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Mini-Review

Comparing the effects of myristic acid- and TAT-conjugated peptides to their native counterparts targeting intracellular pathways mediating myocardial ischemia/reperfusion injury and neutrophil superoxide release

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ABSTRACT

Two commonly used modifications of native peptides to increase peptide permeability through the cell membrane to target intracellular substrates are myristic acid conjugated peptides (MYR) and transactivating conjugated peptides (TAT). However, there is limited literature comparing the effects of these two types of modified peptides in the same bioassay. The objective of this mini-review is to compare the effects of MYR- and TAT-conjugated peptides in two bioassays: 1) Myocardial ischemia-reperfusion injury (I/R) in isolated perfused rat hearts using a mitochondrial fission peptide inhibitor, P110; 2) N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP)-induced superoxide (SO) release from isolated rat neutrophils using a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase peptide assembly inhibitor, Nox2ds. MYR-conjugated P110 (1 μM) exerted sustained improvement in post-reperfused cardiac function combined with reduced infarct size compared to TAT-conjugated P110 (1 μM) or native P110 peptide (1 μM) in hearts subjected to I(30 min)/ R(90 min). Nox2ds data suggested that MYR-conjugated Nox2ds peptide exerted more potent effects and better permeability through the cell membrane to attenuate fMLP-induced SO release from rat neutrophils when compared to TAT-conjugated Nox2ds peptide. However, further metabolism studies are needed to determine peptide half-life between the MYR- and TAT-conjugated peptides to corroborate the pharmacodynamics studies.

KEYWORDS: myristic acid-conjugated peptide, TAT-conjugated peptide, neutrophils, superoxide release, mitochondrial fission, Nox2ds, P110, peptide permeability

INTRODUCTION

The bioavailability of molecular probes and drugs directed at intracellular targets are often limited by their cell membrane permeability. There is an increased need to further develop peptides that target intracellular proteins that control critical pathways under normal and pathologic conditions. In recent years, several methods have been developed to improve cellular accessibility of membrane-impermeant molecules. Currently, two of the most commonly used methods for improving cell permeability of peptides involve conjugation with a fatty acid moiety, myristic acid (MYR), and the addition of a transactivating peptide (TAT) also known as a cell-penetrating peptide (CPP) [1]. Through such a conjugation, the peptide has better access to its intracellular targets, which will increase the efficaciousness of the peptide and reduce the likelihood of adverse drug reactions associated with the agents. Alone, a peptide would normally enter the cell *via* a facilitated diffusion process

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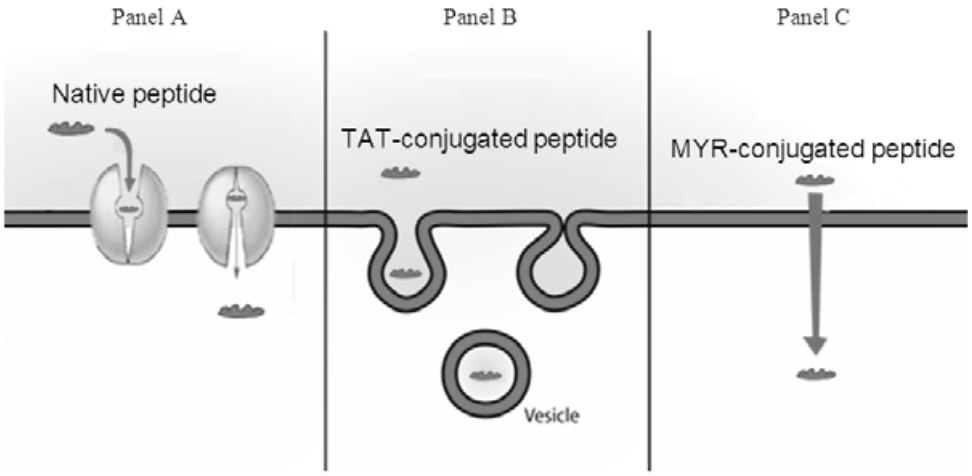

Figure 1. Mechanism of peptide entry through the plasma membrane for native peptide (Panel A), TAT-conjugated peptide (Panel B), and MYR-conjugated peptide (Panel C).

that requires a carrier protein (Figure 1, Panel A). With the addition of TAT or MYR, the peptide is able to cross the plasma membrane *via* endocytotic mechanisms or simple diffusion, respectively (Figure 1, Panels B and C).

The goal of this mini-review is to compare the efficaciousness of TAT-conjugated and MYR-conjugated peptides to their native counterparts in two bioassays: 1) Myocardial ischemia/reperfusion (I/R) injury in isolated perfused rat hearts; 2) Superoxide (SO) release from isolated rat neutrophils also known as polymorphonuclear leukocytes (PMNs).

MYR-conjugated peptides

The ability to increase peptide permeability through a cell *via* MYR conjugation was first described by O'Brian *et al*. [2]. They found that an MYR-conjugated protein kinase C (PKC) octapeptide inhibitor of classical isoforms (alpha, beta 1, beta 2, gamma) in the pseudosubstrate domain of PKC attenuated histone phosphorylation in murine fibrosarcoma cells at 5 μM. By contrast, neither MYR alone nor unconjugated PKC peptide could inhibit PKC-catalyzed histone phosphorylation at ten-fold higher concentrations. Thereafter, MYR-conjugation was further explored in studies by Eichholtz *et al.* [3] using an MYR nonapeptide of the pseudosubstrate domain of PKC (alpha and beta isoforms). They reported a similar trend in human fibroblasts, in which the nonmyristoylated form of the PKC pseudosubstrate peptide was less effective (at least two-fold) at inhibiting the phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) protein compared to the myristoylated form. More recently, Perkins *et al*. [4] showed that myristoylated selective PKC beta II (nonapeptide) and zeta (tredecapeptide) isoform inhibitors of the receptor for activated C kinase (RACK) binding and pseudosubstrate binding domains, respectively, attenuated PKC-mediated SO release in rat PMNs *via* phorbol 12-myristate 13-acetate (PMA), a diacylglycerol mimetic or N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP), a leukocyte chemotactic receptor agonist. This study showed that MYR conjugation improves accessibility towards its peptide targets (e.g. NADPH oxidase) in whole cells. In the aforementioned studies, the effective concentration of myristoylated peptide to inhibit biological activity was between 5-10 μM as opposed to MYR alone or native peptide in the same concentration range which do not show any significant effect. Collectively, the results from these studies suggest that MYR-conjugated peptides are more effective than their native peptide counterparts in accessing intracellular targets which manifest a greater efficacy.

MYR mechanism of action

The putative mechanism of action of myristoylated peptides is thought to involve the following:

First, the myristoylated moiety is thought to insert directly into the plasma lipid bilayer without the need for supplemental moieties for adherence to the surface of the membrane [5]. Second, the cargo sequence attached to the MYR is carried into the intracellular space by simple diffusion (Figure 1, Panel C). Third, once inside the intracellular space, the cargo sequence is thought to be released by a yet undefined cleavage mechanism, allowing the peptide (i.e. cargo sequence) to interact with its intended target [1]. Alternatively, MYR-conjugated peptides may be sequestered in the plasma membrane where they could readily interact with intracellular targets located at or near the plasma membrane (e.g. NADPH oxidase) [1].

TAT-conjugated peptides

The original TAT peptide was an 86 amino acid peptide derived from HIV-1 that Frankel *et al*. [6] reported could permeate the cell membrane. Thereafter, it was discovered that a nine amino acid basic domain sequence from residues 49-57 (RKKRRQRRR) of TAT was effective in facilitating cellular internalization. It was able to permeate the plasma membrane *via* an endocytotic mechanism involving the positive charges on the TAT-carrier protein and the negative charges existing on the lipid bilayer of the plasma membrane [7-10] (Figure 1, Panel B). Pepinsky *et al*. [7] conjugated the aforementioned TAT nonapeptide to a human papillomavirus (HPV) E2 repressor (a 121 amino acid transcription factor inhibitor) which was able to decrease HPV expression in human cervical epithelial cell lines. Later, Rey *et al*. [11] conjugated the TAT nonapeptide with the docking sequence of the Nox2 peptide (CSTRRRQQL), also a nonapeptide that inhibits Nox2 assembly. Nox2 is one of the seven isoforms of NADPH oxidase and is the primary source of SO release from PMNs. It is expressed in many cell types of the cardiovascular system that include endothelial cells, myocytes, platelets and fibroblasts [12]. These studies indicated that TAT nonapeptide conjugation could improve intracellular delivery of cargo peptides of varying sizes.

TAT mechanism of action

The putative mechanism of action of TAT-conjugated peptides has been proposed to occur *via* either an interaction between TAT and glycosaminoglycans (specifically heparin sulfate), or by complexing with the glycerol backbone of the lipid bilayer cell membrane. In both cases, this would lead to macropinocytosis of TAT into the cell. The ability of TAT to enter the cell will facilitate the delivery of the conjugated cargo peptide to gain intracellular access to regulate specific targets [7-10].

Myocardial I/R

Discovering a pharmacological tool that can attenuate clinical myocardial I/R injury has been a primary focus of both clinical and bench research throughout the world, especially in the United States where myocardial infarction (MI) is the leading cause of mortality [13]. The ability of agents to enter the cell effectively to mitigate myocardial- derived reactive oxygen species (ROS) is crucial to attenuate the deleterious effects of myocardial I/R injury. Induction of a myocardial I/R injury allows to test the effectiveness of MYR- or TAT-conjugated peptides. Myocardial I/R injury in animal models was first described by Jennings *et al*. [14], and has been further described more recently by Hausenloy and Yellon [15]. They describe factors that exacerbate myocardial I/R injury, which include the release of ROS during reperfusion after prolonged ischemia. A key source of myocardial I/R-induced ROS is Nox2, the principal NADPH oxidase isoform expressed in mitochondria, particularly in cardiovascular tissue [16]. The ROS release from mitochondria ultimately leads to cardiomyocyte dysfunction and hypershortening, along with mitochondrial fragmentation, a process known as fission [17]. Fragmented mitochondria can serve as a significant source of ROS overproduction and uncoupling of the electron transport chain.

Role of P110, a putative mitochondrial fission inhibiting peptide

Mitochondrial dysfunction is an important contributor to myocardial I/R injury. Reperfusion of blood to previously prolonged ischemic cardiac tissue leads to uncoupling of the mitochondrial electron transport chain. Excessive mitochondrial ROS is significant in heart tissue, since 30% of the heart volume is composed of mitochondria given the high ATP requirement in this organ [15]. This in turn leads to ROS release, further damaging the cardiac myocyte

and corresponding vasculature by the uncoupling of endothelial nitric oxide synthase (eNOS) activity causing further ROS production. Dysfunctional mitochondria will signal excessive mitochondrial fission mediated primarily by the interaction between dynamin related protein-1 (Drp1) and fission protein-1 (Fis1). The results of recent preclinical studies have suggested that slowing mitochondrial fission will minimize myocardial I/R injury [18, 19]. Disatnik *et al*. [18] used TAT-conjugated P110, a mitochondrial fission inhibiting heptapeptide (DLLPRGT) to block the binding between Drp1 and Fis1, to attenuate myocardial I/R injury [18] (Figure 2).

They reported a modest reduction in infarct size (33%) but the study did not include an assessment of cardiac function nor was the efficacy of TAT-conjugated P110 in reducing infarct size compared to the native peptide. Our lab sought to compare the effects of TAT-conjugated P110 to native and MYR-conjugated P110 in a similar *ex vivo* rat I(30 min)/R(90 min) model, on cardiac function and infarct size. In particular, we thought the comparison of TAT- *vs*. MYR-conjugated P110 would be of value, since our previous research showed that MYR-conjugated PKC-epsilon peptide inhibitor was effective in restoring post-reperfused cardiac function and reducing infarct size in *ex vivo* rat and *in vivo* porcine myocardial I/R models [21-23]. By contrast, the Zatta *et al*. [24] study did not show reduced infarct size with TAT-conjugated PKC-epsilon peptide inhibitor with an *in vivo* rat myocardial I/R model. Therefore, we thought it prudent to compare TAT-conjugated and MYR-conjugated P110 to each other, as well as to their native counterpart [17].

Given the improved intracellular access or membrane targeting of MYR-conjugated peptides, we thought that MYR-conjugated P110 would exhibit a larger improvement in post-reperfused cardiac function and a further reduction in infarct size when compared to native and TAT-conjugated P110. We reported that, the MYR-P110 was the most efficacious in reducing infarct size (28 ± 2%) when compared to untreated myocardial I/R controls whose infarct size was 46 ± 3%. The TAT-conjugated P110 and native P110-treated I/R hearts had infarct sizes of 34 ± 3%. Interestingly, our study found that TAT-conjugated P110 initially showed a significant improvement in the maximal rise of post-reperfused cardiac contractility (i.e. dP/dt_{max}) of 56 ± 6% of initial baseline at 35-40 min post-reperfusion. However, this effect was only transient and was not maintained for the duration of the 90 min

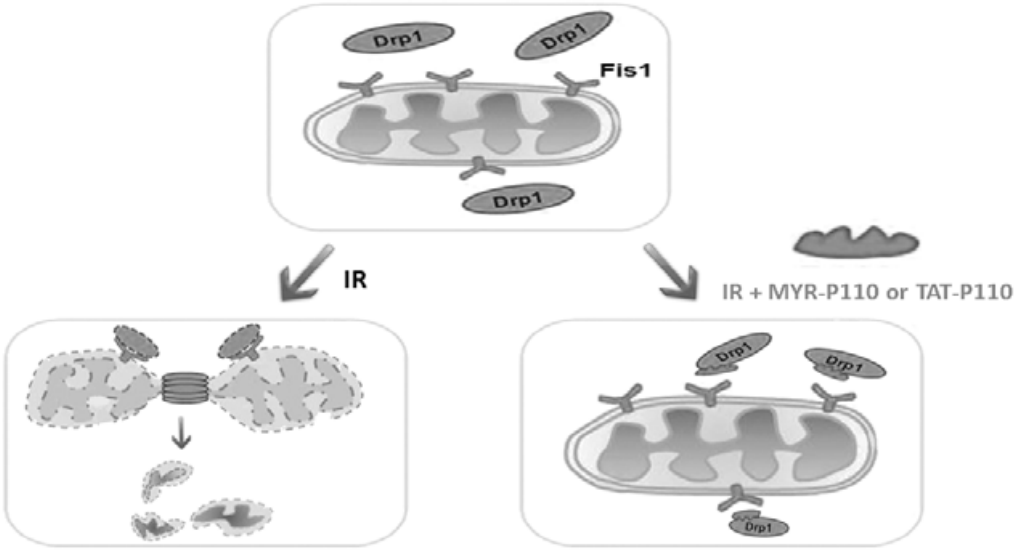

Figure 2. Schematic showing pre-ischemic mitochondria (center), the mechanism of mitochondrial fission in myocardial ischemia-reperfusion (IR) (bottom left) and how MYR-P110 or TAT- P110 can inhibit the interaction between Drp1 and Fis1 to prevent mitochondrial fission in myocardial IR (bottom right). Adapted from Qi *et al*. [20].

post-reperfusion period. The dP/dt_{max} values slowly declined back to 26 ± 5% at the end of the 90 min post-reperfusion period and were not different from untreated control (28 ± 4%) or native P110-treated I/R hearts (35 ± 6%). By contrast, MYR-conjugated P110-treated hearts showed a steady improvement in post-reperfused cardiac function by 25 min eventually raising dP/dt_{max} to 49 ± 7% at 90 minutes post-reperfusion of initial baseline. This effect was significantly different from the untreated control, native P110-treated, and TAT-conjugated P110-treated I/R hearts at the end of the 90 min post-reperfusion period [17]. These results suggest that MYR-P110 is able to maintain the cardioprotective effects for a longer duration of time compared to TAT-P110 perhaps due to enhanced cellular entry or membrane partitioning near its target substrate.

PMN SO release

Role of Nox2ds, a putative Nox2 assembly inhibitor

Nox2 (formerly known as gp91 phox) is the principal isoform of NADPH oxidase responsible for generating SO release in PMNs. Therefore, one would speculate that conjugated MYR or TAT Nox2ds (formerly known as gp91ds), a Nox2 inhibiting peptide that inhibits assembly of $p47^{phox}$ with Nox2 (i.e. gp91 phox), would have an increased effect on inhibiting PMA- or fMLP-induced increase in PMN SO release compared to the native sequence [11, 25] (Figure 3).

Rey *et al*. [11] showed a modest inhibition of PMA-induced SO release in human PMNs (~35%) using a TAT-conjugated Nox2ds (Nox2ds-TAT) peptide. Consequently, our research group decided to test the PMN chemotactic receptor response (i.e. fMLP) on whether MYR-conjugated Nox2ds peptide would exhibit increased inhibition compared to TAT-conjugated Nox2ds peptide. Previously, we have shown that MYR-conjugated peptides inhibited both PMA- and fMLP-induced SO release in rat PMNs up to 70% compared to their native counterparts [4]. We speculated that TAT-conjugated Nox2ds would have limited effectiveness in attenuating PMN SO release due to subeffective intracellular access. Consequently, only a limited inhibition of Nox2 assembly would occur; specifically, the docking sequence (i.e. CSTRRRQQL) of this peptide would only have a modest effect on inhibition of the assembly of Nox2 after stimulation by PMA or fMLP due to limited bioavailability.

Patel *et al*. [27] examined the effectiveness of MYR-conjugated and TAT-conjugated Nox2ds peptides on fMLP-induced rat PMN SO release, compared to native Nox2ds peptide and untreated

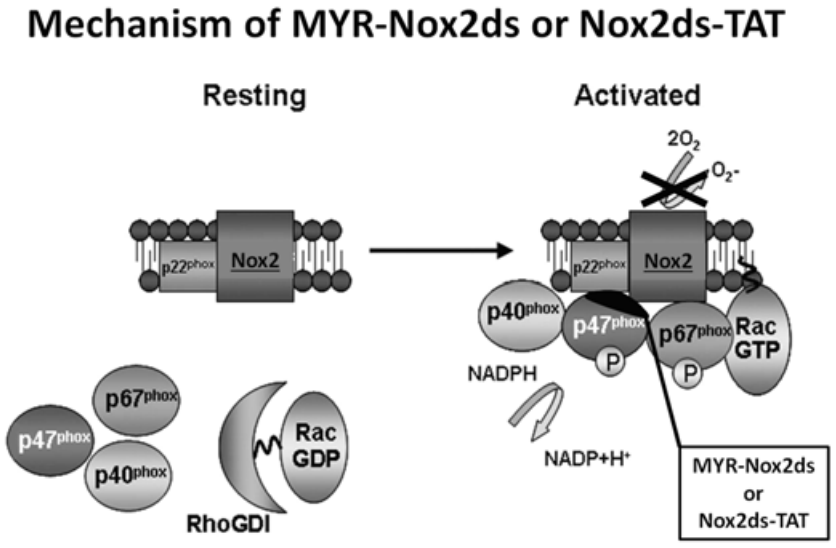

Figure 3. Schematic represents the inactive and active forms of Nox2. Area marked between $p47^{phox}$ and Nox2 indicates assembly inhibition by MYR-Nox2ds or Nox2ds-TAT and cross mark denotes area of superoxide (O_2^-) inhibition. Adapted from Wilkinson and Landreth [26].

control groups. As expected, native Nox2ds did not significantly attenuate fMLP-induced SO release. Concentrations of Nox2ds-TAT below 40 μM were also found to be ineffective to attenuate SO release, while at the highest dose (80 μM), fMLP-induced SO release was only reduced by 36%. By contrast, MYR-conjugated Nox2ds peptide (10 μM) significantly inhibited fMLP-induced PMN SO release by 56%. The results suggest that MYR-Nox2ds formulation was more potent than Nox2ds-TAT at inhibiting fMLP-induced PMN SO release in part *via* enhanced delivery of the cargo sequence to inhibit the assembly of Nox2. Similar to our findings, Nelson *et al*. [1] also showed that MYR-conjugated peptides entered into B-lymphocytes more efficiently than TAT-conjugated peptides without affecting cell viability.

Discussion

Relevance of P110 findings

This data suggests that the MYR-P110 was the best at augmenting the permeability of P110 through the plasma membrane, whereas TAT-conjugated P110 was no different from native P110 control by the end of the reperfusion period. Furthermore, MYR-P110 was able to show effective intracellular delivery as suggested by the continual rise in post-reperfused dP/dt_{max} throughout reperfusion. TAT-conjugated peptide delivery only had a transient effect on post-reperfused dP/dt_{max}, suggesting that this mode of intracellular delivery was less effective than MYR-conjugated peptide in maintaining the improvement in post-reperfused cardiac function throughout the 90 min time period. This is a significant point clinically, because having a drug which produces a longer effect would prove beneficial in not only attenuating heart injury, but also in minimizing dosing intervals. Collectively, this could lead to fewer adverse events and hospital stay time. Moreover, the ability to discharge patients earlier would be fiscally beneficial to health care institutions.

Relevance of Nox2ds findings

Similar to the P110 findings, the Nox2ds study suggests that the myristoylated conjugation was superior in its ability to inhibit Nox2 assembly and subsequently inhibit SO release in rat neutrophils when compared to TAT-conjugated Nox2ds. While Rey *et al*. [11] reported a modest (~35%) but significant decrease in PMA-stimulated SO release in human PMNs with TAT-conjugated Nox2ds, the study by Patel *et al*. [27] showed that MYR can increase the effect of Nox2ds by 1.5 times at $1/8^{th}$ the dose when compared to TAT-conjugated Nox2ds in rat PMNs. The implications of these results are that MYR-conjugated Nox2ds can be an effective tool to inhibit cell injury mediated by inflammation, which occurs in MI and organ transplant patients, among other conditions. Although TAT-conjugated Nox2ds significantly reduced fMLP-induced rat PMN SO release, compared to untreated controls, it was not significantly different from effects induced by native Nox2ds [27]. The data suggests that TAT-mediated intracellular delivery of Nox2ds is not different than carrier-mediated intracellular delivery of native Nox2ds in rat PMNs (Figure 1A). As mentioned previously, using lower doses to obtain a greater effect is advantageous in reducing potential adverse drug effects, which may be seen when drugs are used at higher doses. Moreover, the Patel *et al*. [27] findings further showed that MYR-conjugated Nox2ds peptide did not negatively affect cell viability when compared to untreated controls, which implies that the effects are mediated by inhibiting PMN SO release.

Future studies

Metabolism studies to determine peptide half-life and volume of distribution between TAT-conjugated and MYR-conjugated P110 would be important to correlate the prolonged cardioprotective effect of MYR-conjugated P110 compared to TAT-conjugated P110. This could be accomplished by attaching a fluoroprobe to both types of conjugated P110 peptides and detect fluorescence in the tissue at the end of the experiment as suggested by Nelson *et al*. [1]. Moreover, Nelson *et al*. [1] suggests that attaching a disulfide group between MYR and cargo sequence may facilitate intracellular delivery of MYR-conjugated peptides. Regarding solubility, TAT-conjugated peptides are more soluble in aqueous solutions like de-ionized water and salt buffer solutions. By contrast, many MYR-conjugated peptides require an organic solvent like dimethyl sulfoxide (DMSO) between 1-10 mM to solubilize the MYR-conjugated peptide into solution [1]. DMSO is known to exert potential cytotoxic effects at

final concentrations above 0.05%. Therefore, using DMSO may provide a limitation to increasing MYR-conjugated peptide dosing [28].

Regarding P110, we would like to conduct further studies using 10x concentration (i.e. 10 μM) or sustained application. This would help us ascertain whether TAT-conjugated P110 could elicit improved post-reperfused cardiac contractility throughout the 90 min reperfusion period, compared to the transient effect observed using the 1 μM dose during the first 20 min of reperfusion. Likewise, MYR-conjugated P110 may have superior recovery at higher concentrations when compared to the effects observed at 1 μM.

A possible direction regarding Nox2ds would also involve a comparison of TAT- and MYR-conjugated formulations in myocardial I/R. Our group has previously reported the cardioprotective effects of Nox2ds-TAT on post-reperfused cardiac function, whereby the highest dose (80 μM) elicited the greatest degree of improvement from initial dP/dt_{max} (62%) values [16, 29]. However, no studies evaluating the effects of MYR-Nox2ds on post-reperfused cardiac function have been completed to date. We believe, given the promising results of MYR-Nox2ds on attenuating SO release, that this would correlate with a decreased infarct size and an improved post-reperfused cardiac function in myocardial I/R at a lower dose than native or Nox2ds-TAT peptides. Previously, we have shown that MYR-PKC zeta or beta-2 peptide inhibitors attenuated PMA- or fMLP-induced PMN SO release at concentrations that correlated with improved post reperfused cardiac function, decreased infarct size and PMN infiltration in post reperfused hearts [4, 30-32].

Alternatively, future studies combining MYR-P110 and MYR-Nox2ds could elicit superior and synergistic cardioprotective effects in myocardial I/R using low doses that would be ineffective on their own. This combination seems feasible since the MYR conjugation allows for the drugs to be effective at lower doses. Furthermore, this could maximize clinical outcomes and minimize adverse drug reactions. Similarly, it would be of interest to see if dual-conjugated peptides (i.e. TAT and MYR) would yield superior effects compared to either TAT-conjugated or MYR-conjugated peptides independently. To date, no studies have been performed using a mixture of both conjugations on one cargo peptide sequence in the same bioassay. Additionally, studies comparing the effects of TAT or MYR alone on SO release and post-reperfused cardiac function parameters would be beneficial in determining the actual effect that these conjugates would have independent of their cargo sequence.

CONCLUSION

In summary, the findings from both the myocardial I/R study with P110 and the PMN SO release study with Nox2ds clearly indicate that MYR-conjugated peptides were more effective than TAT-conjugated and native peptides in their respective assays. While it is promising to see that both MYR- and TAT-conjugated peptides exert effects in different types of cells, it is clear that more studies in this area are needed. Studies to date are very limited, and the benefits of using such conjugations for peptide delivery are profound.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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