

C. PRELIMINARY STUDIES

These preliminary studies are designed to demonstrate our progress toward a successful research program. The core of our experimental **approach** relies on *in vivo* digital microscopy and the use of reverse genetics (siRNA). The **feasibility** of this approach has been proven in our preliminary work. By collaborating with seminal experts like Drs. John Wahren and Makoto Suematsu, we will be able to address issues on the forefront of cardiovascular disease in type I diabetes using an advanced interdisciplinary approach. The **innovative** aspects of this study include the sophisticated *in vivo* techniques of cellular and molecular bioimaging, the use of an ELISA for true *in vivo* HO activity, and the application of *in vivo* gene silencing to mechanistically determine the roles of NADPH and HO in vasoprotection during type I diabetes. Dr. Brock is well-suited to lead this research effort as **principal investigator** and has assembled an excellent cadre of basic and clinical researchers, including Drs. Laura Lamps, Makoto Suematsu, John Wahren, and Phyllis Dennerly, with expertise ranging from *in vivo* digital microscopy with fluorescence and experimental therapeutics to pathology and enzymology. Finally, a modern laboratory equipped with spectrophotometers capable of photometrics, kinetics, and spectrum scanning, as well as a high-resolution Zeiss inverted microscope system with fluorescence and digital image processing/analysis, will ensure the appropriate **environment** in which to carry out the proposed work.

In this section, we will sequentially provide data demonstrating that:

- ◆ the administration of C-peptide does not have confounding effects on body weight or blood glucose
- ◆ reductions in type I diabetic vascular function and viability are reversed by C-peptide
- ◆ a single dose of C-peptide enhances NADPH bioavailability in type I diabetes
- ◆ type I diabetes results in reduced G6PD activity with concomitant elevations in cAMP
- ◆ HO is induced by type I diabetes and cobalt protoporphyrin augmented its expression in both parenchymal and vascular cell fractions
- ◆ murine HO-1 is inhibited by hydrodynamically-based transfection of targeted siRNAs

Comment [D1]: Addressing each of the review criteria in this section.

Comment [D2]: Use of bullet list to organize and highlight what will be presented. Sometimes put at end, but usually more effective at beginning.

Reductions In Type I Diabetic Vascular Function are Reversed by C-Peptide

Comment [D3]: Descriptive heading

Preliminary work from our laboratory confirms the protection C-peptide affords the circulation in a model of type I diabetes. These studies characterize the hemodynamic responses of the hepatic vasculature using the same experimental design as described for our murine model of type I diabetes in the previous section. Vascular function, in terms of nutritive blood flow, was assessed using *in vivo* digital microscopy. As shown in Figure 2, nutritive blood flow was detrimentally affected in type I diabetic mice (n=5) compared to controls (n=4). In stark contrast, acute administration (1 hour prior to observation) of C-peptide (n=5) dramatically reversed these adverse responses. These results are very exciting. These data are our first indication that an acute administration of C-peptide improves the vascular function of type I diabetes. The mechanisms by which the treatment of C-peptide is vasoprotective remains unknown. However, we believe that C-peptide enhances NADPH synthesis which, in turn, restores the function of endogenous vasoprotective systems.

NADPH is Elevated in Type I Diabetes by Acute C-peptide Administration

The design of the next series of studies was similar to the previous set of experiments, except they were intended to determine the effect of type I diabetes on NADPH bioavailability. The results from these experiments (Figure 3) are the first known to describe an improvement in NADPH bioavailability by C-peptide during type I diabetes. NADPH levels were measured in mice (n=5) using a single-extract spectrophotometric assay.⁹⁴ The induction of type I diabetes resulted in a tremendous reduction in NADPH bioavailability that was subsequently enhanced with an acute treatment of C-peptide (1 hour prior to observation). Although these studies confirm the depletion of NADPH in the type I diabetic state and its reversal by C-peptide, they fail to clarify whether alterations to NADPH are a result of changes in its synthesis or its consumption. We suspect that the restoration of NADPH was a result of augmented glucose-6-phosphate dehydrogenase (G6PD) protein expression with type I diabetes. These data are the first to suggest that an acute treatment of C-peptide restores NADPH levels during type I diabetes.

Comment [D4]: Selling language

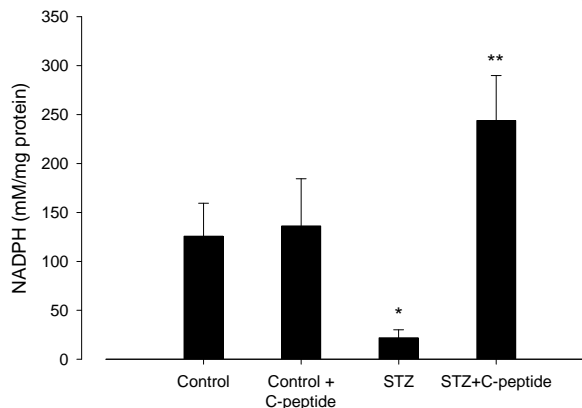


Figure 3. Reductions to NADPH in streptozotocin-induced diabetic mice (STZ) were reversed by C-peptide. Data are expressed as mean±SEM and normalized to protein content. * $p=0.018$ compared to Controls; ** $p=0.004$ compared to STZ

HO Protein Expression is Augmented by Cobalt Protoporphyrin in Parenchymal and Vascular Cells

Although whole organ microsomal preparations reflect the response of an organ to various stressors, they do not provide detailed information regarding the localized cellular responses. As such, we propose to supplement our whole organ assessments with a more comprehensive approach involving cell fractionation. Using a previously described differential centrifugation technique,^{97,98} we were able to separate hepatic parenchymal cells (P) from vascular cells (V). To confirm that our cell fractionation will provide us with an adequate yield for sensitive determination of cell population differences, we induced HO-1 with cobalt protoporphyrin (CoPP; 15 mg/kg, i.p.) and measured HO-1 protein content via immunoblotting 24 hours later (Figure 6). We demonstrated that CoPP induced HO-1 protein expression in both hepatic parenchymal and vascular cells, with a far greater induction observed in vascular cells. These results indicate that our technique of cell fractionation provides an adequate yield for determining differences in hepatocellular responses.

Comment [D5]: Illustrates that proposed methods have already been successfully carried out.