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ARTICLE ADDENDUM

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Peptide translocation through the plasma membrane of human cells: Can oxidative stress be exploited to gain better intracellular access?

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ABSTRACT

Cell-penetrating peptides (CPPs) enter cells primarily by escaping from endosomal compartments or by directly translocating across the plasma membrane. Due to their capability of permeating into the cytosolic space of the cell, CPPs are utilized for the delivery of cell-impermeable molecules. However, the fundamental mechanisms and parameters associated with the penetration of CPPs and their cargos through the lipid bilayer have not been fully determined. This in turn has hampered their usage in biotechnological or therapeutic applications. We have recently reported that the cell penetration activity of poly-arginine CPPs (PACPPs) is dependent on the oxidation status of the plasma membrane of cells. Our data support a model where the positively-charged PACPP binds negatively-charged lipids exposed on the cell surface as a result of oxidative damage. The PACPP then crosses the membrane via formation of inverted micelles with these anionic lipids. This model provides a plausible explanation for the high variability in the cell delivery efficiency of a PACPP often observed in different settings. Notably, taking into account the current literature describing the effects of lipid oxidation, our data point to a highly complex and underappreciated interplay between PACPPs and oxidized membrane species. Overall, a better understanding of oxidation-dependent cell penetration might provide a fundamental basis for development of optimal cell permeable peptides (including cyclic peptides, stapled peptides, peptoids, etc…) and of robust delivery protocols.

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Introduction

CPPs are peptides (generally 5 to 30 amino acids) that enter cells into the cytoplasmic space of live cells without causing cell death.[1] CPPs can be used as carrier agents for a variety of molecular cargos with a wide range of sizes, from small-molecule compounds and short peptides to large molecules such as proteins, polynucleotides, and nanoparticles. Cargo delivery can be achieved by covalent attachment of a CPP to a cargo of interest or by simple by co-incubation.[1] Most CPPs have shown no cell specificity as the peptides penetrate various cell types including plant, insect, and stem cells, as well as both cancerous and noncancerous cells.[2-7] Polyarginine CPPs are one of the most widely used and studied CPPs. Despite the promise of a broad utility *in cellulo* and *in vivo*, general rules of cytosolic penetration have not yet clearly been established. It is widely accepted that PACPPs enter the cell via 2 possible routes: a 2-step process involving endocytosis and endosomal permeation, or a single step process involving direct translocation at the plasma membrane.[8] Various mechanisms by which PACPPs traverse membranes have been proposed and tested.[5,9-12] Yet, these models do not fully explain the variations in cell delivery results. For instances, at the same peptide concentration and in the same cancer cell line, nona-polyarginine (R9) displayed different cell penetration activities. In one result, R9 was trapped in the endocytic vesicles after incubation, but in a similar experiment, the peptide clearly penetrated the cell and distributed throughout the cytosol.[13,14] This variability suggests that the membrane permeation of PACPPs is dependent on parameters that are not controlled during standard tissue culture delivery protocols.

Several reports have demonstrated that fluorescent-labeled CPPs can penetrate cells while irradiated with light during fluorescence microscopy.[15] In particular, it was demonstrated that the reactive oxygen species (ROS) generated by typical fluorophores upon excitation could damage cellular membranes and facilitate the membrane translocation of CPPs.[16,17] While these effects have been exploited to achieve light-dependent cytosolic delivery,[18] light-induced cell penetration represents a potential artifact that might alter the experimental outcome of unsuspecting investigators. Using this notion as a potential

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clue, we hypothesized that endogenous levels of membrane oxidation could modulate the cytosolic penetration of CPPs, both after light irradiation and in the dark (absence of light or low light doses). This hypothesis is plausible because cells are typically cultured at 20% oxygen. This is in sharp contrast to the oxygen tension experienced by cells *in vivo* (e.g. internal tissues are exposed to approximately 2% oxygen).[19] Cultured cells are therefore typically under oxidative stress[20,21] and some level of membrane oxidation can be expected to be present in cells grown in standard incubators.

Summary of results

To test our hypothesis, we examined the interplay between cellular oxidation and cytosolic penetration of peptides using PACPPs as model compounds. We showed that the cytosolic penetration of nona- or trideca-arginine CPPs (r9 and r13 where r is D-Arg, an unnatural arginine residue used to confer protease-resistance to the peptide) was abolished or significantly reduced, respectively, when cells were cultured at 2% oxygen as opposed to 20% oxygen. Cell penetration was also reduced when the cellular growth media was supplemented with antioxidants.[22] Together, these results indicated that reducing oxidative cellular stress diminishes the transport of PACPPs into cells. Conversely, an increase in the oxidation state of the plasma membrane of cells led to an increase in PACPP cytosolic penetration. For instance, r13 efficiently entered cells pretreated with lipophilic oxidants (oxidation conditions were mild and did not affect cell viability and proliferation rates). Overall, the penetration activity of PACPP positively correlates with the oxidation status of the membrane of cells.

Given that lipids are possible targets of both oxidative damage and potential cellular factors interacting with PACPPs, we next tested whether oxidized lipids might be involved in the oxidation-dependent translocation of the peptides. We first established that the cell penetration of TMR-r13 positively correlated with the level of lipid peroxides present in cellular membranes. In particular, a cell line prone to cell penetration by TMR-r13 displayed higher endogenous levels of oxidized lipids than a cell line more resistant to the translocation of TMR-r13. We then observed that E06, a monoclonal antibody that binds oxidized phosphatidylcholine (oxPC) lipids, blocked the intracellular delivery of PACPP after a simple pre-incubation with cells. Furthermore, extracellular addition of the anionic oxPC lipids, PGPC or PazePC, enhanced the cytosolic penetration of PACPPs. Notably, an *in vitro* partitioning assay established that both PGPC and PazePC can transport PACPPs into a milieu of low dielectric constant. This, in turn, is consistent with the notion that PACPPs and anionic oxPC species have the ability to form structures, most likely inverted micelles, that allow transfer of the hydrophilic peptide across a hydrophobic lipid bilayer.

Proposed model based on current data

Our results support a novel model for cytosolic penetration where arginine-rich peptides translocate across the plasma membrane of cells by interacting with anionic lipids exposed on the cell surface as a result of oxidative membrane damage. In particular, oxidized lipids might act as direct mediator of peptide transport. In agreement with the lipid whisker model, lipid oxidation leads to the formation of anionic truncated fatty acid moieties that become exposed on the cell surface (Fig. 1).[23] PACPPs interact with these moieties and inverted micelles form between cationic peptide and anionic lipids. The concentration gradient generated during incubation (e.g., micromolar concentration of peptide outside cells and no CPP inside cells at initial incubation conditions) as well as the membrane potential may then drive the accumulation of PACPPs inside cells.

Other possible models connecting oxidation, lipid damage, and cell penetration

The involvement of oxPC lipids such as PGPC and PazePC provide a plausible explanation for peptide translocation. Yet, due to the complexities of cell membrane composition and oxidation chemistry, it is likely that other oxidized lipids or that other oxidation-dependent processes might also play a role. Below, we present alternative and non-mutually exclusive scenari that might also provide a molecular basis for the oxidation-dependent behavior of PACPPs.

Melikov and co-workers have proposed that phosphatidylserine (PS), an anionic phospholipid normally present in human cell membrane may mediate the translocation of PACPPs.[12] This lipid typically resides in the inner leaflet of mammalian plasma membrane and whether PACPPs can interact with PS on the cell surface remains therefore unclear. Yet, it has been established that membrane oxidation can disrupt the lipid asymmetry of biological lipid bilayers and that the level of PS exposed extracellularly can increase during membrane oxidation.[24] It is therefore possible that upon membrane oxidation, PS partially and transiently relocalizes to the outer leaflet of the plasma membrane. It might then bind PACPPs electrostatically and assist their membrane translocation in a micelle-dependent manner similar to that described for oxPCs (Fig. 2). In this scenario, oxidation does not lead to the formation of new species, but simply influences the level at which an anionic lipid is exposed on the cell surface.

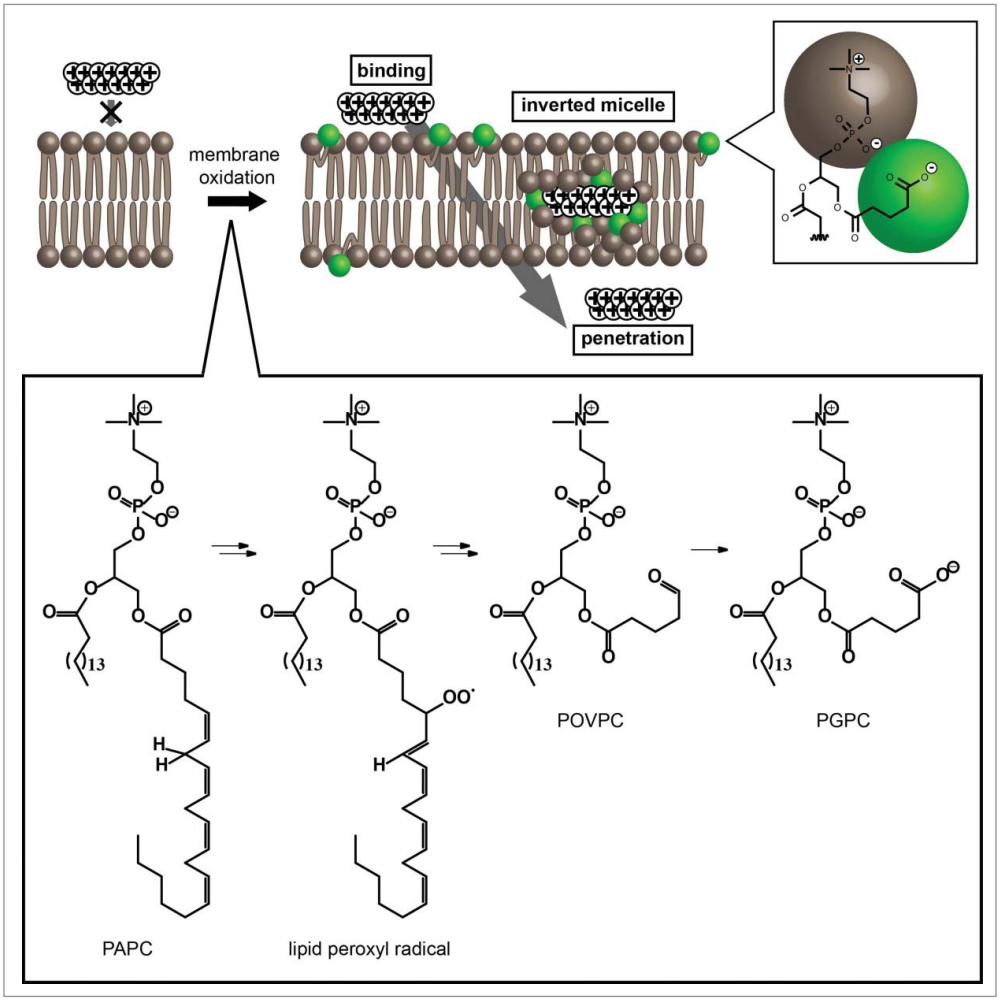

Figure 1. Membrane oxidation can lead to the exposure of anionic phospholipid on the cell surface. In this illustration, the oxidation of arachidonic fatty acid of PAPC yields POVPC. Further oxidation of POVPC forms PGPC. The carboxylate formed is negatively charged and exposed on the surface of the bilayer. The chemical structure of the lipid head and newly formed anionic fatty acyl chain of PGPC is shown and labeled with dark brown and green spheres accordingly.

Another possible scenario relates to the fact that oxidation of polyunsaturated fatty acids generates a wild array of oxidized species, including aldehydes such as malondialdehyde (MDA).[25] The formation of MDA is in fact often used as a quantitative measure of cellular oxidative stress. In addition, MDA reacts with the amino groups of biomolecules to form Schiff-base adducts.[26] In particular, MDA can react with phosphatidylethanolamine (PE), a

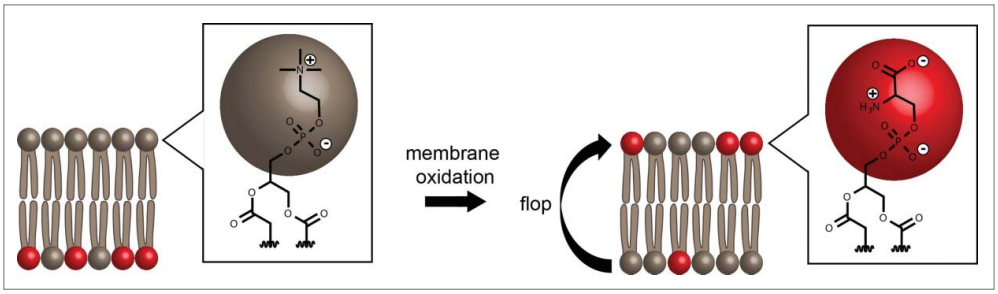

Figure 2. Externalization of PS upon membrane oxidation. A simplified lipid bilayer is represented with only the phospholipid phosphatidylcholine (PC) and phosphatidylserine (PS). The PS asymmetry of the lipid bilayer, maintained by flippases in the plasma membrane of human cells, is potentially disrupted upon membrane oxidation. The chemical structure of the lipid head of PC and PS are shown in the box and labeled with dark brown and red spheres, respectively.

phospholipid containing a positively charged amine in its polar head.[27] Notably, while PE is zwitterionic, a PE-MDA adduct is anionic (reaction with the amino group removes the positive charge of this functional group, leaving a single negative charge on the phosphate). Thus, the by-products of lipid oxidation can lead to the formation of an anionic lipid on the cell surface. This anionic lipid represents therefore another target for possible PACPP binding and translocation (Fig. 3).

It is possible that PACPPs, in addition to binding anionic lipids, exploit oxidation-induced hydrophobic or hydrophilic defects present in the bilayer. Membrane defects can, for example, be formed upon generation of lipids containing truncated fatty acyl chain (anionic or non-anionic). Such species are formed as a consequence of lipid peroxidation and of repair by detoxifying enzymes (eg: phospholipase A2).[28] These oxidized lipids have an inverted cone shape and detergent-like properties. These species form membrane microdomain and change membrane dynamics (e.g. membrane fluidity and lipid packing). These changes are thought to increase the permeability of membranes[29,30] (Fig. 4). Overall we propose that these effects could in principle synergize with inverted micelle formation and further facilitate translocation of PACPPs (Fig. 5).

Overall, the models described herein are speculative and have not been tested to date. Yet, preliminary results, presented in Figure 6, suggest that PGPC, PazePC, PS, and

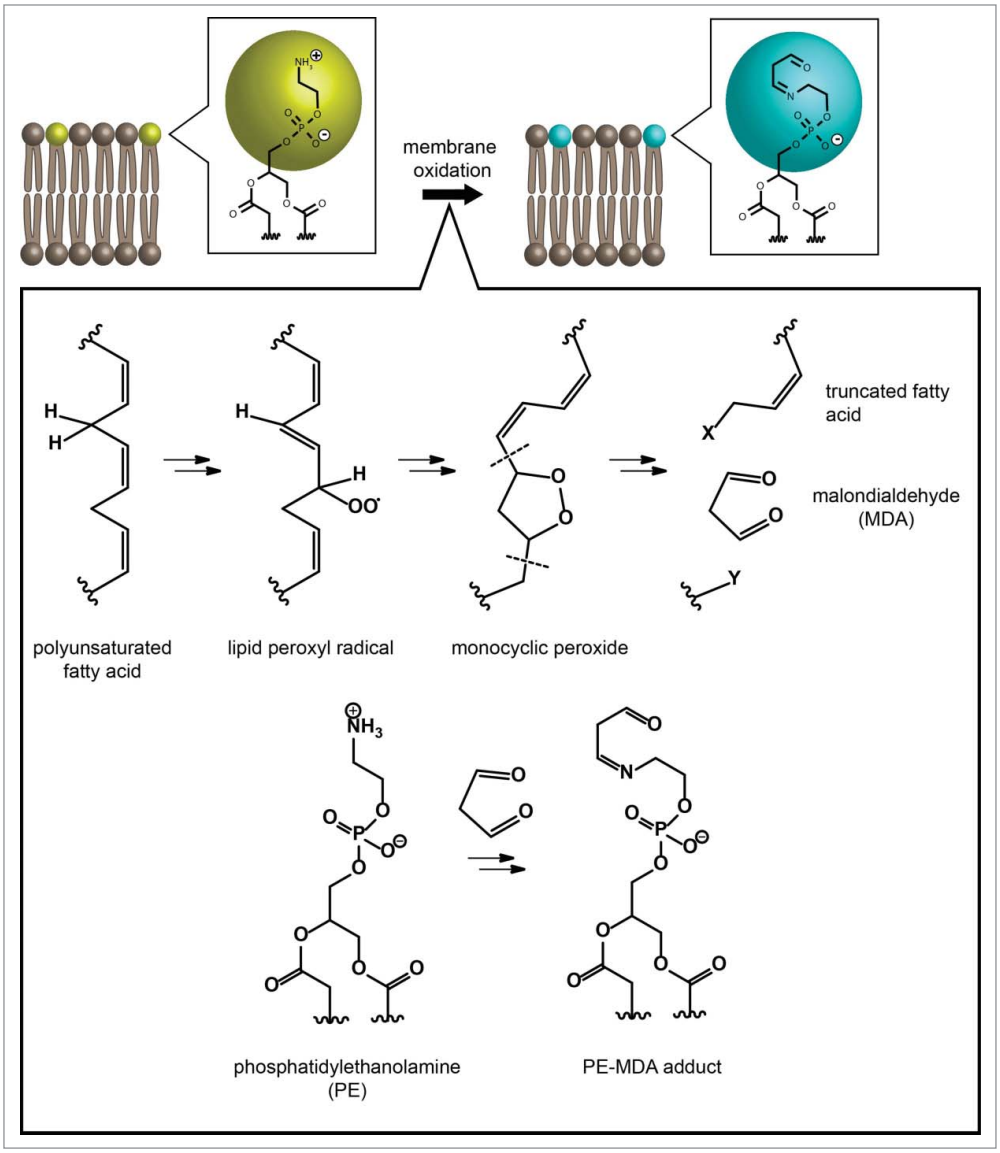

Figure 3. Oxidation-dependent modification of the polar heads of lipids. PE-MDA production is used as an example. MDA is created upon oxidation of polyunsaturated fatty acids. The generated MDA reacts with the amino group of the zwitterionc lipid phosphatidylethanolamine (PE) to form PE-MDA adduct, an anionic species. The chemical structure of the lipid head of PE and PE-MDA is shown and labeled with yellow green and cyan spheres, respectively.

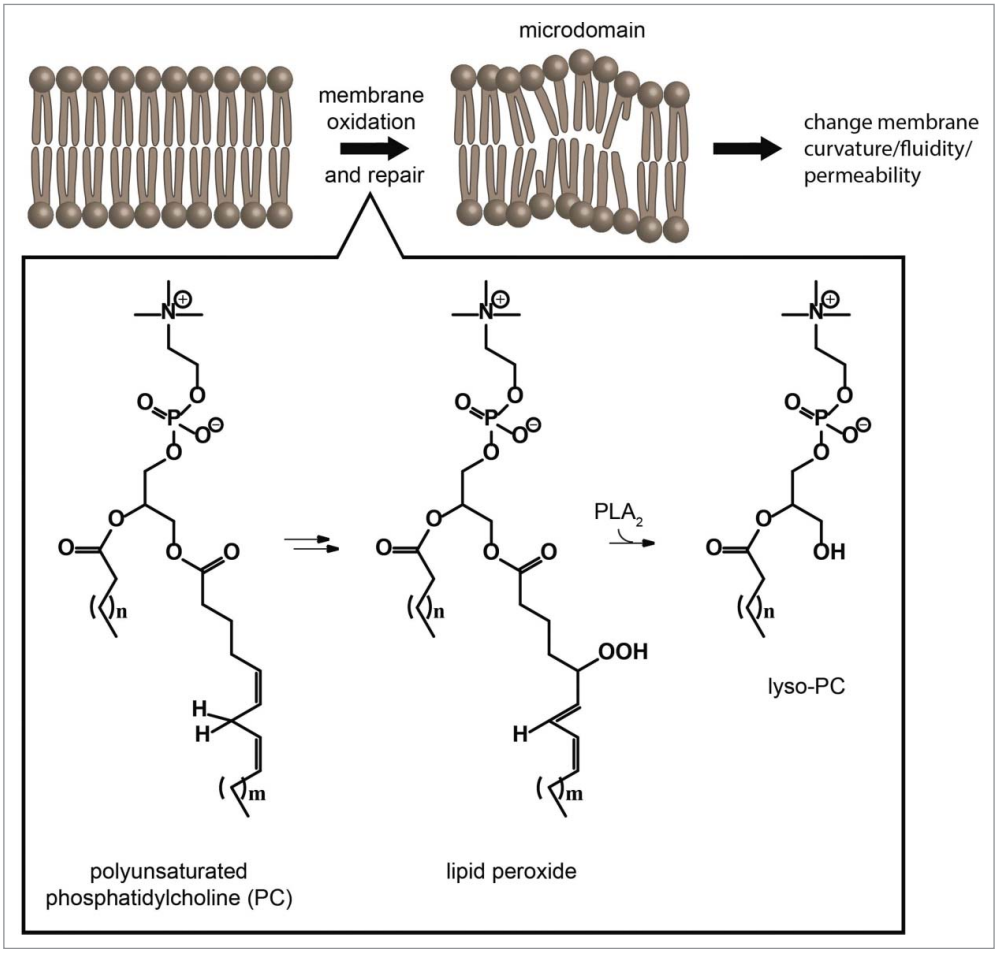

Figure 4. Generation of lysophospholipids and membrane defects upon oxidation. Oxidation of PC is given as an example. The unsaturated fatty acid of PC is oxidized to form lipid peroxide. Phospholipase A2 (PLA_2) cleaves the oxidized fatty acid. The generated lysophosphatidylcholine (lyso-PC) induces the formation of microdomain, leading to alterations in membrane packing, membrane curvature, fluidity, and permeability.

PE-MDA all share a similar behavior *in vitro*. In particular, a simple partitioning assay between water and hexane, show that PGPC, PazePC, PS, and PE-MDA can mediate the transfer of r13 into a milieu of low dielectric constant while the neutral oxidized lipids POVPC and PoxonPC can not. In principle, all these anionic lipids are capable of interacting with PACPPs and bringing them in an environment resembling the lipid bilayer. It is therefore possible that several anionic lipid species, while concomitantly generated during oxidative stress, might act in concert to interact with PACPPs during cell penetration.

What does this mean for cell culture experiments?

The oxidation-dependent activity of PACPPs can explain the variations often observed in PACPP performance in live cell delivery experiments. As described herein, differences in delivery efficiency can arise from distinct oxidation levels of the cell membrane that are generated by exogenous and endogenous factors. Cell culture conditions and media composition can impact cell metabolism and oxidative stress levels. For instance, the antioxidant ascorbic acid (i.e. vitamin C) is present in certain cell culture media but not in others (for example, ascorbic acid is present in CMRL-1066 medium but not in the commonly used media DMEM).[21] In addition, selenium is a cofactor that is essential to the activity of glutathione peroxidase, an enzyme that protect cells from oxidative damage and educes lipid hydroperoxides. Selenium is typically introduced in cell cultures by addition of serum. Yet, the amount of selenium in serum varies from batch to batch and from brand to brand.[31] These variations have been shown to significantly impact the activities of enzymes involving in antioxidant defense pathways.[31] It is therefore conceivable that variations in growth conditions lead to variations in plasma membrane oxidation and in peptide translocation.

Similar oxidation-related principles might explain why certain cell types are more resistant to peptide penetration than others. For instance, it is interesting to note that

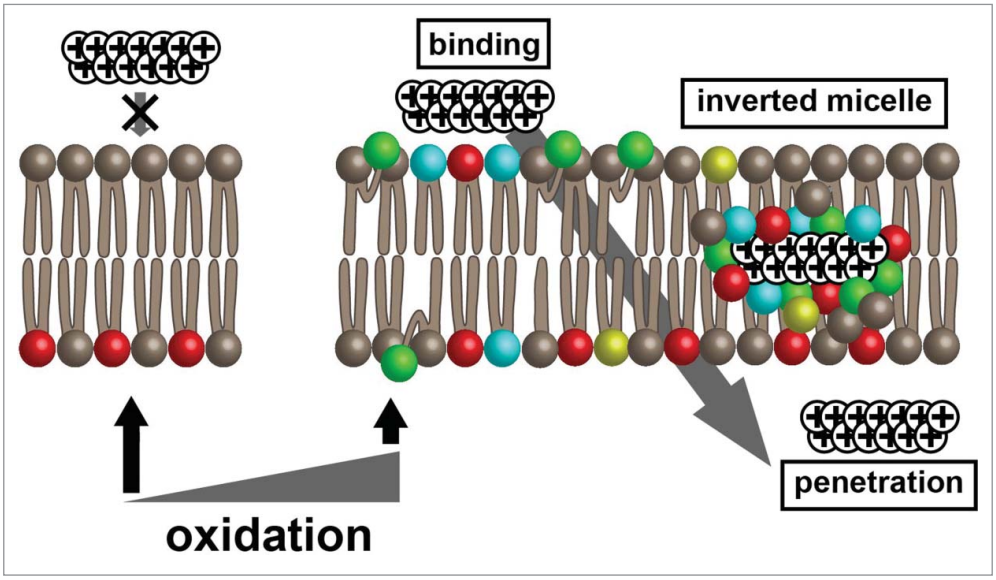

Figure 5. Proposed models for oxidation-dependent cytosolic penetration of polyarginine peptide. A simplified lipid bilayer is represented with only the lipids of PC, PS, PE, PE-MDA, and PGPC. The polyarginine peptide is expressed as a string of positive charges. Under low oxidative stress, the surface of the lipid bilayer is neutral, resulting in no binding and no cytosolic penetration from the peptide. Under high oxidative stress, anionic lipids are present that promote the binding, inverted micelle formation and membrane translocation of the peptide.

endogenous level of antioxidants and activities of antioxidation enzymes differ in various cell types.[32] Whether these differences lead to changes in plasma membrane compositions and structures has not been explored. Yet, one can envision how cells displaying low oxidative damage at the plasma membrane because of a highly active antioxidative defense system would be less susceptible to PACPP penetration than cells more prone to oxidation.[22] Finally, the level of lipid peroxides present in cells increases with the number of passages used in a particular cell culture.[33] It is therefore possible that cells with high passage numbers ("old" cells) differ significantly from freshly cultured cells ("young" cells) in their responses to PACPP treatment.

Many parameters can impact the oxidative status of the cell and this, in turn, will influence peptide translocation outcomes. When comparing the activity of different PACPPs, controlling membrane oxidation is therefore important as different experimental settings might lead to highly variable results. Moreover, favoring mild oxidative cell culture conditions might lead to improvements in PACPP-based delivery applications. However, excessive oxidative damage at the plasma membrane can severely alter cell physiology. Overall, cell culture conditions optimal for both PACPP delivery and cell biology remain to be established.

What does this mean for in vivo *applications?*

It is not clear how the insights we have gained with *in vitro* experiments might apply to how PACPPs behave *in vivo*. This is in part because it is hard to measure or predict what the *in vivo* oxidation status of cells is. One can, however, envision how a PACPP that penetrates cells efficiently *in vitro* might not do so *in vivo* simply because *in vivo* tissues are better protected from oxidative damage (e.g., cells grown at 20% oxygen vs. tissue exposed to 2% oxygen *in vivo*). This would then suggest that cytosolic penetration studies should not be performed with cells grown under oxidative stress (*i.e.*, ambient air) when a goal is to develop cell-permeable peptides for therapeutic applications. In particular, assays performed with cells protected from oxidation (e.g. culture at 2% rather than 20% oxygen) might offer better predictions for how such peptides might perform *in vivo*. Yet, this issue is complicated by the fact that certain tissues are more prone to oxidation than others. For instance, airways are exposed to 20% oxygen. Inflamed tissues and tumors are also sites of elevated oxidative stress.[25,34] How CPPs behave in these tissues based on *in vitro* data is again hard to predict. Yet, it is possible that CPPs have the ability to more readily permeate cells in these environments.

Conclusions and future prospects

More molecular details are still needed to fully elucidate how PACPPs enter cells. It is plausible that PACPPs enter cells by multiple routes while associating with a wide variety of cellular species. Herein, we describe how several oxidized lipid species might play equal roles in this process. The anionic lipids presented are, however,

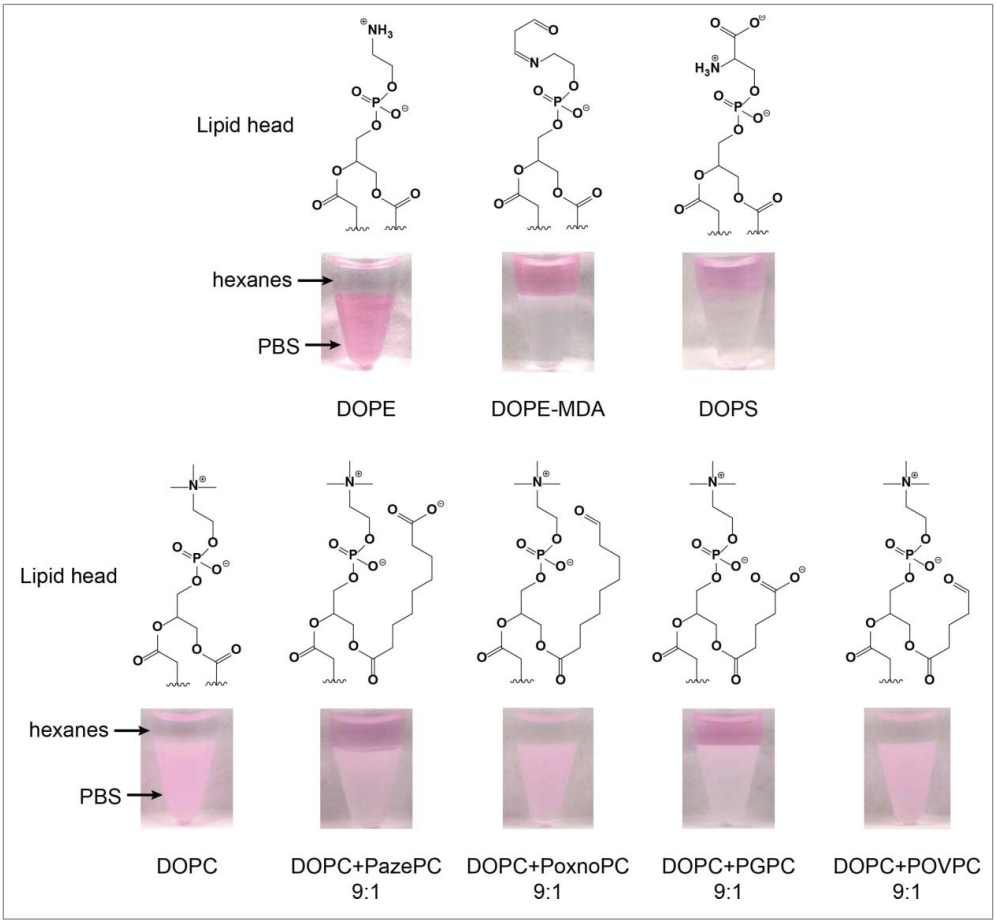

Figure 6. Phospholipids-mediated partitioning of polyarginine peptide into hexane is charge-dependent. The fluorescently labeled PACPP TMR-r13 (5 μM) was mixed with PBS/hexane supplemented with various lipids (total lipid concentration is 3 mM in all samples) as described in Wang et al.[22]

not exclusive candidates and other oxidized species might be involved in the process of peptide translocation. Further testing of the hypotheses described herein will require a thorough examination of the composition of the plasma membrane of cells under various conditions of oxidative stress, something that is currently lacking. Useful protocols for the analysis of oxidized lipids by mass spectrometry have been developed.[35] Sample preparation remain however a challenge. It is for instance important to protect samples from oxidation during analysis so as to not generate artifacts. In addition, selectively purifying the components of the plasma membrane from other cellular membranes is not trivial (one can for instance envision that the membrane of mitochondria contains oxidized lipids that are not present in the plasma membrane and vice versa). Techniques that involve the formation and purification of plasma membrane vesicles might be useful in this respect.[36] However, given that oxidized lipids display a wide range of biophysical properties, it is not clear whether a single purification scheme can capture all the species present in the plasma membrane. For instance, while many oxidized lipids are lipophilic, others are water-soluble. It is therefore possible that these oxidized lipids equilibrate in solution during purification and that they do not partition in the organic solvents typically used to separate lipids from other cell components. To circumvent some of these problems, we are currently developing pull-down protocols, using TMR-r13 as a bait for lipid enrichment. While this approach will not identify all the oxidized lipids present in the plasma membrane, it might help establish which species interact with the peptide during translocation.

While much remains to be done to reveal how a highly hydrophilic and charged peptide such as TMR-r13 crosses a hydrophobic membrane, the interplay between cytosolic penetration and oxidation presented herein should have immediate implications. For instance, inducing oxidative damage to cellular membranes might offer a novel strategy to improve the cell permeability of therapeutic peptides. As indicated by experiments involving co-incubation with PGPC or PazePC,

formulations that include CPPs and oxidized lipids might represent one such strategy.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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