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Anti-inflammatory, analgesic and antioxidant activities of novel kyotorphin-nitroxide hybrid molecules

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ABSTRACT

Mitochondrial oxidative damage contributes to a wide range of pathologies, including ischemia/reperfusion (I/R) injury, cardiovascular disorders and neurodegenerative diseases. Accordingly, protecting mitochondria from oxidative damage should possess therapeutic relevance. In the present study, we have designed and synthesized a series of novel kyotorphin-nitroxide hybrid molecules, and examined their free radical scavenging activities, in addition to their anti-inflammatory and analgesic activities. We have further characterized these compounds in a simulated I/R cellular model. Our findings suggest that the protective effects of kyotorphin-nitroxides partially reside in maintaining optimal mitochondrial function.

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Ischemia/reperfusion (I/R) injury represents tissue damage associated with the return of blood supply following an ischemic period. I/R is a general mechanism of damage relevant to many organs. The events of I/R trigger a systemic inflammatory response that can lead to cellular damage and even organ failure. It is widely felt that reactive oxygen species (ROS) and poly-morphonuclear leukocytes play important roles in mediating I/R injury.[1–3] Numerous treatment strategies have been proposed to reduce I/R injury, such as ischemic pre-conditioning, post-conditioning, controlled reperfusion, and injection/infusion with various therapeutic agents.[4–6] The ultimate therapeutic objective of these treatments is to reduce oxidative stress, inflammation, vascular injury, while simultaneously providing an energy supply to the reperfused organ. Among several cellular events postulated to contribute to reperfusion injury, bursts of ROS occurring during early reperfusion have been shown to contribute to the evolution of a leukocyte-mediated inflammatory reaction that exacerbates reperfusion-induced tissue injury. Accordingly, antioxidant supplementation and anti-inflammatory agents may represent a rationale therapeutic approach to alleviate I/R injury. However, conventional anti-inflammatory antioxidants have limited efficacy due to the difficulty of delivering them to mitochondria in a targeted manner. To address this limitation in our ability to effectively treat I/R, we seek to develop organelle-targeting anti-inflammatory antioxidants to alleviate mitochondrial oxidative damage.

We and others have provided preliminary studies which indicate that nitroxides can attenuate oxidative damage in various I/R experimental models.[7–17] The protective effects of nitroxides are attributed to their antioxidant capacities. In addition to directly scavenging free radicals, nitroxides also attenuate the formation of other reactive oxygen species (ROS) and reactive nitrogen species (RNS). Unlike antioxidants that act in a sacrificial mode, nitroxides can provide a unique type of catalytic protection. Nitroxides undergo one-electron redox reactions to yield the corresponding hydroxylamines and oxo-ammonium cations via electron transfer reactions. The hydroxylamine and oxo-ammonium cations can *com*-proportionate, yielding two nitroxide molecules. The non-radical species might also *com*-proportionate and yield the more stable radical form, thus regenerating themselves. Consequently, nitroxide radical, oxo-ammonium cation, and hydroxylamine can be present simultaneously in the tissue. These three forms can serve as self-restoring antioxidants through continuous exchange, and in this manner confer catalytic protective activity against

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oxidative damage. These unique features of nitroxides highlight a very novel role for this unique class of agents in protecting against I/R injury. Moreover, nitroxides readily penetrate the brain while permeating the cell membrane, which is unique in comparison to many other biological antioxidants (e.g., SOD, glutathione peroxidase, etc.). Thus, this class of agents is unique with respect to therapeutic potential for diseases and injuries related to oxidative stress.

Kyotorphin (KTP; L-Tyr-L-Arg) has been isolated from bovine brain and shown to demonstrate analgesic activity in animal models, but only following central delivery. The therapeutic potential of KTP is primarily hampered in relation to low intestinal absorption. Accordingly, increasing peptide-cell membrane affinity represents a promising strategy to overcome this limitation. Here, our design principle is based on our hypothesis that covalent combination of kyotorphin with nitronyl nitroxide (NN) will improve intestinal absorption of KTP. These constructs result from both a chemical rationale (NN renders KTP more lipophilic) and a physiological rationale (combining analgesic and free radical scavenging capacity). The complexity of tissue injury induced by I/R requires a multi-targeted treatment strategy, which we have adapted in the current studies. In our present study, conjugation of nitronyl nitroxide with KTP through a diamine linker proved to be a simple modification that converted kyotorphin into an effective analgesic agent. Although there is no report about the application of kyotorphin alone for I/R injury, it is known that anesthetics have a protective role against ischemia reperfusion injury.[5] These protective effects have been attributed to pre- and post-conditioning effects in relation to apoptosis. It has been proposed that anesthetics modulate the interaction of PMN with the endothelial cell, and this may contribute their protective effects.[5] We found that compound **9i**,**j** displayed potent analgesic, anti-inflammatory and free radical scavenging activities. Therefore these compounds were further characterized in a simulated I/R cellular model. Our findings suggest that their protective effects are at least partially reflected in the maintenance of mitochondrial function. Here, we report the design, synthesis and preliminary preclinical efficacy evaluation of novel kyotorphin-nitroxide hybrid molecules.

The parental nitronyl nitroxide derivative (NN-7) was synthesized according to Ullman's procedure with minor modification.[14–16] Synthesis was initiated with dinitro compound **1**, followed by the reduction with zinc in an ammonium chloride (Zn/NH_4Cl) buffered solution to yield the intermediate bis(hydroxylamine) compound **3**, which was subsequently subjected to condensation with 4-hydroxybenzylaldehyde to generate the tetramethylimidazoline derivative **4** with moderate yield (52%). Oxidation by PdO_2 yielded compound **NN-5**. Treatment of **5** with $BrCH_2COOC_2H_5$ followed by hydrolysis in the presence of NaOH (2 mol/L) provided **NN-7** in good yield (90%). Synthesis of kyotorphin-nitroxide hybrid molecules (**9a**–**j**) was straightforward, involving a simple coupling followed by deprotection (Scheme S1, SI). We employed hydrazinium monoformate as hydrogen donor, and magnesium as the catalyst, to facilitate the removal of some commonly used protecting groups during peptide synthesis. ESR spectra of these kyotorphin-nitroxide hybrid molecules all exhibited nitronyl nitroxide free radical characteristics with spectra showing a five-line pattern at a 1/2/3/2/1 ratio, and there was no difference among the compounds tested.

The newly synthesized kyotorphin-nitroxide hybrids (**9a**–**j**) were subjected to the tail flick assay to evaluate their analgesic activities. The tail flick is considered a spinal reflex, which is a commonly used acute pain (nociception) model.[18] The analgesic capacities were expressed by the pain threshold variation (PTV), defined as the difference of pain threshold after drug administration minus basic pain threshold/basic pain threshold. All the test compounds except aspirin were prescreened for analgesic activity at a dose of 0.1 μmol/kg. For each animal, PTVs were estimated at 30, 60, 90, 120, 150 and 180 min following vehicle or drug administration.

Our previous in vitro pharmacological studies of the peptide conjugates revealed that bioactivity was dependent on the linker type and the position of conjugation.[17–22] With respect to the anchoring site, and based on our previous studies, two different conjugation positions were considered. Initially, KTP (L-Tyr-L-Arg) was conjugated with nitronyl nitroxide (NN) at the N-terminus to form **9a** or **9b**, respectively. Using the tail flick in vivo pain model, we found that the PTV profile of compound **9b** at a dose of 0.1 μmol/kg was comparable to that of KTP, whereas compound **9a** exhibited moderate analgesic activity. Next, we further tested the importance of the individual amino acids for biological activities using single amino acid analogs. Initially, we attempted to replace Tyr by several Tyr analogs (3-methyltyrosine, 3-hydroxymethyltyrosine, 2-methoxytyrosine, 2-ethyltyrosine) to give compounds **9c**–**f**. Compared to **9a**, placement of a methyl group *ortho* to the phenol hydroxyl of Tyr (compound **9c**) significantly decreased PTV at all test time intervals. Compared to **9a**, *meta*-substitution of the tyrosine aromatic ring with an *O*Me group (**9e**) led to a significant diminution of PTV. Since it was uncertain whether the decreased activity with *O*Me resulted from the electronic effect of oxygen or the increased steric effect due to a larger group, the ethyl-substituted analog **9f** was evaluated. The PTV profile of the ethyl derivative was similar to the *O*Me analog, suggesting that electronic effects of the oxygen atom at the *meta*-position were non-disruptive. Theoretically, the presence of a hydroxylmethyl *ortho* to the phenol hydroxyl in compound **9d** has the potential to disrupt hydrogen bonding or augment hydrogen bonding to active site groups. However, the observed effect of this group was minimal, suggesting that potential H-bonding between the hydroxylmethyl and the phenol oxygen or active site groups did not play a major role. Taken together, the replacement at the phenolic functions of aromatic amino acids was considered detrimental to analgesic activity. We further evaluated replacement of arginine with Lys and Glu. Lys substitution (**9g**) had minimal effects. Compared to **9a**, Glu substitution (**9h**) resulted in a significant effect on PTV, as predicted. Additionally, conjugation of nitronyl nitroxide with KTP through a diamine linker proved to be a simple modification that converted kyotorphin into an effective analgesic molecule. Among all test compounds, compounds **9i**,**j** were shown to possess significant and longer acting analgesic activities as revealed by their PTV profiles. At the dose of 0.1 μmol/kg, a statistically significant increase of PTV was found for **9j**, and it simultaneously induced a long-lasting inhibition in the tail flick assay (Table 1).

Unlike KTP, a dose- and time-dependent inhibition was detected in the tail flick test for our new nitroxide hybrids, and we observed different pharmacokinetic parameters for each compound. For example, the maximal pain threshold for aspirin was observed after 60 min, whereas for kyotorphin-nitroxide hybrids (i.e., **9a**,**g**,**i**,**j**) the value was 120 min post drug administration. The general duration of the analgesic action of these compounds was approximately 180 min. No analgesic effects were observed after oral administration of KTP, NN-7 or saline. To investigate dose-dependency effects, the compounds with significant analgesic activities (**9i**,**j**) were further examined in the same assay. Mice were administrated 0.05, 0.10 and 0.15 μmol/kg of compound **9i**,**j** orally (Table 2). We observed a clear correlation between dose and analgesic activity. For example, even at the lowest dose of 0.05 μmol/kg, the PTV value of compound **9j** was still observed.

All kyotorphin-nitroxide hybrids were further evaluated for anti-inflammatory capacity in a xylene-induced ear edema model. Xylene-induced ear edema is widely used for rapid screening of compounds with anti-inflammatory capacity.[23–28] Each kyotorphin-nitroxide hybrid molecule was initially tested at