

Review Article

# Ionizing Radiation Induced Nitric Oxide Signaling

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## Abstract

Radiotherapy is one of the most widely used forms of cancer therapy today used in the treatment of >60% of adult cancers. Treatment in many cases is limited as with other cancer therapeutics, due to normal tissue toxicity. Thus investigators are looking for ways to enhance the efficacy of radiation or to mitigate the damage to normal healthy tissues. Ionizing radiation stimulation of nitric oxide synthase activity has been studied extensively with conflicting results showing both cytotoxicity and cytoprotection. In this review experimental evidence is summarized suggesting that manipulation of nitric oxide signaling in combination with ionizing radiation, due to the dual nature of the cellular response to nitric oxide, has potential to enhance the anti-tumor efficacy of radiotherapy and mitigate damage to normal healthy tissue.

**Keywords:** Ionizing radiation; Nitric oxide synthase; ROS/RNS; Uncoupling

## Abbreviations

BH<sub>2</sub>: Dihydrobiopterin; BH<sub>4</sub>: Tetrahydrobiopterin; cGMP: Cyclic Guanosine Monophosphate; DSB: Double Strand Breaks; EGFR: Epidermal Growth Factor Receptor; eNOS: Endothelial Nitric Oxide Synthase; GTPCH-I: Guanidine Triphosphate Cyclohydrolase I; HIF1- $\alpha$ : Hypoxia Inducible Factor 1-Alpha; iNOS: Inducible Nitric Oxide Synthase; IR: Ionizing Radiation; nNOS: Neuronal Nitric Oxide Synthase; NO: Nitric Oxide; O<sub>2</sub><sup>-</sup>: Superoxide; ONOO<sup>-</sup>: Peroxynitrite; PKG: Protein Kinase G; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; sGC: Soluble Guanylate Cyclase; VEGFR: Vascular Endothelial Growth Factor Receptor

## Introduction

Ionizing Radiation (IR) is utilized in the treatment of a variety of tumors including breast, colon, lung and prostate. Exposure of mammalian cells to a clinically relevant dose of IR of approximately 2 Gy produces about 3000 DNA lesions: 850 pyrimidine lesions, 450 purine lesions, 1000 single strand breaks and 40 double strand breaks [1]. A hallmark of ionizing radiation is the formation of clustered damage sites, which include double strand breaks, characterized by two or more lesions within one or two helical turns of the DNA. These sites are thought to be the most cytotoxic lesions induced by IR [2,3]. DNA damage sensors within the nucleus detect this damage and initiate signal transduction pathways resulting in activation of cell cycle checkpoints and DNA damage repair. The cell's response involves a number of proteins including, but not limited to, ATM/ATR, DNA-dependent protein kinase, Chk 1/2 and p53, as well as the generation of reactive oxygen species/reactive nitrogen species (ROS/RNS), much of which may be attributable to the activation of nitric oxide synthase (NOS) [4-6].

Nitric Oxide (NO) is a highly diffusible regulator of several physiological processes; playing major roles in vasodilation, neurotransmission and the immune response. NO is produced in cells by Nitric Oxide Synthases (NOS). NOS are a group of calcium/calmodulin responsive enzymes (eNOS (NOS III), nNOS (NOS I), and iNOS (NOS II)) that catalyze the production of NO (and L-citrulline)

through the oxidation of L-arginine. NO has been shown in numerous investigations to be involved in the cellular response to IR. Leach et al. (2002) demonstrated low doses of IR activate a Ca<sup>2+</sup> dependent NOS, while many others have shown up-regulation of iNOS in a wide range of tumor cells and tissues (glioblastoma, breast, head and neck) post IR exposure [7,8].

At low concentrations, << 1 $\mu$ M, NO binds to the heme-containing Soluble Guanylate Cyclase (sGC) resulting in the formation of cGMP leading to Protein Kinase G (PKG) activation. The binding of NO to the heme moiety of sGC is a direct effect of NO, but indirect effects of NOS activation are also observed. The indirect effects occur through the generation of ROS/RNS, often at much higher concentrations of NO, and occur through the interaction of NO with ROS, such as superoxide (O<sub>2</sub><sup>-</sup>), generating different RNS. As NO is relatively stable and can diffuse readily throughout the cell, formation of RNS may be significant in the areas of greatest ROS generation; such as, the mitochondria and the plasma membrane near NADPH oxidases. Biologically relevant signaling from ROS/RNS has been demonstrated to occur through protein S-nitrosylation and tyrosine nitration [9-11].

While numerous studies have demonstrated the antitumor effects of NO, it also promotes angiogenesis and metastasis suggesting that the concentration of NO, location of NO, whether it is an endogenous vs. exogenous source, and the tumor microenvironment, may determine the eventual cellular response. In this review, we will address the role NO plays in the efficacy of radiotherapy in terms of the mode of cell death, the evasion of treatment (radioresistance), involvement in tumor regeneration, and use as a radiosensitizer as well as a possible mechanism for the apparent conflicting results seen in previous studies.

## Ionizing radiation induced NO promotes tumor cell toxicity

NO (or RNS) generated by ionizing radiation has been demonstrated to activate a number of stress proteins, including MAPK and JNK [12,13]. At low doses of IR or with low concentrations of RNS donors, activation of these pathways has been shown to be cytoprotective. We will discuss this more in the next section. At much

higher levels of NO/RNS achieved with induction of iNOS expression or treatment with high levels of NO/RNS donors, cell damaging effects are seen [14,15]. It has been demonstrated that the cytotoxic effects of NO/RNS, at least in part, are due to their direct interaction with DNA and lipids producing DNA and lipid radicals leading to cell cycle arrest and apoptosis [16].

Numerous studies have shown that the biological effects of IR are not only a result of the irradiated cell, but also the neighboring unirradiated cells. Results show that cells in the vicinity of the irradiated cells, bystander cells, respond in a similar fashion to the irradiated cell [17]. Not only are the irradiated tumor cells able to generate NO, but macrophages, being radioresistant, survive, get activated, and produce large amounts of NO [18,19].

Sokolov et al (2005) reported that irradiation of target cells induced  $\gamma$ -H2AX foci, a measure of double strand breaks (DSB) and DSB repair proteins, p53 ATM, Mre11, Rad50 and Nbs1, in bystander cell populations. The mechanism for the DSBs was not elucidated; however, pretreatment with the NO scavenger c-PTIO and Aminoguanidine (AG), a NOS inhibitor, abolished the effect [20]. Similar results have been published demonstrating the bystander effect in human glioblastoma T98G cells, where non-irradiated cells showed micronuclei induction by a process that was also blocked by c-PTIO and AG [21]. Shao et al (2003) went further to demonstrate that both NO and TGF- $\beta$ 1 are involved in the bystander effect in glioma cells. Treatment of the cells with AG reduced TGF- $\beta$ 1 to control levels, suggesting that these two factors are not independent. Further evidence for the involvement of TGF- $\beta$ 1 in the bystander effect has been shown by Arnold et al (1999). In this study they showed that the conditioned media from MCF-7 and MDA-MB-231 cells contained two-fold more TGF- $\beta$ 1 than that of un-irradiated cells [22].

Others have cited the necessity for NF $\kappa$ B induced iNOS resulting in elevated COX-2 expression as being involved in the radiation induced bystander effect [23]. The common denominator in all studies investigating the bystander effect is elevated NOS activity, but the mechanistic details are lacking. Future studies are needed to further elucidate the mechanism and the clinical relevance of the bystander effect.

### **Nitric oxide and nitric oxide synthase activity as a radiosensitizer**

A variety of factors likely play a role in determining the therapeutic outcome of IR including hypoxia and the tumor vasculature. Studies have demonstrated significantly reduced radiosensitivity in tumor cells under conditions of low oxygen [24-27]. NO may increase tumor blood flow and tumor oxygenation, enhancing radiosensitivity of tumors [28,29] however the exact mechanism is unclear [24]. There is substantial evidence in multiple different tumor types showing increased radiosensitivity in tumors treated with NO donors [30-35]. Similar evidence suggests increased NOS activity may also increase radiosensitization as well [36-39].

Evidence to the contrary has been published as well. Our group and others have shown NOS inhibition can sensitize tumors to radiation [40-42]. Treatment of squamous cell carcinoma xenografts with the combination of L-NNA and radiation decreased tumor

blood flow, leading to decreased growth in tumor cells, increased cytotoxicity of tumor cells as well as prolonged survival in mice [40]. NO and NOS activity may also play a dichotomous role in tumor cells and cells in the surrounding microenvironment. All of these studies illustrate the complexity in drawing conclusions in the actions of NO and NOS, either as a product of IR or in combination with IR.

### **Radiation-induced NOS activity is radioprotective in tumor cells**

Although IR activates pathways leading to apoptosis and cell death, there is increasing evidence, at least in a subset of tumor cells, that radiation can also activate proliferative and pro-survival pathways. Our group and others have shown activation of the epidermal growth factor receptor (EGFR) signaling pathway after radiation, a protective mechanism, potentially leading to radio-resistance [43-47]. Lee et al (2008) showed this radiation induced activation of EGFR was dependent on NOS [48]. A potential mechanism for the activation of EGFR may be the cysteine oxidation of SHP2 protein phosphatase, shown to dephosphorylate Tyr992/1173 on EGFR. Studies have demonstrated that Cys453 on SHP2 is S-nitrosylated by a mechanism dependent on the production of reactive oxygen and nitrogen species via NOS, and inhibited post IR [10,49].

Hypoxia-inducible factor 1-alpha (HIF1 $\alpha$ ) can also be stabilized via NO/ROS-dependent cysteine oxidation leading to the activation of pro-survival and proliferative pathways [50,51]. The activation of HIF1 $\alpha$  was also reported to cause the adaptation of glioma tumors to a more radio-resistant phenotype [52]. Matsumoto et al (2007) showed in human glioblastoma cells, treatment with exogenous NO as well as low dose IR contributed to a radioadaptive response. This radioadaptation was dependent on iNOS activity and levels [53].

Our lab has also demonstrated low dose IR and peroxynitrite activates NF- $\kappa$ B. Low dose IR-induced nitration of Tyr181 of I $\kappa$ B $\alpha$  causes I $\kappa$ B $\alpha$  to dissociate from NF- $\kappa$ B, activating the transcription factor. Inhibition of NOS activity with the non-specific NOS inhibitor, L-NNA, blocked nitration of I $\kappa$ B $\alpha$  and NF- $\kappa$ B activation [9]. This evidence suggests a combination of NO plus other ROS/RNS leads to an oxidative environment activating cytoprotective pathways decreasing the efficacy of radiation. A separate study showed that treatment of MCF-10A cells with low levels of RNS donors or co-culturing with activated macrophages results in the Tyr nitration and stimulation of PP2A activity leading to the down-regulation of BRCA1. A consequence is the reduction in homologous recombination DNA repair and enhanced non-homologous end-joining repair thereby promoting chromosomal instability, a hallmark of tumor progression [54]. The above evidence suggests that ROS/RNS may have multiple roles in tumor cell repopulation and acquired radioresistance but the actual mechanisms remain to be determined.

### **Radiation effects on surrounding cells in the tumor microenvironment**

Radiation also activates pro- and anti-survival pathways in surrounding stromal cells such as fibroblasts and endothelial cells in the tumor microenvironment. These activated stromal cells provide cytokines and growth factors necessary for the tumor cells to survive radiation. Sonveaux et al (2003) demonstrated that radiation increased migration and capillary formation of endothelial cells *in vitro* as well as enhanced angiogenesis in matrigel plug assays (*ex*

*vivo*). Treatment with the NOS inhibitor, L-NAME, abolished this radiation induced angiogenic effect [55]. Unpublished data in our lab demonstrates very similar results with 2H11 immortalized mouse tumor endothelial cells. In these experiments L-NNA blocked IR-induced migration of 2H11 cells, suggesting a possible mechanism for repopulation and angiogenesis post IR leading to radioresistance of tumor cells. IR has also been shown to activate vascular endothelial growth factor receptor (VEGFR) in multiple tumor types, enhancing post-irradiation angiogenesis [56,57]. A related phenomenon is that of irradiation induced angiosarcomas. Angiosarcomas are tumors derived from endothelial cells which have activated VEGFR and HIF1 $\alpha$  signaling. These types of tumors typically arise after breast irradiation but can be found in many different sites as well as different tumor types such as meningiomas and sarcomas [58,59]. As mentioned above, VEGFR, EGFR and HIF1 $\alpha$  all can be activated post irradiation via a mechanism dependent on ROS/RNS and NOS activity. Radiation also activates NF- $\kappa$ B and IL-6 production in mast cells and fibroblasts [60,61]. The activation of these cells enhances the inflammatory/pro-survival microenvironment of tumors, potentially leading to decreased efficacy of IR [62,63]

### NOS uncoupling in tumor cells

Important questions that arise from the above discussion on the dual nature of NO mediated cellular responses to IR are what is the mechanism(s) that account for this duality in responses and can this mechanism(s) be therapeutically manipulated to enhance the efficacy of radiotherapy? An area of NO biosynthesis that has yet to be explored in great detail in tumor biology is NOS uncoupling. The synthesis of NO occurs through NOS dimers and requires the substrate arginine along with NADPH and molecular oxygen ( $O_2$ ) as co-substrates. Tetrahydrobiopterin (BH4), FAD and FMN are required cofactors [64]. Tetrahydrobiopterin is a necessary cofactor for the production of NO from NOS. Evidence has shown that NOS's can generate  $O_2^-$  under certain pathophysiological conditions and current research indicates that the level of BH4 is important in regulating the balance of  $O_2^-$  and NO produced by NOS. A BH4 molecule binds in the oxygenase domain of each NOS monomer resulting in two BH4 molecules in the active dimer. In conditions where BH4 levels are low, electron transfer in the active site of the enzyme becomes uncoupled from L-arginine oxidation resulting in the production of  $O_2^-$  instead of NO [65,66]. The uncoupled enzyme therefore becomes a "peroxynitrite synthase", which is produced rapidly by the reaction of  $O_2^-$  with NO produced in the same area.

In diabetes, hypertension and atherosclerosis loss of NO production is a common feature accounting in part for the endothelial dysfunction associated with these inflammatory diseases. Recent experiments showed that under certain inflammatory conditions the cofactor BH4 is limiting and that this results in reduced NO bioavailability. Low levels of BH4 can be a result of the low levels of GTP cyclohydrolase-I (GTPCH-I), the rate-limiting enzyme in the production of BH4, or through direct oxidation of BH4 to dihydrobiopterin (BH2) in the face of enhanced ROS [67,68]. Evidence has shown that NOS's have an equal affinity for BH4 and BH2 but when BH2 is bound the NOS dimer is unstable and  $O_2^-$  production dominates [69].

It has been demonstrated that ischemia reperfusion injury results

in BH4 oxidation and it is ameliorated by exogenous BH4, suppressing NOS-derived superoxide [70]. A cells response to an IR event has been demonstrated to be similar in terms of the inflammatory response to that seen after vascular injury during ischemia reperfusion. Indeed, Berbee et al (2011) showed that mice exposed to 8.5Gy total body irradiation (TBI) displayed significantly decreased BH4 (pmol/mg protein) at 3.5 and 7 days post IR [71]. BH4 is currently being evaluated for protection from post-irradiation vascular oxidative stress [72]. Our lab has evaluated the BH4 precursor, Sepiapterin (SP), in the DSS/AOM induced mouse model of colorectal cancer. Here we demonstrated that animals treated orally with SP showed decreased tumor formation when given SP in conjunction with DSS and AOM. A hallmark of the tumors generated by this model was a reduced BH4:BH2 ratio compared to normal colon tissue, which we were able to increase with SP in the drinking water [73]. We also observed increased levels of cGMP with SP treatment sensitive to inhibitors of the NO-dependent sGC. It has been shown that metastatic breast, colon and lung cancers have increased levels of PDE 2 and/or PDE 5 compared to normal tissue [74]. An alternative approach to stimulating cGMP production through exogenous activators of sGC is through inhibition of PDE's that break down cGMP. Exisulind, sulindac sulfone (a metabolite of the NSAID sulindac), has been shown to have pro-apoptotic effects in SW480 and HT29 cells by inhibiting PDE's leading to elevated cGMP [75,76].

Given that NO signaling in cells is mediated in large part by the generation of ROS/RNS, whether or not NOS is coupled can have significant effects on tumor cell signaling and response to IR. When NOS is fully coupled, NO dependent signaling pathways such as sGC and PKG dominate; whereas when NOS is uncoupled, ONOO $^-$  and other ROS/RNS signaling pathways dominate (Figure 1). As discussed earlier, depending on the cellular environment and concentration, the latter may actually be cytoprotective. The state of NOS coupling may be the reason for the paradoxical effects of NO seen in irradiated tumors.

### Conclusion

In this short review, we have attempted to provide experimental evidence highlighting the role of NO in the cellular fate after exposure

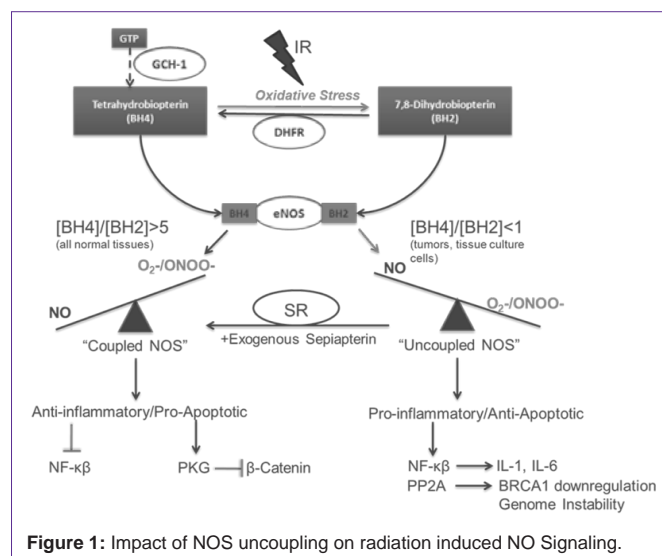


Figure 1: Impact of NOS uncoupling on radiation induced NO Signaling.

to an ionizing event as well as evidence suggesting the potential for manipulating NO signaling in combination with radiotherapy. NO can induce cellular damage by direct interaction with DNA, can protect from radiation-induced cell death and apoptosis by scavenging free radicals and inhibiting caspase activity as well as induction of a host of protective pathways through RNS signaling.

Further research is needed to determine the exact role of NO in the bystander effect as well as the therapeutic potential of NO as a radiosensitizer. Of particular interest to our lab is that radiation-induced ROS may decrease the cellular BH4 levels, leading to a prolonged stress response or even further activation of cytoprotective mechanisms. Future studies are needed to determine the exact role radiation plays in BH4 levels and NOS uncoupling in tumor cells during radiotherapy with the idea of continuing to characterize the dual nature of NO and the conflicting results observed.

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