Activation of Constitutive Nitric-oxide Synthase Activity Is an Early Signaling Event Induced by Ionizing Radiation

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Received for publication, October 26, 2001, and in revised form, January 31, 2002
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M110309200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 18, Issue of May 3, pp. 15400–15406, 2002
Printed in U.S.A.

Ionizing radiation at clinical dose levels activates both pro- and anti-proliferative signal transduction pathways, the balance of which determines cell fate. The initiating and amplifying mechanisms involved in the activation are poorly understood. We demonstrate that one mechanism involves stimulation of constitutive nitric-oxide synthase (NOS) activity. NOS activity of Chinese hamster ovary cells was measured by the arginine → citrulline conversion assay. Irradiation stimulated a transient activation of NOS with maximal activity at 5 min of post-irradiation. Western blot analysis and genetic manipulation by overexpression of wild type or dominant negative NOS mutant identify the radiation-induced isoform as NOS-1. Further evidence that NOS-1 is activated by radiation was the demonstration of radiation-induced cGMP formation in cells transiently transfected with the NO-dependent soluble guanylate cyclase. Protein Tyr nitration, a footprint of peroxynitrite formation, followed radiation exposure and was inhibited by expression of a dominant negative NOS-1 mutant. Radiation-induced ERK1/2 kinase activity, a cytoprotective response to radiation, was also blocked by inhibiting NOS activity. These experiments establish NO-dependent signal transduction pathways as being radiosensitive. Given the lipophilic and relatively stable properties of NO, these results also suggest a possible mechanism by which ionization events in one cell may activate signaling processes in adjacent cells.

Ionizing radiation activates several cytoplasmic signal transduction pathways involving Tyr kinases, protein kinase C, ERK-1/2, ceramide, and Ca2+-homeostatic mechanisms (1–8). Because these pathways represent both pro- and anti-proliferative signals, their relative balance can determine cell fate (2).

The underlying mechanisms by which cytoplasmic ionization events initiate these pathways are not known. There are only a few primary ionization events (~0.2600/Gy/cell) implicating sensitive detecting and amplifying mechanisms. Theoretical calculations rule out secondary products of the initial ionization events (e.g. H2O2 and O2) because the amount formed at clinical radiation doses (1–5 Gy) is much less than that generated by cellular metabolism (14). Recent studies, however, are consistent with amplification mechanisms responsive to low doses of ionizing radiation and involving ROS or RNS (15–17).

In our studies cellular ROS was measured with dihydro-DCF at the single cell level by fluorescence microscopy (15). ROS generation occurred within seconds of radiation exposure (1–10 Gy) and persisted for 2–5 min post-radiation. The amount of ROS generated per responding cell was relatively constant in this dose range. With increasing radiation dose there was a corresponding increase in number of cells generating elevated ROS, suggesting an all-or-nothing response. Genetic and pharmacological analyses indicated that the radiation-induced ROS resulted from the Ca2+-dependent propagation of a reversible permeability transition from one mitochondrion to another. A similar mechanism for enhanced ROS generation resulting from a reversible permeability transition has been described for cardiomyocytes (18).

RNS may also have a significant role in the response of cells to radiation. NO reacts with O2 to diffuse controlled rates competing with endogenous SOD for substrate (19). Inhibiting NOS activity or treating cells with NO scavengers stimulates oxidative stress (20–22). The product of O2 + NO, ONOO–, mostly rearranges to form biologically inert nitrite or reacts with GSH to form the NO donor GSNO (19, 23, 24). However, when the [NO] approaches that of SOD, the resulting high levels of ONOO– produce a number of cell-damaging effects (19, 24). Thus, radiosensitization is observed with exogenous NO donors or after cytokine stimulation of inducible NOS (NOS-2) expression (e.g. Refs. 25 and 26). A cellular footprint of ONOO– formation is the Tyr nitration of proteins (19, 27).

NO is formed during the NOS-catalyzed conversion of arginine to citrulline. Three isoforms of NOS with similar catalytic mechanisms have been described (28). The NOS-1 and -3 isoforms are constitutively expressed in many cell types, and their activities are Ca2+-calmodulin-dependent. NOS-1 and -3 produce relatively low amounts of NO compared with inducible NOS-2. NOS-2 tightly binds calmodulin, and its activity is largely Ca2+-independent. Here we demonstrate that low doses of radiation transiently activate NOS-1. Previous conclusions about radiation-induced ROS based on the use of DCF fluorescence measurements must be modified to include RNS and RNS-dependent downstream signaling.
EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The CHO-K1 cells were grown in RPMI 1640 plus 5% fetal bovine serum and antibiotics. Cells were transfected with the LipofectAMINE PLUS™ kit (Invitrogen) according to the manufacturer’s directions.

Reagents—Reagents and their suppliers are as follows: fluorescent probes (Molecular Probes, Eugene, OR); anti-nitro-Tyr antibodies (Upstate Biotechnology, Inc., Lake Placid, NY); anti-Mn-SOD antibody (Oxix Biochemical, CA); anti-ERK1/2, anti-Myc, anti-SHP-1, and anti-SHP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); secondary antibodies conjugated to alkaline phosphatase (Promega, Madison, WI); and [3H]arginine (ICN Biochemical). Plasmids for rat brain wild type (pnNOS) or dominant negative NOS-1 mutants (pHeme-RedF and pHeme) have been described (29). Plasmids encoding the α- and β-subunits of eNOS were provided by Dr. P. Yuen (30). Dr. C. Susini provided the pSHP-1 wild type and pSHP-1c/s plasmids. The latter Cys453 units of sGC were provided by Dr. P. Yuen (30).

Manufacturer’s protocol with cell lysates prepared from confluent 35-mm forms of the kinases (anti-phospho-p44/42 E10 monoclonal antibody, Cell Signaling Technology, Beverly, MA). Procedures followed the manufacturer’s protocol with cell lysates prepared from confluent 35-mm cell cultures.

ROS/RNS Production—Single cell analysis with a digitized imaging system, radiation with a 90Sr source mounted on the microscope, and dye loading conditions have been described (1, 15). Dihydro-DCF, which becomes fluorescent upon oxidation, is a well characterized dye for detecting ROS/RNS (15, 16, 33–35). The spectroscopic properties of DCF (excitation at 490 nm, emission at 530 nm) do not permit ratio-metric analysis and normalization for the sampling volume. To achieve the latter, cells were simultaneously loaded with fura-2, a Ca2+-sensitive dye, and fura-2 fluorescence was monitored at 530 nm with excitation at 360 nm, the Ca2+-insensitive, isosbestic excitation wavelength of fura-2 (15).

Immunoprecipitation/Western Blot Analyses—Cells from confluent 6-cm dishes were washed once with ice-cold phosphate-buffered saline and scraped into 300 μl of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)). Lysates were incubated overnight with 1 μg of precipitating antibody followed by incubation for 1 h with protein G plus agarose beads (Calbiochem). Beads were washed once in lysis buffer and twice in phosphate-buffered saline before boiling for 5 min in SDS sample buffer. Proteins were analyzed by standard Western blotting methods (7). Where quantitation was indicated, the radiograms were scanned and densities determined with Jandel SigmaScan software.

cGMP Measurements—Cells in 35-mm dishes were transfected with equal amounts (0.5 μg) of plasmids encoding the α- and β-subunits of sGC. Forty eight h after transfection cells were incubated for 30 min with 0.6 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterases and irradiated with 3 Gy. At the designated times, culture medium was aspirated, and cells were lysed with 300 μl of 0.1 M HCl. cGMP levels in the lysates were measured with the non-acetylated version of a competitive ELISA assay kit (Biomol, Plymouth Meeting, PA).

ERK1/2 Kinase Activity—An immune complex kinase assay with myc epitope as substrate was used to measure ERK1/2 kinase activity (7). Activation of ERK1/2 was also assessed by Western blots of cellular lysates with antibody specific for the phosphorylated, activated forms of the kinases (anti-phospho-p44/42 E10 monoclonal antibody, Cell Signaling Technology, Beverly, MA). Procedures followed the manufacturer’s protocol with cell lysates prepared from confluent 35-mm cell cultures.

RESULTS

Radiation Stimulates NOS-1 Activity in Epithelial Cells—CHO-K1 cells were irradiated at a clinically representative dose of 2 Gy. NOS activities were assessed at the indicated post-irradiation times by the amount of [3H]citrulline formed and retained in both irradiated and sham-irradiated cells. In the non-irradiated cells, the amount of cell associated [3H]citrulline was constant over the 30-min period investigated. However, in cells exposed to radiation, NOS activity (cell associated citrulline) was transiently enhanced with a maximal fold activation of 1.58 ± 0.11 at 5 min post-irradiation (Fig. 1A). By 10 min after radiation the fold activation had decreased to 1.33 ± 0.08 compared with non-irradiated samples. By 30 min, NOS activity had returned to basal levels.

Both pharmacological and genetic approaches were used to establish further this assay of cellular NOS activity. l-NMMA is a competitive inhibitor of all three NOS isoforms. As shown in Fig. 1A, incubating cells for 60 min prior to radiation with 1 mM l-NMMA inhibited radiation-induced NOS activity by more than 50%. Basal activity was equally inhibited by this treatment.

Another NOS inhibitor, N′-nitro-L-arginine, also inhibited basal and radiation-stimulated NOS activity (data not shown).

Previous studies (36) demonstrated that CHO-K1 cells express NOS-1. Thus we tested whether we could manipulate basal and radiation-induced NOS activities by overexpression...
of wild type NOS-1 or expression of a dominant negative mutant. CHO-K1 cells were transfected with expression plasmids encoding wild type rat brain NOS-1 (pNOS) or a dominant negative NOS-1 mutant (pHeme-RedF). The mutant protein encoded by pHeme-RedF prevents dimerization of endogenous NOS-1 monomers and thus inhibits NOS-1 activity (29). Relative expression levels of the wild type and mutant proteins detected by Western blot are shown in the inset to Fig. 1B. In the vector-control cells a band that migrates with the same mobility as wild type rat brain NOS-1 is just detectable relative to cells transfected with either pNOS or pHeme-RedF. Immunoreactivity of this band is inhibited by the peptide used to generate the NOS-1 antibody and is also reactive with other anti-NOS-1 antibodies but not antibodies directed against either NOS-2 or NOS-3 (data not shown).

Basal NOS activity of cells overexpressing wild type NOS-1 was not significantly different from vector control cells. However, radiation-stimulated NOS activity was enhanced in the NOS-1-overexpressing cells. In contrast, expression of the dominant negative NOS-1 mutant protein reduced basal NOS activity by 35 ± 3% and almost completely inhibited radiation-stimulated activity (Fig. 1B). Similar results were obtained with another dominant negative NOS-1 expression plasmid, pHeme (see Ref. 29 and data not shown).

To judge the relative quantitative significance of these radiation-induced changes in cellular NOS-1 activity, we compared the fold activation obtained with radiation with that observed after increasing intracellular [Ca$^{2+}$]. Cells were treated with the Ca$^{2+}$-ionophore, ionomycin, to maximally elevate intracellular [Ca$^{2+}$]$^2$ with or without ATP to activate purinergic receptors and as a consequence stimulate a transient increase in cytosolic [Ca$^{2+}$]$^2$. Incubating cells with 10 μM ionomycin or 400 nM ATP for 5 min caused 3.3 ± 0.3- and 1.5 ± 0.2-fold, respectively, increases in NOS activity ($n = 3$ independent experiments). These increases in NOS activity are comparable with those obtained with the radiation doses used here.

A previous study (36) reported that in CHO-K1 cells the protein-Tyr phosphatase, SHP-2, was capable of dephosphorylating and as a consequence activating NOS-1. We tested for a potential role for SHP-2 in radiation-mediated NOS-1 activation. NOS activity was measured by the arginine-citrulline conversion assay with CHO-K1 cells transiently transfected with plasmids encoding either wild type SHP-2 or the dominant negative Cys$^{459} ightarrow$ Ser SHP-2 mutant (32). Results in Fig. 2A show that expression of the Cys$^{459} ightarrow$ Ser SHP-2 mutant like the dominant negative NOS-1 mutant almost completely suppresses radiation-induced NOS activity. Overexpression of wild type SHP-2, on the other hand, further enhanced the observed stimulation of NOS activity by radiation (2.64 ± 0.66 versus $1.55 ± 0.05$). This enhancement in the radiation response was similar to that observed with cells overexpressing wild type NOS-1. The results from experiments with wild type SHP-2 and Cys$^{459} ightarrow$ Ser SHP-2 mutant expression parallel those obtained in previous studies (36). Similar experiments have also been performed with the wild type SHP-1 and Cys$^{459} ightarrow$ Ser dominant negative SHP-1 mutant, but the expression of either protein had no effect on radiation-stimulated NOS activity (Fig. 2B). We also tested whether NOS-1 in CHO-K1 cells was Tyr-phosphorylated as reported previously (36). Multiple attempts with different antibodies directed against phospho-Tyr and with or without immunoprecipitation of NOS-1 were unsuccessful in demonstrating Tyr-phosphorylated NOS-1. Treatment of cells with 1 mM orthovanadate, a Tyr phosphatase inhibitor, for up to 1 h also did not alter the phosphorylation status of NOS-1. These results suggest that some other protein whose activity is modulated by SHP-2 is required for regulating NOS-1 activity after a radiation exposure.

Ionizing Radiation Enhances Cellular cGMP Formation—An important downstream target of NO is the heme-containing sGC. Activation of sGC is therefore an indicator of NO formation. Because we were unable to detect cGMP in CHO-K1 cells even with a highly sensitive acetylation version of the ELISA kit for cGMP, cells were transfected with expression plasmids encoding SHP-1 wild type ($\triangledown$) and dominant negative Cys$^{459} ightarrow$ Ser (C/S) SHP-1 mutant (○), and 48 h post-transfection, cells were irradiated (2 Gy) and NOS activities measured with the arginine → citrulline conversion assay and compared with vector controls (●). The results are the average of triplicate samples ± S.E. of one experiment that is representative of two independent experiments. B, identical experiments except that cells were transfected with plasmids encoding SHP-1 wild type ($\triangledown$) and dominant negative Cys$^{459} ightarrow$ Ser (C/S) SHP-1 mutant (○) and compared with vector controls (●). The results are the average of triplicate samples ± S.E. of one experiment representative of two independent experiments.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Radiation-induced NOS activity is modulated by the Tyr phosphatase, SHP-2. A, cells were transfected with plasmids encoding SHP-2 wild type ($\triangledown$) and dominant negative Cys → Ser (C/S) SHP-2 mutant (○), and 48 h post-transfection, cells were irradiated (2 Gy) and NOS activities measured with the arginine → citrulline conversion assay and compared with vector controls (●). The results are the average of triplicate samples ± S.E. of one experiment that is representative of two independent experiments. B, identical experiments except that cells were transfected with plasmids encoding SHP-1 wild type ($\triangledown$) and dominant negative Cys → Ser (C/S) SHP-1 mutant (○) and compared with vector controls (●). The results are the average of triplicate samples ± S.E. of one experiment representative of two independent experiments.

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$^2$ R. B. Mikkelsen, unpublished data.
Radiation activates soluble guanylate cyclase. CHO cells were transfected with both the α- and β-subunits of sGC. 48 h post-transfection cells were incubated for 20 min with 0.6 mM 3-isobutyl-1-methylxanthine to inhibit cyclic nucleotide phosphodiesterases and then irradiated at 3 Gy (●). Control non-irradiated cells were otherwise treated identically (○). Cell lysates were prepared and cGMP measured as described under “Experimental Procedures.” The results are the average of duplicate samples ± S.E. from one experiment and are representative of two independent experiments. Cells transfected with either vector alone or with an empty vector contained no measurable cGMP.

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Fig. 3. Radiation activates soluble guanylate cyclase. CHO cells were transfected with both the α- and β-subunits of sGC. 48 h post-transfection cells were incubated for 20 min with 0.6 mM 3-isobutyl-1-methylxanthine to inhibit cyclic nucleotide phosphodiesterases and then irradiated at 3 Gy (●). Control non-irradiated cells were otherwise treated identically (○). Cell lysates were prepared and cGMP measured as described under “Experimental Procedures.” The results are the average of duplicate samples ± S.E. from one experiment and are representative of two independent experiments. Cells transfected with either vector alone or with an empty vector contained no measurable cGMP.

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Fig. 4. NOS inhibitors block radiation-induced ROS/RNS generation detected by DCF fluorescence. Experimental details are provided under “Experimental Procedures.” A, the results represent the average ± S.E. of the 6 cells responding (out of 20 total in the field) to radiation (4 Gy). B, no cells exhibited enhanced DCF fluorescence after the radiation exposure and thus the results represent the average ± S.E. for all 20 cells sampled in this experiment. B, cells were pre-equilibrated with 1 mM L-NAME for 20 min prior to radiation (4 Gy). H2O2 was added where indicated to 100 μM as a positive control. Results are from one experiment representative of 2 independent experiments.

Protein Tyr Nitration Increases After Radiation—Protein nitro-Tyr formation is considered to be a marker of ONOO− formation. Protein Tyr nitration was monitored by immunoprecipitation with mouse monoclonal anti-nitro-Tyr followed by immunoblotting with rabbit polyclonal anti-nitro-Tyr. Fig. 5A shows a time course in CHO-K1 cells for protein Tyr nitration after radiation. The protein nitro-Tyr levels of several proteins are transiently increased with maximal Tyr nitration at 5 min post-irradiation. Enhanced protein Tyr nitration after radiation treatment was also observed in a number of other cell lines including RAT-1 fibroblast, U87 glioma, HEK293, and A431 squamous carcinoma cells.

Control experiments (Fig. 5B) with inclusion of competing 10 mM 3-nitro-Tyr ethyl ester in with the immunoprecipitation or Western blot antibodies established the specificity of the antibodies for our cell system (37). Although 3-nitro-Tyr ethyl ester was an effective inhibitor of the immunoprecipitation of Tyr-nitrated proteins, inhibition was incomplete as observed by the developers of these antibodies (27). This contrasts with the complete inhibition in the Western blot analysis and probably reflects the relative differences in the antibody affinities and antigen presentation as discussed previously (27).

A previous study (37) demonstrated that Mn-SOD, a mitochondrial enzyme, is Tyr-nitrated during ischemia reperfusion. Thus, we tested whether Mn-SOD is nitrated following irradiation (Fig. 5C). At the indicated times post-irradiation nitro-Tyr-labeled proteins were immunoprecipitated, fractionated by gel electrophoresis, and resulting blots probed with anti-Mn-SOD. A single band at 26 kDa was revealed, the intensity of which increased and decreased as described with the other...
proteins. To show that this nitration of Mn-SOD is the result of radiation-induced NOS activity, similar experiments were performed with cells expressing the dominant negative NOS-1 mutant, Heme-RedF. As shown in Fig. 5D, the radiation-induced Tyr nitration of Mn-SOD is decreased by greater than 50% in the Heme-RedF-expressing cells compared with vector controls. Radiation-stimulated Tyr nitration of Mn-SOD was also inhibited by the NOS inhibitor L-NMMA (data not shown).

**Radiation Stimulates ERK1/2 by a NO-dependent Mechanism**—An early consequence of irradiation shown in several cell types is the activation of ERK1/2 kinases (3, 5, 7, 37). In cells where it has been examined, the radiation-dependent activation requires Ca²⁺ and an intact mitochondrial electron transport chain (7, 15). As do other cells, CHO-K1 cells exhibit a transient ERK1/2 activation centered at 3–5 min after irradiation and returning to basal levels by 10 min post-irradiation (Fig. 6A). Results in this figure also show that ERK1/2 stimulation is almost completely inhibited when cells are treated with the NOS inhibitor L-NMMA. In contrast, the ineffective stereoisomer D-NAME does not block radiation-induced increases in ERK1/2 activity. 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5-tetramethyl-1H-imidazol-1-oxo-3-oxide, a scavenger of NO (21), also prevents stimulation of ERK1/2 activity, further establishing a role for NO in the radiation-stimulated ERK1/2 activity (Fig. 6B). In an effort to determine whether NO directly activates ERK1/2 or if ONOO⁻ is involved in the activation of ERK1/2, we incubated the cells with a SOD mimetic, MnTBAP (21). Fig. 6B shows that MnTBAP abrogates ERK1/2 activation and implicates ONOO⁻ in the activation of ERK1/2.

Results from these NOS inhibitor studies were confirmed in analyses with cells expressing the dominant negative NOS-1 mutant or overexpressing wild type NOS-1. Activation of ERK1/2 was determined by Western blot analysis of lysates with monoclonal antibody directed against the phosphorylated activated forms of ERK1/2. Cells were transfected as described under "Experimental Procedures," and equal amounts of protein were loaded per lane. These results are from one experiment representative of three independent experiments.
radiation-induced activity by more than 50% in three independent experiments. This substantial but incomplete inhibition compared with that obtained with small molecule inhibitors probably reflects the less than 100% transfection efficiency (82 ± 16%, n = 5 independent experiments with a green fluorescent protein expressing plasmid). In addition, complete inhibition of NOS-1 activity may not be achieved with cells expressing low levels of the dominant negative mutant.

**DISCUSSION**

Previous studies (15–17, 38) have demonstrated that radiation stimulates metabolic ROS/RNS generation, a possible mechanism by which cells sense and amplify cellular ionization events. Evidence presented herein indicates that one component of this mechanism is a constitutive NOS. Both basal and radiation-induced NOS activities of CHO-K1 cells could be manipulated genetically or pharmacologically in a manner consistent with radiation-stimulating NOS-1. The degree of activation obtained with radiation compared favorably with that observed after treating cells with a Ca2+ ionophore or a purinergic receptor agonist. Downstream consequences of NO generation were assayed. We determined that radiation activated NO-dependent sGC in a time frame consistent with the measurements of NOS activity. In addition, radiation exposure also enhanced protein Tyr nitration, a footprint of ONOO– formation. For one protein examined in detail, radiation-induced Tyr nitration of Mn-SOD was inhibited by NOS inhibitors or by expression of a dominant negative NOS-1 mutant. The combined results demonstrate that ionizing radiation at doses of clinical relevance activate NOS-1.

The mechanistic relationship between the radiation-induced reversible mitochondrial permeability transition described previously (15) and NOS-1 activation is unclear. One possibility lies in localized Ca2+ transients that propagate the permeability transition and can simultaneously activate the Ca2+-dependent NOS-1. This mechanism is supported by the recent finding that NOS-1 can associate with mitochondria via its PDZ domain (39) putting NOS-1 at the origin of mitochondria-released Ca2+. Possibly also involved in this process is the ROS-induced ROS release that accompanies the mitochondrial permeability transition and that may be necessary for its propagation at least in cardiomyocytes (18). Previous studies (36) and results presented here also demonstrate that the protein-Tyr phosphatase SHP-2 modulates NOS-1 activity (Fig. 2). Overexpression of wild type SHP-2 enhances whereas the dominant negative SHP-2 mutant blocks radiation-stimulated NOS activity. This appears counterintuitive because ROS and RNS inhibit Tyr phosphatases (e.g. Ref. 40), and one would predict that ROS generated by radiation or metabolism would inhibit SHP-2 and thus NOS-1. Ongoing studies are attempting to resolve the role of SHP-2 in radiation-induced NOS-1 activation.

NO is the prototypic redox signaling molecule being more versatile than either O2 or H2O2 (e.g. Refs. 19, 41, and 42). The best described NO-dependent signal transduction pathway involves sGC and the cGMP-dependent PKG. The role of this pathway in the radiation biology of mammalian cells is not known because most cells cultured in vitro do not express key components of this pathway (Refs. 43–47, e.g. sGC for CHO-K1 cells). However, PKG modulates signaling pathways activated by radiation including ERK1/2, p38, and c-Jun NH2-terminal kinase (43–48) and the transcriptional regulation of proteins whose expression is response-dependent (e.g. c-Fos and p21

RNS also induce a number of reversible protein thiol modifications including S-nitrosylation and formation of sulfenic acids and both intramolecular (S–S) and mixed (S–SR, e.g. glutathiolation) disulfides (41, 51). This of special importance to radiation-induced activation of ERK1/2 which we have shown is RNS-dependent (Fig. 6). Both positive and negative regulatory components of ERK1/2 are potentially involved. The GTP exchange activity of Ras is enhanced by nitrosylation of Cys115 resulting in enhanced downstream signaling and ERK1/2 activity (52, 53). On the other hand by dephosphorylating ERK1/2, protein-Tyr phosphatases inhibit ERK1/2 activation. The catalytic sites of protein-Tyr phosphatases are characterized by a Cys within a consensus motif for S-nitrosylation (54). Oxidation of this Cys inhibits phosphatase activity (40). Thus S-nitrosylation of either Ras or a protein-Tyr phosphatase could result in the enhanced ERK1/2 activity observed after radiation. We are investigating both as mechanisms for the radiation-induced modulation of Tyr kinase-dependent pathways including ERK1/2.

Radiation-induced protein Tyr nitration of several proteins is also observed, but its functional significance is unclear (Fig. 5). In the case of Mn-SOD, high concentrations of ONOO– catalyze nitration on Tyr24 (9) resulting in inactivation. However, results from an in-gel assay for Mn-SOD activity did not indicate an effect of radiation on Mn-SOD activity in the time and dose ranges studied here (data not shown). Tyr-nitrated proteins are preferentially degraded by proteasomes, and this may explain why the radiation-stimulated protein Tyr nitration is transient (10). There is no definitive molecular evidence for mammalian denitrases (11, 12).

The significance of Tyr nitration may lie more as being diagnostic for ONOO– formation. Although high concentrations of ONOO– can cause specific DNA base lesions, ONOO– is highly unstable and mostly isotermizes to relatively innocuous nitrates and nitrites (19). Wink et al. (13) propose that this is a mechanism by which NO protects cells from oxidative DNA damage caused by O2 and H2O2.

The uniqueness of NO as a redox signaling molecule resides in part in its relative stability and hydrophobic properties that permit its diffusion across cell membranes over several cell diameter distances (19). The present study demonstrating radiation-stimulated NO generation suggests mechanisms by which an ionization event in one cell can be sensed in neighboring cells. This may have significance not only for radiotherapy but may represent one mechanism by which cells sense and initiate trans-cellular signaling critical for tissues in responding to localized oxidative events, e.g. metabolic ROS generation, hypoxia, or low level environmental radiation.

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J. Biol. Chem. 2002, 277:15400-15406. doi: 10.1074/jbc.M110309200 originally published online February 20, 2002

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