51

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MITOCHONDRIALLY-TARGETED DRUGS FOR THE PREVENTION OF RADIATION CYSTITIS

Hypothesis / aims of study

Ionizing radiation activates a variety of cytoplasmic signal transduction pathways, some of which are believed to be initiated in mitochondria and mediated by reactive nitrogen and oxygen species (RNS and ROS). The activation of mitochondrial nitric oxide synthase (mtNOS) was suggested as an early radiation event in the urothelial cells that line the urinary bladder [1]. As a consequence, nitric oxide (NO) is produced in excessive amounts and inhibits the mitochondrial respiratory chain, resulting in superoxide (O_2^-) production. The NO then out-competes superoxide dismutase (MnSOD) to react with O_2^- forming high levels of peroxynitrite ($ONOO^-$), which damages complexes I and III of respiratory chain. It has been demonstrated earlier in our lab that the presence of a NOS inhibitor (L-NAME; 100 μM) in the bladder during irradiation is radioprotective [1].Systemic drug administration to protect the bladder against irradiation damage can result in undesirable side effects and may exhibit poor deliverability and short durations of action. A potential solution to this problem is to target drug delivery to the mitochondria. Accordingly, radioprotectors were conjugated to unique peptide isosteres, which drags the prodrug to the negatively charged inner mitochondria membrane and released it overtime, thereby allowing the use of lower drug concentrations (Fig. 1). As a conjugator we used fragments of the membrane-active antibiotic Gramicidin S (GS), bioavailability of which was improved by replacing critical amide bonds with (*E*)-alkene peptide isosteres [2]. These isosteres were designed to distribute the active drug moiety in a timed-release fashion.

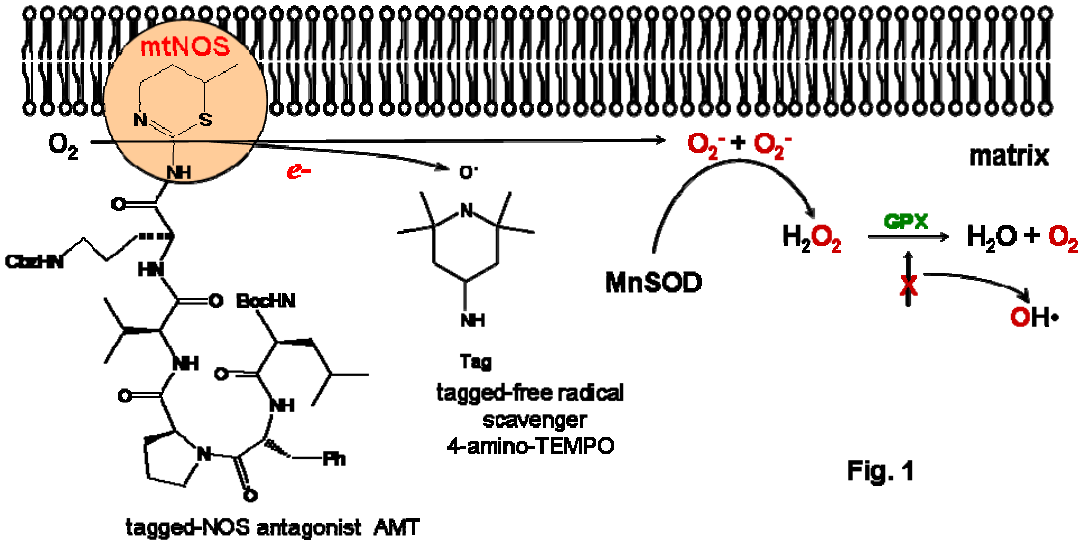


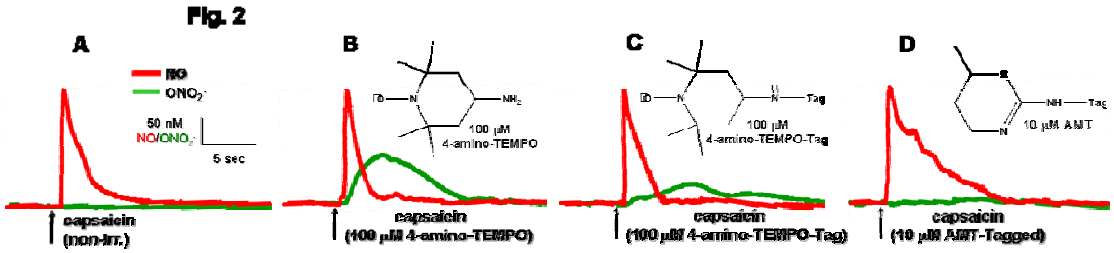
Fig. 1

Study design, materials and methods

Because irradiation results in the increased production of both NO and O_2^- in the urothelium that lines the bladder, we treated dissociated urothelial cells with unconjugated and conjugated 2-amino-6-methylthiazaline (AMT; 10 to 100 μM) or 4-amino-2,2,6,6-tetramethyl-piperidine-N-oxyl (4-amino-TEMPO; 10 to 100 μM) to determine whether inhibition of NO production or scavenging of reactive oxygen species is more protective. Physiological studies were conducted in order to evaluate the effect of peptide-targeted AMT and 4-amino-TEMPO on NO and $ONOO^-$ production in irradiated (20 Gray; 1 Gy = 1 J/kg = 100 rad) urothelial cells. The cells were cultured in 8-well slide chambers for three days and microsensor measurements were taken 24 hours after irradiation. Quantitative mass spectrometry was used to compare effectiveness of different AMT and 4-amino-TEMPO conjugates in penetrating mitochondria.

Results

Mass spectroscopy analysis showed that unconjugated AMT as well as unconjugated 4-amino-TEMPO does not enter the mitochondria, but the targeting peptide successfully directs the NOS antagonist and the nitroxide free radical scavenger to mitochondria. In untreated irradiated cells and cells treated with unconjugated 4-amino-TEMPO (100 μM; Fig. 2B) and unconjugated AMT (100 μM; not shown), agonist (capsaicin, 1 μM) evoked NO production resulted in formation of comparable amounts of $ONOO^-$. In cells treated with high-dose conjugated 4-amino-TEMPO (100 μM; Fig. 2C), $ONOO^-$ production was significantly decreased. However, in non-irradiated cells (Fig. 2A) and cells treated with conjugated AMT (10 μM; Fig. 2D), NO induced $ONOO^-$ formation was not observed or almost completely abolished, respectively (n=6).



Four different peptide conjugates were then tested to determine the effectiveness of their mitochondrial penetration. It was shown that the trisubstituted (*E*)-alkene moiety embedded in XJB-5-241 had a much stronger conformational effect then the less biologically active disubstituted (*E*)-alkene (XJB-5-133) or the GS peptidyl fragment XJB-5-127 (not shown). The concentrations of these three compounds in mitochondria were 103.3 Fmole/10μg mitochondrial protein, 89.9 Fmole/10μg and 50.8 Fmole/10μg, respectively. The least efficacious conjugate was XJB-5-234 (1.45 Fmole/10μg protein); this may be due to the lack of a complete targeting sequence.

Interpretation of results

NO- and $ONOO^-$ microsensor measurements demonstrate that peptide conjugates drag membrane impermeant 4-amino-TEMPO and AMT across the mitochondrial membrane and do not interfere with the protective activity of these drugs. The targeting of a NOS antagonist was more radioprotective than the targeting of a free radical scavenger. A defined secondary structure and an appropriate conformational preorganization is important in accomplishing efficient mitochondrial delivery. The presence of non-hydrolyzable (*E*)-alkene isostere functions in place of labile peptide bonds is also significant for a prolonged mechanism of action.

Concluding message

Our data suggest that the mitochondrial targeting of NOS antagonists or free radical scavengers using a peptide dragging strategy enhances their radioprotective effects and avoids complications associated with systemic administration. Previous studies have demonstrated that when a NOS antagonist and a free radical scavenger were administered as a dual-function molecule, the therapeutic effect was greater then when given together but unlinked. The dual-action drug may locally inhibit both NO and O_2^- production which can occur at the inhibited mtNOS enzyme, thereby preventing $ONOO^-$ formation which preserves the enzyme. Accordingly, future plans include the synthesis and analysis of the therapeutic benefits of targeted dual-action radioprotectors.

References

1. Am J Physiol (2004) 286; H13-H21.
2. Org Biomol Chem (2007) 5; 307-309.

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ANIMAL SUBJECTS: This study followed the guidelines for care and use of laboratory animals and was approved by University of Pittsburgh Institutional Animal Care and Use Commity