Mitochondrial Ca\(^{2+}\) Uptake Drives Endothelial Injury By Radiation Therapy

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**BACKGROUND:** Radiation therapy strongly increases the risk of atherosclerotic vascular disease, such as carotid stenosis. Radiation induces DNA damage, in particular in mitochondria, but the upstream and downstream signaling events are poorly understood. The objective of this study was to define such mechanisms.

**METHODS:** Endothelial-specific MCU (mitochondrial Ca\(^{2+}\) uniporter) knockout and C57Bl6/J mice with or without a preinfusion of a mitoTEMPO (mitochondrial reactive oxygen species [ROS] scavenger) were exposed to a single dose of cranial irradiation. 24, and 240 hours postirradiation, vascular reactivity, endothelial function, and mitochondrial integrity were assessed ex vivo and in vitro.

**RESULTS:** In cultured human endothelial cells, irradiation with 4 Gy increased cytosolic Ca\(^{2+}\) transients and the mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{mt}}\)]\(_{\text{ }}\)) and activated MCU. These outcomes correlated with increases in mitochondrial ROS (\(_{\text{mt}}\)ROS), loss of NO production, and sustained damage to mitochondrial but not nuclear DNA. Moreover, irradiation impaired activity of the ETC (electron transport chain) and the transcription of ETC subunits encoded by mitochondrial DNA (\(_{\text{mt}}\)DNA). Knockdown or pharmacological inhibition of MCU blocked irradiation-induced \(_{\text{mt}}\)ROS production, \(_{\text{mt}}\)DNA damage, loss of NO production, and impairment of ETC activity. Similarly, the pretreatment with mitoTEMPO, a scavenger of \(_{\text{mt}}\)ROS, reduced irradiation-induced Ca\(^{2+}\) entry, and preserved both the integrity of the \(_{\text{mt}}\)DNA and the production of NO, suggesting a feed-forward loop involving [Ca\(^{2+}\)]\(_{\text{mt}}\) and \(_{\text{mt}}\)ROS. Enhancement of DNA repair in mitochondria, but not in the nucleus, was sufficient to block prolonged \(_{\text{mt}}\)ROS elevations and maintain NO production. Consistent with the findings from cultured cells, in C57BL/6J mice, head and neck irradiation decreased endothelium-dependent vasodilation, and \(_{\text{mt}}\)DNA integrity in the carotid artery after irradiation. These effects were prevented by endothelial knockout of MCU or infusion with mitoTEMPO.

**CONCLUSIONS:** Irradiation-induced damage to \(_{\text{mt}}\)DNA is driven by MCU-dependent Ca\(^{2+}\) influx and the generation of \(_{\text{mt}}\)ROS. Such damage leads to reduced transcription of mitochondrial genes and activity of the ETC, promoting sustained \(_{\text{mt}}\)ROS production that induces endothelial dysfunction. Our findings suggest that targeting MCU and \(_{\text{mt}}\)ROS might be sufficient to mitigate irradiation-induced vascular disease.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** calcium ■ carotid stenosis ■ endothelium ■ mitochondria ■ reactive oxygen species
The high prevalence of atherosclerotic vascular disease in adult cancer survivors is due in part to their shared risk factors, including tobacco use and older age. However, data from childhood cancer survivors demonstrate that irradiation is an independent multiplicative risk factor for the development of atherosclerotic disease, implying that irradiation induces molecular events additional to those provoked by established risk factors for atherosclerosis. Yet, these events have been incompletely elucidated.

One major cause of irradiation-induced vascular disease is believed to be acute injury to endothelial cells, which are known to be highly sensitive to irradiation. The injury initiates long-term signaling events that ultimately promote atherosclerosis. Indeed, one study showed that at 4 to 6 weeks after radiation therapy, human carotid arteries exhibited significant impairment of endothelium-dependent dilation in response to NO and prostacyclin, whereas constriction, which is mediated by smooth muscle cells, was unaffected. These findings also support the notion that endothelial dysfunction is a clinically relevant indicator of carotid injury after radiation therapy.

Exposure to radiation leads to an acute burst of reactive oxygen species (ROS) and free radicals; this is followed by chronic elevations in ROS and free radicals, which is known to be highly sensitive to irradiation. The injury initiates long-term signaling events that ultimately promote atherosclerosis. Indeed, one study showed that at 4 to 6 weeks after radiation therapy, human carotid arteries exhibited significant impairment of endothelium-dependent dilation in response to NO and prostacyclin, whereas constriction, which is mediated by smooth muscle cells, was unaffected. These findings also support the notion that endothelial dysfunction is a clinically relevant indicator of carotid injury after radiation therapy.

Here, we sought to determine whether reducing Ca$^{2+}$ entry or reducing mitochondrial ROS production during radiation therapy is sufficient to prevent short- and long-term endothelial dysfunction and to establish the underlying mechanisms, in addition to testing how acute injury induces sustained endothelial dysfunction. Specifically, we investigated whether in vivo scavenging of mitochondrial superoxide or selective endothelial deletion of MCU is sufficient to protect against both the production of excess ROS after irradiation. Irradiation induces the oxidation of Fe/sulfur clusters of ETC subunits from mitochondrial, but not nuclear, DNA and superoxide production driven by a hyperpolarized membrane potential that induced mitochondrial DNA damage and loss of nitric oxide.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| Δψ<sub>mt</sub> | mitochondrial membrane potential |
| AUC | area under the curve |
| ECs | endothelial cells |
| ETC | electron transport chain |
| HCAECs | human coronary artery endothelial cells |
| HUVECs | human umbilical endothelial cells |
| MCU | mitochondrial Ca$^{2+}$ uniporter |
| mitoTEMPO | mitochondrial reactive oxygen species scavenger |
| mtDNA | mitochondrial DNA |
| mtROS | mitochondrial reactive oxygen species |
| MTT | mitochondrial TEMPO |
| mt<sub>DNA</sub> | mitochondrial DNA |
| OGG1 | 8-oxoguanine glycosylase 1α |
| PCR | polymerase chain reaction |

Highlights

- In human endothelial cells, irradiation induced a feed-forward circuit of mitochondrial Ca$^{2+}$ entry and superoxide production driven by a hyperpolarized membrane potential that induced mitochondrial DNA damage and loss of nitric oxide.
- Transcription of ETC (electron transport chain) subunits from mitochondrial, but not nuclear, DNA and activity of the ETC (electron transport chain) were reduced after irradiation but preserved by concomitant mitochondrial superoxide scavenging or MCU (mitochondrial Ca$^{2+}$ uniporter) blockade.
- Enhancement of DNA repair in mitochondria normalized mitochondrial reactive oxygen species and NO production following irradiation.
- Endothelial MCU knockout or infusion with the mitochondrial superoxide scavenger mitoTEMPO (mitochondrial reactive oxygen species scavenger) decreased endothelium-dependent vasodilation and mitochondrial DNA integrity in the carotid artery at 24 and 240 hours after irradiation.

Materials and Methods

All raw data, analytical methods, and study materials that support the findings of this study are available from the corresponding authors upon reasonable request.
Reagents

Phenylephrine (no. 0754) was obtained from Amresco, ace-
tylcholine (no. A6625), and sodium nitroprusside (S0501) were obtained from Sigma-Aldrich, and N(gamma)-nitro-L-
arginine methyl ester (L-NAME, no. 483125) was obtained from EMD Millipore.

Human coronary artery endothelial cells (HCAECs) were kindly provided by Dr Gerene Denning (University of Iowa), and human umbilical endothelial cells (HUVECs, no. PCS-100-013) were obtained from American Type Culture Collection. Endothelial cells (ECs) were grown in endothelial cell medium supplemented with growth factors (no. 1001, ScienCell).

Transfection was performed using Opti-MEM I medium (Gibco, 31985–062) and Lipofectamine 2000 (Thermofisher). MitoTEMPO (mitochondrial reactive oxygen species [ROS] scavenger; no. SML0737) was obtained from Enzo. MitoSOX Red (no. D1168) and MitoTracker Green FM (no. M7514) were obtained from Thermofisher. Tetramethylrhodamine methyl ester (no. T668) was purchased from Molecular Probes.

siRNAs and Plasmids

Scrambled and silencer (si)RNAs against MCU were obtained from Integrated DNA Technologies.

Plasmids encoding mitochondria-targeted (mito-; no. 18706), mutant mitochondria-targeted (mut; no. 18708), and nucleus-targeted (nuc-; no. 18709) OGG1α (8-oxoguanine-DNA glycosylase 1-α; no. 18709) were generated and deposited by Dr Sidransky21 (Addgene). The small-molecule MCU inhibitor RU265 was kindly provided by Dr Woods, Cornell University.

Mice

All experimental procedures were approved by the Institutional Animal Care and Use Committees of both the University of Iowa and the Iowa City VA Health Care System and complied with the standards of the Institute of Laboratory Animal Resource, National Academy of Science. The C57BL/6J male and female mice used in the described experiments were obtained from The Jackson Laboratory (no. 000664). Specific MCU deletions were studied in mice in which exons 5 and 6 of MCU are flanked by loxP sites.23 These mice were purchased from Jackson Laboratory (no. 029817). Endothelial MCU knockout mice (eMCU−/−) were generated by mating MCUfl/fl mice with counterparts carrying a cre second course of tamoxifen treatment.25 Littermate MCUfl/fl mice that lacked the cre allele but were treated with tamoxifen served as controls for all experiments. All mice used were from 12 to 16 weeks of age at the time of treatment; male and female mice were used in equal proportions. Data from male and female mice were analyzed separately initially. The results reported in this article were combined because no difference was seen between the 2 groups regardless of sex. Correct recombination and MCU were confirmed by quantitative reverse-transcription polymerase chain reaction (PCR) and imaging of mitochondrial

Ca2+ transients with Pericam (mitochondria-targeted genetic Ca2+ indicator Pericam).

Radiation Exposure in Mice

When mice were at least 12 weeks of age, they were anesthetized with ketamine and xylazine and received a single dose (12 Gy) of X-rays (irradiation) to the whole brain. This treatment was administered using an XStrahl Small Animal Radiation Research Platform,26 which incorporates a 60 kVp beam of 0.2 mm Al quality for use in Cone Beam CT acquisition and a 220 kVp 0.63 mm Cu quality beam for use in treatment-type irradiation. The dose of 12 Gy was chosen because it is the equivalent of 2 Gy fractions of 40 Gy, which is within the range used in humans for radiation therapy in head and neck cancer. Control animals were sham irradiated (anesthetized and placed in the radiation chamber only).

Mitochondrial ROS Scavenging In Vivo

For scavenging mitochondrial superoxide in mice, mitochondrial TEMPO (MTT)28 was administered for 14 days by continuous infusion, using osmotic mini-pumps, at a dose of 0.7 mg/kg per day. The mini-pumps were implanted 3 days before irradiation. Control animals were implanted with osmotic mini-pumps filled with normal saline.

Measurement of Vascular Reactivity

The carotid and second-branch mesenteric resistance arteries were carefully cleaned of fat and connective tissue and then cut into rings 2 mm in length. These arterial rings were mounted in a small vessel dual-chamber myograph and isotonic tension was measured. The arteries were then equilibrated in Krebs solution bubbled with carbogen at 37 °C and pH 7.4 for 30 minutes and stretched to their optimal physiological lumen diameter for active tension development for 1 hour. The vascular rings were then preconstricted with phenylephrine (3.10-6 M). Once a steady maximal contraction was reached, cumulative concentration-response curves were obtained for acetylcholine (10-8–3×10-5 M) and sodium nitroprusside (10-8–3×10-5 M).

Endothelial Cell Cultures and Treatments

Primary HCAECs and HUVECs were grown in endothelial cell medium at 37 °C and 5% CO2, and used at passages 3 to 5.

siRNA Transfection

ECs were transfected with 5 nmol/L of siRNA duplexes targeting MCU (siMCU; 5′-GCACCUAGAGAAUAACGAUCAGCTC-3′ and 5′-GGGAAAAGGGAUCUAAAGCCTG-3′ at a 1:1 ratio) or scrambled control (5′-CGUAAUCGCUGUAAUACG CGUA-3′, Integrated DNA Technologies) in Lipofectamine RNAiMax reagent following the manufacturer’s instructions. Transfection was performed 48 hours before irradiation. After 48 hours, knockdown efficiency was assessed by Western blotting for MCU. The efficiency of knockdown was consistently >75%.

MTT Treatment

ECs were treated with 10 μM MTT in dimethyl sulfoxide (DMSO) for 18 hours before irradiation. Control cells were treated with DMSO only.
**Irradiation**

Once ECs were 80% confluent, they were exposed to 4 Gy. Ionizing radiation was delivered at 1.29 Gy/min using a cesium-137 γ-ray source in the Radiation and Free Radical Research Core of the University of Iowa. At 24, 48, 120, and 240 hours following irradiation, ECs were collected for analyses of mRNA levels, protein levels, or imaging.

**Cytosolic Ca²⁺ Measurement**

HCAECs were cultured on 35-mm glass-bottom microwell dishes for 24 hours and after that used for analysis. Cells were incubated with the ratiometric indicator dye Fura-2 AM (1 μM, Invitrogen) in endothelial cell medium (endothelial cells phenol-free media) for 10 minutes at 37°C. For recording of cytosolic Ca²⁺ transients, images were acquired continuously for at least 10 minutes every 5 seconds. After short baseline recording (15–20 s), thapsigargin (1 μM) was applied by micropipette injection. The cells were excited alternately at 340 and 380 nm. Fluorescence signal intensity was acquired at 510 nm. Data are presented as peak amplitude or area under the curve (AUC). Imaging was done with Nikon Eclipse Ti2 microscope under ×40 objective. Analysis was performed with NS Elements software (Nikon).

**Assessment of Damage to mtDNA**

Quantitative PCR was used to assay mtDNA damage as described previously. Briefly, total DNA was isolated using Genomic-tips and the Genomic DNA Buffer Set Kit (Qiagen, Valencia, CA). The purified DNA was quantified fluorometrically using Pico Green dsDNA reagent (Molecular Probes, Life Technologies). The Platinum PCR Super Mix (Invitrogen) was used to amplify 20 ng genomic DNA. Specific primers were used to amplify a long fragment of the _DNA_ (8.9 kb) to determine its integrity, as well as a short fragment (139 bp) to monitor changes in _DNA_ copy number and to normalize the data obtained from amplification of the 8.9-kb fragment. Ratios of relative amplification were calculated to compare _DNA_ damage in irradiated ECs to that in nonirradiated ECs; these values were used to express the number of lesions present in DNA, assuming a Poisson distribution, as previously described.

**Assessment of Damage to Nuclear DNA (nucDNA)**

Total genomic DNA was purified from ECs using the DNeasy Blood & Tissue Kit (Qiagen). Quantity and purity of the DNA were determined by spectrometry using a Nanodrop 1000 instrument. SYBR Green PCR Master Mix complemented with Platinum Taq DNA Polymerase, High Fidelity (amplification capacity of 20 kb; Invitrogen) was used to amplify fragments from 20 ng of genomic DNA. Primers that amplify a long fragment of the nuclear gene β-globin (12 kb) were used to assess the integrity of the genomic DNA. Primers that amplify a short fragment of the nuclear gene _ESR1_ (139 bp) were used for normalization to β-globin levels across samples. Data were analyzed using the comparative Ct method. β-globin levels were normalized to those of the short fragment amplified from the _ESR1_ gene and expressed as relative fold change.

**Real-Time PCR**

Total mRNA was harvested from HCAECs using a Qiagen RNeasy Kit. Approximately 1000 ng of RNA was used to synthesize cDNA using SuperScript VILO MasterMix (Invitrogen). Gene expression for ETC subunits was quantified by real-time-quantitative PCR using primers sets: mt-COI (cytochrome c oxidase I): Forward 5’-TCGCAATTCTCAGTTGTCGTC-3’ and reverse: 5’-CTGGTAGGGTTGCAAGTGCA-3’; mt-ND1 (NADH-ubiquinone oxidoreductase chain I): Forward 5’-GCCGCTTACATCCCGCCGCAAGC-3’ and reverse: 5’-GTTTGGGGCTACGCCTCG-3’; NDUF1 (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1): Forward 5’-ATGTTGTTTGGATTCTCC-3’ and reverse: 5’-GCAACCCCTTTTCTTTGC-3’;
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RESULTS

Mitochondrial Ca\(^{2+}\) Entry Is Necessary for Irradiation-Induced ROS Production and Endothelial Dysfunction

Previous studies had reported that irradiation leads to altered cytosolic Ca\(^{2+}\) handling and endoplasmic reticulum Ca\(^{2+}\) loading.\(^{16}\) In this study, we first confirmed that endoplasmic reticulum Ca\(^{2+}\) loading is increased after irradiation in HUVECs 24 hours after irradiation. Knockdown of MCU before irradiation led to a decreased cytosolic Ca\(^{2+}\) transients assessed as thapsigargin-induced peak amplitude (Figure 1B) and AUC (Figure 1C; Figure 1A through 1C). The acute inhibition of the mitochondrial Ca\(^{2+}\) uniporter following irradiation, using the cell-permeable, selective small-molecule inhibitor Ru265, had the same effect (Figure S1A through S1C). At 24 hours after irradiation, the MCU was phosphorylated at Ser 92, indicating that the Ca\(^{2+}\) conducting activity of MCU was increased\(^{35,36}\) (Figure 1D).

To directly test whether irradiation affects mitochondrial Ca\(^{2+}\) entry via MCU, mitochondrial Ca\(^{2+}\) levels were measured using the Pericam. In control HUVECs, irradiation led to increases in both the baseline mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{mt}\)) and PDGF-induced mitochondrial Ca\(^{2+}\) transients (assessed as AUC), at 24 postirradiation (Figure 1E through 1G). The converse was found in cells where MCU had been knocked down using a siRNA-mediated approach. Specifically, in such cells, irradiation led to reductions in the baseline [Ca\(^{2+}\)]\(_{mt}\) and peak amplitude of PDGF-induced mitochondrial Ca\(^{2+}\) transients (AUC; Figure 1E through 1G).

Mitochondrial Ca\(^{2+}\) entry via the MCU complex is driven by the membrane potential (ΔΨ\(_{mt}\)). Thus, we investigated the relationship between mitochondrial Ca\(^{2+}\) entry and the ΔΨ\(_{mt}\). Recordings of HUVECs with tetramethylrhodamine methyl ester at 24 and 240 hours after irradiation confirmed that the mitochondrial membrane is hyperpolarized in control HUVECs but not in counterparts transfected with an MCU siRNA (Figure 1H).

To confirm that an increase in [Ca\(^{2+}\)]\(_{mt}\) following irradiation drives \(_{mt}\) ROS production, we measured mitochondrial superoxide production in primary HCAECs following siRNA-mediated MCU knockdown at 24 and 240 hours after irradiation. In these cells, irradiation failed to induce \(_{mt}\) ROS production or an increase in ΔΨ\(_{mt}\) (Figure 1I and 1J). Sustained increases in superoxide are known to reduce levels of nitric oxide (NO) by scavenging and eNOS uncoupling.\(^{32,38}\) As anticipated, NO levels were reduced in control cells following irradiation, but preserved in counterparts in which MCU had been knocked down (Figure 1K and 1L).

Scavenging \(_{mt}\) ROS Protects From Irradiation-Induced Ca\(^{2+}\) Alterations, \(_{mt}\) DNA Damage, and Loss of NO Production in Endothelial Cells

Next, we tested the ability of the mitochondrial superoxide scavenger MTT to reduce \(_{mt}\) ROS and preserve NO production in HCAECs following irradiation. In irradiated HCAECs, levels of \(_{mt}\) ROS were 15-fold and 20-fold higher than in nonirradiated counterparts at 24 and 240 hours, respectively (Figure 2A and 2B). Pretreatment with MTT completely blocked the increases in \(_{mt}\) ROS at both time points. In accordance with the concept that \(_{mt}\) ROS levels diminish the bioavailability of NO in vascular beds,\(^{30}\) NO production by HCAECs after irradiation was significantly reduced at both time points, and this effect was abolished by pretreatment with MTT (Figure 2C and 2D).

To dissect the relationship between mitochondrial Ca\(^{2+}\) entry and the generation of \(_{mt}\) ROS, we tested the effects of \(_{mt}\) ROS scavenging on ΔΨ\(_{mt}\) and Ca\(^{2+}\) entry. Irradiation led to an elevation in ΔΨ\(_{mt}\) and this was attenuated by MTT (Figure 3A). As predicted based on ΔΨ\(_{mt}\),
in vehicle-treated cells irradiation increased \([\text{Ca}^{2+}]_{\text{mt}}\) (Figure 3B) and MTT pretreatment reduced this effect. Moreover, the peak amplitude of the Ca\(^{2+}\) transient in response to ATP in irradiated cells was reduced in cells treated with MTT (Figure 3C and 3D). Thus, scavenging of superoxide reduces \(\Delta \psi \text{mt}\) as well as \([\text{Ca}^{2+}]_{\text{mt}}\) and Ca\(^{2+}\) entry in response to agonists, whereas deletion of MCU reduces \(\Delta \psi \text{mt}\) and the production of \(\text{mtROS}\). These findings imply an inter-relationship of Ca\(^{2+}\) entry, the production of \(\text{mtROS}\), and \(\Delta \psi \text{mt}\).
Mitochondrial Ca\(^{2+}\) Entry Is Required for Irradiation-Induced \(\text{mtROS}\) Production and Endothelial Dysfunction in Carotid Arteries

We extended the above results from cultured cells to the ex vivo context, examining the extent to which inhibiting mitochondrial Ca\(^{2+}\) uptake in the endothelium of carotid arteries protects against irradiation-induced dysfunction. For this purpose, we established and validated a transgenic model in which MCU is selectively deleted in the endothelium (eMCU\(^{-/-}\), Figure S2). Endothelium-dependent vasodilation at 24 and 240 hours postirradiation was significantly higher in carotid arteries from irradiation-treated eMCU\(^{-/-}\) mice than in those from irradiation-treated wild-type (WT) mice, and similar to that observed in control (no irradiation) WT and eMCU\(^{-/-}\) carotid arteries (Figure 4A). To determine whether loss of MCU in the endothelium prevents the reduction of NO after irradiation, we tested the effect of the NO-scavenger, L-NAME, on acetylcholine-induced vasodilation. In WT carotid arteries at 24 hours postirradiation, L-NAME had only a minimal effect (Figure 4B), suggesting that the decrease in vasodilation after irradiation is due to NO deficiency. In carotid arteries of eMCU\(^{-/-}\) mice, L-NAME impaired endothelium-dependent relaxation to a similar extent regardless of whether or not they had been irradiated (Figure 4C). At 240 hours after irradiation, endothelial dysfunction was detected in WT but not in eMCU\(^{-/-}\) arteries (Figure 4D). Further impairment of endothelium-dependent relaxation in response to L-NAME was seen only in irradiation-treated WT carotid arteries (Figure 4E and 4F), suggesting that the decrease in vasodilation at the later time point is driven in part by factors other than NO deficiency.

Inhibition of Mitochondrial ROS Production Prevents Irradiation-Induced Endothelial Dysfunction and NO Deficiency in Carotid Arteries

To determine whether irradiation-induced production of \(\text{mtROS}\) leads to endothelial dysfunction, we assessed dilation in response to acetylcholine in the carotid artery of C57BL/6J mice subjected to irradiation (12 Gy X-ray) of the head and neck, in the presence and absence of the mitochondrial ROS scavenger mitoTEMPO (MTT). MTT was administered by continuous infusion using a minipump, starting 3 days before irradiation. At 24 hours postirradiation, relaxation in response to acetylcholine was significantly less pronounced in carotid arteries of irradiation compared to sham-treated mice (Figure 5A). At 48 and 120 hours postirradiation, dilation did not differ significantly between the 2 groups (Figure 5B and 5C), yet at 240 hours postirradiation, a decrease in relaxation in response was observed (Figure 5D). Moreover, infusion
with MTT preserved endothelium-dependent relaxation at 24 and 240 hours postirradiation (Figure 5A through 5D). Notably, endothelium-independent dilation of the carotid artery, that is, relaxation in response to sodium nitroprusside, was similar in all groups (Figure 5E), and this was also the case for endothelium-dependent dilation of mesenteric resistance arteries in response to acetylcholine (Figure 5F). These data indicate that irradiation induces sustained increases in mtROS production locally and that these increases lead to a deficiency in endothelial NO production.

In Cultured Cells and Mice, Mitochondrial DNA Damage Drives Sustained mtROS Production and Reduced NO Production After Irradiation

Next, we sought to identify the mechanism by which irradiation induces mtROS production that persists at 240 hours. We reasoned that excessive ROS is a byproduct of dysregulated activity of the ETC. Because mtDNA lacks histones and is located near the ETC, which is the primary source of ROS, the damage to mtDNA from oxidative stress is more extensive and persists longer than that to nucDNA.40,41 Sustained mtDNA damage could then lead to a further increase in the generation of mtROS by altering the transcription of ETC subunits encoded by mtDNA.40 To test this possibility, we measured the damage to both mtDNA and nucDNA in HUVECs after irradiation.

As anticipated, at 24 hours postirradiation, levels of mtDNA damage were high, and this persisted at 240 hours (Figure 6A and 6B). In contrast, nucDNA damage was detected only at 24 hours after irradiation (Figure 6C and 6D). Pretreatment of HUVECs with MTT blocked mtDNA damage at both timepoints but did not provide protection against nucDNA damage at 24 hours. To confirm that mtDNA damage occurs after irradiation in vivo, we also measured mtDNA damage in nucDNA isolated from the carotid artery of mice after irradiation of the head and neck. A significant increase in damage to mtDNA, but not nucDNA, was seen at 24 hours and it persisted at 240 hours postirradiation (comparison was to sham mice). Continuous infusion of MTT prevented mtDNA damage after irradiation (Figure 6E and 6F). These data indicate that excess production of mitochondrial superoxide during irradiation induces mtDNA damage that is sustained through 240 hours and that it correlates with persistent increases in levels of ROS and reductions in levels of NO (Figure 2). Having established a feed-forward circuit between Ca\textsuperscript{2+} entry and mtROS (Figures 1 and 3), we also tested DNA damage postirradiation in MCU knockdown HUVECs. This analysis confirmed that mtDNA damage was significantly reduced through 240 hours (Figure 6H and 6I) but that nucDNA damage was not (Figure 6J and 6K).

We next tested the effects of acute MCU blockade with Ru265 on irradiation-induced endothelial damage.22 As expected, pretreatment of HCAECs with Ru265 blocked mitochondrial Ca\textsuperscript{2+} entry (Figure S1D). Like MCU knockdown, this treatment led to decreases in both ROS levels and mtDNA damage (Figure S1E and S1F). This experiment...
To identify a mechanistic link between mtDNA damage, changes in levels of mtROS, and changes in the transcription of ETC subunits, we performed quantitative reverse-transcription PCR for the ETC subunits mt-COI and mt-ND1, which are transcribed from mtDNA. Exposure to irradiation significantly reduced the transcript levels at 24 and 240 hours (Figure 7A through 7H). Preincubation with Ru265 and MTT prevented this effect (Figure 7A through 7D and Figure 7E through 7H, respectively). In contrast, the transcription of 2 subunits encoded by nucDNA, NDUF1 (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1), and COX11 (cytochrome c oxidase 11), was unaffected by irradiation (Figure S3). The dissociation of the transcription of nucDNA- and mtDNA-encoded ETC subunits predicts that ETC activity is impaired after irradiation. Indeed, the activity of ETC complex 1 was reduced at 24 hours, and this effect was blocked by inhibiting MCU using Ru265 or by scavenging mtROS with MTT (Figure 7I and 7J). These data delineate a complete pathway by which irradiation leads to increased mitochondrial Ca\(^{2+}\) entry via MCU and promotes mtROS production. Increased mitochondrial Ca\(^{2+}\) entry and mtROS production correlate with loss of NO.

In Cultured Endothelial Cells, Enhanced Repair of mtDNA Protects From Irradiation-Induced Endothelial Injury

To determine whether promoting the repair of mtDNA is sufficient to protect against irradiation-induced generation of mtROS and loss of NO, we overexpressed various forms of the enzyme OGG1, which binds to the modified base 8-oxoguanine (produced as a result of oxidative stress) and initiates DNA base-excision repair.\(^42\) Specifically, we expressed a mitochondria-targeted (mito-OGG1), nucleus-targeted OGG1 (nuc-OGG1), and a mitochondria-targeted inactive mutant (R229Q) form (mutt-OGG1). Examination of mtDNA in HCAECs revealed that overexpression of mito-OGG1 abolished mtDNA injury after irradiation, whereas overexpression of mutt-OGG1 or nuc-OGG1 did not (Figure 8A and 8B). Examination of nucDNA, based on the amplification of β-globin in irradiated versus nonirradiated cells, revealed that overexpression of nuc-OGG1 more effectively restored nucDNA integrity at 24 hours than did mito-OGG1 or mut-OGG1. No significant

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**Figure 4.** In ex vivo mouse carotid arteries, endothelium-specific deletion of MCU (mitochondrial Ca\(^{2+}\) uniporter) protects against endothelial dysfunction following head and neck irradiation (IR).

Dilation of carotid artery in response to cumulative doses of acetylcholine (Ach; endothelium-dependent dilation) in the contexts of the indicated treatments at (A–C) 24 and (D–F) 240 h after IR. Effects in (A and D) wild-type (WT) and eMCU\(^{-/}\) mice, (B and E) WT type mice pretreated (or not) for 30 min with the eNOS inhibitor N(gamma)-nitro-L-arginine methyl ester (L-NAME), and (C and F) eMCU\(^{-/}\) mice pretreated (or not) with L-NAME for 30 min. n=5 mice per group. P values were determined by repeated-measures 2-way ANOVA followed by Tukey post hoc test.
irradiation-induced mtDNA damage was present at 240 hours (Figure 8C and 8D).

We then investigated whether mtDNA repair blocks irradiation-induced mtROS production. Overexpression of mito-OGG1 in HCAECs eliminated irradiation-induced mtROS production at both 24 and 240 hours postirradiation (Figure 8E and 8F). In contrast, overexpression of nuc-OGG1 or mutt-OGG1 led to significant increases in mtROS production at both 24 and 240 hours after irradiation.

Lastly, we asked whether enhanced mtDNA repair after irradiation-induced injury alleviates endothelial dysfunction. Specifically, we tested whether OGG1 overexpression attenuates the deleterious effect of irradiation on endothelial NO bioavailability. HCAECs overexpressing mito-OGG1 were protected from irradiation-induced dysfunction, and levels of NO were similar to those in nonirradiated cells at 240 hours. In contrast, the overexpression of neither mutt-OGG1 nor nuc-OGG1 protected from irradiation-induced loss of NO production (Figure 8G and 8H).

**DISCUSSION**

Radiation therapy for head and neck cancer is associated with increased risk of atherosclerotic carotid stenosis after a lag period of several years. Here, we report 4 major new findings related to the molecular mechanisms underlying early irradiation-induced carotid endothelial dysfunction and the transition to chronic injury. First, an in vivo block of mitochondrial Ca\(^{2+}\) entry via MCU or scavenging of mtROS prevented endothelial dysfunction following irradiation. Second, irradiation promoted mitochondrial Ca\(^{2+}\) entry via MCU and production of mtROS. These events were interrelated and led to sustained production of excess mtROS. Third, mtDNA damage was identified as a key mechanism driving mtROS production after irradiation. It impaired the transcription of specifically those ETC subunits that are encoded by mtDNA as opposed to nucDNA, and it reduced ETC activity. Fourth, these adverse events were prevented by scavenging mtROS or blocking MCU, and either of these 2 manipulations or enhanced repair of mtDNA damage prevented the loss of irradiation-induced endothelial NO bioavailability. Collectively, our data delineate a complete pathway by which irradiation disrupts intracellular MCU-mediated Ca\(^{2+}\) handling, causes mitochondrial injury, and leads to endothelial dysfunction. We provide strong support for novel approaches to prevent irradiation-induced
vascular dysfunction, based on targeting downstream effectors of excess mtROS and mtDNA damage. These findings are significant because, despite renewed interest in the side effects of cancer therapies on the vascular system, the exact pathogenesis of irradiation-induced arterial wall injury and atherosclerotic disease remains poorly understood. Such injury is of particular clinical relevance for the carotid artery because its...

Figure 6. In vitro and in vivo irradiation (IR) induces sustained mitochondrial DNA (mtDNA) damage. A and B, Damage to mtDNA in human coronary artery endothelial cells, as assessed by polymerase chain reaction (PCR) assay. mtDNA lesions at (A) 24 and (B) 240 h after IR, in cells treated with mitochondrial TEMPO (MTT) or vehicle starting at 18 h before IR. In (B), pretreatment with 500 µM H2O2 served as a positive control. C and D, Damage to mtDNA in human umbilical endothelial cells, as assessed by PCR for β-globin. Expression of β-globin at (C) 24 and (D) 240 h after IR, in cells treated with MTT or vehicle starting at 18 h before IR. In (D), pretreatment with 500 µM H2O2 served as positive control. E and F, Damage to nucDNA in carotid arteries at (E) 24 and (F) 240 h after IR. G, Copy number of β-globin in the carotid artery at (G) 24 h after IR. H and I, Lesions in mtDNA at (H) 24 and (I) 240 h post-IR. J and K, Damage to nucDNA, as assessed by PCR of β-globin from genomic DNA at (K) 24 and (L) 240 h post-IR. Statistical significance was determined by Kruskal-Wallis test. siMCU indicates silencer for MCU RNA.
injury by irradiation is associated with increased risk of cerebrovascular events.\(^6,8,43,44\) The radiation sensitivity of different parts of the vascular wall has been investigated in the past. Irradiation-mediated injury to smooth muscle cells in the medial layer of this vessel has been proposed as the main driver of atherosclerotic disease postirradiation.\(^45\) However, endothelial cells are deemed more sensitive to irradiation than smooth muscle cells.\(^46,47\)

**Figure 7.** Irradiation (IR)-induced reduction of mitochondrial DNA (mtDNA) transcription and ETC (electron transport chain) activity are prevented by mitochondrial reactive oxygen species (mtROS) scavenging or MCU (mitochondrial Ca\(^{2+}\) uniporter) inhibition.

A–D, Comparison of human coronary artery endothelial cells (HCAECs) pretreated with MCU inhibitor Ru265 (100 \(\mu\)M, 1 h). A and B, Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for MT-COI (cytochrome c oxidase I) with cDNA normalized to 100 ng at 24 (A) and 240 h (B) after IR. C and D, qRT-PCR for MT-ND1 (NADH-ubiquinone oxidoreductase chain 1) at 24 (C) and 240 h (D) after IR.

E–H, Comparison of HCAECs pretreated with mtROS scavenger mitochondrial TEMPO (MTT; 10 \(\mu\)M, overnight). E and F, Quantitative RT-PCR for MT-COI with cDNA normalized to 100ng at 24 (E) and 240 h (F) after IR. G and H, qRT-PCR for MT-ND1 at 24 (G) and 240 h (H) after IR.

I and J, Activity of ETC complex 1 assessed by fluorometric assay at 24 h (I) and 240 h (J) after IR. Statistical significance was determined by Kruskal-Wallis test.
Figure 8. In vitro, enhancement of base-excision repair of mitochondrial DNA (mtDNA) protects cells by sustaining mitochondrial reactive oxygen species (mtROS) levels and reducing NO production.

A and B, Lesions in mtDNA in human coronary artery endothelial cells (HCAECs) transfected with mitochondria-targeted 8-oxoguanine DNA glycosylase (mito-OGG1), mutant mitochondria-targeted OGG1 (mutt-OGG1), or nucleus- targeted OGG1 (nuc-OGG1) for 72 h before irradiation (IR) or sham treatment, as assessed by quantitative reverse-transcription polymerase chain reaction (RT-PCR). Samples were analyzed at (A) 24 and (B) 240 h post-IR (4 Gy, x-ray). n=4 independent experiments. In both panels, samples pretreated with 500 µM H2O2 serve as positive controls.

C and D, Lesions in nuclear DNA (nucDNA) in cells, assessed as in (A and B), at (C) 24 and (D) 240 h after IR. In both C and D, samples pretreated with 500 µM H2O2 serve as positive controls. E and F, MitoSOX fluorescence normalized to mitoTracker fluorescence at (E) 24 and (F) 240 h after IR. G and H, NO production in HCAECs at (G) 24 and (H) 240 h after IR, assessed based on DAF2 diacetate fluorescence (arbitrary units [AU]). NO production was measured after stimulation with PDGF (platelet-derived growth factor). Data were normalized to baseline (before PDGF stimulation) and are plotted as fold difference compared to untreated cells. Statistical significance was determined by Kruskal-Wallis test. DAF2 diacetate indicates diaminofluorescein diacetate.
Numerous studies have reported on molecular pathways that mediate endothelial injury after irradiation in vitro, but in vivo evidence that these pathways can be leveraged to reduce irradiation-induced macrovascular disease as provided in this study is scarce.

Irradiation-induced oxidative stress that subsides within seconds of exposure can lead to the initiation of a self-amplifying cycle, giving rise to long-term ROS production and mitochondrial dysfunction. This study reveals that although endothelial cells contain fewer mitochondria than other cell types and produce only a small proportion of the energy a cell requires under physiological conditions, mitochondrial damage to mtDNA is the key driver of irradiation-induced chronic mtROS production and endothelial dysfunction. These findings are interesting in light of previous observations that excessive mtROS causes sustained inflammatory responses and NFκB (nuclear factor kappa B) activation in human carotid artery specimens months after irradiation. Given that NFκB is activated by mtROS-driven inflammasomes and we posit that the pathway revealed here lies upstream of sustained NFκB activation after irradiation. Our data are also consistent with seminal work by Yakes and Van Houten, indicating that prolonged exposure to an oxidizing agent results in unpaired damage to mtDNA but not nDNA. At 24 hours postirradiation, we detected lesions in both mtDNA and nDNA, but at 240 hours, the mtDNA was fully repaired, whereas the nDNA damage persisted. This is further supported by our finding that when base-excision repair was targeted to mitochondria, it fully protected against radiation-induced elevations in mtROS and reduction of NO. Also, our findings that persistent mtDNA damage drives long-term increases in mtROS production suggest that pharmacological approaches that activate OGG1 could be leveraged to protect against endothelial impairment following radiation therapy.

Previous explanations for the production of mtROS following irradiation were divergent, ranging from increases in the activity of ETC complexes caused by increased ATP demand to reductions in the reentry of protons into the mitochondrial matrix via ETC complex V caused by reversed activity of ATP synthase. In our studies, irradiation led to increased ΔΨmt and this effect was abolished by blockade of MCU. We reason that the change in ΔΨmt by irradiation is driven by indirect effects of Ca2+ in mitochondria, such as increases in metabolic activity, as opposed to a direct effect, given that the entry of positively charged ions would be expected to depolarize the membrane. As a result of the increase in ΔΨmt, the baseline [Ca2+]mt was elevated in control cells after irradiation. An alternative explanation is that, like other states of high oxidative stress, irradiation promotes posttranscriptional modifications that enhance the Ca2+ conductance of the pore-forming subunit of MCU. This is consistent with our discovery that application of the mitochondrial superoxide scavenger MTT reduced ΔΨmt and thereby the driving force for entry of Ca2+ into the matrix, leading to reductions in the baseline Ca2+ concentration and transients (Figure 3). The higher [Ca2+]mt after irradiation may also explain the increase in the mitochondrial transition that was reported in cancer cell lines in the past. Given that MCU inhibition and MTT treatment ultimately reduce mitochondrial levels of superoxide, both preserve integrity of the mtDNA and thus endothelial function.

Taken together, we demonstrate that endothelial dilatory dysfunction after irradiation is driven by mitochondrial injury. We also identify an increased mitochondrial membrane potential ΔΨmt as the upstream effector of the mtROS production and mitochondrial Ca2+ entry that promote each other. Moreover, we establish a feed-forward circuit of mtROS production and mitochondrial Ca2+ uptake using specific inhibitors prevents irradiation-induced vascular dysfunction. These approaches target excess mtDNA damage as common downstream effectors.

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Disclosures
None.

Supplemental Material
Supplemental Methods
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