pH (Low) Insertion Peptide (pHLIP) Targets Ischemic Myocardium

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The pH (low) insertion peptide (pHLIP) family enables targeting of cells in tissues with low extracellular pH. Here, we show that ischemic myocardium is targeted, potentially opening a new route to diagnosis and therapy. The experiments were performed using two murine ischemia models: regional ischemia induced by coronary artery occlusion and global low-flow ischemia in isolated hearts. In both models, pH-sensitive pHLIPs [wild type (WT) and Var7] or WT-pHLIP-coated liposomes bind ischemic but not normal regions of myocardium, whereas pH-insensitive, kVar7, and liposomes coated with PEG showed no preference. pHLIP did not influence either the mechanical or the electrical activity of ischemic myocardium. In contrast to other known targeting strategies, the pHLIP-based binding does not require severe myocardial damage. Thus, pHLIP could be used for delivery of pharmaceutical agents or imaging probes to the myocardial regions undergoing brief restrictions of blood supply that do not induce irreversible changes in myocytes.

Low-Flow Myocardial Ischemia in Isolated Hearts.

In another approach, we evaluated the ability of the pH peptides to selectively target the ischemic myocardium using a model of low-flow global

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ischemia in isolated hearts, where better control of coronary flow can be achieved. As shown by the increased mean intensity of fluorescence, the Alexa 488-Var7 was present in significantly higher amounts in the ventricles of the hearts subjected to low-flow global ischemia than in the normally perfused heart controls (Fig. 2A, C, and D). These results are consistent with the observation that Var7 is selectively bound to ischemic myocardium in situ.

To test whether pH sensitivity is important for pH-LIP binding to ischemic myocardium, we used the pH-insensitive kVar7 and found that the mean intensity of fluorescence for pH-insensitive Alexa 488-kVar7 was significantly less than for the pH-sensitive Alexa 488-Var7. In the normally perfused hearts, however, binding of labeled kVar7 does not differ from Alexa 488-Var7 (Fig. 2B and D), supporting the idea that pH sensitivity is the key for selective binding of pH-LIP to ischemic myocardium.

Using the model of low-flow myocardial ischemia in isolated hearts, we also tested the ability of WT-pHLIP–coated liposomes to target ischemic myocardium. Significantly higher values of the mean intensity of fluorescence reveal more WT-pHLIP liposomes in the ventricular myocardium of the hearts subjected to ischemia than in the normally perfused hearts (Fig. 2E, G, and H). Control liposomes coated with carboxyl-terminated PEG (designed to have the same density of negative charges on the liposome surface as in the case of the pHLIP coating) did not accumulate in ischemic myocardium (Fig. 2F and H), showing that the presence of pHLIP is the agent for targeting.

Histological examination revealed that the pattern of staining with Alexa 488-WT-pHLIP and WT-pHLIP–coated liposomes is different (Fig. 3). Alexa 488-WT-pHLIP stained plasma membranes (Fig. 3D), as had been shown previously in tumor models.
in vivo (9), whereas rhodamine-labeled lipids from the targeted liposomes appear to have reached intracellular organelles like mitochondria (Fig. 3B).

**Isolated Left Atrial Appendages.** We began experiments with isolated atrial appendages by examining the effect of pH on pHLIP binding to the myocardial tissues. With the pH-sensitive Alexa 488-Var7, accumulation of pHLIP (assessed by the mean intensity of fluorescence) was significantly higher when the pH was 6.5 compared with 7.4 (Fig. 4 A, D, and E). An equally bright fluorescence at low pH 6.5 was found with the pH-sensitive Alexa 488-WT (Fig. 4 C and E) but not with the pH-insensitive Alexa 488-kVar7 (Fig. 4 B and E). Thus, pH-sensitive pHLIP variants accumulate in the atrial myocardium to a greater extent at lower pH than at higher pH.

The main goal of this series of experiments was to assess the effects of the pH-sensitive Alexa 488-Var7 pHLIP on the electrical and mechanical activity of paced left atrial appendages in the normal and low pH environment. At pH 7.4, Var7 had no effect either on the force developed or on parameters of transmembrane potential (Fig. 5 A and B; Table S2). The decrease in pH by itself caused a significant reduction of the force developed without any significant change in the parameters of transmembrane potential (Fig. 5 C and D; Table S2). Administration of Var7 at low pH had no additional effect on the force developed or on the transmembrane potential (Fig. 5 D and E; SI Data, Table S2). Thus, exposure to the pH-sensitive Var7-pHLIP did not significantly influence the mechanical or electrical activity in preparations of left atrial appendages of mice.

**Discussion**

During the past few years, several techniques for passive and active imaging and drug delivery in diseased cardiac tissue have been developed (19, 20), including various types of nanoparticles for drug delivery such as liposomes, drug–polymer conjugates, polymeric micelles, dendrimers, nanoshells, and nucleic acid-based carriers (21). Passive drug delivery is based on the fact that the endothelial lining of the blood vessel wall becomes more permeable in infarcted areas so that large molecules and even relatively large nanoparticles (micelles and liposomes) can leave the vascular bed and accumulate inside the infarcted region (22–24). For active targeting, monoclonal antibodies against characteristic components of ischemic myocardium are the most frequently used vector molecules. Cellular components that are normally localized intracellularly but exposed extracellularly due to membrane disruptions in necrotic myocytes (e.g., intracellular cardiac myosin, phosphatidylserine, cytoskeleton) are used as biomarkers (19, 24–27). P-selectin expression, which reaches a peak of up-regulation at 4 h after myocardial infarction, has also been used as a biomarker (28). Analysis of the existing methods for selective drug delivery in diseased cardiac tissue shows that these techniques have a general distinctive feature: they need severe myocardial ischemia or infarction (leaky vasculature for passive delivery or cell apoptosis for active targeting) and are therefore useful only in tissue repair or regenerative strategies.

In contrast, we find in this study that pH-sensitive WT and Var7-pHLIPs and WT-pHLIP-coated liposomes selectively target ischemic myocardium in tissues that are less damaged than is required for the targeting strategies previously used. The results were obtained in two murine ischemia models: regional ischemia induced by coronary artery occlusion and global low-flow ischemia in isolated hearts. Also, we find that the controls, pH-insensitive kVar7 and liposomes coated with PEG, do not target the ischemic myocardium in our models. The selective binding of pH-sensitive pHLIPs in ischemic myocardium is consistent with the original concept of targeting of acidic tumors (5, 8, 9, 29), while it is not a trivial consequence thereof. Importantly, accumulation of pHLIP in ischemic myocardium did not influence its mechanical or electrical activity. This result is in accordance with our previous data showing that the interaction of pHLIP with cellular membranes at both neutral and low pH does not lead to membrane leakage (4, 30) or cellular toxicity (7) over a range of peptide concentrations. A distinctive feature of pHLIP-based targeting of ischemic myocardium is that pHLIP binding does not require severe myocardial damage and should work in the myocardial region undergoing brief restrictions of blood supply that do not induce irreversible changes in cardiomyocytes. Such...
The major problem with medical treatment for angina is that all antianginal drugs have cardiac and noncardiac side effects that significantly restrict their utilization (15). Most of the adverse effects result from the fact that the antianginal drugs affect tissues and organs throughout the body. The results of the present study suggest that pHLIP might be used as a carrier for delivering pharmaceutical agents specifically into ischemic regions during anginal attacks. Although there are no direct measurements of intracellular or extracellular pH in ischemic areas during angina attacks in humans, there is evidence that pH drops to values low enough to allow pHLIP binding to ischemic myocardium: a range of pH values can be estimated indirectly using the fact that angina episodes are sensed as a chest pain, which results from the activation of cardiac sensory neurons by acidosis (31). It has been shown that chest pain caused by myocardial ischemia is mediated by cardiac sympathetic (C-fiber) afferents (32). Specifically, the acid-sensing ion channel 3 (ASIC3), which is expressed at extremely high levels in sensory neurons innervating the heart, functions as a pH sensor (33, 34). The activation threshold for the channel is about 7.0 and the half-activation value is between 6.7 and 6.6 (33–36). Thus, sensing chest pain during angina episode implies pH values within the pHLIP insertion range, which allows us to hypothesize that pHLIP can effectively bind and accumulate in the region of restricted blood flow. Because a decrease in pH occurs very rapidly (within a few minutes) after the onset of ischemia, we suggest that pHLIP might quickly deliver antianginal agents to ischemic regions, decrease the oxygen supply/demand ratio and therefore cell damage, and manage the anginal attack avoiding the multiple side effects of antianginal drugs. Because pHLIP can deliver polar cell-impermeable molecules intracellularly, a number of new therapeutic compounds might be formulated. Moreover, pHLIP-coated liposomes might be used to deliver a mixture of pharmaceuticals to the risk zone. Thus, we assume that pHLIP technology might have a significant impact on the treatment of chronic angina.

pHLIP could deliver therapeutic agents, imaging agents, or both at the same time, acting as a theranostic. The most straightforward cardiac application of pHLIP could be for imaging ischemic regions in myocardium, and might be especially important for the diagnosis of myocardial ischemia in pediatric patients. The problem with current tests is that the ST-segment elevation in ECG used as a sign of myocardial ischemia in adults is often misleading in the young (37), whereas radiography may reveal significant anatomical abnormalities conducive to myocardial ischemia but not myocardial ischemia per se. Obviously, it would be advantageous to have an agent visualizing ischemic myocardium at the stage of basic X-ray examination of children. To diagnose myocardial ischemia in children, exercise or pharmacologic cardiac stress is induced. We expect that pHLIP will target the acidic myocardium during the acute ischemia associated with cardiac stress and deliver contrast agents to any ischemic region, opening new opportunities in use of existing imaging modalities for the diagnosis of myocardial ischemia in children.

In conclusion, the results of our study demonstrate that pHLIPs or pHLIP-coated liposomes target ischemic but not normal regions of myocardium. pHLIP accumulation does not influence either the mechanical or the electrical activity of ischemic myocardium. Importantly, pHLIP recognizes ischemic tissue due to a drop in extracellular pH. Therefore, pHLIP can deliver pharmaceutical agents or imaging probes to the myocardial region undergoing brief restrictions of blood supply, which do not induce irreversible changes in myocytes. This is an important advantage that distinguishes pHLIP-based technologies from other existing methods of ischemic myocardium targeting.

Materials and Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (38) and the rules of the Columbia University Institutional Animal Care and Use Committee. The experimental techniques were generally similar to those described by us previously (39, 40).

Constructs Used in Study. The goal of our study was to evaluate ability of fluorescently labeled pHLIPs and pHLIP-coated liposomes to target ischemic myocardium. Several pHLIP variants were used in the study as follows: WT, ACEQNPY WARYADWLFTTPLLLDLALL VDADEGT; Var7, ACEQNPY WAR YEWLPFETTLLEEL; and kVar7, ACEQNPY WARYKLWLPFTKLLEKL (underlining indicates transmembrane domain in peptides).

WT is a well-characterized pHLIP peptide that targets acidic tissue by virtue of the titration and neutralization of the carboxyl groups in the TM region. Var7 is a recently introduced truncated version of WT, which has a higher pK of insertion (substituting Glu for Asp in the TM) and much faster blood clearance. It was designed for PET-single-photon emission tomography imaging. kVar7 is a pH-insensitive peptide that does not target acidic tissue, because the protonatable Glu residues are replaced by positively charged Lys residues. Each peptide was conjugated with the Alexa 488 fluorescent dye. Additionally, we tested WT-pHLIP-coated and PEG-coated liposomes containing fluorescent rhodamine-PE lipids. The following liposome formulations (mol %) were investigated for targeting of ischemic myocardium: WT-pHLIP-coated liposomes: 90% DOPC, 5% Rho-PE, 5% pHLIP-PE; and PEG-coated liposomes: 90% DOPC, 5% Rho-PE, 5% DSPE-PEG2000-COOH.

The experiments with pHLIP peptides and liposomes were carried out using two murine ischemia models: regional ischemia induced by coronary artery occlusion and global low-flow ischemia in isolated hearts.

Preparation of Constructs. pHLIP peptides were prepared by large-scale solid-phase peptide synthesis using 9-fluorenlymethyloxycarbonyl (fmooc) chemistry at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale by James Elliott (Yale University, New Haven, CT). The detailed description of peptide conjugations with fluorescent dyes and lipids can be found in SI Data. Liposomes of two different compositions coated with pHLIP (5 mol % pHLIP-PE, 5% Rho PE, 90% DOPC) and control liposomes coated with PEG (5% DSPE-PEG2000-COOH, 5% Rho-PE, 90% DOPC) were prepared by extrusion; the details are presented in SI Data.

Local Myocardial Ischemia in Situ. Male C57B mice weighing 23–32 g were anesthetized with sodium pentobarbital (50–70 mg/kg, i.p.; added per need in the course of experiment) and artificially ventilated at a rate of 120 pulse/min with a tidal volume of 0.5 mL (Harvard Apparatus Respirator; model
707. Mice received heparin (1,000 mg/kg, i.p.) and a snare around the left coronary artery was periodically tightened (for 10 min) and reperfused (for 5 s) to imitate an obstructed coronary flow. Tested pHlIP variants (80 μM in 100 μL of PBS) were injected into the femoral vein 3 min before the first occlusion. At the end of the protocol, the heart was excised with the left coronary artery still occluded, the aorta was cannulated, and 1% Evans Blue was injected into the aorta to delineate the area at risk. Frozen ventricles were sectioned and fluorescent or white light images were taken with Nikon SMZ2000 UV dissection microscope. The contrast of fluorescence was measured by comparison of the mean intensities of fluorescence in the area at risk and in the intact myocardium.

**Low-Flow Myocardial Ischemia in Isolated Hearts.** Mouse hearts were excised and placed into a cold (4 °C) physiologic solution of the following composition (in mmol/L): 150 NaCl, 4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5.5 dextrose, and 10 Hepes equilibrated with 100% oxygen (pH 7.4) and promptly mounted for retrograde Langendorff perfusion with the same solution at 37 °C and constant flow rate of 4 ml/min while being continuously paced at a cycle length of 200 ms. Global low-flow ischemia was induced by reducing perfusion rate to 0.2 ml/min during 15 min. pHlIP or liposomes coated with pHlIP in concentration of 1 μM were administered during perfusion at a slow rate. SI Data contain more details of the experimental procedures.

**Isolated Preparations of Left Atrial Appendages.** Preparations of left atrial appendages were mounted epicardial surface up in a chamber perfused with the same physiologic solution as used for experiments with isolated Langendorff perfused hearts. Preparations were paced at a cycle length of 250 ms; tension transducer and conventional microelectrode techniques were used to register isometric developed force and transmembrane potential. Effects of 30-min exposure to 10 μM pHlIP were studied at normal and low pH (7.4 and 6.5, respectively). Further details of experimental techniques can be found in SI Data.

**Statistical Analysis.** The data are presented as mean ± SEM. One- or two-way analysis of the variance for repeated measures were used for statistical analysis, and Bonferroni test was used for post hoc comparisons when appropriate.

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