

BACKGROUND AND SIGNIFICANCE

Background

▪ Function of the ELS

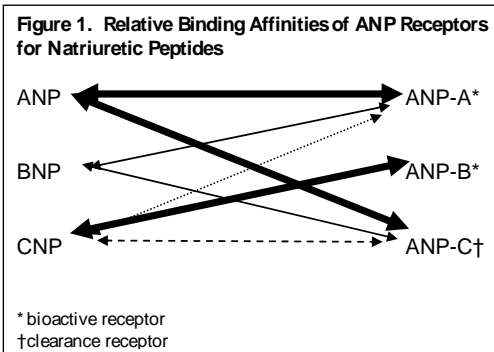
Although the ELS was once thought to represent a vestigial organ of embryogenesis, it is now known to have an essential role in inner ear homeostasis. The systemic administration of hypertonic agents, such as glycerol, has been shown to provoke a transient improvement in hearing in patients with the endolymphatic hydrops associated with Meniere's disease through mechanisms that were initially unclear.⁸ More recent research in animals has demonstrated morphologic changes in the endolymphatic sac associated with the systemic administration of glycerol. These changes include increased metabolic activity, with the secretion of osmotically active glycoproteins into the lumen of the sac and subsequent lysis and breakdown of these substances.⁹ It has thus been suggested that the ELS influences fluid homeostasis in the endolymph system through the modulation of the osmotic milieu of this space, with the secretion of osmotically active substances into its lumen.¹⁰ The exact mechanism through which the ELS is able to regulate this system is unknown, but it is postulated to involve a locally effective paracrine system, possibly involving the cochlear duct or stria vascularis. Dysfunction of the ELS or breakdown of this system is felt to result in the endolymphatic hydrops of Meniere's disease.¹

▪ Atrial Natriuretic Peptides (ANPs) and Their Receptors

Recent studies have shown that atrial natriuretic peptides (ANPs) and ANP-like receptors exist in the inner ear. Their involvement with fluid homeostasis in other organ systems would indicate that they could be involved in normal inner ear fluid regulation. Our current understanding of natriuretic peptides, their specific receptors, and their natriuretic, diuretic, and vasodilator effects are the result of numerous studies already performed on cardiac and renal tissue. ANP is a cardiac hormone with potent natriuretic, diuretic, and vasodilating effects that was first discovered and isolated from mammalian atria in the early 1980s.^{2,3} Subsequently, two additional neuropeptides with similar activity have been described: brain natriuretic peptide (BNP)¹¹ and C-type natriuretic peptide (CNP).¹² Myocardocytes appear to be the major site of synthesis and secretion for ANP; however, immunohistochemical activity of ANP has been observed in a variety of extracardiac tissues, such as the brain, kidney, adrenal medulla, salivary glands, ciliary process of the eye, and the anterior pituitary gland.¹³⁻¹⁷ BNP and CNP, first isolated from porcine brain tissue, have also been located in other sites, including the heart (BNP),¹⁸ kidney (CNP),¹⁹ and gastrointestinal tract (CNP).¹⁹

Three types of natriuretic peptide receptors have been identified and characterized. Two of these, ANP type A (ANP-A) and ANP type B (ANP-B), represent the bioactive receptors.^{20,21} They consist of an extracellular domain for natriuretic peptide binding, a single transmembrane domain, a single ATP-binding domain, and a guanylyl cyclase moiety.²² By activating guanylate cyclase, intracellular cyclic GMP concentrations increase and serve as a second messenger at the cellular level in various target tissues.²³ Recently, a third receptor, known as the C-type receptor (ANP-C), has been identified.²⁴ ANP-C, which is not coupled to guanylate cyclase, is thought to be biologically silent and serve as a specific clearance binding site for natriuretic peptides.²⁵ It has been shown in cardiovascular studies that ANP-C degrades ANP.⁶

Natriuretic peptides (ANP, BNP, CNP) have been shown to exhibit different binding affinities to the three receptor subtypes.²⁶ ANP-A receptors preferentially bind ANP over BNP, with little reactivity for CNP. ANP-B receptors appear to be highly selective for CNP, with an affinity that is 50- to 500-fold higher than for ANP or BNP.²⁷ ANP-C receptors bind all three natriuretic peptides, but with a rank order of ANP>BNP>CNP (See Figure 1).



Comment [D1]: Illustrated with flow chart.

▪ Role of ANPs and ANP Receptors in the Inner Ear

Based on the knowledge that natriuretic peptides are located within extracardiac tissues known to play a role in the maintenance of fluid/electrolyte homeostasis (i.e., ciliary process of the eye, choroid plexus of the cerebral ventricles), it has been postulated that they may also be present in the inner ear and have a similar activity. Lamprecht et al.⁵ and Meyer zum Gottesberge et al.²⁸ demonstrated the presence and localization of ANP receptors within the inner ear of the guinea pig. Using autoradiographic techniques with radiolabeled ANP/CDD (atrial natriuretic peptide/cardioidilantin), receptors were localized to the stria vascularis, the organ of Corti, spiral ganglion cells, and the epithelial layer of the pars rugosa of the ELS.²⁸ In contrast, a separate study by Koch et al. localized ANP receptors only to the stria vascularis, with nonspecific binding within the vestibular organ and ELS.²⁹ Recently, CNP and ANP-B receptor mRNAs were detected in the rat inner ear.³⁰ Since CNP is the major natriuretic peptide in human cerebrospinal fluid,³¹ it may play a more significant role than ANP within the inner ear. A study by Rachel et al. has shown that infusion of ANP directly into the inner ear of Guinea pigs participates in the regulation of vestibular blood flow.³² A more recent study even suggests that epithelial cells of the ELS contain an endogenous hormone, tentatively named saccin, that exerts a strong natriuresis.³³ In 1997, Krause et al. identified the presence of ANP-A and ANP-B receptor subtypes within the inner ear tissue obtained from guinea pigs using reverse transcription PCR.³⁴ They also identified a new guanylyl-cyclase receptor, GC-C, which is normally present in the intestines. Interestingly, their PCR analysis failed to demonstrate the presence of any clearance receptors (ANP-C).

These studies clearly suggest that natriuretic peptides, particularly ANP and CNP, may be involved in the control of fluid and electrolyte homeostasis within the inner ear. However, of these various studies involving the inner ear, none have been performed on human tissue. In addition, the detection of ANP receptors was studied only indirectly, using antibodies against the peptides, rather than directly, using antibodies against any of the three specific receptor subtypes. In a preliminary study (described below), we were able to demonstrate the presence of ANP receptors, with a predominance of ANP-B receptors, in human ELS specimens using histochemical techniques. These findings imply that CNP would be an effective peptide within the human ELS for fluid regulation since its binding affinity is virtually exclusive for the ANP-B receptor. This is consistent with what is known about CNP, which is the major natriuretic peptide in human cerebrospinal fluid^{12,31} and has been shown to stimulate excretion of sodium and water from the kidney.³¹ The identity of ANP-B was then confirmed by RT-PCR. To our knowledge, this was the first study to identify specific natriuretic peptide receptors within the human ELS. Our preliminary studies have also demonstrated physiologic response through receptor upregulation in the rat using ANPs, indicating a peptide/receptor interaction occurs in the inner ear.

Comment [D2]: This is one of the gaps the investigators will address.

Comment [D3]: Use of selling language.

We now plan to confirm the ANP/receptor interaction by administering receptor antagonists and by blocking the clearance receptor and then assessing the effect of ANP administration. We will also confirm that the ANP-B receptor can be upregulated in human ELS tissue after administration of the appropriate peptide by establishing an *in vitro* organ culture model (Aim 1). Confirmation of receptor upregulation in human ELS would be a key step in confirming our hypothesis. We also plan to develop an animal model of endolymphatic sac dysfunction that will confirm that ANP is involved in fluid homeostasis of the inner ear (Aim 2). Thus, results of these aims should support our hypothesis that fluid homeostasis in the endolymphatic system is regulated by a locally effective paracrine system in which ANPs control water and electrolyte activity through their specific receptors in the ELS. These results and future studies to assess the therapeutic potential of ANP administration will help us achieve our long-term objective of understanding the influence of ANPs on inner ear fluid homeostasis in an attempt to develop therapy that abrogates the symptoms of Meniere's disease.

Comment [D4]: These are all gaps that are now being addressed in the proposed project.

Significance

The proposed studies present a novel approach for understanding normal inner ear fluid homeostasis as well as for developing a treatment for the endolymphatic hydrops associated with Meniere's disease. If we are able to demonstrate that ANPs activate the ELS through ligand/receptor interaction, with the subsequent production of osmotically active proteoglycans, it would suggest that ANPs may be able to control water and electrolyte activity within the inner ear. Consequently, direct application or infusion of the peptide into the ELS could be used in patients suffering from

Comment [D5]: Selling language

Comment [D6]: Significance.

endolymphatic hydrops associated with Meniere's disease. The natriuretic effects of the infusions would reverse the fluid overload and alleviate symptoms and restore hearing in these patients. Therapeutic benefit similar to that currently achieved with glycerol could be attained; however, the benefit would be longer lasting because the treatment would emulate the paracrine system believed to be active in the inner ear.

This potential therapy is both practical and reasonable. The ELS is the one portion of the membranous labyrinth that is not surrounded by perilymph; thus, it can be easily approached through a mastoidectomy procedure, allowing direct application of the therapeutic peptide. Likewise, perfusion through the round window via gelfoam pledgets or an FDA-approved microcatheter inner ear delivery system (available through IntraEAR, Cupertino, CA), would offer other reasonable alternatives for delivery of therapy. Given what is already known about the function of natriuretic peptides, and our evidence of ANP receptors in the middle ear, we believe these studies have the potential for significant clinical contributions to the field of hearing loss associated with Meniere's disease.

Comment [D7]: Significance.

B. BACKGROUND AND SIGNIFICANCE

Type I diabetes is a chronic disease that has no definitive cure and is one of the leading causes of cardiovascular disease in the United States. Many individuals first become aware that they have type I diabetes when they develop one of its life-threatening complications. These studies target the role of C-peptide in augmenting NADPH synthesis and, ultimately, how re-establishing NADPH bioavailability will improve type I diabetic vascular dysfunction. Type I diabetics have an anomaly in redox regulation, a result of metabolic imbalances in the cytosol,¹⁵ which precedes chronic vascular disease and endothelial dysfunction. The explanation proposed for such imbalances rests with the interplay between defective endogenous protection and enhanced oxidative stress. This interplay eventually evolves into compromised protection as a consequence of competing NADPH requirements of enhanced glucose metabolism through elevated aldose reductase activity¹⁻³ and NADPH oxidase activity.⁴⁻⁶

NADPH is an important cofactor that plays a critical role in vascular function through the actions of numerous systems, including HO. The purpose of these studies is to understand the role of NADPH, C-peptide, and HO in the pathogenesis of type I diabetic vascular dysfunction. We hypothesize that **activation of NADPH synthesis with C-peptide will re-establish HO activity and directly improve vascular dysfunction in type I diabetes (Figure 1)**. Results from our studies should confirm the roles of NADPH, C-peptide, and HO in the development of cardiovascular disease during type I diabetes. As such, these basic science findings will be directly translatable into improved clinical outcomes for individuals suffering from type I diabetes.

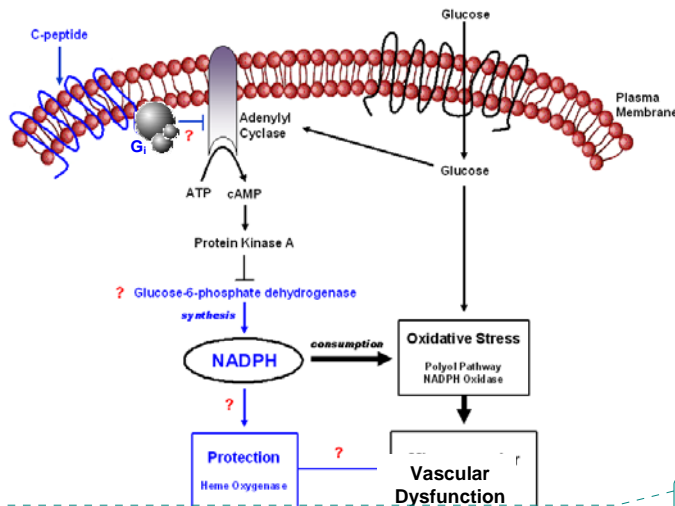


Figure 1. Interactions that are proposed to mediate the manifestation of vascular dysfunction in type I diabetes (black). Included are the pathways that we hypothesize play a role in the vasoprotection afforded the type I diabetic with restored NADPH (blue). The aims of our proposed studies will address the lack of NADPH synthesis and its role in the diminished preservation of vascular function in type I diabetes.

Comment [D8]: These type of illustrations are often used for complicated aims.

The Circulation in Type I Diabetes

Although the focus of previous studies of the diabetic vasculature was on oxidant production, we believe a gap in knowledge exists by ignoring the importance of compromised vasoprotection. We plan to address this lack of understanding by investigating the role of vascular dysfunction in type I diabetes and its relationship with compromised vasoprotection in vivo.

Comment [D9]: Selling language

Comment [D10]: Selling language.

Functional Adaptations Precede Morphologic Changes in the Diabetic Circulation

The integrity of the circulation is pivotal to the function of the tissue it serves. One key feature of the circulation throughout the pathogenesis of type I diabetes is the initiation of functional adaptations prior to any morphological changes.¹⁶ Several investigations have described these functional alterations, including increased vascular permeability, leukocyte accumulation, and diminished nutritive blood flow.¹⁷⁻²² Most functional alterations appear early in the pathogenesis of type I diabetes, with observations as early as a couple of weeks.^{23,24} However, one must take care when interpreting the mechanisms of these alterations since the function of the circulation during type I diabetes can be mediated by a number of factors, including disease duration,²⁵ degree of hyperglycemia,^{26,27} and insulin concentration.²⁸ Despite this, and the generally accepted view that diabetic vascular integrity is mediated by a myriad of common factors,²⁹ the mechanisms involved remain elusive.

SIGNIFICANCE

This project addresses one of the major exploration-mission risk areas: Impaired cognitive and/or physical performance due to motion sickness symptoms or treatments, especially during/after G-level changes. We plan to address the critical question: How effective are other drugs in providing fast relief in mission critical situations and does the drug have unacceptable side effects, particularly the short term effects on attention and/or cognitive function? The results of our study will lead to new countermeasures for SMS that are ready for space-based research trials.

This study also has excellent potential for translational and multidisciplinary research, with numerous applications to clinical scenarios worthy of further investigation, including studies of Sospite syndrome; diagnosis and treatment of motion sickness and other disorders stemming from the neurovestibular apparatus, such as vertigo or tinnitus, that affect preattentive and cognitive function; and investigation of ear dominance, a landmark finding demonstrated by our laboratory that may prove useful in screening for motion sickness susceptibility. Furthermore, the standardized measures of oculomotor function, postural stability, and cognitive performance established by this work will be crucial for determining the effectiveness and quantifying the side effects of potential therapies for vestibular disorders. Finally, the results of our study will advance scientific knowledge by developing a testable model that integrates our current knowledge of both rotary-induced motion sickness and SMS, which may ultimately help physicians treat patients with balance disorders related to inner ear dysfunction.

Our **approach** relies on immediate clinical testing, without any need for animal tests. This approach is reasonable, as it is currently not possible to assess degree of nausea, as opposed to vomiting as an endpoint, in animals (78), and it is necessary to provide adequate study data for subsequent space-based trials. The feasibility of this approach has been proven in our preliminary work, in which we have assessed the effects of rotation and various countermeasures in nearly 75 subjects to date. Our **methods** rely on strong interdisciplinary and multi-institutional components in order to provide a comprehensive study of the efficacy and side effects of drug countermeasures for SMS. As this is a university-based study, we will have an extensive pool of potential study subjects via the student and resident populations of the various colleges. The **innovative** aspects of this study include direct translation to the clinical setting and incorporation of a sensitive battery of tests to detect cognitive impairment and sensory gating. Dr. Dornhoffer is a member of the NSBRI Vestibular Adaptation Team and has successfully led the preliminary study funded by NSBRI, so he is well-suited to lead this research effort as **principal investigator**. Finally, a modern vestibular laboratory (the rotary chair is the only one of its kind in the state and was specially designed for our preliminary NSBRI-funded study) and collaborations with basic researchers, other institutions (NCTR and the University of Arkansas at Little Rock [UALR]), and consultants who are experts in their field (see letter of support from Dr. Lakshmi Putcha, Senior Pharmacologist at the Johnson Space Center) ensure the appropriate **environment** in which to carry out the proposed work.

Comment [D11]: Addressing each of the review criteria.

Significance

We believe that the next step in diabetes research must take advantage of innovative advances in reverse genetics and *in vivo* bioimaging to observe the sequential deterioration of the vasculature and the tissue it serves. The proposed studies are **significant** in that they will serve to enhance our understanding of the complex roles of NADPH, C-peptide, and HO during the onset and progression of cardiovascular disease in type I diabetes. By demonstrating the role of NADPH depletion and the capacity for C-peptide to enhance NADPH synthesis and HO activity, we will be able to establish a means to restore endogenous vasoprotection during type I diabetes. Our results, in combination with continued follow-up, will be immediately translatable to the clinic and will serve to uncover novel therapeutic interventions aimed at improving one of the most noteworthy factors of type I diabetic morbidity and mortality, vascular dysfunction.

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 [D12]: Addressing each of the review criteria in this section.

Comment [D13]: 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 [D14]: 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 [D15]: Selling language

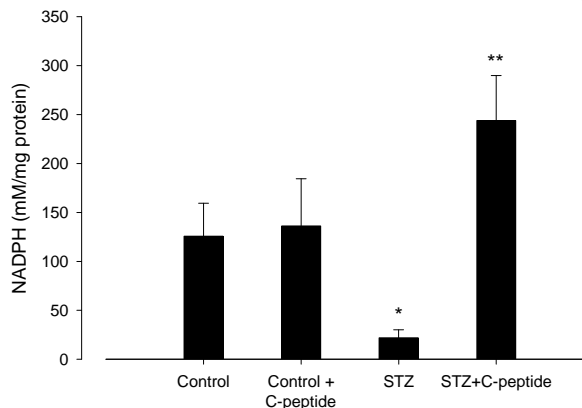


Figure 3. Reductions to NADPH in streptozotocin-induced diabetic mice (STZ) were reversed by C-peptide. Data are expressed as mean±SEM and normalized to protein content. * $p=0.018$ compared to Controls; ** $p=0.004$ compared to STZ

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 [D16]: Illustrates that proposed methods have already been successfully carried out.