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Research Paper

Molecular transporters for peptides: delivery of a cardioprotective εPKC agonist peptide into cells and intact ischemic heart using a transport system, R_7

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Abstract

Background: Recently, we reported a novel oligoguanidine transporter system, polyarginine (R_7), which, when conjugated to spectroscopic probes (e.g., fluorescein) and drugs (e.g., cyclosporin A), results in highly water-soluble conjugates that rapidly enter cells and tissues. We report herein the preparation of the first R_7 peptide conjugates and a study of their cellular and organ uptake and functional activity. The octapeptide ψεRACK was selected for this study as it is known to exhibit selective ε protein kinase C isozyme agonist activity and to reduce ischemia-induced damage in cardiomyocytes. However, ψεRACK is not cell-permeable.

Results: Here we show that an R_7-ψεRACK conjugate readily enters cardiomyocytes, significantly outperforming ψεRACK conjugates of the transporters derived from HIV Tat and from Antennapedia. Moreover, R_7-ψεRACK conjugate reduced ischemic damage when delivered into intact hearts either prior to or after the ischemic insult.

Conclusions: Our data suggest that R_7 converts a peptide lead into a potential therapeutic agent for the ischemic heart.

Keywords: Cardiomyocyte; Molecular transporter; Polyarginine; ψεRACK; R_7; εPKC

1. Introduction

Many drugs, drug candidates, and probe molecules fail because of cellular uptake problems. To circumvent these problems, the physical properties of the agent must be optimized to achieve acceptable levels of passive entry into cells, a process often requiring the synthesis and evaluation of numerous analogues. An emerging and highly effective alternative strategy involves conjugation of the agent to a molecular transporter allowing agents with a wider range of physical properties to enter cells [1]. Peptides derived from HIV Tat [2,3] and from Antennapedia [4] have been used as transporters of otherwise difficult to deliver agents. More recently, we have shown that short oligomers of arginine can be used to enable or enhance uptake of agents into cells that do not enter or do so only poorly in unconjugated form [5]. We have also shown that these transporters enable uptake into skin [6]. Peptides represent a significant and emerging class of therapeutic candidates that often suffer from cellular uptake problems [7]. Here, we describe how a cell-impermeable peptide (ψRACK) that regulates protein–protein interactions involving protein kinase C (PKC) can be delivered into cells and intact heart with retention of biological activity.

PKC comprises a family of signal-transducing serine/threonine kinases. Activation of PKC is associated with translocation of each PKC isozyme to different subcellular sites. This isozyme-specific translocation is mediated, at least in part, by interaction with isozyme-specific anchoring proteins termed RACKs (receptors for activated C-kinase) [8,9]. Because binding of the activated PKC isozyme to its respective RACK determines the function of each isozyme, potentiation of binding to RACK should selec-

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tively increase the cellular activity of the corresponding isozyme. Using rational design, we demonstrated that short PKC-derived peptides that mimic RACKs (termed pseudo-RACK, or ψRACK) induce translocation of their corresponding isozymes, resulting in isozyme-selective translocation and activation of PKC [18–20]. However, this peptide, corresponding to the pseudo-RACK sequence of εPKC, or ψεRACK (HDAPIGYD, εPKC_{85-92}), is incapable of entering into cells [17]. As a first attempt at cellular delivery, the peptide was conjugated to the delivery peptide, penetratin, derived from the *Drosophila* Antennapedia homeodomain protein (RQIKIWFQNRRMKWKK) [22]. The peptides were coupled to the carrier through a reducible cysteine–cysteine disulfide bond, to enable release of ψεRACK (cargo) from the transporter after uptake.

Using this delivery conjugate of ψεRACK, we previously investigated the role of εPKC in the process of ischemic preconditioning. Ischemic preconditioning is a natural biological phenomenon that protects cells from ischemic injury, first described by Murry et al. in 1986 [25]. In their study, they found that hearts that were 'preconditioned' with short bouts of ischemia were more resistant to cell death and functional loss when subjected to a subsequent prolonged ischemic episode. Since their initial discovery, ischemic preconditioning has been a central focus of cardiovascular research. Although many advances towards determining worthy drug targets to mimic a preconditioning effect have been made, very few good candidates have been identified. PKC has been shown by many groups to be involved in preconditioning. However, due to side effects of isozyme non-selective PKC inhibitors and activators, PKC has not been a viable drug target. Upon development of our isozyme-selective PKC activators and inhibitor peptides conjugated to the Antennapedia delivery system, we previously determined that εPKC activation mediates cardioprotection from ischemic damage [16,22]. Treatment of the cells with conjugate (500 nM) prior to and throughout the experiment resulted in a 70% reduction in damage of isolated adult cardiac myocytes during simulated ischemia [19]. Moreover, delivery of ψεRACK as a transgene product provided similar protection from ischemic injury to the intact heart of transgenic mice [19].

Our results suggest that if sufficient quantities of ψεRACK could be delivered into the intact heart, it would have potential as a therapeutic agent. We therefore set out to identify an effective transporter for the ψεRACK peptide. We compared delivery via R_7 or r_7 [5] to Tat and Antennapedia systems. Delivery was tested both in isolated adult rat myocytes and in intact heart, using retrograde perfusion through the coronary arteries and measuring the increased resistance to ischemic damage as an indirect measure of intracellular delivery. We found that delivery of ψεRACK conjugated to R_7 is the most effective system for reducing cardiac damage by ischemia. These results suggest that the R_7 could be more generally used to deliver peptides for fundamental mechanistic studies as well as for therapeutic applications.

2. Results

2.1. Protection from ischemia in isolated cardiomyocytes by ψεRACK delivered by R_7 and Tat- and Antennapedia-derived peptides

Using releasable disulfide conjugates of transporter peptides (as their corresponding trifluoroacetate salts) and ψεRACK, we determined the relative efficacy of R_7 delivery peptide against the Antennapedia- and Tat-derived peptides (Ant-ψεR and Tat-ψεR, respectively; see Table 1 for peptides' composition). In a previous study, the ψεRACK-C-SS-C-Antennapedia conjugate at a concentration of 500 nM was applied to isolated cardiomyocytes for 10 min prior to simulated ischemia and remained throughout the experiment [19]. These conditions resulted in optimal protection yielding 16 ± 5% cell damage, which constitutes a 70% protection as compared with cells subjected to ischemia in the absence of this peptide. The effect of ischemia was assessed by increased membrane fragility as indicated by an increase in trypan blue uptake. In previous studies we demonstrated that this assay directly corresponded to damage assessed by leakage of intracellular enzymes in the medium, staining with propidium iodide, and changes in cell shape [21]. To compare the relative effectiveness of the three delivery systems, we elected to use the conjugates under sub-optimal conditions, applying peptide conjugate at a lower concentration (100 nM) and only during the 10-min incubation prior to the ischemic event. Under these conditions, cell-permeable Antennapedia- and Tat-peptide conjugates of ψεRACK (Ant-ψεR and Tat-ψεR) provided 30% and 25% protection

Table 1
Peptides used in this study

Control peptide conjugates
(Ant-ψεR) (ψεRACK-C-SS-C-Antennapedia)
HO_2C-DYGIPADHC-SS-CRQIKIWFQNRRMKWKK-$CONH_2$
(Tat-ψεR) (ψεRACK-C-SS-C-HIV-Tat)
HO_2C-DYGIPADHC-SS-CRKKRRQRRR-$CONH_2$
(K7-ψεR) (ψεRACK-C-SS-C-Lys_7)
HO_2C-DYGIPADHC-SS-CK_7-$CONH_2$
(NR 1-R_7) (ψεRACK-Arg_7)
NH_2-R_7-DYGIPADHC-CO_2H
(NR 2-R_7) (ψεRACK-aca-Arg_7)
NH_2-R_7-aca-DYGIPADHC-CO_2H
(R_7 alone) (Arg_7)
(R_7-Scr-ψεR) (scrambled ψεRACK-C-SS-C-Arg_7)
HO_2C-IGADHYDPC-SS-CR_7-$CONH_2$
Delivery conjugates
(R_7-ψεR) (ψεRACK-C-SS-C-Arg_7)
HO_2C-DYGIPADHC-SS-CR_7-$CONH_2$
(r_7-ψεR) (ψεRACK-C-SS-C-D-Arg_7)
HO_2C-DYGIPADHC-SS-Cr_7-$CONH_2$

(29 ± 4% and 31 ± 7%, respectively, vs. 39 ± 3% cell damage in the absence of any peptide, $P < 0.05$; Fig. 1). The R_7 conjugate of ψεRACK (R_7-ψεR) caused a 70% protection (there was 16 ± 6% cell damage, $P < 0.05$; Fig. 1), representing a 2.8-fold increase in protection from ischemia relative to the Antennapedia conjugate and a 2.3-fold increase relative to the Tat conjugate (Fig. 1). Control studies indicated that the R_7 peptide alone (R_7 alone) or the R_7 peptide conjugated to a scrambled ψεRACK peptide (R_7-Scr-ψεR) had virtually no effect (the percentage of ischemia-induced damaged cells was 45 ± 4% and 44 ± 3% respectively, vs. 39 ± 3% in the absence of any peptide, P = not significant; ns). Therefore, the protection conferred by only 100 nM R_7 conjugate (R_7) when preincubated with the cells for 15 min prior to prolonged ischemia was similar to the protection provided by the Antennapedia delivery system under optimal conditions (500 nM peptide added 15 min prior to the ischemia as well as during the ischemia) in the previous study described above (16 ± 5% vs. 16 ± 6% cell damage, respectively; Fig. 1 and [19]). The present study demonstrates that the R_7 transporter system enables the efficient delivery of a therapeutically beneficial peptide that by itself could not enter cells. Furthermore, R_7 was a superior vehicle for peptide delivery as compared with the currently available Tat and Antennapedia delivery systems.

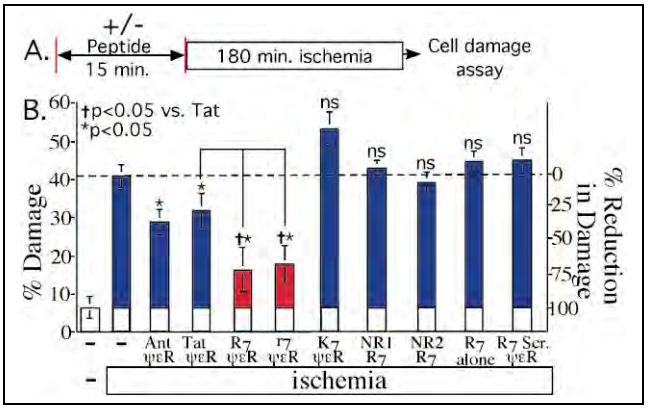

Fig. 1. Comparison of efficacy of peptide delivery systems. Carrier peptides are compared for their ability to confer protection of isolated cardiomyocytes from ischemic damage by delivery of ψεRACK. (A) Schematic of treatment protocol. (B) Protection from ischemic damage. Myocytes were treated with 100 nM of the following carrier peptide-ψεRACK conjugates prior to ischemia: ψεRACK-C-SS-C-Antennapedia (Ant-ψεR), ψεRACK-C-SS-C-Tat (Tat-ψεR), ψεRACK-C-SS-C-Arg_7 (R_7-ψεR), ψεRACK-C-SS-C-Arg_7 (r_7-ψεR), ψεRACK-C-SS-C-Lys_7 (K_7-ψεR), ψεRACK-Arg_7 (NR 1-R_7), ψεRACK-aca-Arg_7 (NR 2-R_7), Arg_7 (carrier peptide alone, R_7-alone), scrambled ψεRACK-C-SS-C-Arg_7 (R_7-Scr-ψεR). Cells were compared to no treatment (−) and no ischemia (normoxia) as controls. Percent of cell damage (left axis) is calculated as increase of percent cells exhibiting decreased membrane integrity. Data are also presented as percent reduction in damage (right axis) as compared to non-treated cells (second bar). Data are mean ± S.E.M. of three experiments.

2.2. *ψεRACK cannot be delivered by any cationic peptide*

To determine if the cationic nature of the R_7 peptide conjugate was responsible for its efficient delivery, a heptamer of L-lysine was used. The releasable polylysine disulfide conjugate of ψεRACK (K_7-ψεR) provided no protection from ischemia (53 ± 6% vs. 39 ± 7% cell damage, P = ns; Fig. 1) and, in fact, appeared to be deleterious. The failure of K_7-ψεR to elicit the desired response indicates, in accord with previous studies [5], that transport is a specific function of the guanidine head groups present in the oligo-arginine.

2.3. *Delivery of ψεRACK by the D-isomer of R_7 (r_7)*

We have previously shown that the D-isomer of the oligo-arginine sequence, denoted r_7, can also be transported across cellular membranes [5]. The activity of the D-isomer is important for biological applications because it provides greater resistance to protease degradation of the peptides. Therefore, the protection rendered by ψεRACK peptide conjugated to r_7 was determined. The r_7ψεRACK conjugate (r_7-ψεR) provided a 60% reduction in ischemia-induced cell damage (20 ± 5% vs. 39 ± 7%, $P < 0.05$; Fig. 1). These data show that the D-isomer of the transport peptide is almost equally effective as the L-isomer at facilitating peptide delivery.

2.4. *Delivery of ψεRACK by non-releasable constructs of R_7*

To determine the necessity of release of the ψεRACK peptide, two non-releasable (NR) constructs were synthesized and evaluated. NR 1-R_7 and NR 2-R_7 are direct conjugates of the delivery peptide to ψεRACK, the key difference between the two being an ε-aminocaproic acid spacer group present in NR 2-R_7. There was no significant reduction in ischemia-induced cell damage when either NR 1-R_7 or NR 2-R_7 were pre-incubated with the myocytes (39 ± 3% and 43 ± 1% vs. 39 ± 7%, P = ns, respectively; Fig. 1). These results indicate that, under the conditions employed here (100 nM peptide applied 10 min prior to the prolonged ischemia only), release of the parent peptide is essential for its biological activity

2.5. *Delivery of R_7-C-SS-C-ψεRACK to whole hearts, prior to ischemic insult*

The suitability of the ψεRACK as a cardioprotective agent for clinical use and the effectiveness of the R_7 transport system were next evaluated in a whole organ model. Using retrograde perfusion via the coronary arteries, ψεRACK-C-SS-C-R_7 (R_7-ψεR) was administered into rat heart over a period of 20 min prior to no-flow global ischemia (45 min). After ischemia, hearts were reperfused for 30 min and fractions were collected at 150-s intervals

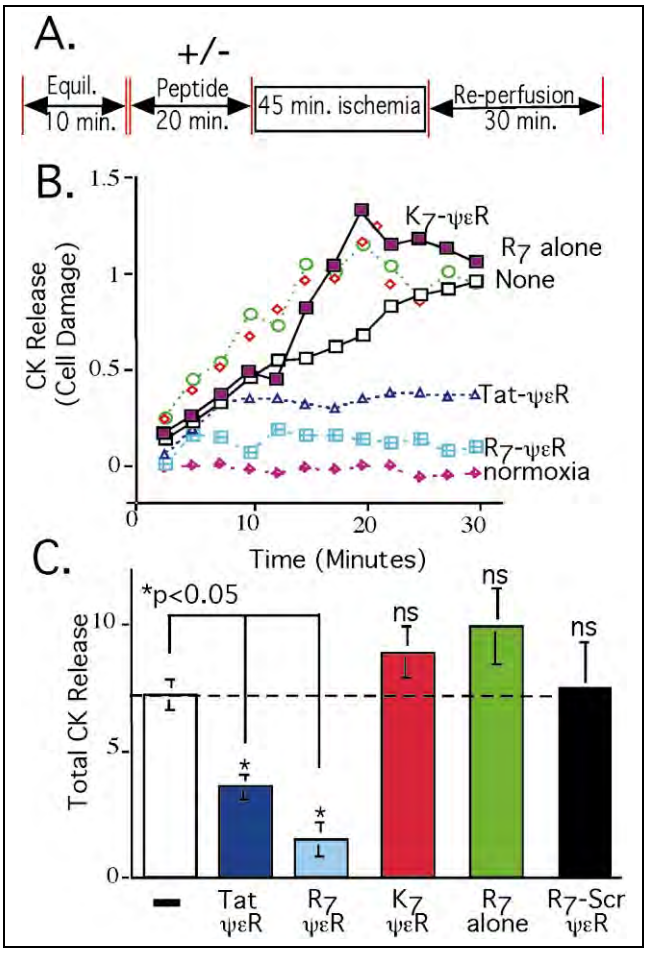

Fig. 2. Delivery of ψεRACK to whole hearts prior to an ischemic insult confers protection from ischemic damage. (A) Schematic of treatment protocol. Whole hearts were perfused with peptides 20 min prior to 45 min global ischemia. Hearts were then reperfused for 30 min. Ischemia-induced cell damage was determined by measuring the creatine phosphokinase enzyme present in the perfusate during reperfusion. Creatine phosphokinase is a cytosolic enzyme in cardiac myocytes and its presence in the perfusate is proportional to the number of cardiomyocytes damaged by the ischemic period. (B,C) Protection from ischemic damage. Whole hearts were treated with 500 nM of the following carrier peptide ψεRACK conjugates prior to ischemia: ψεRACK-C-SS-C-Tat (Tat-ψεR), ψεRACK-C-SS-C-Arg_7 (R_7-ψεR), ψεRACK-C-SS-C-Lys_7 (K_7-ψεR), Arg_7 (R_7 alone), scrambled ψεRACK-C-SS-C-Arg_7 (R_7-Scr-ψεR). Treated hearts were compared to no treatment and no ischemia (normoxia) as controls. Data are represented as cell damage as a function of time during reperfusion (B) and total cell damage during the reperfusion period (C) ($n = 4$, $^*P < 0.05$, ns = not significant).

during reperfusion (Fig. 2A). Cardiac damage was assessed by measuring release of the intracellular cardiac-specific enzyme creatine phosphokinase (CK) during the reperfusion period (Fig. 2B). Typically, ischemic events result in the release of large quantities of CK compared to a heart maintained under normoxic conditions. As seen in Fig. 2C, ψεRACK-C-SS-C-R_7 (R_7-ψεR) caused a 78% reduction in CK release measured during 30 min of reperfusion (1.5 ± 0.7 vs. 7.2 ± 0.6, $P < 0.05$). Under the same conditions, ψεRACK-C-SS-C-Tat caused a 50% reduction in CK release (3.6 ± 0.1 vs. 7.2 ± 0.6, $P < 0.05$). As with the isolated myocytes, there was no protection at any of the times tested with R_7 alone, scrambled ψεRACK-C-SS-C-R_7 or ψεRACK-C-SS-C-K_7 (9.9 ± 1.5, 7.5 ± 1.8, and 8.9 ± 1.0, P = ns, respectively; Fig. 2C). These data demonstrate that delivery of the ψεRACK peptide to whole organs prior to an ischemic event significantly reduces ischemia-induced cell damage. Furthermore, they show that the R_7 delivery peptide surpasses Tat peptide-mediated delivery not only in an isolated cell system, but also in whole organ delivery.

2.6. *Delivery of R_7-C-SS-C-ψεRACK to whole hearts after an ischemic insult*

Patients arriving *after* a cardiac ischemic event is a clinically common situation. Therefore, we determined whether R_7-ψεRACK confers protection from ischemia when applied *after* 45 min of global ischemia. Hearts were treated with R_7-ψεRACK for the first 20 min of the 60-min reperfusion period and CK release was measured to assess cardiac damage over time (Fig. 3A). Hearts treated with R_7-ψεRACK had less damage at all time points within 15 min after peptide delivery (Fig. 3B). There was a 48% reduction in CK release compared to untreated hearts (12 ± 2 vs. 21 ± 2, $P < 0.05$; Fig. 3C). Taken together, these data indicate that treatment of whole hearts with ψεRACK conjugated to R_7 confers pro-

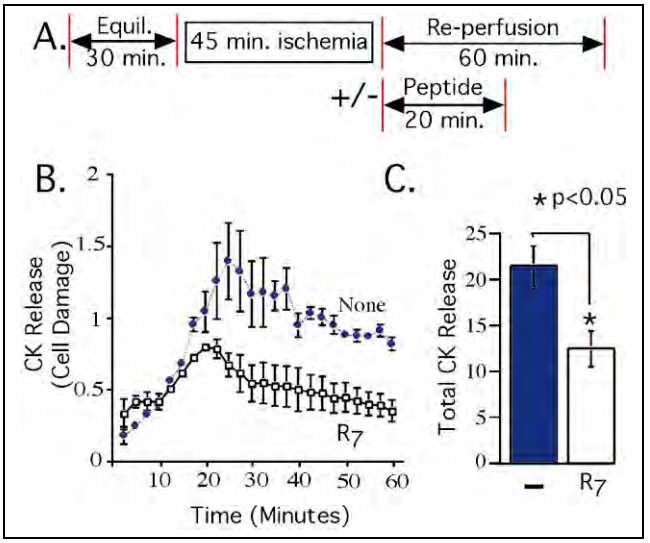

Fig. 3. Delivery of ψεRACK to whole hearts *after* an ischemic insult confers protection from ischemic damage. (A) Schematic of treatment protocol. Whole hearts undergo 45 min of global ischemia. Hearts are then perfused with or without peptide for 20 min followed by 40 min perfusion without peptide. Ischemia-induced cell damage determined by creatine phosphokinase activity in perfusate collected during reperfusion (as in Fig. 2). (B,C) Protection from ischemic damage. Whole hearts are treated with 500 nM of ψεRACK-C-SS-C-Arg_7 (R_7-ψεR) after an ischemic insult and compared to untreated hearts as a control. Data are represented as cell damage as a function of time during reperfusion (B) and total cell damage during the reperfusion period (C) ($n = 4$; $^*P < 0.05$, ns = not significant).

tection when applied both prior to and after an ischemic insult.

3. Discussion

In this study, we successfully transported a cell-impermeable octapeptide, ψεRACK, into isolated cardiac myocytes and into intact heart using the oligo-arginine (R_7) conjugated to a biologically active peptide (ψεRACK) through a simple Cys–Cys disulfide bond. ψεRACK delivery by R_7 (ψεRACK-C-SS-C-R_7) was demonstrated by the increased resistance of isolated cardiomyocytes and intact heart to damage induced by prolonged ischemia. Although it is possible that cardioprotection is promoted by a release of cardioprotective agents from a few cells that obtained ψεRACK-C-SS-C-R_7, our data are more consistent with the possibility that the 70% protection of the intact heart from ischemia indicates that at least 70% of the cardiac muscle received the therapeutic peptide. In that case, our data suggest that ψεRACK-C-SS-C-R_7 crossed the endothelial cells of the blood vessels and several muscle cells and fibroblasts to exert its full effect, demonstrating that R_7 can convert peptides that act intracellularly into viable drug candidates.

Of the four delivery peptides tested (Antennapedia-derived peptide, Tat-derived peptide and the R_7 and r_7), the R_7 and r_7 conjugates of ψεRACK (100 nM) conferred the highest levels of protection from ischemia-induced damage in isolated cardiac myocytes (70 and 60% reduction; Fig. 1). The Tat-derived conjugate (Tat) was equal to the Antennapedia conjugate in delivery of the ψεRACK activity, but was two-fold less effective than the R_7. The product of the non-reducible cross-linking of ψεRACK to R_7 (NR 1-R_7 and NR 2-R_7) was biologically inactive under the same assay conditions, indicating that the delivery system, in this case, must release ψεRACK in order for the latter to function biologically. ψεRACK conjugated to oligo-lysine (K_7-ψεR) was also inactive demonstrating that the guanidine head groups present in these transport peptides are required for transport activity. The apparent deleterious effect of the polylysine conjugate is most likely an artifact of the transport peptide's inherent toxicity.

Importantly, cardioprotection was conferred to whole hearts treated with ψεRACK conjugated to R_7 prior to ischemia (Fig. 2). These data suggest the potential use of ψεRACK-R_7 as a therapeutic agent in situations where the timing of an ischemic episode can be predicted. In many procedures such as open heart surgery, bypass surgery, and heart transplantation, this is indeed the case. Of even greater clinical importance, we found that delivery of ψεRACK using R_7 rendered the heart more resistant to ischemic and reperfusion damage when the peptide was applied *after* the ischemia and during the reperfusion stage (Fig. 3). These findings suggest a potential use for these peptides in even more common clinical settings, such as acute cardiac ischemia. Acute myocardial ischemia is responsible for up to 500 000 deaths per year in the USA alone and nearly 14 million worldwide. Because more than half of these deaths occur in the hospital, an improved therapeutic intervention such as the one described here could save many lives.

Studies in the last few years identified many peptides that interfere with protein–protein interaction inside cells (e.g., those that affect β-adrenergic G-protein signaling [10], Lyn function [11], protein kinase A signaling [12–15], and PKC signaling [23]). Traditionally, these peptides are not considered drug candidates; more often, they are used as leads for high throughput screening of non-peptide compounds or to provide a proof-of-principle for gene delivery approaches. Here, we show that R_7 can transport peptides through the blood vessels into an intact organ, demonstrating that such peptide conjugates could themselves be used as drugs. Moreover, such delivery of a peptide results in transient biological activity and therefore, unlike gene delivery, is completely reversible. Although the stability of the peptides in the blood could be a concern in general, the demonstrated effectiveness of the D-isomer (r_7) provides an effective solution to this problem. In addition, the ability to deliver the peptide nearby the target organ, such as the case for intra-coronary delivery, is likely to reduce the effects on other organs. Finally, peptide drug instability in the blood could be overcome, if sufficient delivery of the peptide conjugate occurs during its first pass through the organ. In fact, there might be an advantage if the peptides are not very stable in the blood, as this will reduce their effects on organs other than the heart. In summary, through the use of R_7, ψεRACK is converted into a potential therapeutic agent for the treatment of acute ischemic heart disease both before and after the ischemic event. If confirmed in humans, ψεRACK delivered by R_7 would provide an effective therapeutic agent for the treatment of ischemic heart disease.

4. Significance

The ability to enable or enhance the uptake of agents into cells or tissue can have major implications in the development of new drugs or in the resurrection of previously non-viable drug candidates. Peptides in particular are often limited as potential drug candidates due to inherently poor bioavailability and rapid degradation. The work described herein demonstrates that by ligation to an oligoguanidine transporter moiety, a peptide agonist of εPKC, unable by itself to penetrate cells, can be delivered across multiple layers of tissue and into cells and subsequently released to act at its intracellular target. This uptake phenomenon is a specific function of the guanidine head groups present in the transporter peptide and is not simply due to its cationic nature. Cleavage of a disulfide bond between the peptide transporter and peptide cargo

allows for intracellular release of the free peptide cargo and in vivo activity. This overall approach provides a general method for the in vivo delivery of bioactive agents including notably peptides that alone have limited potential as therapeutic agents.

In this specific study, transporter-enabled delivery of a peptide cargo through the coronary arteries demonstrates the opportunity provided by this cellular delivery method to introduce therapeutic agents designed to protect the myocardium from known ischemic episodes. Prophylactic treatment for patients at risk from ischemic heart disease, therapies for coronary artery bypass surgery and transplant preservation can now be realized given the capabilities of this delivery technology and the biological activity of these peptides. With the advent of this delivery technology, many new therapeutic agents become available.

5. Materials and methods

5.1. Peptides

Peptides were synthesized using a PE Biosystems model 433A Automated Peptide Synthesizer. The acid- or amide-linked resins, appropriately protected amino acids (arginine was protected as its 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Pbf) derivative, tryptophan as its *N*-*t*-butoxycarbonyl (*t*-BOC) derivative, and cysteine as its *S*-trityl (Trt) derivative) and synthesizer reagents were purchased from Bachem or Novabiochem. Peptides were prepared using the standard FastMoc protocol on either a 0.1 mmol or 0.25 mmol scale.

Non-cysteine-containing peptides were cleaved from resin supports using 95% trifluoroacetic acid (TFA):5% triisopropylsilane for 4 h. Peptides containing cysteine were cleaved using 85% TFA, 5% thioanisole, 2.5% triisopropylsilane, 2.5% phenol, 2.5% water, and 2.5% ethanedithiol for 4 h. In all cases, the final products were obtained in analytical purity (ESI-TOF MS and analytical HPLC) using a Varian Model HPLC system fitted with a Varian C18-packed reverse phase column.

5.2. Peptide conjugates

Disulfide conjugates were prepared by the following general procedure [24]: 2,2′-dithiobis(5-nitropyridine) (5 eq) was dissolved in a minimal amount of 3:1 acetic acid:water over a period of 30 min at room temperature. Once the reagent had mostly dissolved, HS-C-R_7-$CONH_2$ (1 eq) was added as a solution in 1 ml of 3:1 acetic acid:water. The orange solution slowly developed a bright yellow and was permitted to stir overnight at room temperature. The reaction was quenched by removal of the solvent in vacuo. The residue was reconstituted in an equal volume mixture of water and ethyl acetate. The layers were separated and the aqueous phase was extracted with ethyl acetate until no yellow color remained. The aqueous layer was concentrated in vacuo to provide the activated disulfide, *N*-Pys-SS-CR_7-$CONH_2$, which was used directly without further purification.

The *N*-Pys-SS-CR_7-$CONH_2$ in 2 ml of argon-degassed water was treated with HS-CHDAPIGYD-CO_2H (1 eq). Upon addition of the second peptide, a bright yellow color was immediately apparent. After stirring for 16 h at room temperature, the reaction was diluted with ethyl acetate. The phases were separated and the aqueous layer was extracted with ethyl acetate until no yellow color remained. The water was removed via lyophilization and the residue purified by RP-HPLC (C-18 column using a 5–50% acetonitrile:water gradient over 30 min) to furnish the desired peptide in typically $>60\%$ overall yield. The identity and purity of the individual conjugates was established using analytical RP-HPLC in conjunction with ESI-TOF MS. All conjugates employed in this study were used as their corresponding trifluoroacetate salts bearing a full complement of counterions.

5.3. Ischemia-reperfusion of isolated adult rat heart and creatine kinase assay

Ischemia of isolated adult cardiac myocytes and peptide treatments were carried out as previously described [19]. For whole organ ischemia, hearts from adult male Sprague–Dawley rats (250–300 g) were isolated and perfused on a Langendorff apparatus. Perfusion was performed at constant pressure of 85 mm Hg at 37°C using Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 3 mM $CaCl_2$ and 5 mM glucose (pH 7.4)). The perfusate was then oxygenated by continuous bubbling of 95% O_2/5% CO_2. After the initial l0-min equilibration period, hearts were perfused for 20 min with 500 nM of the indicated peptide conjugate in the buffer solution, followed by 45 min of no-flow simulated ischemia. Immediately following ischemia the hearts were subjected to 30 min of reperfusion. Samples of coronary venous outflow were collected every 2.5 min during the reperfusion period (total of 12 fractions). Creatine phosphokinase (CK) release was assayed using a calorimetric determination kit (Sigma) to measure the extent of cardiac injury. Where indicated, ψεRACK peptide conjugate was perfused into the heart only after the 45 min no-flow ischemia, for the first 20 min of reperfusion. CK release was monitored during this period and an additional 40 min of reperfusion, and assayed as above.

Acknowledgements

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