Activation of eNOS in endothelial cells exposed to ionizing radiation involves components of the DNA damage response pathway.
Activation of eNOS in endothelial cells exposed to ionizing radiation involves components of the DNA damage response pathway

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Abstract

In this study, the involvement of ataxia telangiectasia mutated (ATM) kinase and heat shock protein 90 (HSP90) in endothelial nitric oxide synthase (eNOS) activation was investigated in X-irradiated bovine aortic endothelial cells. The activity of nitric oxide synthase (NOS) and the phosphorylation of serine 1179 of eNOS (eNOS-Ser1179) were significantly increased in irradiated cells. The radiation-induced increases in NOS activity and eNOS-Ser1179 phosphorylation levels were significantly reduced by treatment with either an ATM inhibitor (Ku-60019) or an HSP90 inhibitor (geldanamycin). Geldanamycin was furthermore found to suppress the radiation-induced phosphorylation of ATM-Ser1181. Our results indicate that the radiation-induced eNOS activation in bovine aortic endothelial cells is regulated by ATM and HSP90.

Key words

Ionizing radiation, endothelial nitric oxide synthase, ATM, HSP90, DNA damage response
Introduction

Nitric oxide (NO), which is derived from L-arginine in an oxidizing reaction catalyzed by NO synthase (NOS), serves multiple functions including vasodilation, neurotransmission, and immune defense [1]. In mammalian cells, three distinct isoforms of NOS have been identified: neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Among these isoforms, eNOS is the major source of NO production in endothelial cells [2]. We have previously demonstrated that ionizing radiation increases eNOS activity, and that endogenous NO production increases tissue perfusion in a murine squamous cell carcinoma SCCVII tumor model, leading to radiation-induced tumor reoxygenation [3].

Tumor oxygenation status has been shown to be strongly correlated with the therapeutic outcome after radiotherapy, and NO-mediated modulation of tumor blood flow may be a promising approach for improving cancer radiotherapy [4]. Therefore, to optimize clinical radiotherapy, it is important to understand the underlying mechanisms of radiation-induced eNOS activation. Moreover, Sonveaux et al. showed that radiation-induced eNOS activation facilitates angiogenesis in vascular endothelial cells after X-irradiation [5], and NO-mediated protein nitration has been shown to have an influence on some signal transduction pathways e.g. IκBα on NFκB pathway [6] or TIMP-1 on Akt pathway [7]. These data indicate that radiation-induced NO plays various roles in determining the characteristics of solid tumors.

Several serine (Ser) and threonine (Thr) residues of the eNOS protein can be phosphorylated. In particular, the phosphorylation of serine 1179 of eNOS (eNOS-Ser1179) by Ser/Thr kinases is a critical step for the activation of the protein [8]. Ionizing radiation induces DNA double strand breaks (DSBs) and subsequently induces an adaptive response toward ionizing radiation, known as the DNA damage response (DDR). In the DDR, ataxia telangiectasia mutated (ATM) kinase plays roles as a key regulator of DNA repair, cell-cycle
arrest, apoptosis, senescence, transcription, and metabolic signaling. Ser/Thr kinases such as Akt, protein kinase A (PKA), and AMP-dependent protein kinase (AMPK), as well as checkpoint kinase (Chk) 1/2 have been shown to be activated after DNA damage [9]. It has also been reported that Akt, PKA, AMPK, and Chk1 are candidate Ser/Thr kinases for eNOS-Ser1179 phosphorylation [10,11].

Recent studies have demonstrated that the heat shock protein 90 (HSP90), a chaperone protein involved in protein trafficking and folding, stabilizes the MRE11–RAD50–NBS1 (MRN) complex which serves as a primary sensor of DSBs and participates in ATM activation [12]. HSP90 is a constitutively expressed molecular chaperone that guides the normal folding, intracellular disposition, and proteolytic turnover of many key regulators of cell growth and survival.

These reports have led us to speculate that ATM and HSP90 may be involved in the radiation-induced eNOS activation during DDR. In this study, we successfully achieved our aim of testing the above hypothesis by evaluating the activity and phosphorylation of eNOS in bovine aortic endothelial cells (BAECs) exposed to X-rays.
Material and methods

Cell culture and X-irradiation

BAECs were obtained from Cell Systems (Kirkland, WA) and maintained in Dulbecco’s Modified Eagle Medium (Life Technologies Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Cells between passages 5 and 9 were used. X-Irradiation was performed with a PANTAK HF-350 X-ray generator (Shimadzu, Kyoto, Japan) at 2.55 Gy/min. In some experiments, cells were treated with Ku-60019 (Selleck Chemicals, Houston, TX) and geldanamycin (Focus Biomolecules, Fort Washington, PA) 1 h prior to X-irradiation.

NOS activity assay

NOS activity was measured using a NOS Activity Assay Kit (Cayman Chemical, Ann Arbor, MI) based on the conversion of $[^{14}\text{C}]$ L-arginine (Perkin-Elmer, Boston, MA) to $[^{14}\text{C}]$ L-citrulline by NOS. In brief, 5 µl protein extract was added to reaction buffer in a total volume of 50 µl and incubated for 60 min at 37°C. The reaction was terminated by the addition of 400 µl stop buffer. Samples were then passed through a cation-exchange resin and $[^{14}\text{C}]$-radioactivity in the flow-through was measured using a liquid scintillation counter (LSC-6100; Hitachi-Aloka, Tokyo, Japan). NOS activity was calculated as follows: NOS activity (pmol/µg/min) = $[^{14}\text{C}]$-radioactivity in the flow-through (CPM)/total protein amount (µg)/specific radioactivity of $[^{14}\text{C}]$ L-arginine (Bq/pmol). The NOS activity obtained from each sample was normalized to that of the nonirradiated sample. To measure eNOS activity, the assays were performed in the presence or absence of a general NOS inhibitor $\text{N}^\text{G}$-nitro-L-arginine methyl ester (L-NAME; DOJINDO, Kumamoto, Japan), or an
iNOS-specific inhibitor 1400W (Wako Pure Chemical Industries, Osaka, Japan).

**SDS-PAGE and western blotting**

Immunoblot analysis was performed according to the previous report [13]. The following antibodies were used for western blotting and immunocytochemistry: eNOS, eNOS-pSer1179 (Catalog nos, ab66127 and ab87750, Abcam, Cambridge, MA), ATM-pSer1981 (Catalog no, 200-301-500, Rockland Immunochemicals, Gilbertsville, PA), HSP90α (Catalog no, 8165, Cell Signaling Technology, Beverly, MA), eNOS-pThr497, and actin (Catalog nos, sc-19827 and sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA). After probing with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), bound antibodies were detected with Western Lightning® Plus-ECL (Perkin-Elmer).

**Immunocytochemistry**

Immunocytochemistry was performed as previously described [13]. Briefly, cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% goat serum. Cells were then incubated with anti-ATM-pSer1981 antibody at 4°C overnight, after which cells were incubated with Alexa Fluor® 488-conjugated secondary antibody (Life Technologies Corporation) for 1 h in the dark. Coverslips were counterstained with 300 nM 4’,6’-diamidino-2-phenylindole (DAPI) (Life Technologies) and mounted with Prolong® Gold antifade reagent (Life Technologies). Images were acquired with an LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) controlled by Zeiss Efficient Navigation software.
**Statistical analysis**

All values are expressed as means ± S.D. of three to five independent experiments. Differences between groups were evaluated by Student’s t-test (two-sided) and differences were considered to be significant at P <0.05.
Results

Ionizing radiation increases NOS activity

The effect of radiation on NOS enzymatic activity in BAECs was evaluated. As shown in Fig. 1A, NOS activity gradually increased in cells exposed to 10 Gy of X-rays. The NOS activity in BAECs at 24 h after irradiation was 1.6±0.4 times higher than that of nonirradiated cells. Furthermore, NOS activity was upregulated at 12 h after irradiation with 5, 10, and 15 Gy of X-rays (Fig. 1B). These findings indicate that NOS activation is induced by ionizing radiation. To determine the NOS isoform responsible for radiation-induced NO production, the effects of a global NOS inhibitor (L-NAME) and an iNOS-specific inhibitor (1400W) on NOS activity were assessed. In the absence of NOS inhibitors, ionizing radiation increased NOS activity in whole BAEC lysates. L-NAME treatment significantly inhibited radiation-induced NOS activation, whereas 1400W did not (Fig. 1C). It was also found that 1400W completely inhibits the activity of iNOS purified from murine macrophages at 10 μM (data not shown). Taken together, these results indicate that iNOS is not involved in radiation-induced NOS activation in BAECs.

Ionizing radiation alters eNOS phosphorylation status

Since the activities of the constitutively-expressed NOSs are known to be finely regulated by posttranslational modifications such as Ser/Thr phosphorylation [1], the phosphorylation status of eNOS following irradiation was evaluated. Western blot analysis revealed that eNOS-Ser1179, which is especially known as a critical activation site for eNOS activity, was phosphorylated 3 h after irradiation, and that this phosphorylation was sustained up to 24 h after irradiation (Figs. 1D and E). In contrast, the other phosphorylation site eNOS-Thr497, an inhibitory one for eNOS activity [14], was dephosphorylated 12 h and 24 h
after irradiation. These results suggest that ionizing radiation induces eNOS activation by regulating its phosphorylation status.

**ATM phosphorylation modulates eNOS activation after irradiation**

It is well known that radiation-induced DSBs trigger the autophosphorylation of ATM-Ser1981 and that this autophosphorylated ATM is an active form of the kinase, which triggers DDR by downstream signal transduction. Immunocytochemical analysis showed that phosphorylation of ATM-Ser1981 in BEAC nuclei was increased 1 h and 6 h after irradiation, and was completely abolished by the ATM inhibitor Ku-60019 (Fig. 2A). KU-60019 was also found to inhibit eNOS-Ser1179 phosphorylation 6 h after irradiation in a concentration-dependent manner (Fig. 2B). Furthermore, 10 μM Ku-60019 significantly inhibited the increase in NOS activity 12 h after irradiation (Fig. 2C). These results suggest that ATM phosphorylation plays a role in Ser1179 phosphorylation of eNOS and NOS activation after irradiation.

**HSP90 is involved in ATM phosphorylation and subsequent eNOS activation after irradiation**

Several studies have shown that HSP90 is involved in the repair of radiation-induced DSBs through DDR [12,15]. To confirm the role of HSP90 in radiation-induced eNOS phosphorylation in BAECs, the effect of an HSP90 inhibitor (geldanamycin) on radiation-induced ATM-Ser1981 phosphorylation and eNOS-Ser1179 phosphorylation was evaluated. Immunocytochemical analysis clearly showed that geldanamycin treatment results in the inhibition of ATM-Ser1981 phosphorylation 6 h after irradiation (Fig. 3A), suggesting that ATM-Ser1981 phosphorylation is modulated by HSP90 modulates after irradiation. Western blot analysis showed that radiation-induced eNOS-Ser1179 phosphorylation (Fig. 3B) and
radiation-induced NOS activation (Fig. 3C) are substantially inhibited by geldanamycin at concentrations ≥20 nM. These results suggest that HSP90 regulates eNOS phosphorylation through the phosphorylation of ATM.

**Phosphorylated ATM translocates to cytoplasm after irradiation**

As shown in Figure 1E, the phosphorylation of eNOS-Ser1179 begins to increase 3 h after irradiation and peaks 6 h after irradiation. The phosphorylation of ATM-Ser1981, however, is known to occur to initiate DNA repair within several minutes after irradiation [16]. This indicates that a substantial time lag exists between eNOS phosphorylation and ATM phosphorylation after irradiation, although this ATM phosphorylation was strongly involved in phosphorylation of eNOS-Ser1179 and NOS activity. Accordingly, the time course of phosphorylation of ATM-Ser1981 and its intracellular distribution in irradiated BAECs was evaluated. As shown in Figure 4A, western blotting demonstrated that ATM phosphorylation is increased 5 min after irradiation, and high-level phosphorylation persists for at least 360 min after irradiation. Immunocytochemical analysis, on the other hand, indicated that the phosphorylation of ATM-Ser1981 was observed only in the nucleus and not in the cytoplasm 10 min and 60 min after irradiation. Interestingly, ATM-Ser1981 phosphorylation in the cytoplasm were clearly detected 180 min and 360 min after irradiation (Fig. 4B). These findings suggest that radiation-induced eNOS activation is associated with the cytoplasmic translocation of phosphorylated ATM-Ser1981.
Discussion

The aim of this study was to clarify the involvement of DDR in radiation-induced eNOS activation. Our findings demonstrate that the phosphorylation of eNOS-Ser1179 and the activation of eNOS after irradiation are modulated by ATM. Figure 1A shows that NOS activity is upregulated 12 h and 24 h after 10 Gy of X-irradiation. Furthermore, radiation-induced NOS activation was inhibited by a global NOS inhibitor (L-NAME), but not by an iNOS-specific inhibitor (1400W), and phosphorylation of eNOS-Ser1179 begins to increase 3 h after irradiation (Fig. 1E). The dose of 20 mM of L-NAME which we used in this study seems to be relatively high. However, same concentration was adopted in other studies [17,18] and was also necessary for inhibition of radiation-induced NOS activation in ex vivo samples from SCCVII tumors in our previous report [3]. In this experiment, L-NAME at 20 mM showed significant inhibition against the increase of NOS activity induced by X-irradiation, but substantial NO activity still remained; the enzyme responsible for this rest activity was unclear at present. Nevertheless, the present study disclosed that the increased NOS activity was at least derived from constitutive NOS but not iNOS. Phosphorylation of eNOS-Ser1179 is reported to stimulate the flux of electrons within the catalytic domain, leading to eNOS activation [1]. On the other hand, it was found in this study that the dephosphorylation of phosphorylated eNOS- Thr497 occurs 12 h after irradiation; this dephosphorylation occurs considerably later than the phosphorylation of eNOS-Ser1179 (Fig. 1E). This dephosphorylation has been reported to facilitate the flow of electrons from NADPH at the catalytic site of eNOS [2], and is thus indicative of an enhancement of eNOS activity. The precise mechanism of this dephosphorylation process is not yet clear, but eNOS-Thr497 phosphorylation reportedly occurs to some extent under basal conditions. Together with the results of both phosphorylation sites, eNOS-Ser1179 phosphorylation and eNOS-Thr497 dephosphorylation contribute its NOS
activation coordinately, resulting in the belated increase of NOS activity at 12 h.

In ischemia/reperfusion [19] and oxidative stress such as H₂O₂ exposure [20], the phosphoinositide 3-kinase (PI3K)/Akt pathway is involved in the phosphorylation of eNOS-Ser1179. From recent research, it is becoming increasingly clear that Akt is not the only protein kinase that phosphorylates eNOS-Ser1179. Other protein kinases, including protein kinase A (PKA), protein kinase G (PKG), AMPK, and Chk have also been shown to be associated with eNOS-Ser1179 phosphorylation [11,21,22,23]. In our study, the strong inhibitory effect of the ATM inhibitor Ku-60019 on the phosphorylation of ATM (Fig. 2) demonstrated that the phosphorylation of eNOS-Ser1179 is tightly regulated by ATM kinase. PI3K inhibitors (wortmannin and LY294002) and a Chk2 kinase inhibitor (2-arylbenzimidazol derivative [24]) were found not to inhibit phosphorylation of eNOS-Ser1179 in BAECs (data not shown), suggesting that the PI3K/Akt pathway and Chk2, a known downstream kinase of ATM, are unlikely to be involved in the radiation-induced phosphorylation of eNOS-Ser1179 in BAECs. Therefore, it is likely that radiation-induced ATM indirectly induces the phosphorylation of eNOS-Ser1179 via the activation of a kinase such as PKA, PKG, or AMPK. Furthermore, the direct phosphorylation of eNOS-Ser1179 by active ATM cannot be ruled out: an in silico prediction of site-specific kinase-substrate relationships using KinasePhos 2.0 [25] indicated the possibility of direct phosphorylation of eNOS-Ser1179 by active ATM itself (http://kinasephos2.mbc.nctu.edu.tw/index.html) (data not shown).

Phosphorylation levels of eNOS-Ser1179 were found to begin increasing 3 h after irradiation and to peak 6 h after irradiation (Figs. 1D and 1E). On the other hand, ATM-Ser1981 phosphorylation occurred immediately (within 5 min) after irradiation (Fig. 4A), while it took 3 h for the translocation of phosphorylated ATM-Ser1981 from the nucleus to the cytoplasm (Fig.4B). In irradiated BAECs, eNOS phosphorylation occurred considerably later than ATM
phosphorylation, and eNOS phosphorylation coincided with the cytoplasmic translocation of active ATM. In endothelial cells, eNOS expression and localization is known to be mainly cytoplasmic [1], and thus the cytoplasmic translocation of active ATM after irradiation may trigger the activation of other Ser/Thr kinases responsible for the phosphorylation of eNOS-Ser1179 in the cytoplasm. Regarding the mechanisms of this ATM translocation, Hinz et al. have reported that Ca²⁺-dependent signaling is involved in the translocation of ATM to the cytoplasm and cell membrane after irradiation [26]. Previous studies have demonstrated irradiation-induced elevation of intracellular Ca²⁺ concentrations in Jurkat cells, keratinocytes, and MOLT-4 cells [27,28]. Radiation-induced elevation of intracellular Ca²⁺ concentration may thus be linked with the cytoplasmic translocation of ATM observed in this study.

Vast numbers of HSP90 client proteins are involved in modulating cellular signaling pathways [29]. Thus far, it has been reported that HSP90 stimulates the NO production through the association with Akt and eNOS directly [30] and the aid to Akt-driven phosphorylation of eNOS protein [31]. In accordance with these studies, the present study revealed that geldanamycin inhibited eNOS phosphorylation and its activation following irradiation (Figs. 3B and 3C). Interestingly, Geldanamycin was also shown to attenuate nuclear ATM phosphorylation after irradiation (Fig. 3A), suggesting a regulatory role for HSP90 upstream of ATM phosphorylation. Recently, Park et al. have shown that nuclear HSP90 interacts with the MRN complex required for ATM activation at DSB sites [12]. This study may lend support to our results showing that the radiation-induced phosphorylation of eNOS-Ser1179 in this study occurred independently of the PI3K/Akt pathway and that geldanamycin was found to abolished not only NOS activity but also the phosphorylation of ATM-Ser1981 (Fig. 3).

In conclusion, this study provides new insights into the mechanism by which the HSP90/ATM pathway is involved in the activation of eNOS in irradiated BAECs. Notably, we
demonstrated for the first time that DDR is involved in the eNOS-Ser1179 phosphorylation. Further study is needed to identify the kinases responsible for the observed radiation-induced eNOS phosphorylation. The data reported here is an important contribution to a clearer understanding of the mechanisms by which radiation-induced tumor reoxygenation influences the radiosensitivity of solid tumors during fractionated radiotherapy.

Acknowledgements

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**FIGURE LEGENDS**

**Fig. 1. NOS activity is increased by ionizing radiation thorough the phosphorylation of eNOS-Ser1179.**

Relative NOS activity following irradiation was assessed using a NOS activity assay. (A) Time-course assessment of NOS activity in BAECs exposed to 10 Gy of X-rays. After irradiation (IR), BAECs were harvested at the indicated times, and NOS activity was measured. N=6. (B) NOS activity measured 12 h after 5, 10, and 15 Gy of X-rays. N=3. (C) The effect of a general NOS inhibitor (L-NAME) and an iNOS-specific inhibitor (1400W) on NOS activity in irradiated and nonirradiated BAECs. BAECs were harvested 12 h after IR and NOS activity was measured in the presence of 0.1% DMSO, 20 mM L-NAME, or 10 μM 1400W. Error bars represent S.D. *P <0.05 vs. nonirradiated; #P <0.05 vs. L-NAME. N=4. (D) Representative time-course images of western blot analyses of eNOS-pSer1179, eNOS-pThr497, eNOS, and actin expression levels. After IR, BAECs were harvested at the indicated times and the phosphorylation status of eNOS was analyzed. (E) Quantification of western blotting analyses. The eNOS-Ser1179 phosphorylation level measured in each sample was normalized to that of 0 h. Error bars represent S.D. *P <0.05, **P <0.01 vs. 0 h. N=3.

**Fig. 2. ATM inhibition attenuates eNOS phosphorylation after irradiation.**

(A) Representative confocal microscopic images of ATM-pSer1981 expression after irradiation (IR) in the presence or absence of Ku-60019. After 1 h of pretreatment with Ku-60019 (10 μM), BAECs were irradiated and incubated for 1 h or 6 h followed by being fixed with 4% PFA. ATM-pSer1981 was then visualized by immunofluorescence staining. *Green: ATM-pSer1981; blue: DAPI. Bar = 20 μm. (B) Representative images of western blotting analysis of eNOS-pSer1179, eNOS, and actin expression levels after irradiation. After 1 h of pretreatment
with Ku-60019 (10 μM), BAECs were irradiated and incubated for 6 h and the phosphorylation status of eNOS was analyzed. (C) NOS activity was measured after irradiation in the presence or absence of Ku-60019. After 1 h of pretreatment with Ku-60019 (10 μM), BAECs were irradiated and then incubated for 12 h. Error bars represent S.D. *P <0.05 vs. IR(+)/Ku-60019(-). N=3.

Fig. 3. HSP90 inhibition attenuates the phosphorylation of ATM and eNOS after irradiation.
(A) Representative confocal microscopic images of ATM-pSer1981 after irradiation (IR) in the presence or absence of geldanamycin. After 1 h of pretreatment with geldanamycin (500 nM), BAECs were irradiated and then incubated for 1 h or 6 h. ATM-pSer1981 expression was visualized by immunofluorescence staining. Green: ATM-pSer1981; blue: DAPI. Bar = 20 μm.
(B) Representative images of western blotting analyses of eNOS-pSer1179 and eNOS expression levels after irradiation. After 1 h of pretreatment with geldanamycin (500 nM), BAECs were irradiated. BAECs were then incubated for 6 h and the phosphorylation status of eNOS was analyzed. (C) NOS activity after irradiation in the presence or absence of geldanamycin. After 1 h of pretreatment with geldanamycin (500 nM), BAECs were irradiated. BAECs were incubated for 12 h and NOS activity was measured. Error bars represent S.D. *P <0.05; **P <0.01 vs. IR(+)/geldanamycin(-). N=3.

Fig. 4. Phosphorylated ATM-Ser1981 translocates from the nucleus to the cytoplasm after irradiation.
(A) Representative images of western blotting analyses of ATM-pSer1981, ATM, and actin expression levels. After irradiation (IR), BAECs were harvested at the indicated times and the
phosphorylation status of ATM was analyzed. (B) Representative confocal microscopic images of ATM-pSer1981 expression after irradiation. After IR, BAECs were incubated for the indicated times and ATM-pSer1981 expression was then visualized by immunofluorescence staining. *Green:* ATM-pSer1981; *blue:* DAPI. Bar = 20 μm.
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Fig. 2. Nagane et al.
**Fig. 3.** Nagane et al.

**A**

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**ATM**
pSer1981

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**B**

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**C**

Relative NOS Activity

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Fig. 4. Nagane et al.