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DNA damage induced by nitric oxide during ionizing radiation is enhanced at replication

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ABSTRACT

Nitric oxide (•NO) is a very effective radiosensitizer of hypoxic mammalian cells, at least as efficient as oxygen in enhancing cell death *in vitro*. •NO may induce cell death through the formation of base lesions which are difficult to repair, and if they occur within complex clustered damage common to ionizing radiation, they may lead to replication-induced DNA strand breaks. It has previously been shown that 8-azaguanine and xanthine result from the reaction of guanine radicals with nitric oxide. We have now shown that adenine radicals also react with •NO to form hypoxanthine and 8-azaadenine. Cells irradiated in exponential growth in the presence of •NO are twice as radiosensitive compared to those irradiated in anoxia alone, whereas confluent cells are less radiosensitive to •NO. In addition, the numbers of DNA double strand breaks observed as γH2AX staining following radiosensitization by •NO, are higher in exponential cells than in confluent cells. DNA damage, detected as 53BP1 foci, is also higher in HF-19 cells expressing Cyclin A, a marker for cells in S and G2 phases of the cell cycle, following radiosensitization by •NO. RAD51 foci are highest in V79-4 cells irradiated in the presence of •NO compared to in anoxia, 24 h after radiolysis. This work presents evidence that radiosensitization of cells by •NO is in part through the formation of specific DNA damage, difficult to repair, which in dividing cells may induce the formation of stalled replication forks and as a consequence replication-induced DNA strand breaks which may lead to cell death.

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Introduction

Radiotherapy is a common treatment regime for cancer; however survival is strongly correlated with tumour oxygenation as hypoxic cells are more difficult to kill. Ionizing radiation induces DNA damage in particular by reaction of the bases [1] with radiolytically formed hydroxyl radicals (·OH) to form adducts with the bases. Oxygen increases radiosensitivity, typically by a factor of 2–3, in part by reacting with these base radicals to form long-lived peroxyl radicals, which may interact with the adjoining sugar, and abstract a proton from the sugar residue forming a single strand break (SSB) [2]. In addition SSB are formed by the direct reaction of ·OH with the sugar moiety [3]. However, double strand breaks (DSB) are thought to be the main contributor to the lethal DNA damaging cellular effects. These are formed directly from ionizing radiation or through reaction with ·OH. Additionally SSB are generally repaired within one hour but when base modifications occur close to sites of SSB, repair can be difficult [4]. These clustered damage sites account for ~30% of damage induced by low linear energy transfer (LET) radiation [5] and if the SSBs within a clustered site encounter a replication fork, they may result in replication-induced DNA DSB [6,7] (Fig. 1).

For over 60 years the role of •NO as a radiosensitizer of mammalian cells has been investigated, for example [8–15]. However, the mechanisms of its action still remain largely unknown (see [16] for a recent review). One proposal is that •NO may react with DNA radicals formed by ionizing radiation [11,13,17–19] and in doing so form modified bases which may induce cytotoxic DNA damage. Indeed the rate of reaction of •NO with 2′-deoxyguanosine monophosphate (dGMP) hydroxyl radical adducts is of the order of 10^9 M^{-1} s^{-1} (pH 5.5, [13]), with kinetics very similar to those of oxygen [20], which has long been established as a radiosensitizer of mammalian cells [21]. In comparison to the reaction with O_2, the reaction of •NO with a free radical would generate a non-radical species and thus prevent further chain reactions, which occur with peroxyl radical products from equivalent reactions with O_2.

The identification of potential products formed in the reaction of DNA base radicals with •NO is necessary. Early studies have

Abbreviations: •NO, nitric oxide; ·OH, hydroxyl radical; dA, 2′-deoxyadenosine; 8oxoA, 8-oxoadenine; FaPyA, 4,6-diamino-5-formamidopyrimidine; HX, hypoxanthine; 8azaA, 8-azaadenine; dGMP, 2′-deoxyguanosine monophosphate; 8azaG, 8-azaguanine; SSB, single strand break; DSB, double strand break; LET, linear energy transfer; AP, apurinic/apyrimidic.

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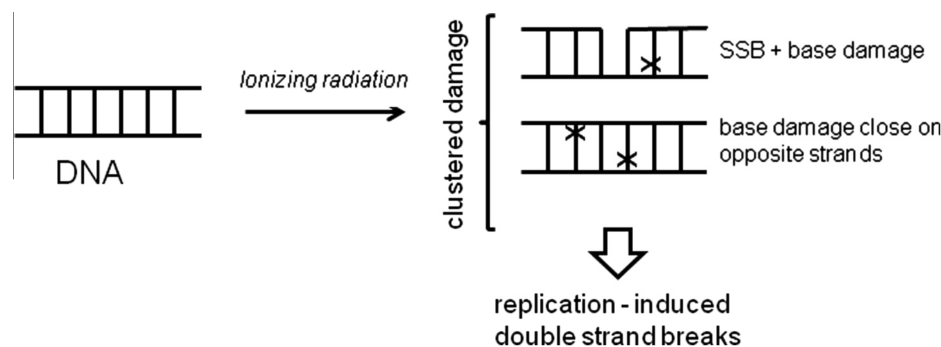

Fig. 1. Schematic of formation of clustered base damage in DNA following ionizing radiation which may lead to replication-induced double strand break.

proposed the formation of a nitroso adduct of uracil from the reaction of •NO with uracil-(·OH) [13]. In addition ·OH-adducts of guanine/dGMP react with •NO to form xanthine and 8-azaguanine (8azaG) modifications [19]. Studies with other DNA bases have not been carried out. A full understanding of the chemistry of reactions of •NO with other bases and nucleotides would be beneficial to gain a better understanding of the role by which •NO acts as a radiosensitizer.

Formation of base modifications, as a result of reactions with •NO, may be difficult to repair by conventional base excision repair, as they may not be recognized by cellular endonucleases, although this needs to be established. Previous studies measured the enhanced formation of γH2AX staining, as a marker of DSB, in hamster fibroblast cells and human breast cancer cells [13] and human prostate cancer cells [14] following radiosensitization by •NO. In V79-4 hamster fibroblasts following radiolysis in the presence of •NO it was found that the maximum number of DSB detected as γH2AX foci were formed at times >30 min when DSB are conventionally detected and at numbers 2-fold higher than those induced in anoxia alone [19]. The results suggested that the damage induced by •NO may be slow to repair. In addition, if these lesions are formed in clustered damage sites together with SSB as shown in Fig. 1 then subsequently formed DSB may arise during replication.

In this study we have investigated further if radiation-induced damage by •NO results in the formation of enhanced levels of replication-induced DSB. We also discuss preliminary investigations into changes which occur to adenine during γ-radiolysis in the presence of •NO under hypoxia and their potential role in replication-induced DSB.

Materials and methods

Materials

Dipotassium orthophosphate, potassium dihydrogen orthophosphate, sodium citrate and methanol (LC–MS grade) were obtained from Fisher, UK. 8-Azaadenine (8azaA) was obtained from MP Biomedicals, UK. DNA bases, nucleotides and nucleosides, bovine serum albumin (BSA), fish skin gelatin (FSG), paraformaldehyde (PFA), propidium iodide (PI), RNAase and cell culture solutions were obtained from Sigma–Aldrich (Poole, UK). Foetal calf serum (FCS) was obtained from Biosera, East Sussex, UK, phosphate buffered saline (PBS) tablets from Oxoid, UK and dissolved 1 tablet/100 ml. Anti phospho-histone H2AX mouse monoclonal IgG1 was obtained from Merck Millipore, USA. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 633 goat anti-rabbit IgG were obtained from Invitrogen, UK. Anti-phospho 53BP1 rabbit monoclonal antibody was obtainedfrom AbCam, UK and mouse anti-RAD51 was obtained from GeneTex, source Bioscience UK. Vectashield and anti-Cyclin A mouse monoclonal antibody were obtained from Vector Laboratories, UK. N_2O and N_2 were supplied by BOC and 1% •NO, balanced N_2 and 1% O_2, balanced N_2 from BOC special gases (Guildford, UK).

Generation of hydroxyl radicals by γ-irradiation

Radiolysis of water generates near equal amounts of oxidizing radicals (·OH) and reducing electrons (e^-_{aq}) along with low yields of protons (H^+), H·, H_2O_2 and H_2. Saturating solutions with N_2O before irradiation allows for e^-_{aq} generated through the ionization of water to be converted into ·OH (Eq. (1)) increasing the ·OH yield to ~0.6 μM Gy^{-1}. This also ensures that only the chemistry of ·OH is studied.

$$e^-_{aq} + N_2O + H^+ \rightarrow {}^{\bullet}OH + N_2 \quad (1)$$

Irradiations were carried out in a Caesium-137 GSR D1 irradiator (Gamma-service Medical GmbH, Leipzig, Germany) at dose rates confirmed by Super Fricke dosimetry [22].

Nitric oxide handling

•NO gas was handled as described previously [13]. Anaerobic conditions were maintained throughout the experimental procedure to prevent the formation of $•NO_2$ from reaction of •NO with O_2. Saturated solutions of 1% •NO/99 % N_2 in PBS are ~18 μM •NO at 25 °C [23] and 0.1% •NO is ~1.8 μM When the concentration of •NO required was <1% the gas was mixed with N_2O using a flow mixer and flow into the solutions was continued throughout the irradiation time, through ports in the side of the irradiator using PEEK tubing and stainless steel needles.

Reaction of adenine radicals with nitric oxide

Adenine (0.5 mM) was dissolved in potassium phosphate buffer (10 mM, pH 7.6) and saturated with N_2O for 10 min. Samples were then irradiated at 13 Gy/min for 2 min intervals with and without •NO (~1.8 μM) as described above. Products were analyzed by HPLC (Waters 2695, Watford, UK) equipped with a photodiode array detector (Waters 2996) and mass spectrometer (Waters micromass ZQ) and compared to commercially available standards. Chromatography used a Hichrom RPB column (3.2 × 250 mm, 5 μm) with a flow rate of 0.5 ml/min. Separation was achieved using 10 mM formic acid and methanol with a gradient of 3–10% methanol in 9 min and returning to starting conditions over 0.1 min. Mass spectrometry was used in electrospray positive or negative mode at 3 kV, with a cone voltage of 22 V.

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