide and plasminogen responses. We have validated that alpha enolase is present on the surface of the HEK293 cells being cultured in our lab (Figure 1) and are moving forward with our cross linking studies with a particular interest in cross-linked products in the size range corresponding to alpha enolase subunits.

Figure 1. Surface staining of Alpha Enolase

FreeStyle 293-F cells (Invitrogen) were grown in serum-free chemically defined medium. Alpha enolase was revealed with FITC labeled anti-alpha enolase antibody followed by fixation and FACS analysis.

Green: anti-alpha enolase; Red: unstained



We have also continued our studies of the *in vivo* effects of C-peptide. To test our delivery system of C-peptide in mice, C-peptide was continuously infused using an implanted osmotic pump (Figure 2A & B) to deliver an amount of C-peptide equivalent to 0.25 U of insulin per day to diabetic mice that are also receiving insulin. We were monitoring circulating C-peptide to determine if our delivery process was effective and made adjustments according to the results obtained over 30 days of treatment. The pumps are expected to function for about 30 days, so we were replacing pumps at that interval to complete the 90 days of our actual experiments.

Figure 2A

C-peptide and Atherosclerosis

LDLR^{-/-}GP mice

Low density lipoprotein receptor deficient mouse with viral glycoprotein expression on the beta cells

Diabetes is inducible with an injection of LCMV (lymphocytic choriomeningitis virus)

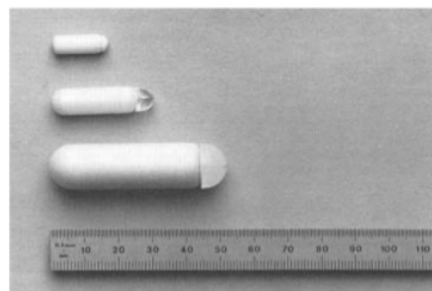




Figure 2B. The osmotic pump in place

We have completed the first full 3 month protocol of delivering C-peptide as well as insulin to diabetic LDLR^{-/-} mice who are also being fed a high fat, high cholesterol (Western) diet. The mice were then sacrificed and the aortas examined for the amount of plaque visible. We are still in the process of analyzing the data but 4 of the aortas are shown below. Figure 3 was a control (insulin therapy only). The other 3 figures show aortas from mice that received both insulin and C-peptide therapy. Figure 4 & 5 received a normal amount of C-peptide (1 fold) and Figure 6 received a higher amount (1.5 fold) C-peptide. Preliminarily, it seems from this small sample that there is a trend toward less arterial plaque when combining C-peptide with insulin therapy to treat diabetic patients on a Western diet.

There were still technical problem with glycemic control and several mice died because of hypoglycemia. This complication was due to the mice losing weight because of being diabetic and most became less than 20 grams in weight. The subcutaneous insulin pellets that are available for long-term (~1 month) insulin release are meant to deliver a dose that is calculated for mice weighing over 20 grams. The pellets therefore released too much insulin for the size of the mice and they became hypoglycemic. Efforts were made to rescue the animals (injections of glucose, removal of the pellets) but several were lost due to this cause. Changes have been made wherein we give the mice twice daily insulin injections instead of using the subcutaneous insulin pellets. So far, with this change, we have not lost any more mice because of hypoglycemia.

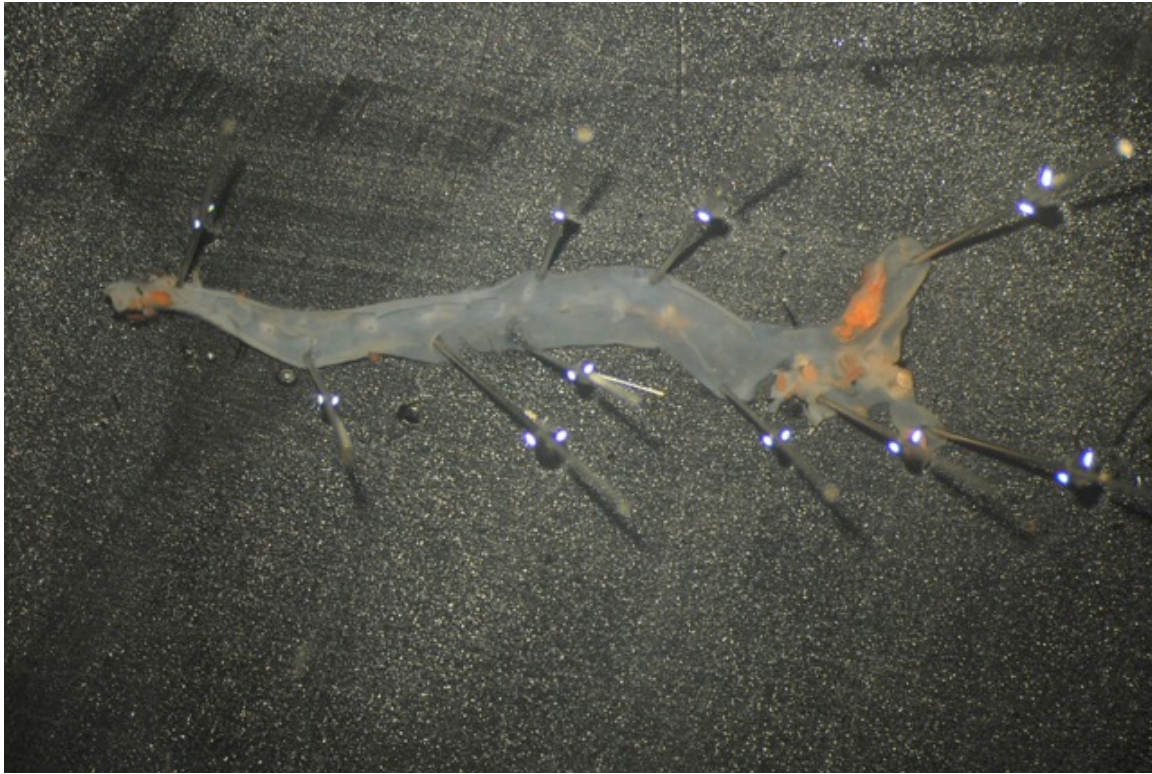


Figure 3. Insulin Only. Mouse aorta stained with Sudan IV Solution, cut open and pinned to dissection board (plaques are stained orange). Magnification 100X



Figure 4. Insulin and C-peptide (1 fold)

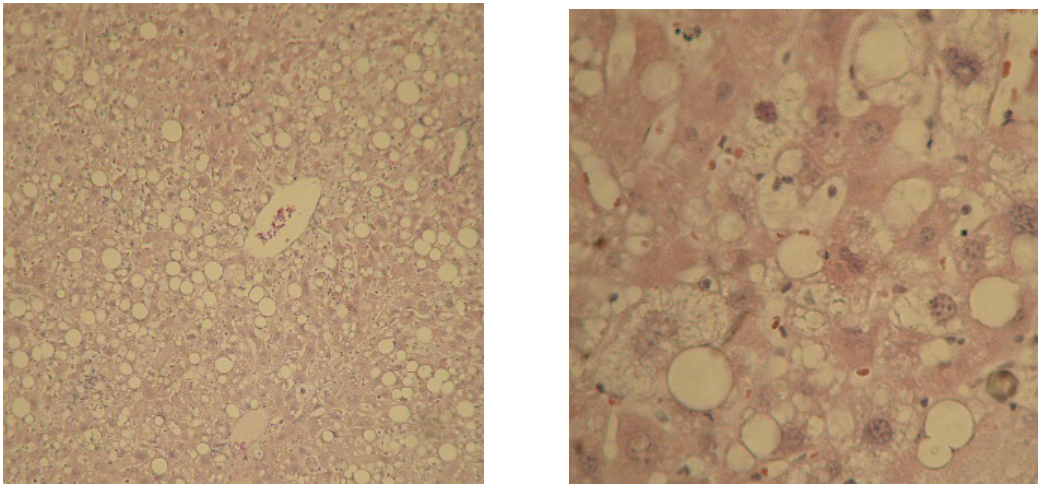


Figure 5. Insulin and C-peptide (1 fold)



Figure 6. Insulin and C-peptide (1.5 fold)

Another problem that we are seeing with this model is the development of fatty livers from the high fat, high cholesterol diet. At the conclusion of the first full 3 month protocol, approximately 85% of the animals, regardless of therapeutic treatment, had some degree of this condition with about 20% so severe that they died or had to be sacrificed before the conclusion of the experiment and therefore excluded from the results. [Figure 7](#) shows a section of liver from one of these severely effected animals. Because of this loss, we have to increase the number of animals per group to reach statistical significance.



[Figure 7](#). H & E of mouse liver showing fatty deposits from diabetic animal fed on a high fat, high cholesterol diet for 3 months. Panel on left, magnification 100X, Panel on right, magnification 400X.

KEY RESEARCH ACCOMPLISHMENTS:

We have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)- κ B pathway and a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells. Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it was hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes.

Certainly a 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. This is the focus of our current work.

Although certainly preliminary, it seems from the small sample tested so far that there is a trend toward less arterial plaque when combining C-peptide with insulin therapy to treat diabetic patients on a Western diet.

REPORTABLE OUTCOMES: Manuscripts (4 publications)

1. Luppi P, Cifarelli V, Wahren J: C-peptide and long-term complications of diabetes. *Pediatric Diabetes* 12:276, 2011.
2. 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.
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.
4. 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.

CONCLUSION:

The previous view that C-peptide is merely an inert by-product of insulin biosynthesis seems no longer tenable. Results from studies in T1D patients and animal models demonstrate that C-peptide in replacement doses exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. Even a cautious evaluation of the available evidence presents the picture of a previously unrecognized bioactive peptide with therapeutic potential in an area where no causal therapy is available today. (See also Luppi P, Cifarelli V, Wahren J. *C-peptide and long-term complications of diabetes. **Pediatric Diabetes** 2011; 12: 276–292).*

Undoubtedly, the identification of the mechanism whereby C-peptide interacts with cell membranes, delineation of its intracellular signaling pathways in different cell types, and further evaluation of its transcriptional effects will enhance our understanding of C-peptide bioactivity.

On the clinical side studies of long duration (>6 months) are performed to document the robustness of its beneficial effects on the different types of long-term complications in order to define its possible role in the therapy of T1D.

Despite the fact that our knowledge is still incomplete, we believe there are several lines of evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in T1D may be beneficial in the treatment of long-term complications. Even though the nature of the peptide's interaction with the cell membrane is only partially understood, its intracellular signaling characteristics and end effects are now well established for many cell systems not only by us but also by other valuable investigators.

The So What Section:

What is the implication of this research?

The World Health Organization estimated that 5–10% of all diabetes cases are T1D, i.e., 11–22 million worldwide and ~1 million only in US, where, with an incidence that increases by about 3% per year, it was the primary cause of diabetes in children less than 10 years of age. Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are T1D. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. T1D is associated with a high morbidity and premature mortality due to complications, like cardiovascular diseases, nephropathy, retinopathy and neuropathy. T1D was estimated to cause \$10.5 billion in annual medical costs and an additional \$4.4 billion in indirect cost. \$1 of every \$7 dollars of US health is spent for medical care of diabetes without considering the loss of productivity. C-peptide administered regularly in physiological quantities might be an additive to insulin able to reduce those complications.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etio-pathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.

Review Article

C-peptide and long-term complications of diabetes

Luppi P, Cifarelli V, Wahren J. C-peptide and long-term complications of diabetes.

Pediatric Diabetes 2011; 12: 276–292.

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Key words: C-peptide – neuropathy – nephropathy – signaling – type 1 diabetes

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C-peptide or connecting peptide is the 31-amino-acid segment that links the A and B chains of proinsulin and serves to promote the efficient folding, assembly and processing of the insulin molecule in the β -cell endoplasmic reticulum in the course of insulin biosynthesis (Fig. 1) (1). Equimolar amounts of insulin and C-peptide are subsequently stored in secretory granules of the β -cells and eventually released into the portal and systemic circulations. Unlike insulin, the C-peptide escapes hepatic retention and circulates at a concentration approximately 10-fold higher than that of insulin. The peptide is primarily catabolized by the kidneys and the biological half-life of the C-peptide is more than 30 min in adult humans, compared to 3–4 min for insulin (2).

Soon after its discovery in 1967, several investigators evaluated C-peptide for possible insulin-like effects but none were found. The apparent lack of physiological

effects and consideration of the C-peptide structural variability and limited sequence conservation between species (3) led to the general view that the peptide was devoid of physiological effects other than its role in insulin biosynthesis (4). Instead, interest focused on the fact that it is co-secreted with insulin and that plasma concentrations of C-peptide effectively reflect the endogenous insulin secretion (5). As a marker of insulin secretion, C-peptide has been of great value in furthering our understanding of the pathophysiology of both type 1 diabetes (T1D) and type 2 diabetes (T2D). Even though C-peptide left the scientific limelight in the mid 1980s, interest in the possibility that the peptide may exert physiological effects remained. This notion was supported by the clinical observation of long standing that, compared to T1D patients in whom β -cell function ceases totally, those patients who retain a low but detectable level

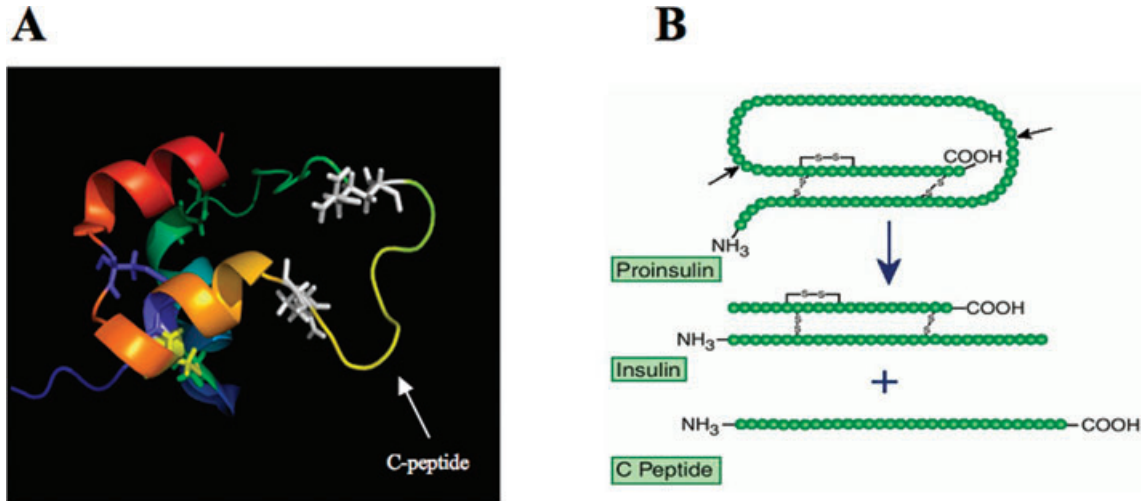


Fig. 1. Proinsulin C-peptide molecule. C-peptide is the peptide segment connecting insulin A and B chains, and a product of proinsulin cleavage in the secretory granules, generated in the pancreatic β -cells as part of normal insulin production. In **A**, a tridimensional image of the proinsulin molecule, from which C-peptide is cleaved by endoproteases at the level of the two Arginine residues linking the B-chain and C-peptide and at the level of the lysine–arginine residues linking the C-peptide and the A-chain (in white) is shown. C-peptide is then secreted into the bloodstream in equimolar amount with insulin in response to elevated blood glucose levels. In **B**, a schematic view of the proinsulin molecule is shown. The black arrows indicate sites of cleavage by proteases at the level of arginine residues. C-peptide circulates at low nanomolar concentrations in healthy individuals, but is absent in most patients with type 1 diabetes (T1D).

of C-peptide are less prone to develop microvascular complications of the eyes, kidneys, and peripheral nerves (6, 7). This view has recently received support from a study involving a large cohort of T1D patients treated uniformly at one medical center. The results show that a remaining C-peptide level above 0.06 nM confers a statistically significant protective effect against the development of microvascular complications independently of glycemic control, duration of diabetes, age, and sex (8). Moreover, pancreas or islet transplantation with restoration of endogenous insulin and C-peptide secretion are known to be accompanied by improvement of diabetes-induced abnormalities of nerve function, endothelial function, and both structural and functional changes of the kidneys (9, 10).

During the last 10–15 yr the above indirect evidence for physiological effects of C-peptide has been supported by a series of studies providing robust and direct evidence that C-peptide is in fact a biologically active peptide in its own right. In this review, we aim to present molecular studies showing binding of C-peptide to cellular membranes, activation of specific signaling pathways, and end effects of critical importance for several cell functions. In addition, clinical studies involving administration of C-peptide in replacement doses to T1D patients will be presented, which highlight beneficial effects on nerve and kidney function. Finally, new aspects of C-peptide physiology and therapeutic possibilities will be discussed, in particular, with regard to the anti-inflammatory characteristics of the peptide.

T1D as an inflammatory disease

Although T cells are recognized to play a central role in the autoimmune destruction of the insulin-producing β -cells (11), recent studies indicate that components of the innate immune system, including natural killer cells, monocytes, and inflammatory mediators have a much broader role in the pathogenesis of T1D and associated vascular complications than the previously recognized components (12–15). The primary role of monocytes in the early stages of T1D pathogenesis has been demonstrated by showing that these cells are the first to accumulate in the pancreatic islets of prediabetic BioBreeding (BB) rats (16). Subsequent T and B lymphocyte infiltration is dependent upon prior monocyte invasion of the islets (16), suggesting that monocytes and secreted inflammatory mediators might contribute to the early induction and amplification of the autoimmune assault against the pancreatic β -cells (17). A more generalized inflammatory response, with activation of monocytes and presence of oxidative stress has been found in the peripheral circulation of T1D patients. This inflammation is characterized by the elevation of plasma levels of several inflammatory biomarkers, such as interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α , and C-reactive protein. Such inflammatory reactions have been detected both in recently diagnosed T1D children as well as in adult T1D patients well after the onset of diabetes (18–22). These findings demonstrate that a generalized inflammatory response is present already

in the very early stages of diabetes (23, 24). Many of the reported inflammatory changes are detected at the level of monocytes, which show upregulation of the adhesion molecule CD11b (Mac-1) (24) and have aberrant constitutive and lipopolysaccharide (LPS)-stimulated expression of cyclooxygenase (COX)-2, a defect which may predispose to a chronic inflammatory response in T1D (15). The vascular endothelium represents a likely target of this inflammatory response by inducing endothelial cell activation, alteration of endothelial function, and monocyte adhesion eventually leading to overt vascular damage in the later stages of T1D. Indeed, inflammation is now considered a major component in the development of T1D-associated vascular dysfunction (20, 25, 26).

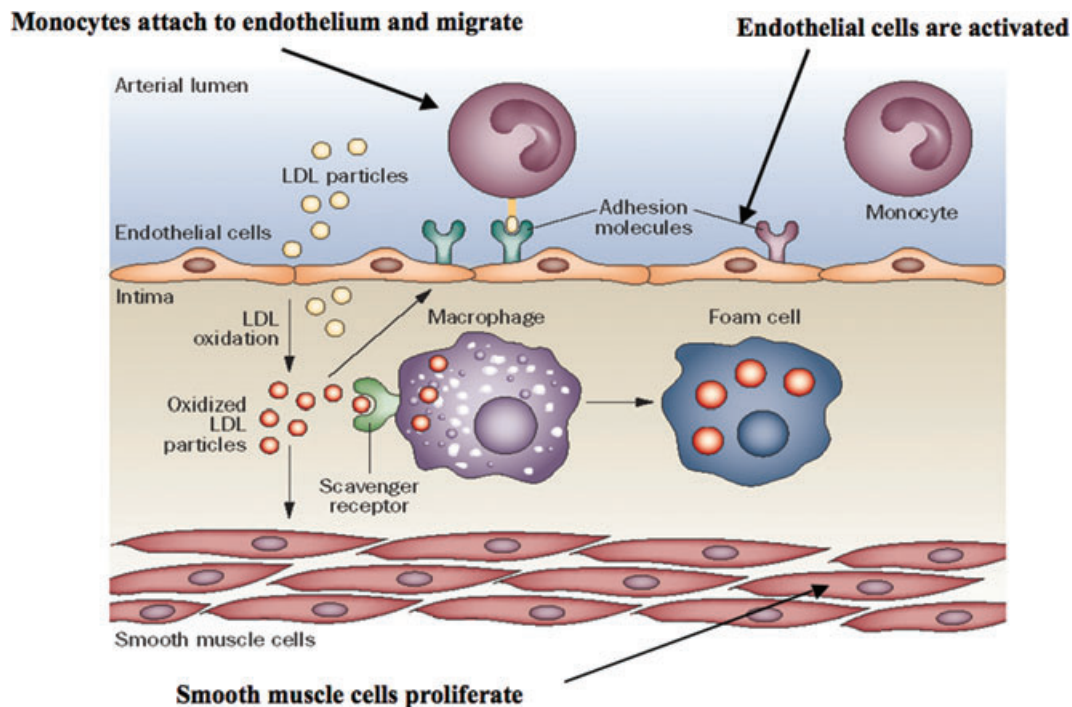
C-peptide and endothelial function

Under conditions of hyperglycemia or other inflammatory insults, endothelial cells become activated by changing the expression of adhesion molecules on the cellular surface, increasing secretion of pro-inflammatory cytokines, and allowing adhesion and migration of circulating monocytes and other leukocytes through the endothelial layer, a first step toward

atherosclerosis plaque formation (Fig. 2) (27–29). C-peptide is now emerging as a molecule displaying potential beneficial effects on the dysfunctional endothelium, as shown in several *in vivo* and *in vitro* models of inflammation-mediated vascular injury. Although the mechanism(s) by which the C-peptide exerts its cytoprotective effects on the endothelium are not entirely understood, it has been reported that C-peptide can influence the activation of different signaling pathways that ultimately modulate or shut down inflammatory responses. In the sections below, we present recent findings in this area.

Effect of C-peptide on nitric oxide and blood flow

In recent years one of the most studied physiological effects of C-peptide is its action on the synthesis of biologically active substances secreted by endothelial cells in both physiological and pathological conditions. Nitric oxide (NO) represents one of these molecules, whose primary functions are to modulate the vascular tone and reactivity (30) and to downregulate inflammatory responses (31). The level of endothelial NO is tightly regulated to ensure maintenance



From Rocha, V. Z. & Libby, P. (2009) *Nat. Rev. Cardiol* 6, 399-409

Fig. 2. Origin of vascular disease in diabetes. Exposure of endothelial cells to high glucose causes inflammatory changes culminating in the upregulation of cell adhesion molecules (i.e., vascular cell adhesion molecule (VCAM)-1) and secretion of pro-inflammatory cytokines i.e., interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1. As a consequence, monocytes and other circulating leukocytes adhere to the activated endothelial cells by interacting with the upregulated adhesion molecules. Subsequently, leukocytes migrate to the subendothelial space, phagocytose oxidized LDL, and become foam cells. Smooth muscle cells proliferate and migrate from the media to the intima of the vessel wall where the new atherosclerotic plaque is developing.

of the adequate vascular tone and endothelial function.

Endothelial cells produce NO in response to elevation of intracellular Ca^{2+} concentrations, which in turn stimulate endothelial NO synthase (eNOS) (32). Under the hyperglycemic conditions typical of diabetes, Ca^{2+} entry in endothelial cells is impaired as a consequence of increased inflammation and oxidative stress (33). This impairment in Ca^{2+} homeostasis affects synthesis of NO (34) leading to attenuation in endothelium-derived vasodilation. *In vitro* exposure of endothelial cells to C-peptide induces release of NO (35–37), an effect responsible for the increased blood flow that C-peptide consistently causes in a number of tissues *in vivo*. In T1D patients, forearm blood flow, measured by venous occlusion plethysmography, showed a concentration-dependent increase in response to C-peptide infusion in the range 0–1 nM (38); no additional circulatory effect of C-peptide occurred when the concentration was raised above 1 nM, in keeping with the demonstration that the binding curve for C-peptide reaches saturation at approximately 1 nM (see below). The stimulatory effect of C-peptide on blood flow is abolished when an eNOS blocker is co-infused with the peptide (39), confirming that the effect is mediated via augmented NO release. The effects of C-peptide on the large artery endothelial function have been studied in T1D during reactive hyperemia, using ultrasound measurement of brachial artery blood flow and diameter (40). Shear stress-induced arterial dilatation, as evaluated by reactive hyperemia, was reduced in the patients. C-peptide administration resulted in a 35% increase in the basal blood flow but did not alter the reactive hyperemia, suggesting that C-peptide exerts its effects primarily on the distal resistance vessels. Forearm vascular dynamics and C-peptide effects have also been studied during exercise. Rhythmic forearm exercise in T1D patients during C-peptide infusion to physiological concentrations resulted in increased blood flow and capillary diffusion capacity, while vascular resistance decreased to levels similar to those for healthy subjects (41). These results and those from a study involving the rat hindquarter model (42) indicate that the effects of C-peptide on skeletal muscle involve facilitation of capillary recruitment.

The effects of C-peptide on blood flow have been demonstrated not only for forearm tissues, mostly skeletal muscle, but also for skin (43), kidney (44), peripheral nerve (45), and myocardium (46, 47). These findings are in keeping with the hypothesis of impaired endothelial function in T1D (48) that is partly corrected by augmented NO availability secondary to C-peptide replacement. The studies on effects of C-peptide on myocardial blood flow involved patients with T1D without signs of heart disease. The left ventricular

myocardial blood flow was reduced in the basal state compared to healthy controls and C-peptide elicited marked increases in basal blood flow (46) and during adenosine-induced myocardial hyperemia (47). Both studies also demonstrated C-peptide mediated improvements in left ventricular performance, as indicated by augmented rates of both contraction and relaxation as well as by increased left ventricular ejection fraction and stroke volume, effects that may be related not only to increased myocardial blood flow but also to myocardial Ca^{2+} influx and Na^+, K^+ -ATPase stimulation.

Besides being related to the NO-mediated direct effects on the resistance vessels, the circulatory effects of C-peptide also involve rheological factors. Erythrocyte deformability is known to be impaired in diabetes (49, 50), with potentially negative effects on the microcirculation. It now emerges that C-peptide is able to ameliorate the altered erythrocyte deformability in T1D. This effect is blockable by ouabain, indicating that it is mediated by a restoration of erythrocyte Na^+, K^+ -ATPase activity (51).

Effect of C-peptide on inflammation and endothelial dysfunction

Several findings support the idea that C-peptide affects leukocyte–endothelium interactions by reducing upregulation of cell adhesion molecules typically observed under inflammatory conditions. The first evidence of this effect is from Scalia et al. (36), who demonstrated that pretreatment with C-peptide to rats injected with the inflammatory agents thrombin or N^G -nitro-L-L-arginine methyl ester (L-NAME), causing acute endothelial dysfunction, resulted in reduced expression of ICAM-1 and P-selectin on the mesenteric microvascular endothelium. As a consequence, the number of rolling, adhering, and transmigrated leukocytes also decreased upon C-peptide administration to the animals. In another model of vascular injury, C-peptide decreased polymorphonuclear leukocyte (PMN) infiltration in isolated rat hearts following ischemia-reperfusion injury (52). PMN infiltration induces endothelial and myocardial injury by releasing cytotoxic substances such as oxygen-derived free radicals, inflammatory cytokines, and proteolytic enzymes. By reducing PMN infiltration to the myocardium, C-peptide restored cardiac contractile function and postreperfusion coronary heart flow (52). These findings have been recently recapitulated *in vitro* in a model of high glucose-endothelial dysfunction in which adhesion of the monocytic cell line U-937 to high glucose-stimulated human aortic endothelial cells (HAEC) *in vitro* decreased by 50% after addition of physiological concentrations of C-peptide, an effect not detected

when C-peptide was heat-inactivated (53). C-peptide was shown to reduce expression of both VCAM-1 mRNA and protein expression in high glucose-treated HAEC. In the same model, C-peptide was also demonstrated to reduce high glucose-induced secretion of IL-8 and MCP-1 by HAEC to the basal levels measured under normal glucose concentrations (53). These two chemokines are essential to promote leukocyte adhesion to endothelial cells. Conversely, when C-peptide was added to the medium containing normal glucose levels, it failed to significantly reduce VCAM-1 expression and IL-8 or MCP-1 secretion from HAEC suggesting that the most meaningful biological effects of C-peptide on the endothelium are observable under conditions of vascular insult or damage.

Potential intracellular pathways mediating the anti-inflammatory effects of C-peptide

The nuclear factor κ B pathway

A likely candidate for C-peptide-mediated anti-inflammatory effects in the vasculature is represented by the signal transduction pathway requiring translocation of the transcription factor nuclear factor (NF)- κ B, a major player in mediating inflammatory responses in a variety of cells (54). In the unstimulated state, NF- κ B exists as a heterodimer composed of p50 and p65 subunits bound to I κ B in the cytoplasm (Fig. 3). Upon activation, for example after cellular exposure to high glucose, I κ B is phosphorylated and degraded, thus causing release of the p50/p65 components of NF- κ B. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of a gamut of genes involved in the inflammatory response, such as pro-inflammatory cytokines, cell-surface adhesion molecules, and chemokines, including IL-8 and monocyte chemoattractant protein (MCP)-1 (Fig. 3) (54). In a recently published paper, it was demonstrated that physiological concentrations of C-peptide reduce high glucose-induced activation of NF- κ B in cultured HAEC, by decreasing translocation of the NF- κ B canonical components p65 and p50 into the nucleus (53). By reducing NF- κ B nuclear translocation, C-peptide might reduce adhesion molecule expression as well as secretion of inflammatory cytokines, such as IL-8 and MCP-1 in cultured HAEC (53). It is not known which NF- κ B-dependent upstream signaling events are affected by C-peptide in endothelial cells; examples are reactive oxygen species (ROS) generation and I κ B kinase, an enzyme that elicits phosphorylation of the cytosolic NF- κ B inhibitor I κ B α . This latter upstream event regulates NF- κ B translocation from the cytoplasm to the nucleus. In vascular smooth muscle cells (VSMC), C-peptide reduces high glucose-induced proliferation; a key event in atherogenesis, by reducing phosphorylation of I κ B α , a pathway likely to be

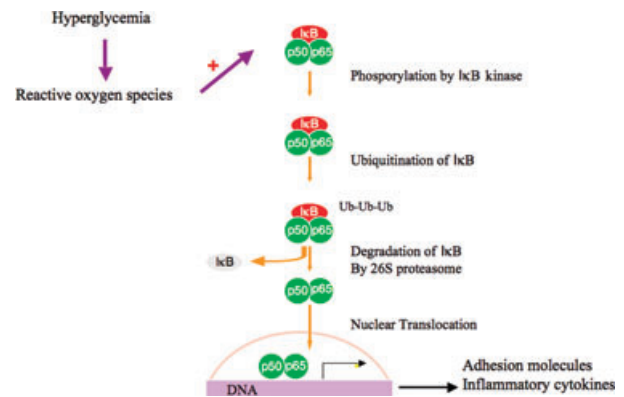


Fig. 3. The nuclear factor (NF)- κ B is a potential target for the anti-inflammatory activity displayed by C-peptide. NF- κ B exists as a heterodimer composed of p50 and p65 subunits bound to I κ B in the unstimulated state. Upon cellular activation, i.e., after exposure of endothelial cells to high glucose, increased production of reactive oxygen species (ROS) mediates phosphorylation and degradation of I κ B, thus causing the release of p50/p65 components of NF- κ B. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of genes involved in the regulation of leukocyte responses, such as pro-inflammatory cytokines and cell-surface adhesion molecules expression. C-peptide decreases nuclear translocation of p65/p50 subunits in high glucose-activated endothelial cells (53). C-peptide might act at different levels along the NF- κ B pathway, such as regulating ROS production, or modulating I κ B phosphorylation, or even physically interacting with the p65/p50 subunits in the nucleus preventing their DNA binding.

targeted in endothelial cells (55). Another possibility is that C-peptide directly interacts with NF- κ B p65/p50 subunits at the nuclear level, preventing DNA binding.

Other studies also demonstrate the importance of C-peptide in modulating the NF- κ B activation and inflammation in the central nervous system. T1D patients may suffer impairments in learning, memory, problem solving, and mental and motor speed with primary diabetic encephalopathy recognized as a late complication of T1D (56). In the type 1 BB/Worcester (BB/Wor) rats (rat model of human T1D), cognitive impairment is associated with apoptosis-induced neuronal loss in the hippocampus, an event associated with NF- κ B and receptor for advanced glycation end products (RAGE) activation (56). C-peptide replacement therapy to these diabetic rats reduced NF- κ B and RAGE expression in the hippocampi leading to a decreased production of inflammatory cytokines, such as TNF- α , IL-1 β , IL-2, and IL-6 (56) and prevention of the cognitive dysfunction and hippocampal neuronal loss (57).

The peroxisome proliferator-activated receptor γ pathway

Another signaling pathway that is affected by C-peptide is the one mediating activation of peroxisome proliferator-activated receptor γ (PPAR- γ) (58), a member of the nuclear receptor superfamily

of ligand-activated transcription factors. In addition to its function in adipogenesis and increasing insulin sensitivity, PPAR- γ regulates the expression of several genes involved in inflammation and vascular disorders, such as atherosclerosis (59), by either controlling the gene transcriptional machinery or interacting with other transcription factors such as activator protein (AP)-1, signal transducers and activators of transcription (STAT), and NF- κ B (60). By neutralizing NF- κ B activation, PPAR- γ modulates a constellation of inflammatory events crucial for the initiation of vascular diseases (61). Although the effect of C-peptide on the activation of PPAR- γ has not been investigated in the vasculature, there is evidence that C-peptide decreases systemic inflammation in an animal model of sepsis by increasing DNA binding of PPAR- γ in the lung of endotoxin-treated mice, an event associated with inhibition of the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 (62).

C-peptide and nerve function in T1D

Diabetic polyneuropathy, the most common microvascular complication of diabetes mellitus, occurs in both T1D and T2D (63). In T1D, the neuropathy tends to progress more rapidly and results in a more severe disorder than in T2D (64). The underlying pathogenetic mechanisms are multiple and thought to involve genetic predisposition as well as metabolic abnormalities consequent to hyperglycemia, such as oxidative stress, accelerated polyol pathway metabolism and generation of advanced glycosylation end products (65). The available data suggest that in the case of T1D other factors, notably C-peptide deficiency, may also play a role (66). In the diabetes control and complications trial (DCCT) study it became evident that intensive as compared to conventional insulin therapy markedly reduces the development of clinical neuropathy (67). Yet, in the intensively treated group, with near-normal blood glucose levels, the cumulative prevalence of overt neuropathy and/or grossly abnormal nerve conduction after 5 yr approached 40%, pointing at the possibility that factors other than hyperglycemia also contribute to the progressive deterioration of nerve function in this disorder. Further support for this view is provided by studies of nerve function and structure in animal models of diabetes and nerve biopsy samples from patients. The nerve abnormalities in type 1 animals, who lack C-peptide, include impairment of nerve Na⁺,K⁺-ATPase, and eNOS activities, resulting in intra-axonal sodium accumulation and reduced endoneurial blood flow (66). Gradually, structural changes appear, involving axonal atrophy and characteristic nodal and paranodal abnormalities that contribute to the progressive deterioration of nerve conduction velocity (NCV) (68, 69). In contrast, in

T2D, with normal or elevated levels of C-peptide and where hyperglycemia is the primary pathogenetic factor, the functional and structural abnormalities of the peripheral nerves are less marked and show a different pattern, including milder axonal degeneration and no or only minimal nodal and paranodal abnormalities (70, 71). The discrepancies in diabetes-induced structural changes between T1D and T2D are also demonstrated in nerve biopsy samples from patients, where the structural abnormalities in T2D patients follow the normal pattern of ageing whereas patients with T1D present with significant nodal and paranodal structural changes (68). Thus, it is conceivable that the lack of C-peptide in T1D contributes to the development of the more severe nerve dysfunction and structural abnormalities in this disorder.

Clinical studies

C-peptide replacement was given for 3 months in a double-blind, placebo-controlled study including 46 patients with approximately 10-yr diabetes duration and reduced sensory and motor nerve conduction velocities (NCV) but no overt symptoms or signs of neuropathy (72). Sensory (sural) but not motor (peroneal) NCV increased gradually during the study; the increase after 3 months was 2.7 m/s, corresponding to an 80% correction of the initial deficit (Fig. 4). Vibration perception thresholds showed only a small increase above normal at baseline but decreased significantly during treatment, consistent with an improved sural nerve function.

The above observations have been extended in a subsequent double-blind clinical trial involving 161 T1D patients with manifest diabetic peripheral neuropathy (73). The patients received either a replacement dose of C-peptide, a dose three times higher or placebo. Sensory NCV improved similarly

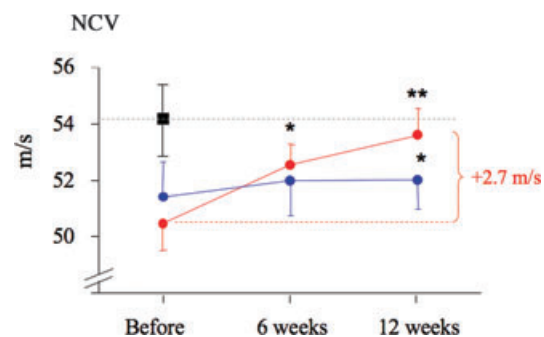


Fig. 4. C-peptide and sensory nerve conduction velocity (NCV) in patients with type 1 diabetes (T1D) and early stage neuropathy. Change in sural NCV after 6 and 12 wk of C-peptide treatment (red symbols) or placebo administration (blue symbols). ** indicates $p < 0.01$ for the increase above baseline NCV in the C-peptide-treated group. The difference between the C-peptide and placebo groups at 12 wk was statistically significant ($p < 0.05$). Scr = Scrambled C-peptide. Adapted from Ref 38.

within the two C-peptide-treated groups and the number of positive responders was significantly greater among the patients receiving C-peptide ($p < 0.03$). The improvement in comparison to placebo was approximately 1 m/s in the least severely diseased half of the patients ($p < 0.02$). The neurological impairment score and vibration perception also improved within the C-peptide-treated group. Glycemic control improved slightly but similarly in the three study groups. In keeping with these findings, an improvement in temperature perception thresholds has been reported after 3 months of C-peptide replacement therapy (74). Metabolic control and blood pressure were unchanged in all of the above studies, indicating that these factors were not responsible for the improvement. The observed improvement in sensory NCV and vibration and temperature perception may be seen as favourable considering the relatively short treatment periods and previous experience of aldose reductase inhibitors (75).

Deficient autonomic nerve function may be evaluated in patients as reduced heart rate variability (HRV) during deep breathing, a measurement that, with a high degree of reproducibility, primarily reflects vagal function. In T1D patients, autonomic dysfunction can be ameliorated by C-peptide in replacement doses; short-term infusion of C-peptide is reported to significantly increase HRV, while no change was seen after saline infusion (76). The heart rate brake index after a tilting manoeuvre was also improved after C-peptide for 3 h. A similar, though less marked improvement was seen after 3 months of C-peptide administration in T1D patients (74).

Animal studies

Positive effects of C-peptide on motor and sensory NCV have been demonstrated in two animal models of T1D. In BB/Wor rats, showing spontaneous development of type 1-like diabetes, C-peptide administration (homologous C-peptide in replacement dose by continuous subcutaneous infusion) prevents the development of NCV deficits when the peptide was given from 1 wk after the onset of diabetes (Fig. 5) (77, 78). In addition, C-peptide elicited an increase in NCV and partially corrected the NCV deficit when treatment was commenced at a time when diabetes-induced abnormalities had become established (after 5 months of diabetes) (Fig. 5) (77). The C-peptide concentrations reached in these studies were in the low physiological concentration range (0.5–0.7 nM). Similarly, in streptozotocin-diabetic rats receiving rat C-peptide in replacement doses from 6 to 8 wk after induction of diabetes, the peptide gave rise to 80 and 60% corrections of the sensory (saphenous) and motor (sciatic) NCV, respectively (45). Scrambled

Nerve Conduction Velocity

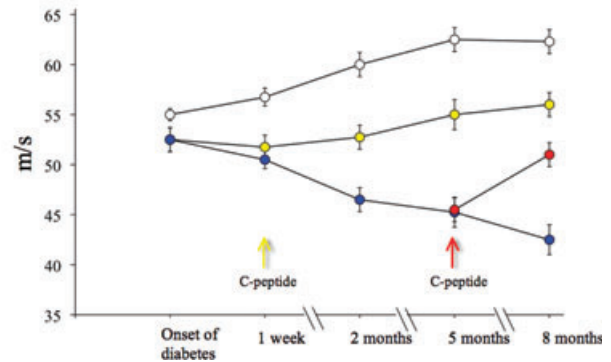


Fig. 5. C-peptide and nerve conduction velocity (NCV) in diabetic Biobreeding/Worcester (BB/Wor) rats. NCV in diabetic (blue symbols) and healthy (open symbols) BB/Wor rats given rat C-peptide in replacement dose by subcutaneous pump infusion starting 1 wk after onset of diabetes (yellow symbols) or after 5 months of diabetes (red circles). The NCV declined progressively in the untreated diabetic animals ($p < 0.01$), but increased in the C-peptide infused animals ($p < 0.05$ – 0.01). Data from Ref 77.

C-peptide, a control peptide with the same 31 residues assembled in random order, was without effect.

Several factors may contribute to the observed effect of C-peptide on NCV. Direct measurements of nerve blood flow, using the hydrogen clearance technique, have demonstrated that endoneurial blood flow is substantially reduced both in diabetic BB/Wor rats and in streptozotocin-diabetic rats (Fig. 6) (45, 79). Continuous-rate subcutaneous infusion of rat C-peptide in replacement doses for 2 wk or 2 months resulted in 52 and 75% correction, respectively, of the endoneurial perfusion deficit (Fig. 6). C-peptide effects on both endoneurial blood flow and NCV were abrogated by an eNOS blocker and the scrambled C-peptide had no effect. Altogether, the findings indicate that C-peptide in physiological

Nerve blood flow

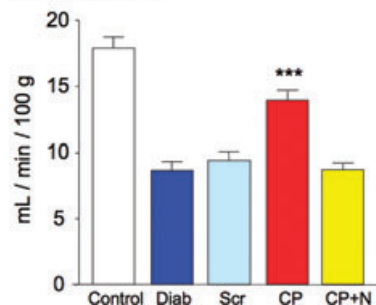


Fig. 6. C-peptide and nerve blood flow in diabetic rats. Endoneurial blood flow in streptozotocin-diabetic rats was measured using a hydrogen elimination technique. Findings are shown for healthy control rats (open bar), for diabetic untreated rats (blue bar), diabetic rats treated with C-peptide (red bar), and diabetic rats given both C-peptide and an endothelial nitric oxide synthase (eNOS) blocker NG-nitro-L-arginine (L-NNA) (yellow bar). Adapted from Ref 45.

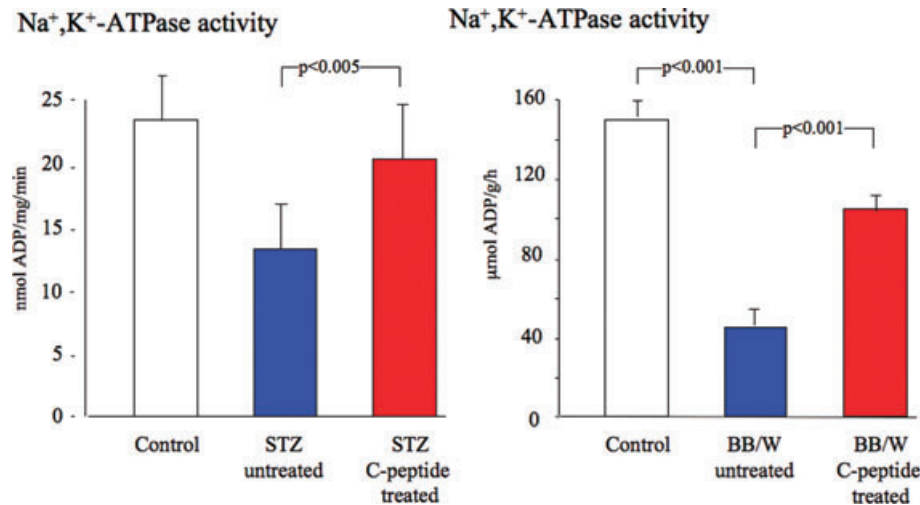


Fig. 7. C-peptide and nerve Na⁺,K⁺-ATPase activity in diabetic rats. Sciatic nerve Na⁺,K⁺-ATPase activity in healthy control rats (open bars), untreated diabetic rats (blue bars), and animals that received C-peptide for 5 wk after induction of diabetes (red bars) (left panel, streptozotocin-diabetic rats) or 2 months after diabetes onset (right panel, Biobreeding/Worcester (BB/Wor) rats). Data from Refs 77 and 80.

concentrations improves nerve function in T1D via a NO-sensitive vascular mechanism mediating vasodilation of the nerve blood vessels. In contrast to the above findings, a previous study has suggested that C-peptide reduces total nerve blood flow in streptozotocin-diabetic rats (80). However, the method for estimation of nerve blood flow was based on the microsphere entrapment technique, which has technical limitations when used in a small tissue such as the rat sciatic nerve (81).

Additional effects of C-peptide, demonstrable in *in vivo* animal studies, may contribute to its beneficial influence on nerve function. Decreased Na⁺,K⁺-ATPase activity in peripheral nerve tissue is a characteristic abnormality in T1D. It is associated with increased inactivation of Na⁺-channels, intra-axonal sodium accumulation (82), and swelling of the paranodal region during the early phase of the disorder (83). C-peptide in physiological concentrations prevents or partially corrects the diabetes-induced reduction in nerve Na⁺,K⁺-ATPase activity both in streptozotocin-diabetic animals (80) and in BB/Wor rats (77), thereby contributing to improved electrolyte balance and partial correction of the paranodal swelling (Fig. 7) (77). At a later stage in the development, structural changes involving axonal atrophy and abnormalities of the nodal and paranodal apparatus occur (84). These include a progressive disruption of the paranodal myelin sheath, resulting in lateralization of the Na⁺ ion channels of the large myelinated fibres, termed axoglial dysjunction, and eventually resulting in paranodal demyelination (84). The latter changes occur to a much lesser extent, or not at all, in T2D (70), and recent evidence suggests that they are the result of impaired C-peptide action rather than hyperglycemia (78). Accordingly,

C-peptide treatment of diabetic BB/Wor rats resulted in marked improvements in nodal, paranodal, and axonal structural changes and in increased repair activity. Thus, 8 months of C-peptide treatment resulted in near total prevention of axoglial dysjunction and paranodal demyelination (85). Likewise, marked improvements in structural abnormalities were observed when C-peptide was given from 5 to 8 months after disease onset; axoglial dysjunction and paranodal demyelination improved significantly, axonal degeneration decreased and nerve fibre regeneration increased fourfold (77). A schematic representation of the different mechanisms, whereby C-peptide may exert beneficial effects on peripheral nerve dysfunction and structural abnormalities, is shown in Fig. 8.

Painful neuropathy is a debilitating consequence of diabetes, which is at least partly a consequence of

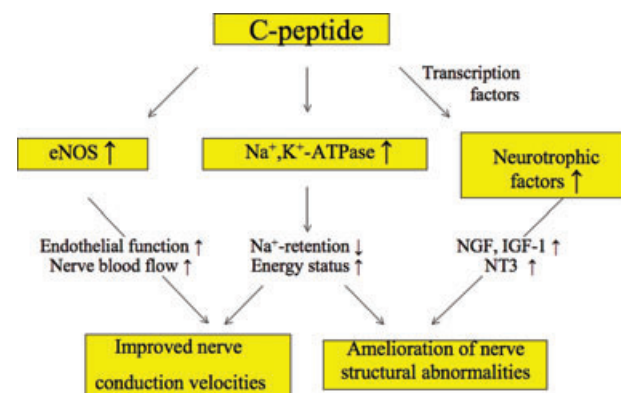


Fig. 8. C-peptide effects on nerve function and structure in diabetes. Schematic representation of the different mechanisms, whereby C-peptide may improve nerve function and ameliorate nerve structural abnormalities secondary to type 1 diabetes (T1D).

damage to the unmyelinated and the small myelinated nociceptive fibres (86) and to reduced neurotrophic support by, e.g., nerve growth factor (NGF). Degeneration of these fibres initially leads to high-firing frequencies and spinal sensitization, which is experienced by the patient as hyperalgesia. Replacement of C-peptide from the onset of diabetes in rats completely prevents thermal hyperalgesia as well as degeneration and loss of unmyelinated fibres (87). These findings are accompanied by improved regulation of gene expression of different neurotrophic factors, e.g., NGF and insulin-like growth factor-1 (IGF-1), and their receptors in the dorsal root ganglion of the rats after 8 months of treatment. Experimental evidence thus supports the notion that C-peptide may be useful in alleviating painful neuropathy in animals but clinical trials will be required to determine if this is true for the human painful neuropathy.

C-peptide and the kidneys in T1D

Early signs of diabetic nephropathy include glomerular hyperfiltration and loss of renal functional reserve, findings which are accompanied by renal hypertrophy and glomerular enlargement due to mesangial matrix expansion. Within a few years the structural changes develop further, glomerular expansion continues and, in addition, there is thickening of the basement membrane (88). An early sign is microalbuminuria (30–300 mg/24 h), frequently in combination with hypertension. Subsequently, the albumin excretion may accelerate and the condition develops into overt diabetic nephropathy with gradually decreasing glomerular filtration and macroalbuminuria (>300 mg/24 h). The prevalence of nephropathy is higher in T1D than in T2D patients and end-stage renal insufficiency is more common in T1D (89). In the former group, proteinuria is consistently accompanied by advanced glomerular structural changes, whereas there is no clear-cut link between urinary albumin excretion and glomerulopathy in T2D patients (90).

Studies in patients with T1D

The short-term effects of C-peptide administration on renal functions have been studied in young T1D patients without overt signs of renal disease (44). C-peptide, infused at rates sufficient to achieve physiological plasma concentrations, resulted in a decreased glomerular filtration rate and slightly increased renal plasma flow. These observations have been extended in a double-blind randomized study in T1D patients with incipient nephropathy receiving C-peptide for 4 wk (91). After 2 and 4 wk in the group receiving C-peptide, the glomerular filtration rate had decreased and at the end of the study there

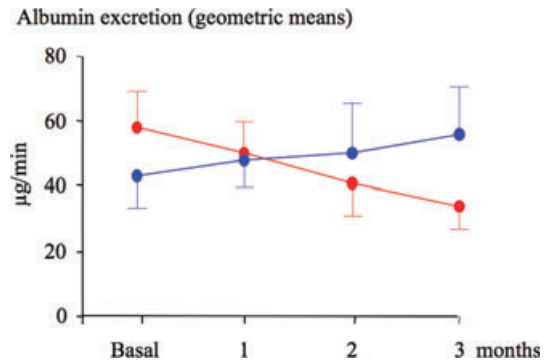


Fig. 9. C-peptide and albumin excretion in urine in type 1 diabetic (T1D) patients. Urinary albumin excretion (geometric means) in T1D patients with early stage nephropathy. The patients received C-peptide plus insulin (red symbols) or placebo plus insulin (blue symbols) for 3 months. Albumin excretion was significantly different between C-peptide and placebo-treated patients after 2 ($p < 0.05$) and 3 ($p < 0.01$) months, respectively. Data from Ref 74.

was a significant reduction in albumin excretion to approximately half the basal value (Fig. 9).

The above findings have been further explored in a study involving C-peptide administration for 3 months (74). A double-blind, placebo-controlled, randomized, crossover study design was used and patients with early stage nephropathy received C-peptide plus insulin and insulin plus placebo for 3 months. Pre-study urinary albumin excretion rates were on average 60 µg/min and all patients were normotensive. During the C-peptide-treatment period, urinary albumin excretion decreased progressively to values significantly below those observed during the pre-study period. The reduction was significant after 2 months, and at the end of the 3-month study period the decrease amounted to approximately 40%. When the patients received insulin only, albumin excretion did not change significantly. All patients remained normotensive throughout the study and glycemic control improved slightly but to the same extent during the two treatment periods. Taken together, the evidence supports the view that C-peptide in replacement doses has the capacity to reduce glomerular hyperfiltration and to decrease urinary albumin excretion in early stage T1D nephropathy. Needed now are long-term studies in patients with clinically manifest nephropathy to further define C-peptide's therapeutic potential in this disorder.

Animal studies

The influence of C-peptide on renal function has been evaluated in streptozotocin-diabetic rats (92). The animals showed glomerular hyperfiltration and urinary protein leakage prior to C-peptide administration. Renal functional reserve, evaluated during intravenous glycine infusion, was reduced in the diabetic compared

to normal rats. Short-term (90 min) C-peptide infusion was followed by an immediate and almost full correction of the glomerular hyperfiltration. The renal functional reserve was almost completely restored and urinary protein excretion decreased significantly (92). The specificity of the C-peptide effect was attested to by the finding that scrambled C-peptide had no effect.

The influence of more prolonged C-peptide administration on renal function and morphology has also been examined by Samnegard et al. (93). When C-peptide was given intravenously as replacement dosage for 14 days, the glomerular filtration rate and renal functional reserve were almost completely normalized and urinary albumin excretion decreased significantly in the C-peptide-treated rats and was similar to that in the controls. Examination of renal morphology revealed that C-peptide significantly diminished the diabetes-induced increase in glomerular volume; in the C-peptide-treated rats, the glomerular volume exceeded that of the controls by no more than 23% (ns) compared with 63% in the untreated diabetic group. When increasing doses of human C-peptide were administered to diabetic rats, C-peptide was found to dose-dependently lower the augmented glomerular filtration rate and albumin excretion. Renal function in healthy animals was unresponsive to C-peptide infusion (94). Further examination of the glomeruli revealed that it was primarily the diabetic-induced hypertrophy of the glomeruli and in particular the mesangial matrix that was inhibited by C-peptide (95).

Several recent studies have offered clues as to the physiological mechanism(s) involved in the beneficial effects of C-peptide on renal function and structure in diabetic nephropathy. Thus, C-peptide has been found to elicit a constriction of the afferent glomerular arteriole and a relaxation of the efferent arteriole in diabetes (96, 97). In addition, C-peptide administration results in an inhibition of tubular Na^+ reabsorption (96). Taken together, these effects will contribute to a reduction of the elevated glomerular filtration rate and a diminished urinary albumin excretion. The extent to which these effects are mediated by C-peptide's influence on either or both of renal eNOS (98) and glomerular and tubular Na^+, K^+ -ATPase (99, 100) is not known. Early indications of the background to C-peptide's beneficial effects on diabetes-induced renal structural abnormalities have been observed in diabetic mice; C-peptide has been found to reduce the glomerular expression of the profibrotic cytokine transforming growth factor-beta ($\text{TGF}\beta$) and type IV collagen (101). Moreover, C-peptide has been found to reverse the structural changes of tubular cells induced by $\text{TGF}\beta$ (102). Overall, there is much evidence in support of a renoprotective effect of C-peptide in the nephropathy of T1D.

How C-peptide works

Membrane binding and internalization of C-peptide

Although C-peptide exerts a variety of effects in different cell types, relatively little is known regarding exactly how C-peptide achieves its intracellular activities in target cells. It was initially thought that C-peptide exerted its effects via nonchiral mechanisms rather than by stereo-specific receptors or binding sites (80), although specific binding of C-peptide to cultured rat pancreatic β -cells was demonstrated in 1986 (103). Subsequently, stereo-specific binding of C-peptide to cellular membranes has been confirmed in several human cell types including human renal tubular cells, human fibroblast and saphenous vein endothelial cells (Fig. 10) (104, 105). Furthermore, C-peptide binding reaches full saturation at 0.9 nM; thus, in healthy subjects, receptor saturation is already achieved at physiologic levels (105). Although a putative C-peptide receptor has not been identified on human cell membranes, it has been suggested to be a G-protein-coupled receptor, as deduced from the inhibitory effects of pertussis toxin on C-peptide binding and intracellular signaling (105, 106).

More recently, C-peptide was shown to cross plasma membranes, localizing in the cytoplasm of HEK-293 cells and Swiss 3T3 fibroblasts (106), where it was detected up to 1 h after its uptake. Nuclear localization of C-peptide in HEK-293 cells and Swiss 3T3 fibroblasts has also been demonstrated by the same group (106). Specifically, C-peptide can be detected in the nucleoli where it promotes transcription of genes encoding for ribosomal RNA (107). Luppi et al. (108) also investigated the process of internalization of C-peptide and its subcellular localization in live HAEC

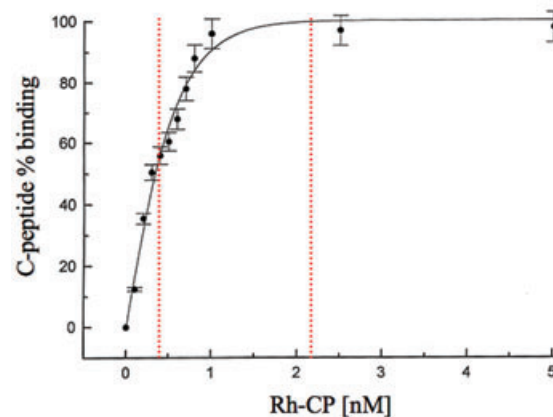


Fig. 10. Binding of rhodamine-labelled C-peptide to all membranes of the renal tubular cells. Fractional saturation of the membrane-bound ligand is presented as a function of the C-peptide concentration in the surrounding medium. The area between the red dotted lines represents the physiological concentration range. Data from Ref 105.

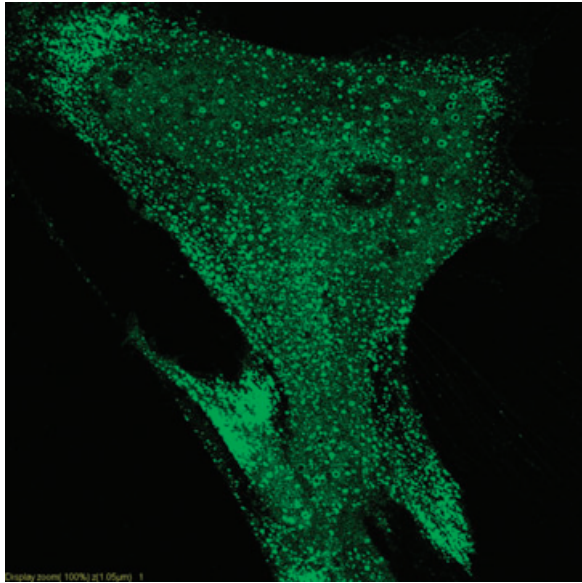


Fig. 11. C-peptide internalizes in human vascular endothelial cells as punctate structures. Internalization of C-peptide has been studied by using AlexaFluor488-labeled C-peptide probe in live-cell imaging using a laser scanning confocal microscopy (108). The green punctate-staining corresponds to the C-peptide probe localized at the periphery of the cell and in the cytoplasm.

and umbilical artery smooth muscle cells (UASMC) by using confocal laser scanner microscopy. They found that C-peptide internalizes to punctate structures localized at the level of the cellular membrane and in the cytoplasm (Fig. 11) (108). Internalization of C-peptide was minimal after 5 min, clearly detectable after 10 min, resulted in bright staining after 30 min, and was completed by 1 h (Fig. 11) (108). By using specific organelle reporter dyes, it was established that the punctate structures identifies with the specific endocytic organelles early endosomes. C-peptide eventually trafficked to lysosomes in live HAEC and UASMC (108). Identification of the subcellular compartments in which C-peptide localizes upon its entry into target cells is important as these compartments likely represent the sites of intracellular signaling activity of C-peptide. In one scenario, it can be envisaged that C-peptide after its binding to the specific receptor at the level of the plasma membrane gets activated, begins the signaling activity, and is quickly internalized into early endosomes where it continues to signal. Localization of C-peptide to early endosomes does not exclude trafficking of C-peptide to other subcellular sites upon its entry into target cells. As an alternative scenario, the signaling activity of the activated C-peptide–receptor complexes is localized at the plasma membrane. Internalization of the C-peptide–receptor complexes to early endosomes just represents a sorting station for internalized activated receptor–peptide complexes on their way to lysosomal degradation.

C-peptide and intracellular signaling

When exposed to C-peptide in physiological concentrations, renal tubular and endothelial cells show a prompt elevation of intracellular Ca^{2+} concentrations (Fig. 12) (35, 99, 109). C-peptide also elicits phosphorylation of phospholipase C (PLC) and several protein kinase C (PKC) isoforms, notably, α , δ , and ϵ , in different tubular cells and fibroblasts (100, 110, 111). PI3-kinase activation has been observed in fibroblasts, myoblasts, renal tubular cells, and lymphocytes (58, 112, 113). Activation of one or several components of the mitogen-activated protein kinase (MAP-kinase) system mostly via Rho A is consistently observed in all examined cell types following exposure to C-peptide at physiological concentrations (3, 110–112, 114, 115). A schematic overview of C-peptide signaling is shown in Fig. 13.

C-peptide has been found to mimic insulin effects in myoblasts and neuroblastoma cells by increasing autophosphorylation of the insulin receptor, stimulation of phosphoinositide 3 (PI3)-kinase but not Akt and phosphorylation of MAP-kinase (112, 116). Glucose utilization and glycogen synthesis are stimulated in myoblasts (112) and in human skeletal muscle strips (117). The mechanism by which these effects are elicited is not apparent; besides the binding of C-peptide to a specific G-protein-coupled receptor causing activation of PI3-K γ , there could be crosstalk between insulin and C-peptide ligand-receptor complexes or interaction between C-peptide and receptors with catalytic activity, as suggested by the finding that C-peptide attenuates the activity of protein tyrosine phosphatase in myoblasts (112).

Endothelial nitric oxide synthase (eNOS)

There is *in vitro* evidence that C-peptide elicits release of NO in endothelial cells in a concentration and time-dependent manner (35). The effect, which has a rapid onset, is abolished in a Ca^{2+} -free medium and in the presence of an NO synthase inhibitor, suggesting that stimulation of eNOS via a Ca^{2+} -dependent signal is involved. In addition, increased expression of eNOS mRNA (36) and eNOS protein (37) has been observed after exposure of lung and aortic endothelial cells to C-peptide; eNOS expression is enhanced via a MAP-kinase-dependent transcriptional activation (37). The above *in vitro* observations are consistent with the finding that C-peptide administration to T1D patients and in animal models of T1D results in concentration-dependent increases in blood flow in skeletal muscle (38, 41), skin (43), peripheral nerve (45, 79), and myocardium (46, 47). The observations support the hypothesis that C-peptide administration in T1D partly corrects the diabetes-induced abnormality in eNOS activity and expression.

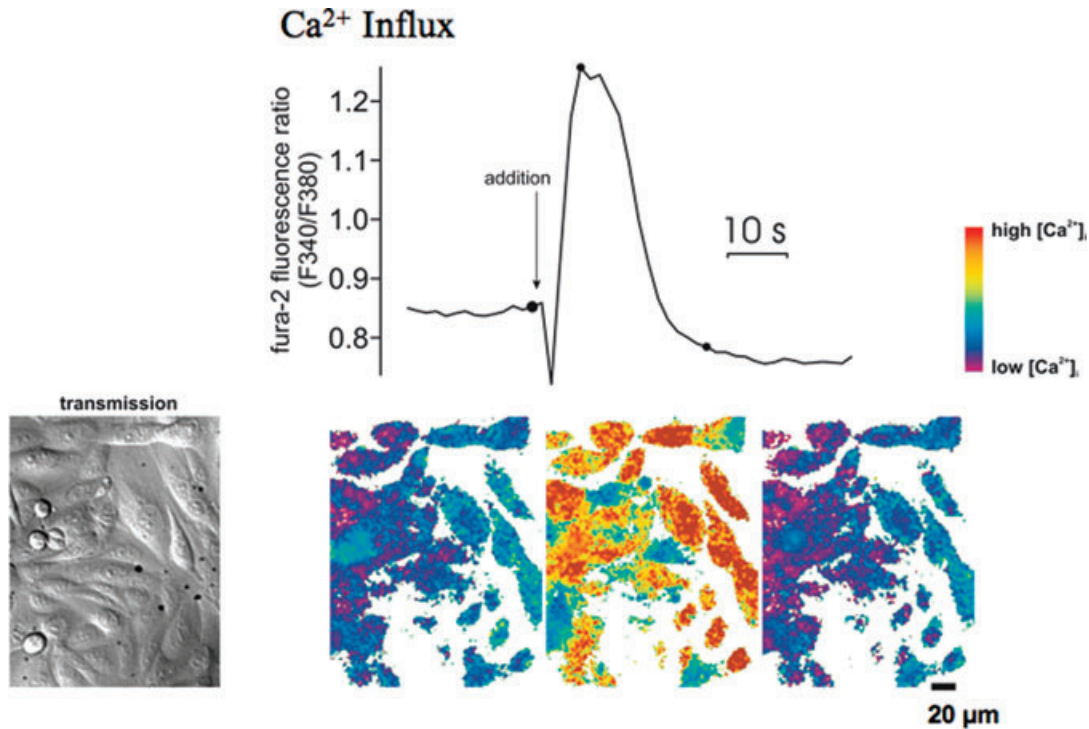


Fig. 12. Monitoring of $[Ca^{2+}]_i$ in fura-2/AM-loaded human renal tubular cells stimulated with 5 nM human C-peptide. Top panel: the trace of the 340/380 fluorescence ratio. Bottom panel: images of the cells in transmission light (first panel) and in a color code (next three panels) representing $[Ca^{2+}]_i$ at the time points shown by spot indications in the trace above. From Ref 109.

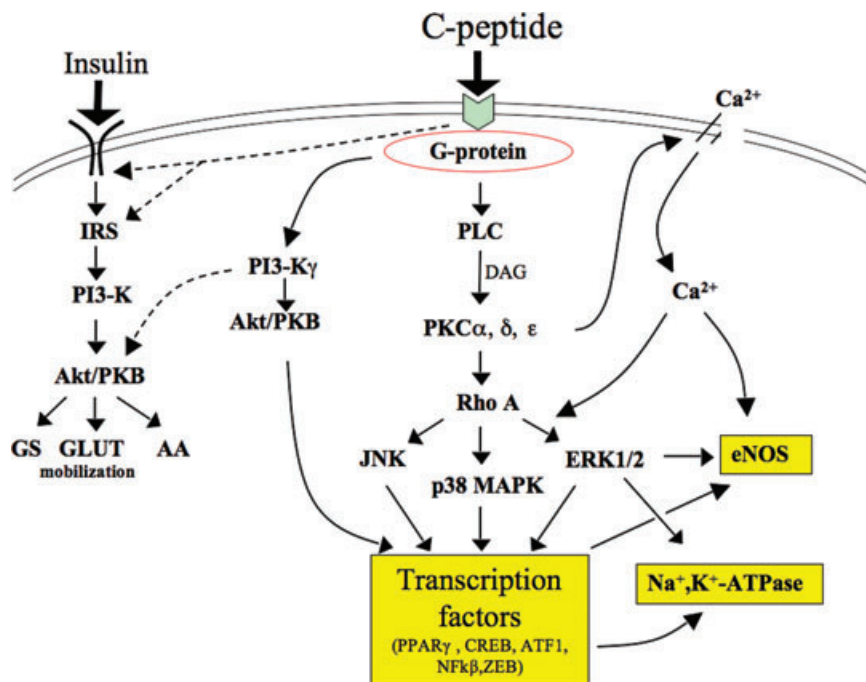


Fig. 13. Intracellular signaling by C-peptide C-peptide interaction with cell membranes results in activation of a pertussis toxin sensitive G-proteins. Subsequently, there is influx of Ca^{2+} into the cell and activation of endothelial nitric oxide synthase (eNOS), resulting in NO formation and local vasodilatation. Phospholipase C (PLC) and specific isomers of protein kinase C (PKC) are also activated as well as the mitogen-activated protein kinase (MAPK) complex. As a result, there is activation of Na^+, K^+ -ATPase enzyme activity, but also DNA binding of several transcription factors, resulting in augmented eNOS mRNA formation and increased eNOS protein synthesis. Phosphoinositide 3-kinase (PI3-K) γ is also activated giving rise to peroxisome proliferator-activated receptor γ (PPAR- γ)-mediated transcriptional activity as well as augmented lymphocyte chemotaxis. In addition, there is evidence to indicate that C-peptide may interact synergistically on the insulin signaling pathway as indicated by dotted lines.

$\text{Na}^+, \text{K}^+ \text{--ATPase}$

C-peptide exerts a direct stimulatory effect on $\text{Na}^+, \text{K}^+ \text{--ATPase}$ in renal tubular segments and tubular cells under *in vitro* conditions (99, 100). The effect is concentration-dependent at physiological concentrations. It is blockable by pertussis toxin and dependent on Ca^{2+} . *In vivo* studies of C-peptide's effects on sciatic nerve $\text{Na}^+, \text{K}^+ \text{--ATPase}$ activity confirm a stimulatory influence (77, 80). Moreover, red blood cell $\text{Na}^+, \text{K}^+ \text{--ATPase}$ activity is reduced in patients with T1D; the reduction is proportional to the decrease in C-peptide levels (118) and can be corrected by C-peptide administration (51) or after islet cell transplantation and restoration of endogenous C-peptide secretion (9). A secondary effect of the decreased red cell $\text{Na}^+, \text{K}^+ \text{--ATPase}$ activity is impaired deformability of the cells, which is corrected after exposure to C-peptide (119). Altogether, the evidence provides strong support for the existence of a direct relationship between C-peptide levels and $\text{Na}^+, \text{K}^+ \text{--ATPase}$ activity in renal and nerve tissue and in red blood cells under both *in vitro* and *in vivo* conditions.

C-peptide–insulin interaction

Insulin occurs in a hexameric form in the β -cells or in vials for insulin therapy but is biologically active only in monomeric form. Interactions between C-peptide and insulin oligomers have been studied using surface plasmon resonance and mass spectrometry. Unexpectedly, it was discovered that C-peptide influences the disaggregation of insulin by binding to insulin oligomers, with dissociation constants in the μM range (120). In accordance with this finding, mass spectrometry revealed that insulin hexamers in solution became non-detectable in the presence of C-peptide. Hence, C-peptide apparently binds to and causes disaggregation of hexameric insulin, increasing the availability of the biologically active monomeric insulin. Similarly, subcutaneous injection of an insulin and C-peptide mixture in T1D patients has been found to result in a more rapid appearance of insulin in plasma and more marked stimulation of glucose utilization compared to injection of insulin only (120). Thus, these results may present a molecular role for C-peptide in increasing the bioavailability of insulin by promoting the disaggregation of oligomeric insulin.

Structural conservation and cellular effects of C-peptide

The considerable structural variability among the C-peptides of 33 examined species – ranging from the Atlantic hagfish to the human (3) – was earlier considered an argument that C-peptide is unlikely to exhibit a

defined biological activity. The mammalian C-peptide has typically 31 residues and includes four to five acidic residues. The central region varies with regard to its amino acid sequence and number of residues. Among the 20 known mammalian forms, nine residues localized to the N- and C-terminal segments show 90% or greater conservation. These are Glu1, Glu3, Gln6, Val7, Glu11, Leu12, Leu26, Glu27, and Gln31 (3). The conservation of the terminal residues Glu1 and Gln31 may be a consequence of the processing of proinsulin and the codon for Gln6 precedes an exon/intron junction. Glu3, Glu11, and especially Glu27 are known to be important for the cellular effects of C-peptide in as much as substitution of one or all of these with Ala results in substantial loss of biological activity as measured by MAPK phosphorylation (3, 104). Even though the species-dependent structural variability of C-peptide is considerable, C-peptide is not unique in this regard among bioactive peptides. Several peptide hormones show a similar degree of structural variability as C-peptide, e.g., parathyroid hormone and relaxin (121).

Summary and what next for C-peptide?

Undoubtedly, there is much more to learn about C-peptide. Identification of the mechanism whereby C-peptide interacts with cell membranes, delineation of its intracellular signaling pathways in different cell types, and further evaluation of its transcriptional effects will enhance our understanding of C-peptide bioactivity. On the clinical side further studies of longer duration (>6 months) will be required to document the robustness of its beneficial effects on the different types of long-term complications in order to define its possible role in the therapy of T1D. Nevertheless, despite the fact that our knowledge is still incomplete, there are several lines of unputdownable evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in T1D may be beneficial in the treatment of long-term complications. Even though the nature of the peptide's interaction with the cell membrane is only partially understood, its intracellular signaling characteristics and end effects including its action on eNOS, $\text{Na}^+, \text{K}^+ \text{--ATPase}$, and several transcription factors are now well established for many cell systems and by different investigators. Results from studies in T1D patients and animal models demonstrate that C-peptide in replacement doses exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. The previous view that C-peptide is merely an inert by-product of insulin biosynthesis seems no longer tenable. Even a cautious evaluation of the available evidence presents the picture of a previously unrecognized bioactive peptide with

therapeutic potential in an area where no causal therapy is available today.

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FOXO1 Mediates the Autocrine Effect of Endothelin-1 on Endothelial Cell Survival

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Chronic hyperglycemia exerts a deleterious effect on endothelium, contributing to endothelial dysfunction and microvascular complications in poorly controlled diabetes. To understand the underlying mechanism, we studied the effect of endothelin-1 (ET-1) on endothelial production of Forkhead box O1 (FOXO1), a forkhead transcription factor that plays an important role in cell survival. ET-1 is a 21-amino acid peptide that is secreted primarily from endothelium. Using adenovirus-mediated gene transfer approach, we delivered FOXO1 cDNA into cultured human aorta endothelial cells. FOXO1 was shown to stimulate B cell leukemia/lymphoma 2-associated death promoter (BAD) production and promote cellular apoptosis. This effect was counteracted by ET-1. In response to ET-1, FOXO1 was phosphorylated and translocated from the nucleus to cytoplasm, resulting in inhibition of BAD production and mitigation of FOXO1-mediated apoptosis. Hyperglycemia stimulated FOXO1 O-glycosylation and promoted its nuclear localization in human aorta endothelial cells. This effect accounted for unbridled FOXO1 activity in the nucleus, contributing to augmented BAD production and endothelial apoptosis under hyperglycemic conditions. FOXO1 expression became deregulated in the aorta of both streptozotocin-induced diabetic mice and diabetic db/db mice. This hyperglycemia-elicited FOXO1 deregulation and its ensuing effect on endothelial cell survival was corrected by ET-1. Likewise, FoxO1 deregulation in the aorta of diabetic mice was reversible after the reduction of hyperglycemia by insulin therapy. These data reveal a mechanism by which FOXO1 mediated the autocrine effect of ET-1 on endothelial cell survival. FOXO1 deregulation, resulting from an impaired ability of ET-1 to control FOXO1 activity in endothelium, may contribute to hyperglycemia-induced endothelial lesion in diabetes. (*Molecular Endocrinology* 26: 1213–1224, 2012)

Endothelin-1 (ET-1) is a 21-amino acid peptide that is produced primarily by vascular endothelial cells. ET-1 acts to regulate vascular tone via its two cognate receptors, ET_A, which is expressed in smooth muscle cells, and ET_B, which is present on endothelial cells (1). Binding of ET-1 to the ET_A receptor causes sodium retention in smooth muscle cells, the effect of which accounts for vasoconstriction. In contrast, binding of ET-1 to the ET_B receptor stimulates nitric oxide (NO) production in endothelial cells, the effect of which contributes to vasodi-

lation. These two opposing effects of ET-1 act in concert to regulate blood pressure, contributing to the fine-tuning of microvascular circulation (1, 2). Deregulation in endothelial ET-1 production is associated with the pathophysiology of a variety of vascular diseases including hypertension, atherosclerosis, diabetes, and ischemic heart disease (3–9).

Apart from its action on vascular tone, ET-1 exerts a cytoprotective effect on cell survival. This antiapoptotic action of ET-1 is observed in a number of cell types in-

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Abbreviations: BAD, BCL2-antagonist of cell death protein; BAK, BCL-2 homologous killer; BAX, BCL-2-associated X protein; BCL-2, B cell leukemia/lymphoma 2; CHIP, chromatin immunoprecipitation; ET-1, endothelin 1; FOXO1, forkhead box O1; HAEC, human aorta endothelial cell; ICR, Institute for Cancer Research; NO, nitric oxide; pfu, plaque-forming unit; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; STZ, streptozotocin; TBP, TATA box binding protein; WGA, wheat-germ agglutinin.

cluding cardiac myocytes (10), human umbilical vein endothelial cells (11), renal carcinoma cells (12), ovarian carcinoma cells (13), and prostate epithelial cells (14). A prevailing notion is that ET-1 signaling through AKT/protein kinase B (PKB) contributes to the prosurvival effect of ET-1 on cells. Consistent with this notion is the observation that ET-1 binding to its cognate receptors results in the activation of the phosphatidylinositol 3-kinase (PI3K)-AKT/PKB signaling cascade in a variety of cell types (10, 13, 15). Nevertheless, the underlying mechanism and physiology by which ET-1 mediates its antiapoptotic effect on endothelial cells remains elusive.

To characterize the ET-1 signaling pathway in endothelial cell survival, we studied the effect of ET-1 on Forkhead box O (FOXO)-1 production in human aorta endothelial cells (HAEC). FOXO1 belongs to a subfamily of transcription factors that is characterized by a highly conserved winged-helix DNA binding motif, termed forkhead domain, which includes FOXO1, FOXO3, FOXO4, and FOXO6 (16, 17). These forkhead proteins are substrates of Akt/PKB and serum- and glucocorticoid-induced kinase, playing important roles in mediating insulin or IGF-I action on the expression of genes involved in cell growth, metabolism, and differentiation (16–18). Its orthologs abnormal Dauer formation 16 in *Caenorhabditis elegans* and dFoxO in *Drosophila* contribute to the regulation of oxidative stress and longevity (19–21). Insulin (or IGF-I) exerts some of its inhibitory effect on target gene expression via a conserved insulin-responsive element (5'-TG/ATTTT/G-3') in the promoter (16, 17, 22). In the absence of insulin (or IGF-I), FOXO1 proteins reside in the nucleus and bind as a *trans*-activator to insulin-responsive element, enhancing promoter activity. In response to insulin (or IGF-I) stimulation, FOXO1 proteins are phosphorylated by AKT/PKB through the PI3K-dependent pathway, resulting in FOXO1 nuclear export and inhibition of target gene expression (23–29). This phosphorylation-dependent subcellular redistribution serves as an acute mechanism for insulin (or IGF-I) to regulate FOXO1 transcriptional activity in cells (16–18, 22, 30). Based on the observation that ET-1 promotes AKT/PKB activation, we hypothesized that FOXO1 mediates the autocrine effect of ET-1 on endothelial cell survival. We tested this hypothesis and addressed the underlying physiological significance *in vitro* using HAEC and *in vivo* using both streptozotocin (STZ)-induced insulin-deficient and insulin-resistant *db/db* models.

Materials and Methods

Cell Culture and adenovirus transduction

HAEC were obtained from Cambrex (Lonza Cambrex Bioscience Walkersville Inc., Walkersville, MD) and cultured in

endothelial basal medium-2 (EBM-2) supplemented with endothelial growth media SingleQuots (Cambrex) in the absence or presence of 100 nM ET-1, as described (31, 32). HAEC were transduced with adenoviral vectors at a defined dose of 100 plaque-forming units (pfu) per cell. The adenoviral vectors used were as follows: Adv-CMV-FOXO1 expressing wild type FOXO1 (1.0×10^{11} pfu/ml) and the null adenovirus Adv-null (1.25×10^{11} pfu/ml), as described (33). Adenoviral vectors were produced in human embryonic kidney 293 cells and purified as described (34).

Animal studies

Institute for Cancer Research (ICR) mice (female, 8 wk old) were purchased from Taconic (Germantown, NY). Male *db/db* mice and heterozygous *db/+* littermates (6 wk old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were fed standard rodent chow and water *ad libitum* in sterile cages with a 12 h-light, 12-h dark cycle. To induce diabetes, mice were ip injected with STZ at the dose of 240 mg/kg (Sigma-Aldrich, St. Louis, MO). A group of age- and sex-matched ICR mice without STZ treatment was used as normal control. Blood glucose levels were measured using Glucometer Elite (Bayer, Indianapolis, IN). One week after STZ administration, diabetic mice were stratified by blood glucose levels and randomly assigned to two groups. One group received insulin therapy and the other group was mock treated with PBS as diabetic control. Insulin therapy was established by implanting the LinBit insulin implant (LinShin Canada Inc., Toronto, Canada) for providing sustained basal insulin release. Diabetic mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine as described (35), followed by inserting one LinBit insulin pellet sc under the middorsal skin, according to the manufacturer's instruction. In addition, each diabetic mouse in the insulin therapy group received a once-daily sc insulin injection (0.25–0.5 IU, Humulin N; Eli Lilly & Co., Indianapolis, IN) in the morning for improving postprandial blood glucose control. When hyperglycemia was corrected after 1 wk of insulin treatment, the entire aorta (ascending aorta, aortic arch, and descending aorta) was surgically removed from individual mice. The ascending aorta was used for immunohistochemistry, using anti-FOXO1 antibody as described (34). The remaining portion of the aorta (aortic arch and descending aorta) was used for isolation of total RNA for real-time quantitative RT-PCR. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine.

RNA isolation and real-time quantitative RT-PCR

Individual aorta were homogenized in 200 μ l Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was prepared following the manufacturer's instruction and subjected to real-time quantitative RT-PCR using specific primers against FOXO1 or β -actin mRNA, as described (36).

Immunofluorescent microscopy

HAEC (5×10^4 cell/well) were seeded onto six-well dishes (Corning Inc., Corning, NY) containing sterile human fibronectin 22-mm-round glass coverslips (BD Bioscience, Bedford, MA) and grown as previously described (31). After 24 h incubation, HAEC were serum starved for 6 h, followed by the addition of 100 nM insulin (Invitrogen) or 100 nM ET-1 (Sigma Aldrich) into

culture medium. Control wells of cells were mock treated with serum-free EBM-2 medium. As additional control, HAEC were cultured with both ET-1 (100 nM) and ET-1 receptor inhibitor IRL1038 (50 μ M; Sigma-Aldrich). After 30 min incubation, cells were washed with PBS and fixed in 4% paraformaldehyde (USB Corp., Cleveland, OH), followed by anti-FoxO1 immunocytochemistry, as described (36).

FOXO1 O-glycosylation assay

This assay relies on the property of wheat-germ agglutinin (WGA) in binding with high-affinity with O-glycosylated proteins (37). To study FOXO1 O-glycosylation, HAEC were cultured in the presence of 5.6 or 25 mM glucose for 24 h. Cell protein lysates (250 μ g protein) were incubated with 30 μ l of succinylated WGA-agarose beads (Vector Laboratories, Burlingame, CA) for 2 h at 4 C. WGA-agarose bead-bound proteins were washed with PBS buffer and subjected to immunoblot analysis using anti-FOXO1 antibody.

Immunoblot assay

HAEC ($\sim 2 \times 10^6$ cells) were lysed in 400 μ l of M-PER supplemented with 4- μ l Halt protease inhibitor cocktail (Pierce, Rockford, IL), followed by centrifugation at 13,000 rpm for 10 min. Subsequent preparation of nuclear and cytosolic fractions for immunoblot analysis was described (33). Aliquots of protein extracts (20 μ g) were resolved on 4–20% SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride membranes, which were subjected to immunoblot assay using the following antibodies: rabbit anti-FOXO1 antibody (1:3000 dilution, developed in our own laboratory) (38); rabbit antiphosphorylated FOXO1 (Ser256, 1:3000 dilution; Cell Signaling Technology, Danvers, MA); rabbit anti-B cell leukemia/lymphoma 2 (BCL-2; 1:2000 dilution, sc-783; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-BCL-2-associated X protein (BAX; 1:1000 dilution, sc-6236; Santa Cruz Biotechnology); rabbit anti-BCL-2 homologous killer (BAK; 1:2000 dilution, catalog no. 04-443; Millipore Upstate, Billerica, MA); and rabbit anti-B cell leukemia/lymphoma 2-associated death promoter (BAD) antibody (1:2000 dilution, catalog no. 04-432; Millipore Upstate). Mouse monoclonal anti-TATA box binding protein (TBP) antibody (1:5000 dilution, catalog no. ab818; Abcam, Cambridge, MA) was used for detecting control nuclear TBP protein in the immunoblots. The protein bands were visualized by chemiluminescent Western detection reagents (Pierce, Rockford, IL). The intensity of the protein bands was quantified by densitometry using the NIH Image software (National Institutes of Health, Bethesda, MD).

Caspase-3 assay

HAEC ($\sim 2 \times 10^5$ cells) were seeded in six-well dishes. After 24 h incubation, cells were transduced with Adv-null or Adv-FOXO1 vector (100 pfu/cell). After 24 h incubation in presence or absence of 100 nM ET-1, cells were collected for the determination of caspase-3 activity using a caspase-3 activity assay kit (catalog no. QIA70; Calbiochem, Gibbstown, NJ).

Chromatin immunoprecipitation (ChIP) assay

A ChIP was performed to study the interaction between FOXO1 and BAD promoter in HAEC as described (34). HAEC (2×10^5 cells) were cultured in EBM-2 medium supplemented

with 5.6 or 25 mM glucose in the absence or presence of 100 nM ET-1. After 24 h incubation, cells were cross-linked with 1% formaldehyde, followed by sonication in a Microson 100-W Ultrasonicator (Structure Probe, West Chester, PA) at 30% of maximum power for five consecutive cycles of 20-sec pulses. After centrifugation at $18,000 \times g$ for 10 min, the supernatant was incubated with 5 μ g polyclonal rabbit anti-FoxO1 antibody that was generated in our laboratory (38), followed by immunoprecipitation using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). The immunoprecipitates were analyzed by PCR assay to detect coimmunoprecipitated DNA using the BAD promoter-specific primers (forward, 5'-GAC-CTGGGTCTTCAGAAATAG-3', reverse, 5'-CACAGAA-GAAGTGAGGAAGTC-3') flanking the DNA region (–1465/–864 nt) of the human BAD promoter. As an off-target control, a pair of primers (forward, 5'-CTTCCTCGCC CGAAGAGCGC-3' and reverse, 5'-GGGCTCGGGTCCCCGGTGACG-3') flanking the human BAD coding region (+721/+1160 nt) was used.

BAD promoter activity assay

The mouse BAD promoter was amplified from C57BL/6J mouse genomic DNA by PCR using primers (5'-AGTG-GTCTTTCCTACCCAGG-3' for forward reaction and 5'-TCCATTTGGATCCTGGAGG-3' for reverse reaction). The DNA fragment flanking the mouse BAD promoter (–1246/+1 nt) was cloned into the pGL3-basic vector encoding the Firefly luciferase gene (Promega, Madison, WI). The resulting plasmid pBAD-Luc was cotransfected with pGL4.75 encoding the Renilla luciferase gene (Promega) into HAEC, followed by transduction with the Adv-FOXO1 or the control Adv-null vector (100 pfu/cell). After 24 h incubation in serum-free medium in the presence or absence of ET-1 (100 nM), cells were subjected to dual-luciferase activity assay for determining BAD promoter activity.

Statistical analysis

Data were presented as mean $\pm$ SEM. Statistical analysis was performed using a two-tailed unpaired Student's *t* test. Pair-wise comparisons were performed to study the significance between different conditions. *P* < 0.05 was considered statistically significant.

Results

FoxO1 expression is up-regulated in aorta endothelium of diabetic mice

To determine the impact of hyperglycemia on endothelial FoxO1 expression *in vivo*, we studied the expression of FoxO1 in the aorta of diabetic mice. ICR female mice (body weight 26.7 ± 1.8 g at 8 wk of age) were injected ip with STZ (240 mg/kg, *n* = 8). Two days after STZ administration, all STZ-treated mice developed severe diabetes (blood glucose levels 518 ± 32 mg/dl), in comparison with sex- and age-matched controls (blood glucose levels 123 ± 14 mg/dl, *n* = 6). Mice were killed 2 wk after STZ injection, and the aorta of individual mice were subjected to anti-FoxO1 antibody immunohistochemistry.

As shown in Fig. 1, intensive FoxO1 immunostaining was detected in the aortic endothelium in STZ-induced diabetic mice but not in normal control mice. As a control, we applied preimmune rabbit serum to immunohisto-

chemistry. The aortic endothelium of both normal and STZ-induced diabetic mice was stained negative (data not shown). To corroborate this finding, we isolated total

RNA from the aorta of STZ-induced diabetic *vs.* control mice, followed by real-time quantitative RT-PCR assay. An 8-fold induction in FoxO1 mRNA expression was detected in the aorta of STZ-induced diabetic mice (Fig. 1G). In contrast, aortic FoxO3 and FoxO4 mRNA levels remained unchanged in STZ-induced diabetic *vs.* control groups. Furthermore, we subjected the aorta of individual mice to anti-FoxO1 immunoblot assay, demonstrating that aortic FoxO1 protein levels were significantly up-regulated in STZ-induced diabetic mice (Fig. 1H). These data indicate that FoxO1 expression in the aorta becomes deregulated in response to prevailing hyperglycemia in diabetic mice.

To underpin the above conclusion, we determined aortic FoxO1 production in *db/db* mice, a commonly used genetic model of obesity and type 2 diabetes. When compared with age- and sex-matched lean control mice ($n = 6$), male *db/db* mice ($n = 6$) exhibited morbid obesity (body weight 43.1 ± 3.4 *vs.* 23.8 ± 1.8 g in the control group) and hyperglycemia (blood glucose levels 272 ± 41 *vs.* 128 ± 18 mg/dl, $P < 0.001$) at 10 wk of age under *ad libitum* conditions. Furthermore, *db/db* mice, as opposed to control mice, were associated with significantly increased FoxO1 production in the aorta (Fig. 1I).

We hypothesized that FoxO1 deregulation in the aorta is secondary to hyperglycemia in diabetic mice. To address this hypothesis, we rendered ICR mice diabetic after an ip dose of STZ as above. Diabetic mice were stratified by the degree of hyperglycemia (blood glucose 500 ± 26 mg/dl) and randomly assigned to two groups ($n = 6$ /group), which received insulin therapy or PBS injection. One group of nondiabetic mice ($n = 6$) was included as normal control (blood glucose 137 ± 8 mg/dl).

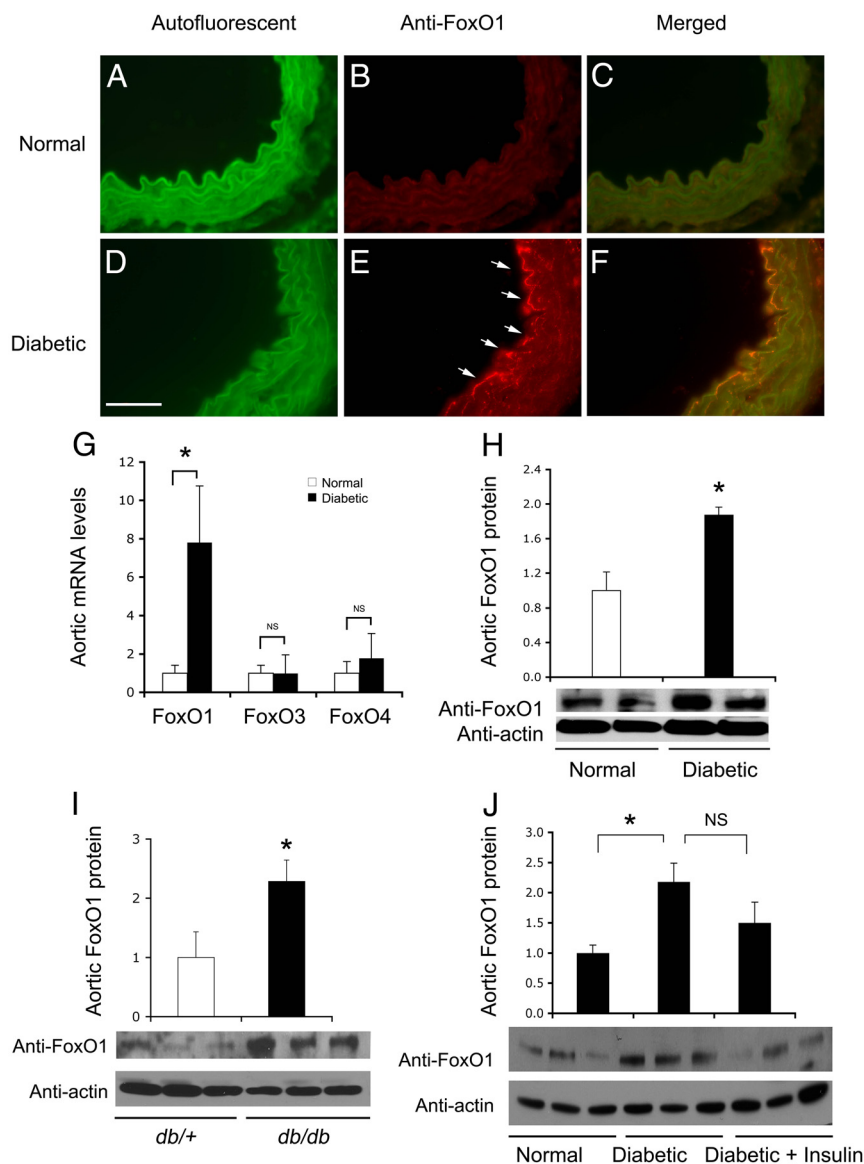


FIG. 1. Endothelial FoxO1 expression in the aorta of diabetic mice. ICR mice (female, 8 wk old) were rendered diabetic by administration of a single dose of STZ (240 mg/kg). STZ-induced diabetic ($n = 8$) and control ($n = 6$) mice were killed 2 wk after STZ administration. Aorta were isolated from normal (A–C) and diabetic (D–F) groups for anti-FoxO1 immunohistochemistry, followed by immunofluorescent microscopy. The elastic fibers of the aorta were autofluorescent green (A and D). FoxO1 was immunostained red in the endothelium (B and E). Merged images were shown in C and F. In addition, aliquots of aortic RNA were subjected to real-time quantitative RT-PCR for the determination of endothelial FoxO1, FoxO3, and FoxO4 mRNA levels using β -actin mRNA as control (G). The ascending aorta was homogenized for the preparation of total protein lysates, which were subjected to anti-FoxO1 immunoblot assay for determining aortic FoxO1 protein levels (H). Likewise, ascending aortas of *db/db* ($n = 3$, male, 10 wk old) and age- and sex-matched control ($n = 3$) were subjected to an anti-FoxO1 immunoblot assay (I). In addition, ICR mice (female, 8 wk old) were rendered diabetic by STZ these mice were randomly assigned to two groups ($n = 6$) receiving insulin therapy or PBS buffer. A group of nondiabetic mice ($n = 6$) was used as a normal control. After 1 wk of insulin therapy, the mice were killed under *ad libitum* condition for determining aortic FoxO1 protein levels (J). Bar, 50 μ m. *, $P < 0.05$ *vs.* control by ANOVA.

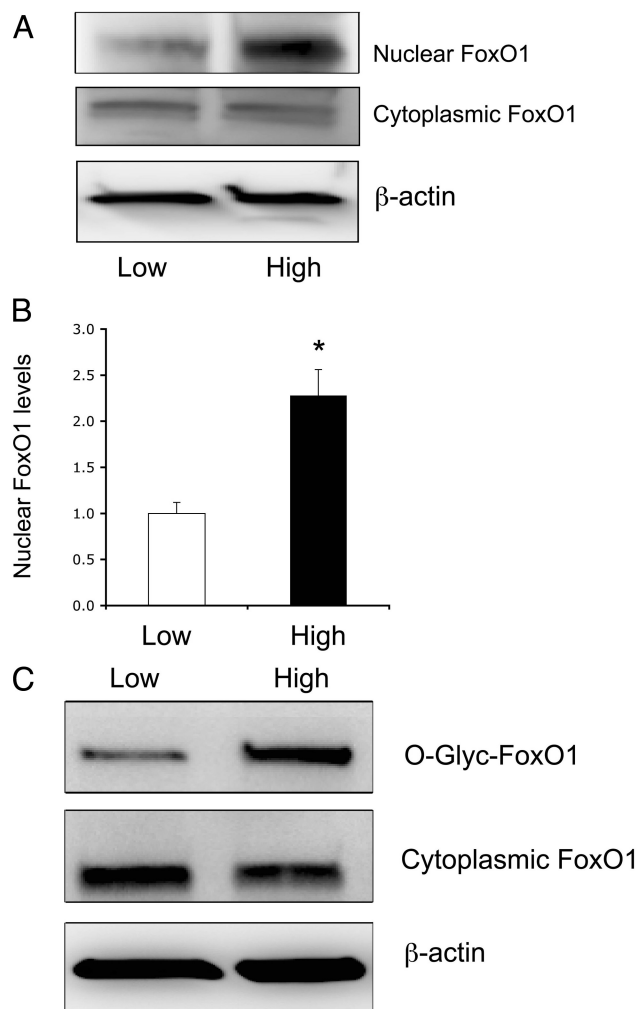


FIG. 2. FOXO1 undergoes O-linked glycosylation under hyperglycemia conditions. HAEC were cultured in EBM-2 under low- (5.6 mM) or high (25 mM)-glucose conditions. After 24 h incubation, cytoplasmic and nuclear fractions of cells were prepared and subjected to immunoblot for the determination of FOXO1 subcellular distribution (A). The induction of FOXO1 nuclear protein levels in response to high glucose was determined (B). In addition, aliquots of cells were subjected to WGA affinity chromatography for the isolation and detection of O-glycosylated proteins by anti-FOXO1 immunoblot assay (C). Data were from three independent experiments. *, $P < 0.05$ vs. control.

Insulin therapy was instituted by implanting the LinBit insulin implant under the middorsal skin of individual diabetic mice for providing basal insulin release, followed by once-daily sc insulin injection (0.25–0.5 IU per mouse) in the morning for controlling postprandial blood glucose levels. After 1 wk of insulin therapy for reducing hyperglycemia to the normal range (127 ± 58 mg/dl), the mice were killed for the isolating aortas, which were subjected to anti-FoxO1 immunoblot assay. FoxO1 protein levels were significantly up-regulated in the aorta of the STZ-induced diabetic mice (Fig. 1J). This effect was ameliorated but not to basal levels by insulin therapy, presum-

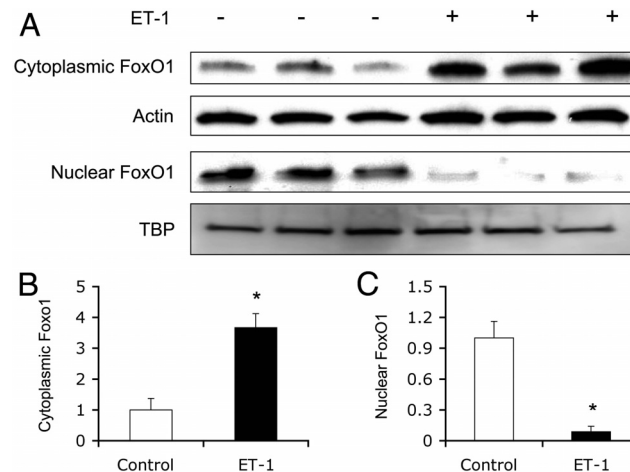


FIG. 3. ET-1 promotes FOXO1 nuclear exclusion in HAEC. HAECs ($\sim 1 \times 10^6$ cells) were transfected with an Adv-FOXO1 vector (100 pfu/cell) in EBM-2 supplemented with 5.6 mM glucose. After 24 h incubation, cells were serum starved for 6 h and then were mock treated or treated with 100 nM ET-1 for 30 min. Cells were collected for the preparation of cytosolic and nuclear fractions, followed by immunoblot analysis using anti-FOXO1 antibody (A). The relative amount of FOXO1 in the cytoplasm was determined using actin as a control (B). Likewise, the relative amount of FoxO1 protein in the nucleus was quantified using nuclear TBP protein as a control (C). Data were from three independent experiments. *, $P < 0.001$ vs. control.

ably due to inadequate blood glucose control in diabetic mice.

Hyperglycemia promotes FOXO1 O-glycosylation and nuclear localization

To recapitulate the above finding, we determined FOXO1 production in cultured HAEC under normal *vs.* hyperglycemic conditions. HAEC were cultured in EBM-2 containing 5.6 mM glucose (low glucose) or 25 mM glucose (high glucose). After 24 h incubation, cells were subjected to immunoblot analysis for the determination of FOXO1 protein levels in the nucleus *vs.* cytoplasm (Fig. 2A). FOXO1 was significantly up-regulated along with its predominant localization in the nucleus of HAEC under high-glucose conditions (Fig. 2B). In contrast, cytosolic FOXO1 protein levels remained unchanged, regardless of glucose concentrations in culture medium.

To probe the underlying mechanism, we hypothesized that FOXO1 may be O-glycosylated in response to hyperglycemia. This hypothesis derived from previous observations that O-glycosylation of FOXO1 promotes its nuclear localization and enhances its transcriptional activity (37, 39, 40). To address this hypothesis, we studied the ability of FOXO1 to undergo O-linked glycosylation in HAEC that were cultured in the presence of 5.6 or 25 mM glucose, using WGA affinity chromatography as described (37). WGA binds with high affinity to O-glycosy-

lated proteins, allowing their fractionation from native polypeptides. This assay detected a marked elevation in FOXO1 O-glycosylation in HAEC exposed to hyperglycemia (Fig. 2C).

Effect of ET-1 on FOXO1 subcellular distribution in HAEC

To determine the effect of ET-1 on FOXO1 subcellular distribution, we treated HAEC with ET-1 for 30 min, followed by the separation of nuclear and cytosolic fractions. Aliquots of protein extracts (20 μ g) were subjected to immunoblot analysis. In response to ET-1, FOXO1 was translocated from the nucleus to cytoplasm (Fig. 3).

To ascertain these findings, we incubated HAEC in the absence or presence of ET-1 (100 nM) for 30 min, followed by anti-FoxO1 immunocytochemistry. FOXO1 was predominantly nuclear in the absence of ET-1 (Fig. 4, A–C). In response to ET-1, FOXO1 detected mainly in the cytoplasm (Fig. 4, D–F). As a control, we treated HAEC with insulin (100 nM) for 30 min, followed by immunocytochemistry. FOXO1 was preferentially localized in the cytoplasm (Fig. 4, G–I), correlating with the ability of FOXO1 to undergo insulin-dependent phosphorylation and nuclear exclusion (16, 17). As an additional control,

we incubated HAEC in both ET-1 and its receptor inhibitor IRL1038, an agent that binds selectively to ET-1 receptor and blocks ET-1 signaling (41). ET-1-mediated FOXO1 redistribution was reversed in the presence of its receptor inhibitor (Fig. 4, J–L).

ET-1 promotes FOXO1 phosphorylation in HAEC

To address the mechanism by which ET-1 promoted FOXO1 redistribution from the nucleus to cytoplasm, we determined the ability of ET-1 to promote FOXO1 phosphorylation. Our hypothesis is that ET-1 stimulates phosphorylation of FOXO1, resulting in its nuclear exclusion. Implicit in this hypothesis is the findings that ET-1 functions through the PI3K-AKT axis to regulate its target gene expression (10, 13, 15). To address this hypothesis, we cultured HAEC in the absence or presence of ET-1 (100 nM), followed by an immunoblot analysis of FOXO1 using anti-phospho (S256)-FOXO1 antibody. This assay detected a 2.5-fold induction of FOXO1 phosphorylation in HAEC in response to ET-1 (Fig. 5).

To determine the ability of ET-1 to promote FOXO1 phosphorylation under hyperglycemic conditions, we cultured HAEC in the presence of 25 mM glucose for 24 h, followed by treatment with ET-1 (100 nM) for 30 min.

Cells were subjected to immunoblot assay for detecting phosphorylated and total FOXO1 protein. ET-1-mediated induction of FOXO1 phosphorylation was impaired in the presence of hyperglycemia (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>).

FOXO1 mediates ET-1 action on endothelial cell survival

To investigate whether FOXO1 mediates ET-1 action on endothelial cell survival, we determined the impact of FOXO1 on the expression of key functions in cell survival and apoptosis, including BCL-2, BAD, BAX, and BAK. BCL-2 is a prosurvival factor whose expression confers a cytoprotective effect on cell survival (42, 43). In contrast, BAD, BAX, and BAK are associated with proapoptotic activities (44–46). HAEC were transduced with an Adv-FOXO1 vector or a control Adv-null vector at a fixed dose of 100 pfu/cell in the absence or presence of ET-1 (100 nM). After 24 h incubation, cells

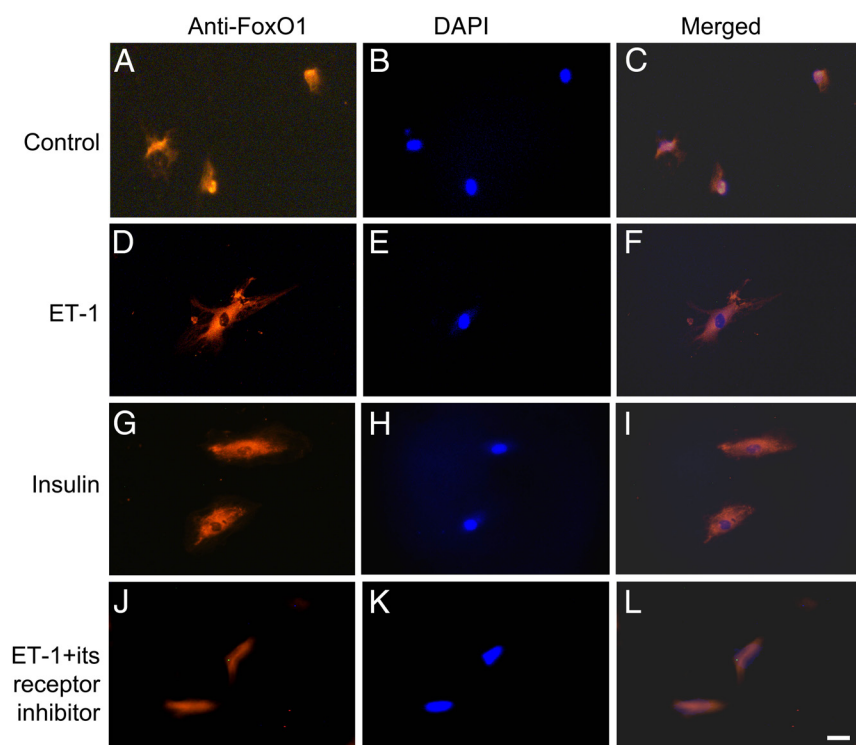


FIG. 4. Effect of ET-1 on FoxO1 subcellular distribution. HAEC were cultured in EBM-2 supplemented with 5.6 mM glucose. After 24 h incubation, cells were serum starved for 6 h, followed by incubation in absence (control, A–C) or presence of ET-1 (100 nM) (D–F) in serum growth factor-free medium for 30 min. As controls, aliquots of cells were treated with insulin (100 nM) (G–I) or ET-1 (100 nM) plus its receptor inhibitor (50 μ M) (J–L) in culture medium for 30 min. Each condition was run in triplicate. Cells were subjected to anti-FoxO1 immunocytochemistry. Bar, 10 μ m. DAPI, 4'6-diamidino-2-phenylindole.

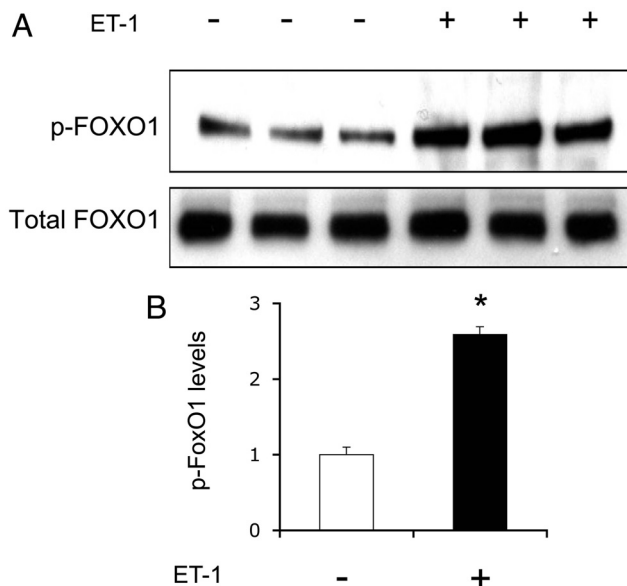


FIG. 5. ET-1 mediates FOXO1 phosphorylation. HAEC were transduced with an Adv-FOXO1 vector (100 pfu/cell) in EBM-2 supplemented with 5.6 mM glucose. After 24 h incubation, cells were serum starved for 6 h, followed by incubation in the absence or presence of 100 nM ET-1 for 30 min. Cells were collected for the preparation of total protein lysates, which were analyzed by immunoblot assay using anti-phospho FOXO1 and anti-FOXO1 antibodies (A). The relative abundance of phosphorylated FOXO1 of total FOXO1 proteins in cells was determined (B). Data were from three independent experiments. *, $P < 0.001$ vs. control.

were subjected to immunoblot analysis (Fig. 6A). Adenovirus-mediated production of FOXO1 resulted in a significant reduction of BCL-2 protein levels in cultured HAEC. This effect was reversed by the addition of ET-1 into culture medium (Fig. 6B). In contrast, the protein levels of BAD and BAX, two important members of the proapoptotic subfamily, were up-regulated in response to FOXO1 production in HAEC (Fig. 6A). Treatment of ET-1 significantly attenuated FOXO1-mediated induction of BAD and BAX production to a greater and lesser extent, respectively, in HAEC (Fig. 6, C and D). In contrast, no significant differences in BAK protein levels were detected in response to FOXO1 production in HAEC, irrespective of the addition of ET-1 into the culture medium (Fig. 6, A and E). These results suggest that FOXO1 contributes to endothelial regulation of BAD production by ET-1 in HAEC.

To corroborate these findings, we determined the effect of FOXO1 on caspase-3 activity in cultured HAEC in response to ET-1 action. HAEC were transduced with a control Adv-null or an Adv-FOXO1 vector in the absence and presence of ET-1 in culture medium. After 24 h incubation, cells were collected for the determination of caspase-3 activity. This assay detected a 2-fold induction of caspase-3 activity in FOXO1 vector-treated HAEC (Fig. 6F). This effect was reversed to normal by the inclu-

sion of ET-1 in culture medium, suggesting that ET-1 signaling through FOXO1 plays an important role in regulating endothelial cell survival.

FOXO1 targets BAD gene for *trans*-activation

To gain insight into the mechanism of FOXO1-mediated induction of endothelial cell apoptosis, we hypothesized that FoxO1 targets the BAD gene for *trans*-activation. To address this hypothesis, we performed sequence analysis, revealing two consensus FOXO1 binding motifs in the promoter of the *BAD* gene. Such consensus FOXO1 binding sites are present in the BAD promoter of human and rodent origins (Supplemental Fig. 2), suggesting an evolutionally conserved mechanism by which FOXO1 mediates the effect of ET-1 on endothelial BAD production and cell survival. To determine the molecular association of FOXO1 with the BAD promoter DNA, we performed a ChIP assay on HAEC that were cultured in serum-free medium under normal and higher glucose conditions in the absence or presence of ET-1. This assay allows the detection of protein-DNA interaction in living cells, as described (33, 47). FOXO1 was shown to bind to its target site within the BAD promoter in cultured HAEC under high-glucose conditions, and this effect was abolished by the inclusion of ET-1 in culture medium (Fig. 7).

To determine the ability of FoxO1 to trans-activate BAD promoter activity, we cloned the mouse BAD promoter (1.2 kb) into the pGL3-basic vector encoding the *Firefly* luciferase gene, resulting in plasmid pBAD-Luc. We cotransfected HAEC with pBAD-Luc and pGL4.75 encoding the *Renilla* luciferase gene as a control, followed by the transduction with the Adv-FOXO1 or control Adv-null vector. After 24 h incubation in serum-free medium in the absence or presence of ET-1, cells were subjected to dual-luciferase activity assay for determining the BAD promoter activity. As shown in Fig. 7, FOXO1 stimulated BAD promoter activity, and this effect was counteracted by ET-1, correlating with the ability of ET-1 to phosphorylate and promote FOXO1 trafficking from the nucleus to the cytoplasm (Fig. 4).

ET-1 protects against hyperglycemia-elicited FOXO1 deregulation in HAEC

To understand the underlying pathophysiology of FOXO1-mediated induction of BAD production in endothelial cells, we incubated HAEC in culture medium supplemented with 5.6 or 25 mM glucose in the absence and presence of ET-1 (100 nM). After 24 h incubation, cells were harvested for the determination of FOXO1 protein levels by immunoblot assay. As shown in Fig. 8, cytosolic FOXO1 protein levels remains unchanged under different conditions. Instead, we detected a 2-fold increase in nu-

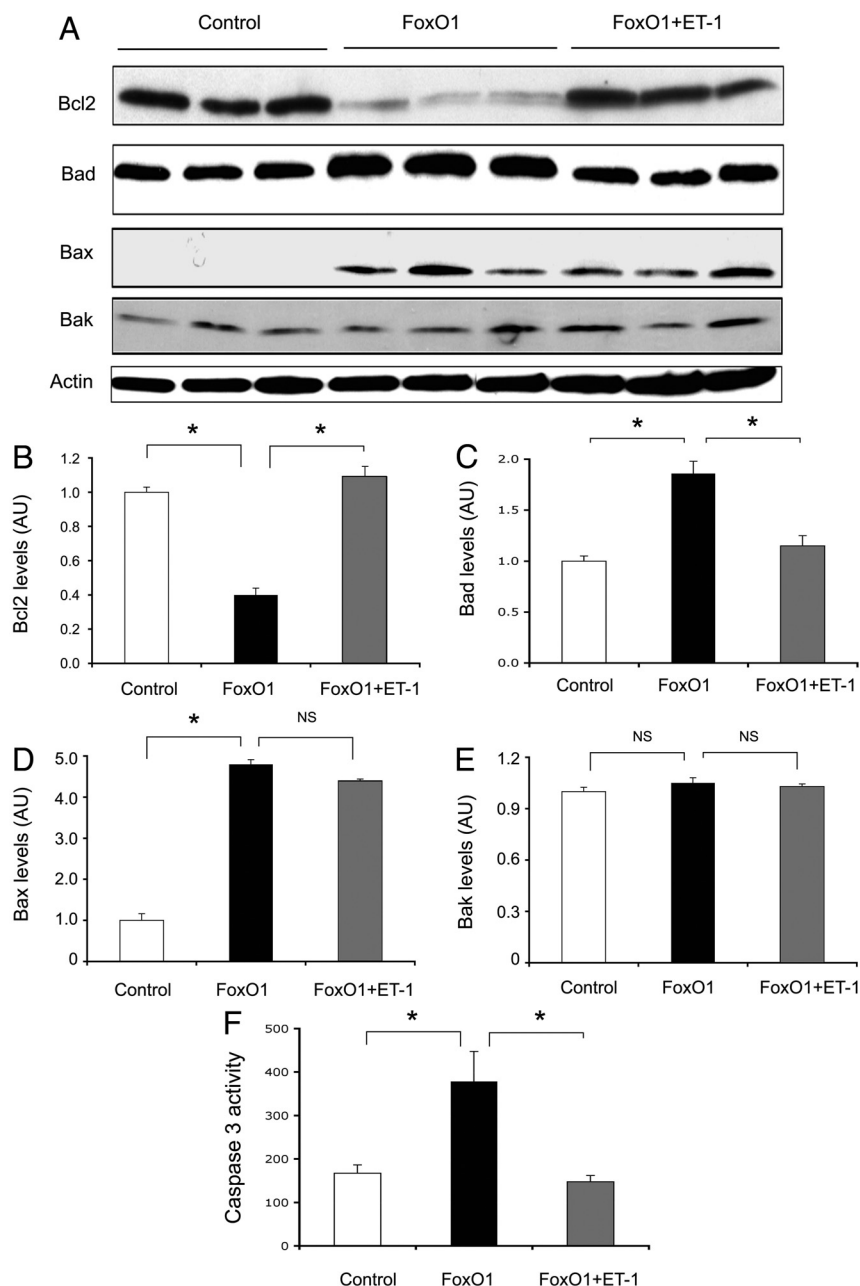


FIG. 6. Effect of FOXO1 on endothelial gene expression. HAEC were transduced with a control Adv-null or Adv-FOXO1 vector (100 pfu/cell) in the absence or presence of ET-1 (100 nM). After 24 h incubation, cells were collected for the preparation of total protein lysates, which were subjected to immunoblot analysis (A). The relative protein abundance of BCL-2 (B), BAD (C), BAX (D), and BAK (E) was determined. In addition, aliquots of HAEC were treated with control or FOXO1 vector subjected to caspase-3 activity assay (F). Data were from three independent experiments. *, $P < 0.05$. NS, Not significant; AU, arbitrary unit.

clear FOXO1 protein levels in HAEC under higher glucose conditions, indicating that endothelial FOXO1 expression became deregulated in response to hyperglycemia. This hyperglycemia-induced FOXO1 deregulation was reversed to normal by the addition of ET-1 into culture medium. As control, we subjected HAEC to a real-time quantitative RT-PCR assay. FOXO1 mRNA levels remained unchanged in response to hyperglycemia or ET-1 treatment (Supplemental

Fig. 3), consistent with the idea that ET-1 modifies FOXO1 activity at the post-translational levels.

Discussion

ET-1 is a potent vasoconstrictor that plays a pivotal role in vascular homeostasis. ET-1 acts via its specific receptors ET_A on smooth muscle cells and ET_B on endothelial cells to regulate vascular tone and integrity in both autocrine and paracrine manners (1, 2). Although it has been shown that ET-1 also possesses antiapoptotic function (11), the underlying mechanism and physiology is not well understood. In this study, we investigated the mechanisms by which ET-1 exerts its autocrine effect on endothelial cell survival. We studied the effect of ET-1 on endothelial expression of FOXO1 in cultured HAEC as well as in the aortic endothelium of diabetic mice. We show that FOXO1 stimulated the production of BAD, a key proapoptotic protein, via selective binding to the BAD promoter. This effect was accompanied by decreased BCL-2 production and increased caspase-3 activity, contributing to endothelial cell apoptosis in HAEC with unbridled FOXO1 activity. Under hyperglycemic conditions, endothelial FOXO1 activity was significantly up-regulated, culminating in its increased nuclear localization in HAEC. Similar profiles of FOXO1 deregulation were seen in the aorta of hyperglycemic mice. This finding was reproduced in both STZ-induced diabetic mice and genetically diabetic *db/db* mice. An increased FoxO1 production accounted for its enhanced transcriptional activity, which in turn

promoted BAD production and endothelial apoptosis in HAEC. In response to ET-1, FOXO1 became phosphorylated, resulting in FOXO1 trafficking from the nucleus to cytoplasm. This effect contributed to inactivation of FOXO1 activity and suppression of BAD production and inhibition of endothelial cell apoptosis. Although ET-1 exerts its cytoprotective effect on cell survival via the

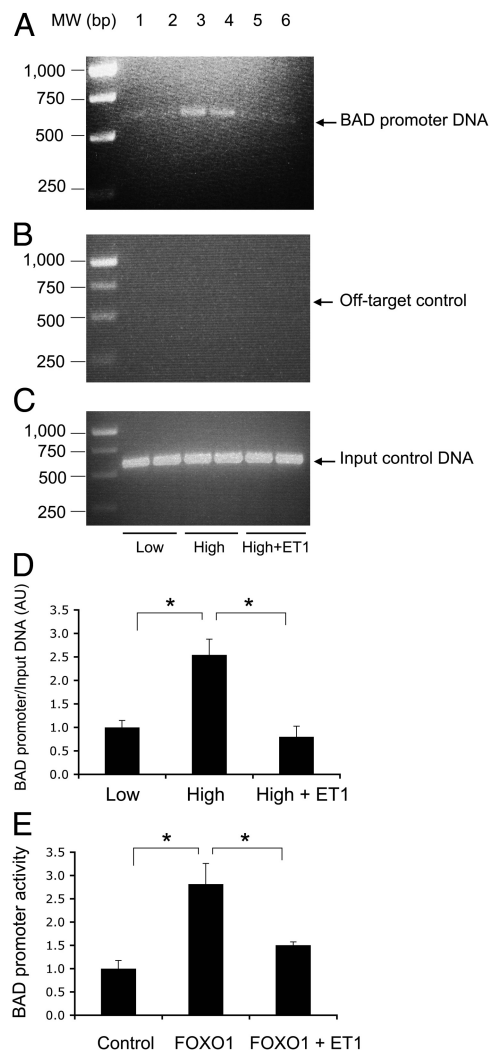


FIG. 7. Molecular association of FOXO1 with BAD promoter. HAEC (2×10^6 cells) were cultured under low-glucose (5.6 mM, lanes 1 and 2), high-glucose (25 mM, lanes 3 and 4), and high-glucose (25 mM) plus ET-1 (100 nM) (lanes 5 and 6) conditions. After 24 h incubation, cells were cross-linked with 1% formaldehyde and subjected to a ChIP assay using anti-FOXO1 antibody. The resulting immunoprecipitates were analyzed by PCR using specific primers flanking the FOXO1 target site (–1465/–864 nt) within the BAD promoter (A). MW, Molecular weight. As a negative control, the immunoprecipitates were subjected to PCR analysis using a pair of off-target primers flanking the coding region of BAD cDNA (+721/+1160 nt) (B). As a positive control, aliquots of input DNA samples (10 μ l) were used in the same PCR assay (C). The amount of BAD promoter DNA immunoprecipitated by anti-FOXO1 IgG relative to input control DNA was determined (D). In addition, HAEC were cotransfected with pBAD-Luc expressing the BAD promoter-directed *Firefly* luciferase reporter system and the pGL4.75 expressing the *Renilla* luciferase gene, followed by transduction with an Adv-FoxO1 or Adv-null vector (100 pfu/cell). After 24 h incubation in serum-free medium with or without ET-1, cells were subjected to a dual-luciferase assay for determining the BAD promoter activity, defined as the ratio of *Firefly* to *Renilla* luciferase activities (E). Data were from two independent experiments, each in duplicate. *, $P < 0.05$ vs. control.

PI3K-Akt cascade (11, 12, 14, 48, 49), the downstream signaling events remain obscure. Our studies suggest that ET-1 signaling through FOXO1 regulates endothelial cell survival.

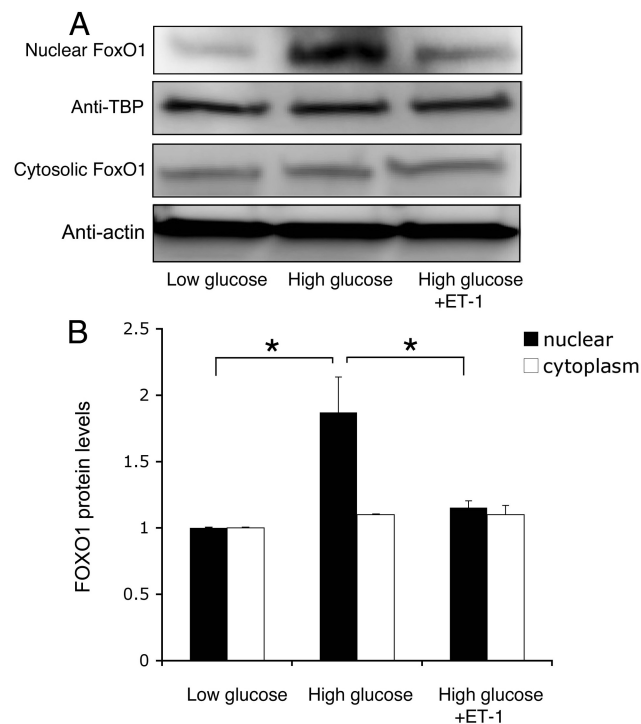


FIG. 8. ET-1 inhibits glucose induction of FOXO1 production. HAECs (2×10^6 cells) were cultured in the presence of low (5.6 mM) or high (25 mM) glucose without and with ET-1 addition of 100 nM in culture medium. After 24 h incubation, cells were subjected to immunoblot analysis for the determination of nuclear vs. cytoplasmic FOXO1 levels (A). The relative amounts of FOXO1 proteins in the nucleus and cytoplasm were quantified using actin as control (B). Data were from three independent experiments. *, $P < 0.05$ vs. control.

Endothelial dysfunction secondary to chronic exposure to hyperglycemia contributes to microvascular complications in poorly controlled diabetes, but the underlying mechanism remains obscure. Tanaka *et al.* (50) showed that FOXO1 activity is significantly up-regulated in endothelial cells in response to hyperglycemia. This effect promotes inducible NO synthase-dependent peroxynitrite generation, which leads to lipid peroxidation and endothelial NO synthase dysfunction. In the present study, we show that FOXO1 underwent O-linked glycosylation in HAEC with prior exposure to hyperglycemia. O-glycosylation is known to interfere with FOXO1 phosphorylation and nuclear export (37, 39, 40). This effect resulted in increased FOXO1 nuclear localization, accounting for its enhanced activity and contributing to the induction of cellular apoptosis in HAEC under hyperglycemic conditions. Such posttranslational modification of FOXO1 has been detected in other cell types, in which O-glycosylation of FOXO1 enhances its transcriptional activity and induces its target gene expression under hyperglycemic conditions (37, 39, 40). Our data together with others suggest that endothelial FOXO1 deregulation, resulting from an impaired ability of ET-1 to keep

FOXO1 activity in check, may be a causative factor for the pathogenesis of endothelial dysfunction in diabetes. In support of this notion, Tsuchiya et al. (51) showed that genetic ablation of *FOXO1*, *FOXO3*, and *FOXO4* genes in the endothelium protected against the development atherosclerosis in low-density lipoprotein receptor deficient mice.

ET-1 synthesis and secretion in endothelial cells is regulated via both the PI3K and MAPK pathways. Two independent studies show that acute treatment of bovine aortic endothelial cells with dehydroepiandrosterone, an adrenal steroid with beneficial effects on insulin sensitivity, results in a marked induction of ET-1 secretion (52, 53). This effect is abolished by the MAPK kinase inhibitor PD98059, suggesting that dehydroepiandrosterone-mediated stimulation of endothelial ET-1 production is via the MAPK-dependent mechanism (52, 53). Likewise, Reiter et al. (54) reported that endothelial ET-1 production is regulated through the PI3k-Akt-FOXO1 signaling pathway. They demonstrate that FOXO1 stimulates ET-1 production in HAEC and this effect is counteracted by green tea polyphenol epigallocatechin gallate, a mechanism that is thought to account for the beneficial effect of epigallocatechin gallate on endothelial function (54–57). Here we show that ET-1 inhibited FOXO1 activity by promoting its phosphorylation and nuclear exclusion. Phosphorylated FOXO1 is targeted for ubiquitination and proteolytic degradation in the cytoplasm (58–60). Together these data illustrate a feedback control mechanism by which endothelial FOXO1 activity is fine-tuned by ET-1. Revelation of the FOXO1 feedback loop in endothelial cells underscores the importance of the ET-1-AKT-FOXO1 axis in endothelial integrity, suggesting that a circuit breakdown in the endothelial FOXO1 feedback loop may be a contributing factor for ET-1 deregulation and endothelial dysfunction in diabetes. Consistent with this conjecture are the observations that endothelial ET-1 production becomes deregulated, culminating in the development of hyperendothelinemia in human subjects with metabolic syndrome (3, 6, 7, 61–64) and animal models with diabetes (5, 65).

We show that FOXO1 was up-regulated at both the mRNA and protein levels in the aorta in the presence of prevailing hyperglycemia in diabetic mice. These *in vivo* results seemed at odds with *in vitro* data showing that FOXO1 was regulated in response to hyperglycemia mainly at the posttranslational levels in HAEC. Apart from hyperglycemia *in vivo*, endothelial cells in the aorta were associated with impaired insulin signaling in diabetic mice. Because FOXO1 is a target of Akt/PKB downstream of insulin action, it is plausible that this discrepancy in endothelial FOXO1 regulation is compounded by altered insulin action in the aorta of diabetic mice. Studies

are warranted to further delineate the signaling pathway that governs FOXO1 regulation in endothelial cells.

It is noteworthy that FOXO1 deregulation in the aorta of diabetic mice was reduced, but not to baseline, after insulin therapy. This was presumably attributable to inadequate blood glucose control by insulin therapy in diabetic mice. Attempts with frequent blood glucose monitoring and insulin injection resulted in hypoglycemic episodes, a scenario that is encountered with intensive insulin therapy in clinics. Accordingly, we provided one morning bolus of insulin for controlling postprandial blood glucose excursion, in combination with basal insulin release from the insulin implant that was implanted under the middorsal skin of diabetic mice. Our results provided the proof of concept that FoxO1 deregulation in the aorta of diabetic mice is reversible after the reduction of hyperglycemia with insulin therapy.

In conclusion, we elucidate a mechanism by which ET-1 exerts its autocrine effect on endothelial cell survival. We show that ET-1 acts through AKT/PKB to promote FOXO1 phosphorylation and nuclear exclusion. This effect serves as a fine-tuning mechanism for keeping endothelial FOXO1 activity in check. Deregulation in endothelial FOXO1 production in the aorta may play a role in linking prevailing hyperglycemia to endothelial dysfunction, contributing to microvascular complications in poorly controlled diabetes.

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