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Original Contribution

Reduction of paraquat-induced renal cytotoxicity by manganese and copper complexes of EGTA and EHPG

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Abstract

Superoxide anion generation plays an important role in the development of paraquat toxicity. Although superoxide dismutase mimetics (SODm) have provided protection against organ injury involving generation of superoxide anions, they often suffer problems, e.g., regarding their bioavailability or potential pro-oxidant activity. The aim here was to investigate and compare the therapeutic potential of two novel SODm, manganese(II) and copper(II) complexes of the calcium chelator ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) and of the contrast agent ethylenebis(hydroxyphenylglycine) (EHPG), against paraquat-induced renal toxicity in vitro. Incubation of renal NRK-52E cells with paraquat (1 mM) for 24 h produced submaximal, yet significant, reduction in cellular viability and cell death and produced significant increases in superoxide anion and hydroxyl radical generation. Manganese and copper complexes of EGTA (10–100 μM) and EHPG (30–100 μM) reduced paraquat-induced renal cell toxicity and reduced superoxide anion and hydroxyl radical generation significantly. Manganese complexes displayed greater efficacy than copper complexes and, at equivalent concentrations, manganese complexed with EHPG provided the greatest protection. Furthermore, these metal complexes did not interfere with the uptake of [*methyl*-^{14}C]paraquat into NRK-52E cells, suggesting that they provided protection against paraquat cytotoxicity via intracellular mechanisms. These complexes did not display cytotoxicity at the concentrations examined. Together, these results suggest that manganese and copper complexes of EGTA and EHPG, and especially the manganese–EHPG complex, could provide benefit against paraquat nephrotoxicity.

Keywords: Cytotoxicity; Cu(II)-EGTA; Cu(II)-EHPG; Hydroxyl radical; Kidney; Mn(II)-EGTA; Mn(II)-EHPG; NRK-52E; Oxidative stress; Paraquat; Renal; Superoxide anion; Superoxide dismutase mimetics; Free radicals

Paraquat (1,1′-dimethyl-4,4′-bipyridium dichloride, also known as methyl viologen) is a widely used broad-spectrum and fast-acting herbicide. However, it is extremely toxic, causing fatalities due to accidental or intentional poisoning, prevalently in developing countries [1,2]. Paraquat poisoning causes severe multiple organ failure, with the degree of poisoning dependent on the route of administration, the amount administered, and the duration of exposure. It is rapidly distributed within the body with highest concentrations accumulating within the kidneys, where it produces early and severe nephrotoxicity [3]. Additionally, as it is primarily excreted unchanged via the kidneys, the consequent reduction in renal function increases plasma concentrations by up to fivefold, which contributes to paraquat toxicity in other organs, especially the lungs [4,5]. Ultimately, respiratory failure, in the presence of nephrotoxic acute renal failure, is responsible for most deaths caused by paraquat [5–7]. Therefore, maintaining renal function in patients suffering from paraquat poisoning remains a therapeutically important treatment strategy.

Generation of reactive oxygen species (ROS), such as superoxide anions, plays a major part in the development of paraquat-induced toxicity [8,9] and especially nephrotoxicity [10,11].Current research has therefore focused on the therapeutic potential of antioxidants against paraquat-induced toxicity, especially those that can degrade superoxide anions such as superoxide dismutase (SOD) [12]. Unfortunately, the

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effectiveness of exogenously administered antioxidant enzymes such as SOD in vivo has been hindered by factors such as its poor bioavailability, low stability, or rapid hydrolysis in the blood and problems regarding its immunogenicity [13–15]. Furthermore, SOD itself can have pro-oxidant effects at higher concentrations [16–19], for example, via the possibility that Cu^{2+} derived from Cu/ZnSOD may facilitate the generation of oxidative stress in the presence of glutathione [17] or via the ability of superoxide to both initiate and terminate lipid peroxidation [19]. Subsequently, many different types of SOD mimetics (SODm) have been synthesized [15,20], including manganese(III) tetrakis(4-benzoic acid) porphyrin (MnTBAP) and M40401 (a manganese-containing SODm), which have been shown to reduce paraquat-induced lung and brain injury in the mouse and rat, respectively [21,22]. However, SODm also suffer problems regarding bioavailability and toxicity, e.g., their poor stability in vivo and pro-oxidant activities [23,24].

The calcium chelator ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA) has recently been shown to possess significant SOD activity [25] and has provided benefits in models of multiple sclerosis and Alzheimer disease [26,27]. Another chelator, ethylenebis(hydroxyphenylglycine) (EHPG), which is used as a contrast agent in imaging and as a transferrin mimic in the study of manganese transport [28,29], also exhibits SODm properties when complexed with manganese (Mn) (II) or copper (Cu) (II) [25]. A major advantage of the potential use of these agents for the treatment of disease is that EGTA and EHPG have already been used therapeutically [26–29]. Furthermore, Mn(II) and Cu(II) complexes of EGTA and EHPG are stable in solution and have a good safety profile in that they do not promote pro-oxidant activities [25].

To date, the protection afforded by Mn and Cu complexes of EGTA and EHPG on ROS generation and subsequent injury and death of renal cells has not been investigated. The aims of this study were to: (i) confirm the role of ROS in the development of paraquat-induced renal (NRK-52E) cell cytotoxicity and (ii) investigate and compare the therapeutic potential of EGTA and EHPG and their Mn(II) and Cu(II) complexes against paraquat-induced renal cytotoxicity in vitro. The mechanisms by which metal complexes of EGTA and EHPG could protect NRK-52E cells against paraquat toxicity were investigated, both by assessing the ability of these complexes to reduce ROS generation by paraquat and by examining the ability of the complexes to alter the uptake of paraquat into renal cells.

Materials and methods

Unless otherwise stated, all compounds used in this study were purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK). Mn(II) and Cu(II) were obtained from Sigma–Aldrich in the form of Mn(II) sulfate and Cu(II) chloride ($MnSO_4$ and $CuCl_2$). Mn(II) and Cu(II) complexes of EGTA and EHPG were synthesized as described previously [25].

Culture of NRK-52E cells

NRK-52E cells, which maintain characteristics of renal proximal tubular cells in culture [30], were obtained at passage 24 from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and used between passages 28 and 60. Cells were routinely cultured in 80-cm^2 Nunc flasks (Fisher Scientific, Loughborough, Leicestershire, UK) and grown in Dulbecco's modified Eagle's medium (DMEM; Cambrex BioScience, Wokingham, Berkshire, UK), supplemented with 10% (v/v) fetal bovine serum (FBS; Biosera, Ringmer, East Sussex, UK), 1% nonessential amino acid solution, 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified 5% carbon dioxide atmosphere. Culture medium was changed every 48 h. NRK-52E cells were subcultured at 90% confluence using a trypsin (0.1% w/v)/versene (0.02% w/v) mixture. For experiments, cells were subcultured and grown on 6- or 24-well Nunc plates (Fisher Scientific) in DMEM as described above except for the substitution of 5% (v/v) FBS. DMEM as described above but containing only 1% (v/v) FBS was used for incubations involving paraquat, metal complexes of EGTA and EHPG, or their individual components.

Measurement of paraquat toxicity

Confluent cultures of NRK-52E cells on 24-well plates were incubated for 24 h with increasing concentrations of paraquat (0.003–1 mM). In detail, culture medium was replaced with incubation medium, which consisted of DMEM containing 1% (v/v) FBS and the required concentration of paraquat. At the end of the incubation period, cellular viability and cell death were determined as described below. From these data, a concentration of 1 mM paraquat was chosen, as this produced a submaximal, but significant, reduction in cellular viability (approximately 90% reduction in mitochondrial respiration compared to untreated controls) and a significant increase in cell death (approximately 80% of the lactate dehydrogenase released by cells treated with Triton X-100).

Effects of Mn(II) and Cu(II) complexes of EGTA and EHPG on paraquat-induced toxicity in NRK-52E cells

To investigate the effects of the metal complexes of EGTA and EHPG on cellular injury and death caused by paraquat, confluent NRK-52E cells were preincubated (30 min at 37°C) with 900 μl DMEM containing 1% (v/v) FBS and increasing concentrations of Mn(II)-EGTA, Cu(II)-EGTA, Mn(II)-EHPG, or Cu(II)-EHPG (0.3–100 μM). After 30 min, 100 μl of a 10 mM stock solution of paraquat or its vehicle (DMEM containing 1% (v/v) FBS) was added to each well to provide a final concentration of paraquat of 1 mM. The concentration range of the complexes used was based on those of similar metal-based SODm which have been shown to provide protection of rat proximal cells against oxidative stress [31,32]. It was also assumed, based on previous investigations [25] and on the dosing profile of previous experiments using SODm and renal cells