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Statins Use a Novel Nijmegen Breakage Syndrome-1–Dependent Pathway to Accelerate DNA Repair in Vascular Smooth Muscle Cells

Melli Mahmoudi, Isabelle Gorenne, John Mercer, Nicola Figg, Trevor Littlewood, Martin Bennett

Abstract**—**Although the hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) are widely used in atherosclerosis to reduce serum cholesterol, statins have multiple other effects, including direct effects on cells of the vessel wall. Recently, DNA damage, including telomere shortening, has been identified in vascular smooth muscle cells (VSMCs) in human atherosclerosis. Although statins reduce DNA damage in vitro, the mechanisms by which they might protect DNA integrity in VSMCs are unknown. We show that human atherosclerotic plaque VSMCs exhibit increased levels of double-stranded DNA breaks and basal activation of DNA repair pathways involving ataxia telangiectasia–mutated (ATM) and the histone H2AX in vivo and in vitro. Oxidant stress induced DNA damage and activated DNA repair pathways in VSMCs. Statin treatment did not reduce oxidant stress or DNA damage but markedly accelerated DNA repair. Accelerated DNA repair required both the Nijmegen breakage syndrome (NBS)-1 protein and the human double minute protein Hdm2, accompanied by phosphorylation of Hdm2, dissociation of NBS-1 and Hdm2, inhibition of NBS-1 degradation, and accelerated phosphorylation of ATM. Statin treatment reduced VSMC senescence and telomere attrition in culture, accelerated DNA repair and reduced apoptosis in vivo after irradiation, and reduced ATM/ATR (ATM and Rad3-related) activity in atherosclerosis. We conclude that statins activate a novel mechanism of accelerating DNA repair, dependent on NBS-1 stabilization and Hdm2. Statin treatment may delay cell senescence and promote DNA repair in atherosclerosis. **(*Circ Res*. 2008;103:717-725.)**

Key Words: atherosclerosis ■ vascular smooth muscle ■ aging ■ oxidant stress

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Human atherosclerotic plaques demonstrate evidence of DNA damage, including expression of oxidized guanosine residues, DNA strand breaks, and activation of DNA repair enzymes.[1,2] Although DNA damage is seen in both vascular smooth muscle cells (VSMCs) and macrophages, the mechanisms underlying DNA damage and its biological consequences are unknown. For example, DNA damage can promote apoptosis and premature cell senescence (reviewed elsewhere[3]), both of which are prominent in VSMCs in human atherosclerosis.[2,4] Conversely, accelerating DNA repair may prevent or reduce accumulated DNA damage, preventing apoptosis or cell senescence.

DNA damage induces a cascade of activated proteins that act as sensors and effectors of the damage response, to stall the cell cycle allowing repair to occur, to promote repair, or to induce apoptosis if damage is severe. DNA damage activates Nijmegen breakage syndrome (NBS)-1, a ubiquitously expressed 754-aa protein and key regulator of the MRE11/RAD-50/NBS-1 (MRN) complex.[5,6] MRN promotes early processing of double-strand breaks (DSBs) via DNA binding and nuclease activities, functions as a DSB sensor, and also recruits the ataxia telangiectasia–mutated (ATM) protein to DSBs, followed by ATM activation.[7–9] ATM is normally present as inactive dimers, but DSB exposure induces autophosphorylation at Ser1981, dimer dissociation, and kinase activation. ATM has multiple downstream substrates that mediate cell senescence, growth arrest and apoptosis, including histone (H)2AX. ATM accumulates at DSBs, as marked by nuclear foci of phosphorylated ATM and Ser139 phosphorylation of H2AX flanking the site of DNA damage.[10] Phosphorylated H2AX (γ-H2AX) facilitates the local assembly of checkpoint and DNA repair factors and is a robust marker of DSBs.[11]

The hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used in atherosclerosis to reduce serum cholesterol. More recently, statins have been reported to reduce DNA damage in vitro and also in vivo, after a variety of insults. There are multiple mechanisms by which statins might reduce DNA damage, including reduction of oxidative stress,[12–15] inhibition of prenylation of proteins involved in inducing DNA damage,[16] and inhibition of downstream signaling from damaged DNA.[17,18] We, therefore, examined the effect of statin treatment on DNA damage and repair pathways in human VSMCs. We identify a novel

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mechanism by which statins accelerate DNA repair involving NBS-1 and Hdm2. Importantly, statins can inhibit cell senescence, accelerate DNA repair, and reduce DNA damage in atherosclerosis in vivo.

Materials and Methods

Cell culture, preparation of nuclear extracts, immunoprecipitation, protein electrophoresis, Western blotting, and immunohistochemistry were as previously described.[2] See the expanded Material and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Small Interfering RNA

VSMCs were transfected with small interference (si)RNA using an Amaxa Nucleofector II (Amaxa, Ge) using the following sequences for Hdm2: sense, r(CUG UCU UAA AUG AGA AGU A)dTdT; and antisense, r(UAC UUC UUU AAG ACA G)dAdG.

Comet Assay

For analysis of DNA strand breaks, 30 000 cells per condition were centrifuged, suspended in 80 μL of low melting point agar, transferred to a slide coated with normal melting point agar, covered, and incubated for 10 minutes at 4°C. Cells were then lysed in alkaline buffer (2.5 mol/L NaOH, 100 mmol/L EDTA, 10 mmol/L Tris, pH 10), electrophoresed for 30 minutes in 0.3 mol/L NaOH, 1 mmol/L EDTA at 24 V and 0.29 A, neutralized in 0.4 mol/L Tris buffer, pH 7.5, and then stained with ethidium bromide. For each slide, 5 random pictures were taken using an Olympus BX51 microscope at $\times$10 magnification, and the comet length of 50 cells was counted using NIH Image analysis software.

Mouse DNA Repair

Mouse experiments were approved by the local Animal Ethics Committee and subject to United Kingdom Home Office licensing. C57Bl-6 mice underwent sublethal total body irradiation in a sealed chamber, using caesium gamma irradiation, at a dose of 7.5 Gy for 20 minutes. Mice were euthanized at differing times from 0 to 48 hours, and tissues were processed for histology.

Atherosclerosis in Rabbits

The study protocol was approved by the Internal Review Board of the Mount Sinai School of Medicine. Aortic atherosclerosis was induced in male New Zealand White rabbits (age, 3 months; weight, 3.5$\pm$0.2 kg) by a combination of a 9-month high-fat diet, enriched with 0.2% cholesterol (Research Diets Inc, New Brunswick, New Jersey), and aortic double-balloon denudation injury, as described previously.[19] At 9 months, rabbits were randomized to high-fat diet, low-fat diet, or low-fat diet+statin for a further 6 months. For full details, see the online data supplement.

Results

Human Atherosclerotic Plaque VSMCs Show DNA Damage and Chronic Activation of DNA Repair Pathways

We first examined human atherosclerotic plaques with a range of disease severity (American Heart Association Grade I to IV) for activation of DNA repair pathways and DNA damage, using immunohistochemistry for phosphorylation of ATM/ATR (ATM and Rad3-related) substrates (a measure of enzyme activity) and the phosphorylated site (Ser139 of H2AX, colabeled with α-smooth muscle actin to identify VSMCs). Phosphorylation of ATM/ATR substrates and H2AX in VSMCs increased with disease severity in vivo (Figure 1A), reaching 70% to 90% of intimal VSMCs in advanced disease. Normal aorta showed minimal expression in medial VSMCs. VSMCs cultured from plaques for several passages retained increased basal ATM and H2AX phosphorylation and increased nuclear foci of these proteins (Figure 1B and 1C), indicating their recruitment to sites of DNA damage and increased DNA strand breaks compared with normal VSMCs (Figure 1D), as assessed by alkaline electrophoresis (comet assay). These results in cultured cells suggest that DNA breaks and associated induction of DNA repair systems are intrinsic to plaque VSMCs and not just in response to the local plaque environment.

Atorvastatin Accelerates DNA Repair in Human VSMCs

To examine whether statin treatment reduces VSMC DNA damage, we administered the potent statin atorvastatin and studied oxidant stress and DNA damage and repair after treatment with the free radical–generating agent tert-butyl hydroperoxide (t-BHP). t-BHP induces DNA breaks in many cell types[20,21] and can induce both "stress-induced premature senescence," and accelerate telomere shortening of human VSMCs.[2] t-BHP increased oxidant stress, DNA breaks and P-ATM and γ-H2AX expression in normal VSMCs to levels seen in plaque VSMCs, suggesting that plaque VSMCs might be subject to chronic oxidant stress (data not shown). Indeed, we have previously shown that plaque VSMCs generate higher levels of reactive oxygen species (ROS) than normal VSMCs in culture.[2] Importantly, atorvastatin did not reduce oxidant stress over 6 hours over a range of concentrations (online data supplement, Figure I). DNA repair was assessed after 1 hour of incubation followed by removal of t-BHP and monitoring strand breaks over time. t-BHP-induced DNA breaks were repaired by 6 hours, as indicated by the normalization of comet tails (Figure 2A). Atorvastatin did not reduce DNA damage (at 1 hour), but significantly accelerated DNA repair, with complete repair by 4 hours (Figure 2A).

To examine the effect of atorvastatin on activation of DNA repair pathways, we studied the induction of P-ATM and γ-H2AX using time points from 0 to 5 hours (Figure 2B) or 0 to 45 minutes (Figure 2C). Phosphorylation of these proteins was induced by t-BHP at 1 hour (P-ATM) or 5 hours (γ-H2AX) (Figure 2B). Atorvastatin accelerated ATM phosphorylation to 15 minutes (Figure 2C) and γ-H2AX phosphorylation to 1 hour (Figure 2B). Mevalonate, an intermediate of cholesterol synthesis downstream of HMG-CoA reductase, reversed the atorvastatin effects, confirming that atorvastatin was acting through HMG-CoA reductase inhibition (Figure 2B). Atorvastatin accelerated P-ATM foci formation at 15 to 30 minutes (Figure 2D), which paralleled the changes seen on Western blotting (Figure 2D).

Acceleration of DNA Repair by Atorvastatin Requires NBS-1

To examine how atorvastatin accelerates DNA repair, we examined signaling molecules upstream of ATM and H2AX, focusing first on the MRN complex and, in particular, NBS-1. Although NBS-1 is an ATM substrate, NBS-1 is required for efficient ATM recruitment to DNA damage sites and its activation.[8] NBS-1 expression and posttranslational modification are also important regulators of DNA repair.[22] NBS-1

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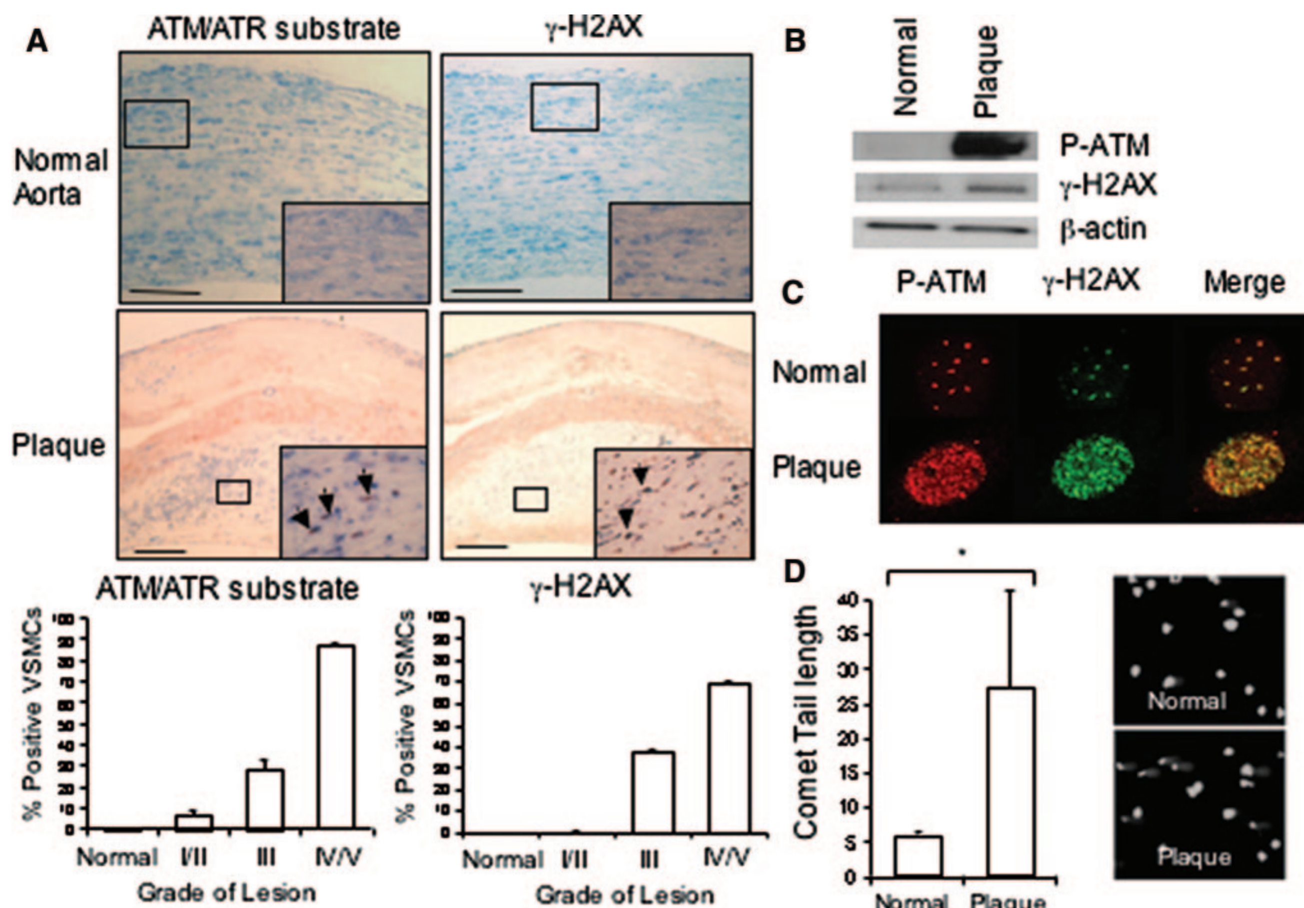

Figure 1. Human atherosclerotic plaques demonstrate persistent markers of DNA damage and repair. A, Immunohistochemistry for phosphorylation of ATM/ATR substrates or γ-H2AX (brown) in human normal aorta or atherosclerotic plaques, colocalized with α-smooth muscle cell actin–positive VSMCs (blue). Arrows indicate double-positive cells. Insets show high-power fields. Scale bar=100 μm. Graphs demonstrate percentages of VSMCs positive for each marker with increasing severity of atherosclerosis assessed by American Heart Association Grade I to V (n=8). B, Western blot for P-ATM and γ-H2AX in cultured normal or plaque VSMCs. C, Immunocytochemistry demonstrating nuclear foci of P-ATM and γ-H2AX in normal and plaque VSMCs under basal conditions. D, DNA strand breaks quantified (left) and demonstrated (right) by comet tail length in normal or plaque VSMCs. Data are means±SEM (n=4). *$P<0.05$.

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showed minimal basal expression in VSMCs but appeared 15 minutes after t-BHP treatment in atorvastatin-treated cells (Figure 2C). Atorvastatin also accelerated induction of NBS-1 and γ-H2AX after treatment with the topoisomerase inhibitor etoposide (from 5 hours to 1 hour) and accelerated DNA repair (supplemental Figures II and III), confirming that its effect was not limited to oxidant stress-induced DNA damage. Neither RAD50 nor MRE were induced by atorvastatin (Figure 2C).

To determine whether NBS-1 was required for the atorvastatin effect, we studied cells with differing expression levels of NBS-1. Wild-type human dermal fibroblasts (HDFs), HDFs lacking NBS-1 ($HDF^{nbs1-/-}$ cells), and $HDF^{nbs1-/-}$ cells containing retrovirus-mediated constitutive expression of NBS-1 ($HDF^{nbs1+/+}$ cells) were incubated with t-BHP. Similar to VSMCs, atorvastatin accelerated ATM phosphorylation in HDFs (to 30 minutes) (Figure 3A) and H2AX phosphorylation from 7 to 2 hours (data not shown), confirming that the atorvastatin effect was not confined to VSMCs. Atorvastatin did not accelerate ATM or H2AX phosphorylation in $HDF^{nbs1-/-}$ cells or $HDF^{NBS+/+}$ cells (Figure 3A and data not shown). t-BHP induced DNA damage in wild-type HDFs, with recovery by 7 hours; atorvastatin accelerated DNA repair in HDFs with recovery by 2 hours (Figure 3B). In contrast, $HDF^{nbs1-/-}$ cells showed defective DNA repair, with incomplete repair even at 7 hours, which was not affected by atorvastatin (Figure 3B). DNA repair was rapid in $HDF^{nbs1+/+}$ cells, with complete repair by 2 hours, but was not also accelerated by atorvastatin. Thus, the ability of atorvastatin to accelerate DNA repair requires NBS-1 and is seen in other mesenchymal cells.

NBS-1 Induction by Atorvastatin Requires Hdm2

The induction of NBS-1 by atorvastatin at 15 minutes (Figure 2C) suggests that NBS-1 regulation is determined by post-translational mechanisms. Indeed, atorvastatin did not alter NBS-1 mRNA expression (data not shown). NBS-1 regulates the nuclear import of MRE11 and RAD50[6] via the importin αKPNA2.[23] Statins can inhibit synthesis of isoprenoid intermediates, which serve as lipid attachments for numerous intracellular signaling molecules. We, therefore, examined NBS-1 expression in cytoplasmic and nuclear fractions after

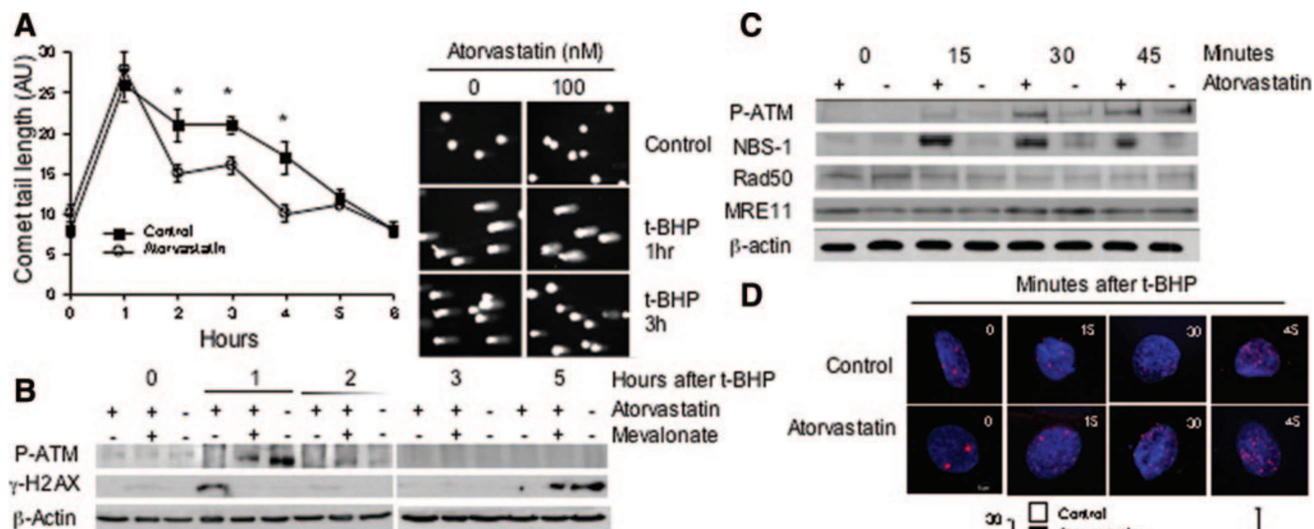

Figure 2. Atorvastatin accelerates DNA repair and activation of DNA repair pathways. A, Human VSMCs were pretreated with 100 nmol/L atorvastatin for 48 hours before administration of 80 μmol/L t-BHP for 1 hour. Left, DNA comet tail length was determined by alkaline agarose gel electrophoresis and quantified using NIH Image (data are means±SEM, **P*<0.05, n=3). Right, Representative images of comet tails 1 or 3 hours after t-BHP treatment or controls. B, Western blot for P-ATM and γ-H2AX 0 to 5 hours after t-BHP with or without atorvastatin and with or without 100 μmol/L mevalonate pretreatments. C, Western blot for P-ATM and MRN complex proteins (NBS-1, MRE11, Rad50) 0 to 45 minutes after t-BHP with or without atorvastatin pretreatment. D, Formation of nuclear foci of P-ATM 0 to 45 minutes after t-BHP with or without atorvastatin pretreatment. Graph quantifies ATM-P foci per nucleus (data are means±SEM, **P*<0.05, n=4).

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treatment with atorvastatin. Atorvastatin rapidly induced the appearance of nuclear NBS-1 (Figure 3C). However, cytoplasmic NBS-1 was undetectable even before t-BHP treatment, indicating that increased nuclear NBS-1 after atorvastatin was not attributable to increased import (Figure 3C). Statins can also inhibit proteasomal activity, although the potency of this effect is unclear.[24] We, therefore, incubated VSMCs with lactacystin, an inhibitor of proteasomal degradation, and compared with atorvastatin. Like atorvastatin, lactacystin induced NBS-1 appearance 15 minutes after t-BHP treatment. However, atorvastatin did not demonstrate significant chymotrypsin-like activity (supplemental Figure IV), indicating that although atorvastatin results in inhibition of NBS-1 degradation, which ultimately occurs through the proteasome, atorvastatin is not a direct proteasomal inhibitor.

Statins can induce Ser166 phosphorylation on the ubiquitin ligase Hdm2,[17] enhancing its nuclear localization and increasing p53 degradation.[17,25,26] Hdm2 phosphorylation also alters its E3 ligase or protein-degrading activity or substrate specificity, independent of Hdm2 localization, binding to its substrate or Hdm2 levels.[27–29] The ability of Hdm2 to bind to NBS-1 at DSBs and inhibit DNA repair requires the NBS-1 binding domain but is apparently independent of its ubiquitin ligase activity.[30] However, statins also reduce Hdm2 half-

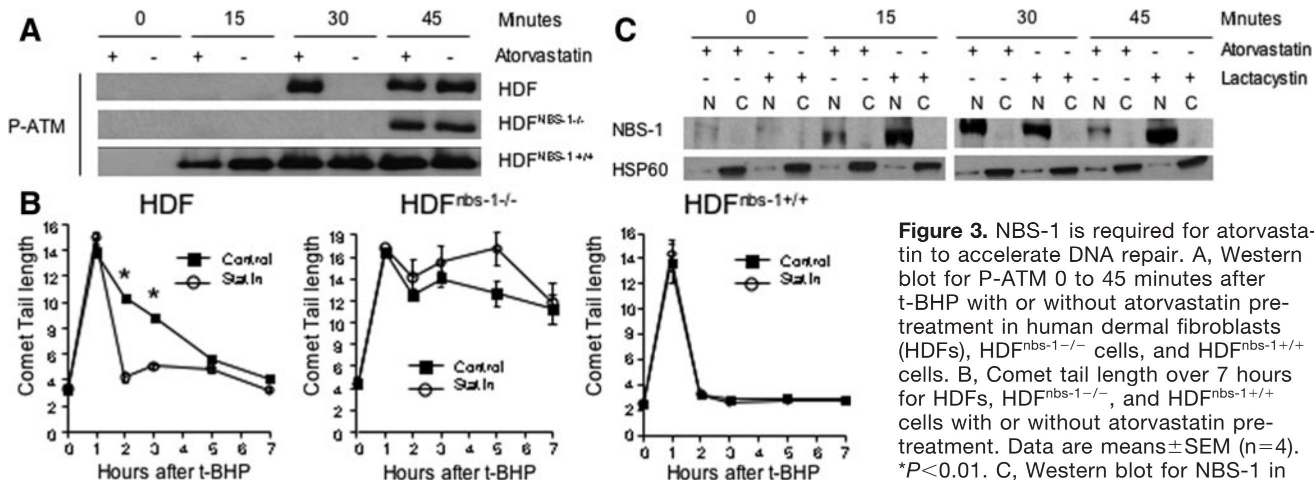

Figure 3. NBS-1 is required for atorvastatin to accelerate DNA repair. A, Western blot for P-ATM 0 to 45 minutes after t-BHP with or without atorvastatin pretreatment in human dermal fibroblasts (HDFs), $HDF^{nbs-1-/-}$ cells, and $HDF^{nbs-1+/+}$ cells. B, Comet tail length over 7 hours for HDFs, $HDF^{nbs-1-/-}$, and $HDF^{nbs-1+/+}$ cells with or without atorvastatin pretreatment. Data are means±SEM (n=4). **P*<0.01. C, Western blot for NBS-1 in nuclear (N) and cytoplasmic (C) subfractions 0 to 45 minutes after t-BHP with or without atorvastatin or lactacystin pretreatments for 48 hours. The cytoplasmic marker heat shock protein 60 (HSP60) is shown as a control.

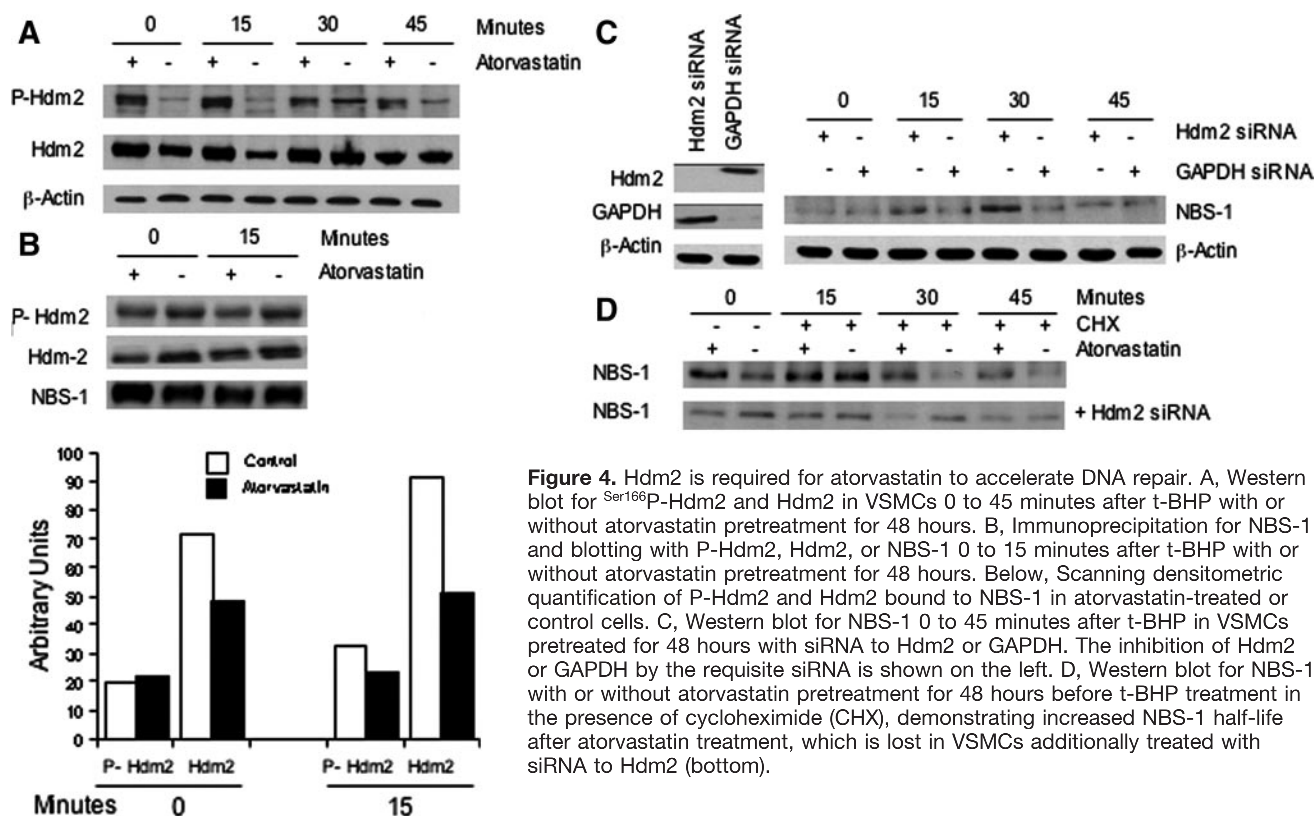

Figure 4. Hdm2 is required for atorvastatin to accelerate DNA repair. A, Western blot for ^{Ser166}P-Hdm2 and Hdm2 in VSMCs 0 to 45 minutes after t-BHP with or without atorvastatin pretreatment for 48 hours. B, Immunoprecipitation for NBS-1 and blotting with P-Hdm2, Hdm2, or NBS-1 0 to 15 minutes after t-BHP with or without atorvastatin pretreatment for 48 hours. Below, Scanning densitometric quantification of P-Hdm2 and Hdm2 bound to NBS-1 in atorvastatin-treated or control cells. C, Western blot for NBS-1 0 to 45 minutes after t-BHP in VSMCs pretreated for 48 hours with siRNA to Hdm2 or GAPDH. The inhibition of Hdm2 or GAPDH by the requisite siRNA is shown on the left. D, Western blot for NBS-1 with or without atorvastatin pretreatment for 48 hours before t-BHP treatment in the presence of cycloheximide (CHX), demonstrating increased NBS-1 half-life after atorvastatin treatment, which is lost in VSMCs additionally treated with siRNA to Hdm2 (bottom).

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life,[17] and it is unclear whether subsequent alterations in Hdm2 expression or phosphorylation alter expression of Hdm-2-bound proteins. In VSMCs, atorvastatin pretreatment for 48 hours induced robust ^{Ser166}Hdm2 phosphorylation, which normalized 30 minutes after DNA damage (Figure 4A), with minimal changes in total Hdm2 expression. Using immunoprecipitation, atorvastatin pretreatment reduced NBS-1 binding to Hdm2 by 32% at time 0 and 44% at 15 minutes after t-BHP (Figure 4B), suggesting that changes in NBS-1 bound to Hdm2 determined NBS-1 expression. To prove that the effect of atorvastatin on NBS-1 required Hdm2, we incubated VSMCs with siRNA to HDM2 and studied NBS-1 induction. Hdm2 inhibition accelerated the appearance of NBS-1 after DNA damage with similar kinetics to atorvastatin (Figure 4C). Furthermore, atorvastatin markedly extended NBS-1 half-life after cycloheximide treatment (from 7 to 14 minutes). siRNA to Hdm2 also significantly extended the half-life of NBS-1 (to 32 minutes), with no additional effect of atorvastatin (Figure 4D). These data suggest that the effect of atorvastatin on NBS-1 is mediated through inhibition of Hdm2-mediated degradation of NBS-1.

Atorvastatin Inhibits Telomere Shortening, Accelerates DNA Repair, Reduces VSMC Death, and Inhibits DNA Damage Markers in Atherosclerosis

Although atorvastatin accelerated DNA repair in VSMCs in vitro, these data do not indicate whether atorvastatin can prevent physiologically relevant DNA damage or accelerate DNA repair in vivo. We, therefore, incubated VSMCs with low-concentration t-BHP, which we have previously shown accelerates telomere shortening in culture.[2] Senescence-associated β-galactosidase activity and telomere shortening were accelerated by t-BHP, but attenuated by atorvastatin treatment (Figure 5A). In endothelial progenitor cells, statins protect telomere function in part by induction of TRF-2.[31] In contrast, in VSMCs, atorvastatin did not alter telomerase activity or expression of the telomere-associated proteins TRF1 or -2, POT1, or PARP-1 (data not shown). Furthermore, atorvastatin reduced telomere shortening and accelerated DNA repair in VSMCs expressing ectopic hTERT,[2] the catalytic subunit of telomerase (data not shown), indicating that the effect of atorvastatin was not mediated by telomerase expression or activity or expression of telomere-associated proteins.

To examine the effect of atorvastatin on the kinetics of DNA repair and cell death after acute DNA damage in vivo, we administered atorvastatin to mice for 2 weeks before sublethal irradiation, harvested organs 0 to 48 hours later, and studied γ-H2AX and apoptosis. In pilot studies, the intestine demonstrated the most reproducible DNA damage and repair response to this regime. Atorvastatin pretreatment did not reduce γ-H2AX expression at 1 hour but significantly reduced γ-H2AX expression after 2 to 4 hours indicating more rapid DSB repair. At 48 hours, statin administration markedly reduced apoptosis (Figure 5B).

In contrast to acute effects on DNA damage and repair after irradiation, DNA damage in atherosclerosis develops slowly over months (animals) or years (man) and resolves equally slowly.[32] To examine the effects of atorvastatin on DNA damage markers in atherosclerosis, we studied rabbits that had undergone a balloon injury followed by high-fat feeding

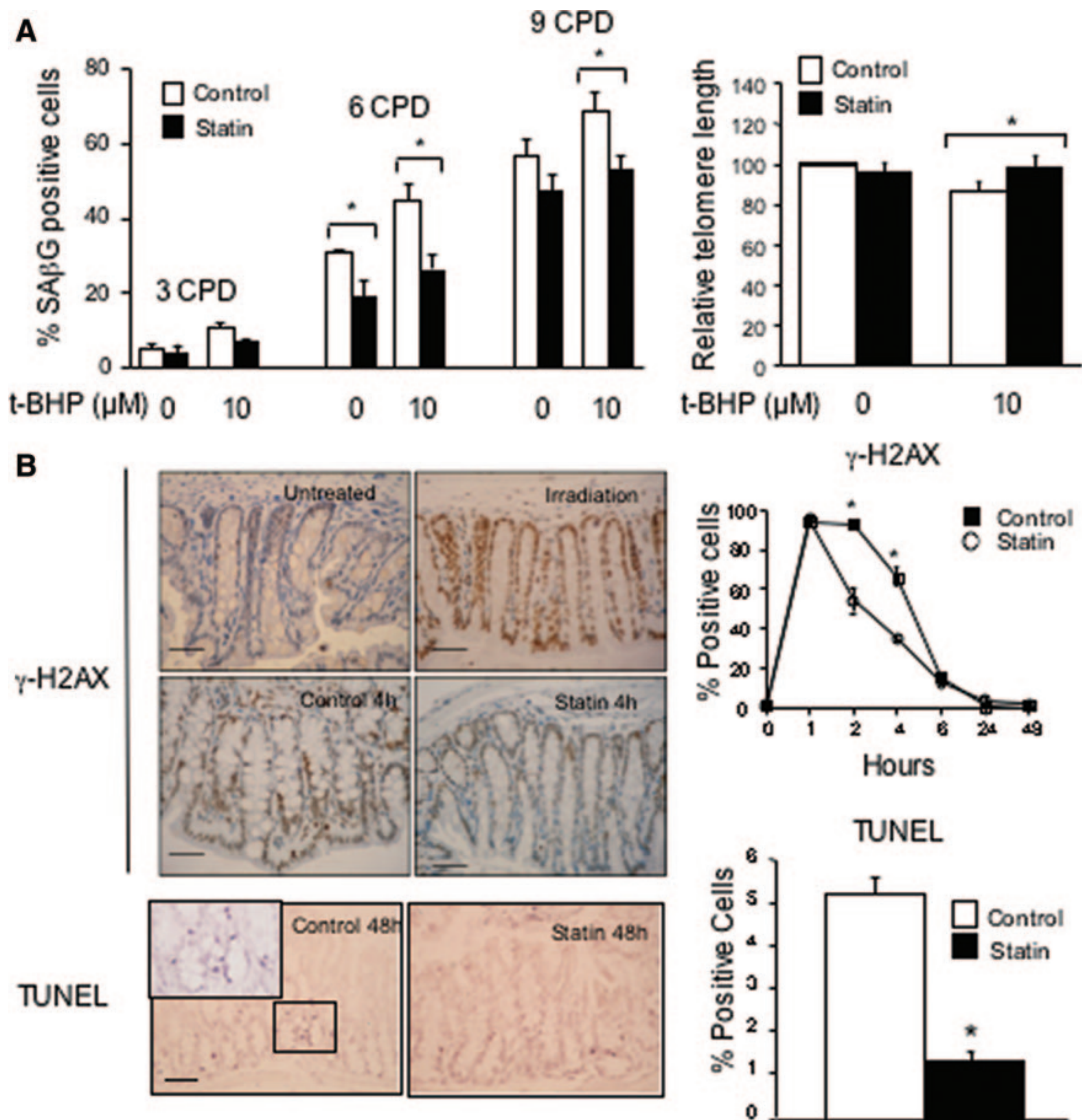

Figure 5. Statins inhibit cellular senescence and accelerate DNA repair in vivo. A, VSMCs were cultured for 9 cumulative population doublings (CPD) with or without 10 μmol/L t-BHP and with or without 100 nmol/L atorvastatin (n=4 cultures). Cells were stained for senescence-associated β-galactosidase activity at 3 to 9 CPD (left), and telomere length was estimated by quantitative PCR of telomere length:36B4 ratio[48] at 9 CPD (right) (n=4). B, Immunohistochemistry (left) and quantification (right) of γ-H2AX (0 to 48 hour) or TUNEL (for apoptosis) at 48 hours in gut of mice undergoing whole-body irradiation with or without pretreatment with atorvastatin. C57Bl6J mice were administered 100 μg/kg atorvastatin per day or methylcellulose (carrier) control by daily gavage for 14 days. Mice were then irradiated with 7.5 Gy, and organs were harvested from 0 to 48 hours (n=4 for each treatment and time point). Scale bars represent 50 μm.

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to develop a preexisting lesion (Figure 6A). After 9 months, rabbits were treated either with a low-fat diet or low-fat diet+statin, and ATM/ATR substrate expression was examined after a further 6 months. Statin treatment reduced the percentage of cells demonstrating ATM/ATR substrate phosphorylation (Figure 6B) associated with plaque regression (change in vessel wall area=−13±4% (low-fat diet+statin) versus 2.5±3% (low-fat diet), $P<0.05$). In both mice and rabbits, statin administration did not reduce serum lipids (data not shown).

Discussion

DNA damage is present in both the circulating cells and atherosclerotic plaques of patients with coronary artery disease. Blood cells from coronary artery disease patients have a higher micronucleus index (a marker of genetic instability) than healthy controls, which correlates with disease severity,[33,34] and increased DNA strand breaks, oxidized pyrimidines, and altered purines.[35] Human plaques show markers of DNA damage, including DNA strand breaks, expression of 8-oxo-G (an oxidative guanine modification), and activation of base excision or nonspecific DNA repair enzymes. Plaques also show critically short telomeres in the fibrous cap,[2] the areas most prone to plaque rupture. DNA damage is also directly correlated with extent of atherosclerosis in experimental animals. For example, cholesterol feeding of rabbits induces 8-oxo-G staining,[32] DNA strand breaks and apopto-

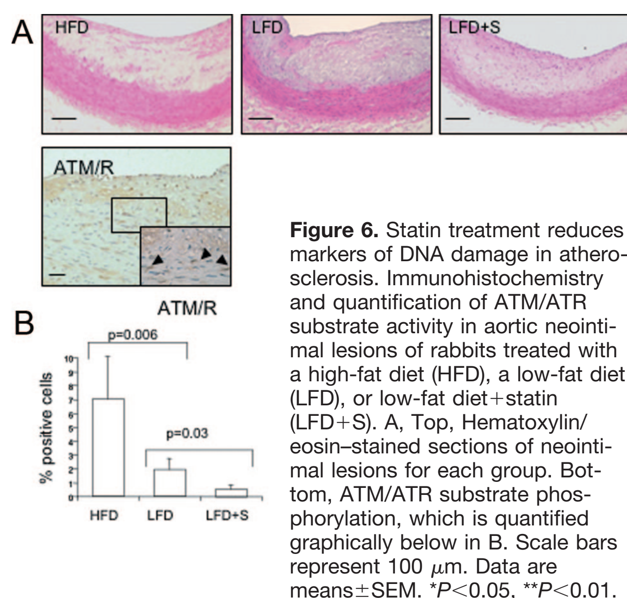

Figure 6. Statin treatment reduces markers of DNA damage in atherosclerosis. Immunohistochemistry and quantification of ATM/ATR substrate activity in aortic neointimal lesions of rabbits treated with a high-fat diet (HFD), a low-fat diet (LFD), or low-fat diet+statin (LFD+S). A, Top, Hematoxylin/eosin–stained sections of neointimal lesions for each group. Bottom, ATM/ATR substrate phosphorylation, which is quantified graphically below in B. Scale bars represent 100 μm. Data are means±SEM. *$P<0.05$, **$P<0.01$.

sis, with increased expression of the DNA repair enzymes poly(ADP-ribose) polymerase 1, DNA-dependent protein kinase, and x-ray repair cross-complementing 1 (XRCC1), and p53 and p53 activation (Ser15 and Ser392 phosphorylation).[1,36] Finally, there is evidence indicating that DNA damage can directly promote atherosclerosis from diseases such as Werner syndrome, which is characterized by defects in DNA repair pathways and accelerated aging including atherosclerosis. Werner protein plays an integral role in base excision repair and at telomeres.[37] Although these data indicate that DNA damage is evident in atherosclerosis, it is not known whether damage can be repaired or prevented, and the mechanism of any effect.

In human plaques, we demonstrate that VSMCs show markers of activation of DNA repair pathways (ATM/ATR substrates) and DSBs (γ-H2AX phosphorylation) in vivo, whose frequency increases with disease severity. Plaque VSMCs in culture maintain ATM and γ-H2AX phosphorylation, organized into nuclear foci at sites of DNA damage, and DNA strand breaks. These findings suggest either that plaque VSMCs continue to generate high levels of DNA-damaging free radicals, even when separated from their local environment, or that their DNA repair pathways are insufficient to repair the accumulated damage, or both. Indeed, we have previously shown that plaque VSMCs show multiple features of cellular senescence, both in vivo and in vitro,[2] and both plaque VSMCs and replicatively senescent normal VSMCs synthesize higher levels of ROS.[2]

Although DNA damage is extensive in atherosclerosis, there has been little study of DNA repair in this disease. Indeed, although DNA strand breaks can normalize within weeks of dietary lipid lowering, 8-oxoG immunoreactivity persists for much longer (12 to 24 weeks) in rabbits,[32] suggesting that DNA repair is inefficient in atherosclerosis. The antioxidant effects of 10 μmol/L atorvastatin on ROS production have been described in VSMCs, acting via alterations of growth factor-induced NADPH or catalase activities, or downregulation of angiotensin II type 1 receptor.[13] In contrast, we found that atorvastatin at a much lower concentration (100 nmol/L) neither reduced the basal production of free radicals in human VSMCs nor altered the extent of initial DNA damage after (external) oxidant stress. However, 100 nmol/L atorvastatin accelerated DNA repair. This effect occurred with other agents inducing DNA damage (eg, etoposide) and other cell types, indicating that it is not limited to VSMCs and oxidant stress. The accelerated DNA repair coincided with the earlier appearance of P-ATM and NBS-1 and earlier formation of P-ATM foci at sites of DNA damage to facilitate DNA repair. The rapid appearance of NBS-1 15 minutes after DNA damage after atorvastatin pretreatment suggested that atorvastatin may be working through NBS-1. Because NBS-1–null human VSMCs are not available, we used HDFs, a mesenchymal cell type with growth control mechanisms that are very similar to VSMCs. Atorvastatin accelerated DNA repair in HDFs even quicker than in VSMCs, associated with the accelerated appearance of P-ATM. $NBS^{-/-}$ HDFs showed markedly impaired DNA repair kinetics that were not affected by atorvastatin. In contrast, constitutive overexpression of NBS-1 rapidly repaired DNA breaks, with the same kinetics as atorvastatin. This suggests that NBS-1 expression levels immediately after DNA damage are rate-limiting for DNA repair in VSMCs and HDFs, an important finding with real therapeutic potential.

Although statins can reduce DNA damage in cultured cells[38] and in vivo in both animals and humans with atherosclerosis, after ionizing radiation or cytotoxic drugs,[12,14,39] the mechanisms underlying this effect are unknown and are likely to be multiple. Statins can reduce oxidant stress,[13] inhibit protein prenylation,[16] and inhibit downstream signaling from damaged DNA,[17,18] all of which may suppress DNA damage or the damage response. Statins can also lead to Ser166 phosphorylation on the ubiquitin ligase mdm2, which enhances its nuclear localization and its interaction with p300, and inhibits its interaction with $p19^{ARF}$, thus increasing p53 degradation.[25,26] We find that statins lead to robust phosphorylation of Hdm2, reduced association of Hdm2 with NBS-1, and reduced NBS-1 degradation. Despite the likely multiple proteins affected by statin treatment, Hdm2 was required for atorvastatin to accelerate DNA repair, because Hdm2 knockdown mimicked the atorvastatin effect and prevented any additional effect of atorvastatin. Previously, Ser166 phosphorylation of Hdm2 was shown to increase its ubiquitin ligase activity and thereby reduce expression of proteins binding to mdm2, such as p53.[17] In contrast, our study is the first to demonstrate that Hdm2 phosphorylation can reduce degradation of the Hdm2-bound protein, NBS-1. The precise mechanism of this effect is not clear; for example, in previous studies, although mdm2 bound NBS-1 at DSBs and inhibited DNA repair, this effect required the NBS-1 binding domain of mdm2 but was independent of its ubiquitin ligase activity.[30]

For the effect of atorvastatin on DNA repair to be significant clinically, statins must inhibit physiological meaningful DNA damage. ROS induce DNA strand breaks and base and nucleotide modifications, particularly in sequences with high guanosine content,[40] such as telomeres.[41] Telomere shortening induces a DNA damage response characterized by acti-

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vation of the same proteins involved in oxidative DNA damage, including ATM, NBS-1, and H2AX.[42,43] We have previously shown that oxidative DNA damage (using t-BHP) dose-dependently accelerates telomere shortening in VSMCs.[2] Here, we find that atorvastatin prevents appearance of SAβG and telomere shortening in VSMCs. Although the effect on telomere shortening could be multifactorial, telomerase expression and expression of telomere-associated proteins was unchanged, and cells containing constitutive telomerase expression still showed accelerated DNA repair after atorvastatin treatment. Thus, we consider it most likely that statins accelerate repair of damaged telomeres, most likely also through NBS-1. Indeed, NBS-1 associates with TRF2 in human telomeres,[44] and is required for telomere maintenance in eukaryotic[45] and yeast cells.[46]

To examine DNA repair acutely in vivo in whole animals, we subjected mice to whole-body irradiation and examined the kinetics of induction and reduction of γ-H2AX (as a marker of DSBs and their repair) and apoptosis. Atorvastatin pretreatment markedly accelerated the return to normal γ-H2AX expression and reduced apoptosis in intestinal mucosa. Although statins can reduce the long-term sequelae of radiotherapy,[47] this is the first study to demonstrate that accelerated DNA repair may underlie some of this effect.

Finally, we show that long-term statin treatment in rabbits with established neointimal lesions reduces expression of ATM/ATR substrate activity. Although this effect was partially seen with lipid lowering, there was an additional effect with low dose statin, independent of any statin effect on serum lipids. Although it is impossible to determine the contribution that accelerating DNA repair (measured over hours) has on a process taking months to achieve, the studies indicate an additional beneficial action of statin treatment on DNA damage in atherosclerosis.

In summary, we have identified a novel mechanism by which statins accelerate DNA repair, via Hdm2 phosphorylation, NBS-1 stabilization, and more rapid ATM and H2AX phosphorylation. Statins attenuate DNA damage, cell senescence, and telomere shortening in VSMCs and may thereby promote plaque stability in atherosclerosis.

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Disclosures

None.

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