The Methyl Xanthine Caffeine Inhibits DNA Damage Signaling and Reactive Species and Reduces Atherosclerosis in ApoE−/− Mice

John R. Mercer, Kelly Gray, Nichola Figg, Sheetal Kumar, Martin R. Bennett

Objective—Caffeine remains one of the most widely consumed drugs in the world. Caffeine has multiple actions, including inhibition of the DNA damage response, and its metabolites, 1-methylxanthine and 1-methyluric acid, are potent antioxidants. Combined, these properties can exert direct effects on cell proliferation, cell death, inflammation, and DNA repair, all important processes that occur in atherosclerosis.

Methods and Results—We first examined the effects of caffeine on mouse vascular smooth muscle cells. Caffeine inhibited activation of the DNA damage response regulator ataxia telangiectasia mutated protein and its downstream targets. Caffeine delayed DNA repair, had a concentration-dependent effect on cell proliferation, and protected against apoptosis. In vitro caffeine reduced oxygen consumption and decreased generation of reactive oxygen species. In vivo caffeine reduced DDR activation in vascular and nonvascular tissues, reduced reactive nitrogen species and serum levels of the DNA adduct 8-oxo-guanine, and inhibited atherogenesis in fat−fed ApoE−/− mice. Reduction in atherosclerosis was independent of the effects on blood pressure and serum lipids but associated with reduced cell proliferation and ataxia telangiectasia mutated protein activation.

Conclusion—The Methyl Xanthine caffeine inhibits the DNA damage response in vitro and in vivo, regulates both cell proliferation and apoptosis after DNA damage, inhibits reactive species, and reduces atherogenesis in ApoE−/− mice. (Arterioscler Thromb Vasc Biol. 2012;32:2461-2467.)

Key Words: caffeine ■ DNA damage ■ atherosclerosis ■ reactive oxygen species

DNA damage is present in human atherosclerosis, particularly in advanced plaques; for example, atherosclerotic plaques show increased expression of the DNA adduct 8-oxo-G, which is the major oxidative lesion in the genome. The cell has evolved an array of proteins to sense, transduce, and mediate a response to DNA damage, termed the DNA damage response (DDR). DDR activation ultimately protects the cell from further damage, by inducing cell cycle arrest to allow DNA repair to occur, or it may induce apoptosis or senescence if damage is excessive. Human plaques show increased expression of DDR markers, including phosphorylated forms of the ataxia telangiectasia–mutated protein (ATM) and H2AX, and activated ATM and ATM-related (ATM/R) kinase substrates, which correlate with increased severity of the atherosclerotic lesion. Inhibition of ATM is associated with increased atherosclerosis indicating that DNA damage may promote atherosclerosis directly and the DDR may be protective.

Reactive oxygen species (ROS) and reactive nitrogen species within the plaque are major stimuli for DNA damage, and the DNA damage induced by reactive species (RS), including superoxide and hydrogen peroxide, have been implicated in atherosclerosis. ROS regulate cell proliferation, apoptosis, and migration, as well as inflammatory gene expression, low-density lipoprotein oxidation, and inactivation of vasoactive compounds, such as endothelial nitric oxide. Although RS generation is a fundamental component of metabolism and signal transduction, unchecked RS can provoke a variety of damage within the cell. For example, RS can induce single- and double-strand DNA breaks and chromosomal translocations and aberrations that contribute to genomic instability and cancer. The atherosclerotic plaque is abundant in RS, as the proinflammatory milieu is a source of intracellular ROS generated through NADPH oxidase and also by conversion of molecular oxygen to superoxide from the mitochondrial respiratory chain. Reactive nitrogen species, such as nitrotyrosine, are generated from nitric oxide and superoxide to produce peroxynitrite that can be equally damaging. Extracellularly, oxidative bursts from monocyte/macrophages are thought to initiate one of the first steps in endothelial dysfunction that leads to atherosclerosis, whereas loss of respiratory chain function is implicated in the ageing of vascular smooth muscle cells (VSMCs). Caffeine (1,3,7-trimethylxantine) is one of the most frequently ingested drugs in the world. However, caffeine
(0.1–10 mmol/L) is an inhibitor of ATM that can suppress cell cycle arrest and abolish DNA damage–induced delays in the cell cycle.\textsuperscript{7,18} Caffeine also suppresses epidermal growth factor and the prosurvival factor Akt,\textsuperscript{19} which is essential for suppression of apoptosis in vascular cells.\textsuperscript{20} These actions would suggest that caffeine might promote atherosclerosis. However, when metabolized by cytochrome p450 1A2 the derivatives of caffeine are potent antioxidants.\textsuperscript{21} In particular, 1-methylxanthine and 1-methyluric acids (1-MUA) are particularly effective as scavengers of the hydroxyl radical,\textsuperscript{22,23} and their activity as antioxidants is comparable with glutathione and significantly higher than ascorbic acid.\textsuperscript{24}

Caffeine has multiple other actions, which may also affect atherogenesis. Caffeine is an A2A adenosine receptor antagonist; these receptors are coupled to G-protein pathways that regulate cAMP production, a key second messenger system for intracellular signal transduction. cAMP activity is mediated through protein kinases and is required for a multitude of housekeeping functions, including carbohydrate and lipid metabolism. Both platelets and endothelial cells express adenosine receptors, and A2A is upregulated and sensitized in response to caffeine blockade.\textsuperscript{25} As caffeine mediates a unique role as a DDR inhibitor and an antioxidant, we examined its effect on atherosclerosis.

### Materials and Methods

#### Primary Cell Cultures and Animal Experiments

Primary murine VSMC cultures were obtained from whole C57Bl/6 apolipoprotein E (ApoE)\textsuperscript{−/−} mouse aortas and identified as described previously by immunocytochemistry for \(\alpha\)-smooth muscle actin and calponin.\textsuperscript{26} All animal experimental procedures conformed to animal ethical committee approval and UK Home Office licensing. Caffeine (Sigma C8960) was administered in the drinking water at an escalating dose (100–400 \(\mu\)g/g) and then maintained at 400 \(\mu\)g/g until study end. Mice were cycled by \(\text{CO}_2\) overdose and perfusion fixed with 5\% buffered formaldehyde.

#### Lipid, Caffeine, and 8-oxo-G Analysis

Whole blood was drawn after an overnight fast and taken before high fat feeding. Serum lipid profiles and caffeine levels were assayed using commercial enzymatic assays (Boehringer). Serum 8-oxo-G levels were assayed using an Oxiselect competitive ELISA protocol (Cell Biolabs).

#### Immunohistochemistry of Aorta and Aortic Root

Murine hearts were postfixed in 10\% formalin, embedded in paraffin, and sectioned from the apex to the coronary artery sinuses as landmarks. 5-\(\mu\)m sections of aortic root plaques were examined at the point of maximum plaque size. Sections were processed for immunohistochemistry, as previously described, using antibodies to \(\alpha\)-smooth muscle actin, Mac-3, Ki67, cleaved caspase-3, and ATM/R substrates.\textsuperscript{26} For cleaved caspase-3 or Ki-67, all positive cells were scored and expressed as a percentage of the total number of cells in each plaque. Cell-type stains, such as smooth muscle actin and mac-3, were scored for the entire plaque based on pixel intensity and expressed as a percentage of the total number of pixels per plaque.

#### Comet Assessment of DNA Damage

DNA damage analysis was performed using Comet assay, as previously described,\textsuperscript{27} using digital quantification with Comet Assay IV software (Perceptive Instruments Ltd, United Kingdom).

### Western Blotting

Western blots were performed using whole cell lysates for phospho-ATM, ATM/R substrate, and \(\gamma\)-H2AX (Cell Signaling Technology) 1:100 primary Ab and 1:500 for secondary Ab, as previously described.\textsuperscript{26}

### Time-Lapse Videomicroscopy

Time-lapse videomicroscopy analysis of cell proliferation and cell death was performed, as described previously.\textsuperscript{27,28} Fluorescently labeled oxidized LDL (BT-920) was obtained from Biomedical Technologies, MA.

### Seahorse XF24

The XF24 is a solid state bioanalyzer that simultaneously measures the 2 major energy-yielding pathways in live, intact cells: oxygen consumption as a measure of aerobic respiration and mPpH as a measure of glycolysis. These variables are quantified over time within microchambers created inside a 24-well microplate. Cells were seeded at 40000/100 \(\mu\)L volume of DMEM containing 2.5 mmol/L caffeine or control. After 24 hours the cells were exchanged into HEPES–buffered Krebs solution at pH 7.4. The plate was inserted into the XF24 analyzer, calibrated, and repeated 40 times using a 3-minute mix, 2-minute wait, and a 3-minute measure cycle. The basal bioenergetic capacity of the cells was then assessed. Respiratory chain inhibitors or uncouplers at 1 \(\mu\)mol/L final concentration were automatically injected using ports A–D in 100 \(\mu\)L volume. Oxygen consumption ± caffeine (2.5 mmol/L) was then obtained.

### Statistical Analysis

Student \(t\) test was used for data after an expected Gaussian distribution and Mann-Whitney rank-sum test used under nonbinominal conditions.

### Results

#### Caffeine Blocks ATM Activation and Signaling in VSMCs In Vitro

To examine whether caffeine can affect the DDR, we first examined stress responses in VSMCs cultured from ApoE\textsuperscript{−/−} mice. t-BHP (tert-butyl-hydroperoxide) is a hydrogen peroxide analogue that induces DNA damage and the DDR.\textsuperscript{29} We tested a range of doses of caffeine from 2.5 mmol/L down to 0.25 \(\mu\)mol/L. 2.5 mmol/L caffeine provided a robust reduction in \(\gamma\)-H2AX expression after t-BHP (Figure I in the online-only Data Supplement) and was thus used for subsequent experiments. To examine the time course of DDR suppression, murine VSMCs were preincubated for 1 hour with 2.5 mmol/L of caffeine and 50 \(\mu\)mol/L of t-BHP added for 1 hour. Activation of phospho-ATM and \(\gamma\)-H2AX occurred rapidly after t-BHP, peaked at 2 hours, but was attenuated by caffeine (Figure 1A). DNA damage induces assembly of multiprotein complexes at double-strand breaks, resulting in visible foci of recruited proteins. Immunocytochemistry confirmed that t-BHP induced phospho-ATM and \(\gamma\)-H2AX foci above basal levels, which were also prevented by caffeine (Figure 1B and 1C). ATM/R activity was examined using an antibody that detects the ATM/R consensus sequence in target substrates that contain a phospho-Ser/Thr preceded by Leu or similar hydrophobic amino acids at the −1 position and followed by glutamine at the +1 position. Caffeine suppressed multiple bands identified as ATM/R substrates (Figure 1D). Finally,
Caffeine Inhibits Atherosclerosis in ApoE<sup>−/−</sup> Mice

Caffeine inhibits the DNA damage response. A, Western blot of phospho–ataxia telangiectasia–mutated protein (p-ATM) and γ-H2AX in murine aortic vascular smooth muscle cells (VSMCs) after treatment with t-BHP (tert-butyl-hydroperoxide) in control cells or after caffeine pretreatment (+). B, Graph of p-ATM foci basally, after t-BHP treatment, or after 1 hour preincubation with caffeine and then 50 μmol/L t-BHP. C, Immunocytochemistry of t-BHP–induced p-ATM or γ-H2AX foci and p-ATM or γ-H2AX foci colocalized, (scale bar, 10 μm). D, Western blot of ataxia telangiectasia–mutated protein (ATM)/R substrates in control cells or in the presence of caffeine (+); arrows indicate loss of downstream activated targets. E, Western blot of downstream ATM targets ser15p53 and Thr68Chk2 in control cells or after caffeine (+) (*P<0.01).

Caffeine reduces DNA repair and induces growth arrest but protects against apoptosis. A, Comet assay for DNA damage in vascular smooth muscle cells (VSMCs) treated with 50 μmol/L t-BHP (tert-butyl-hydroperoxide) with 1 to 4 hours of recovery ± caffeine. B and C, Time-lapse videomicroscopy analysis of the percentage of VSMCs undergoing proliferation (B) or apoptosis (C) over 24 hours (n=3) (Caffeine: +25 μmol/L, +250 μmol/L, ++ 2.5 mmol/L) (t-BHP: +50 μmol/L) (*P<0.05). NS indicates not significant.

Figure 1. Caffeine inhibits the DNA damage response. A, Western blot of phospho–ataxia telangiectasia–mutated protein (p-ATM) and γ-H2AX in murine aortic vascular smooth muscle cells (VSMCs) after treatment with t-BHP (tert-butyl-hydroperoxide) in control cells or after caffeine pretreatment (+). B, Graph of p-ATM foci basally, after t-BHP treatment, or after 1 hour preincubation with caffeine and then 50 μmol/L t-BHP. C, Immunocytochemistry of t-BHP–induced p-ATM or γ-H2AX foci and p-ATM or γ-H2AX foci colocalized, (scale bar, 10 μm). D, Western blot of ataxia telangiectasia–mutated protein (ATM)/R substrates in control cells or in the presence of caffeine (+); arrows indicate loss of downstream activated targets. E, Western blot of downstream ATM targets ser15p53 and Thr68Chk2 in control cells or after caffeine (+) (*P<0.01).

we examined the ATM target proteins p53 and checkpoint kinase 2; caffeine suppressed phosphorylation of both p53 and checkpoint kinase 2 (Figure 1E). We also examined whether caffeine inhibited other stress-induced kinases, including p38 mitogen–activated protein kinase<sup>30</sup> and Akt. BHP induced P-Akt, which was not inhibited by any concentration of caffeine; however, increasing concentrations of caffeine were less effective, suggesting a DDR-independent effect of caffeine to suppress cell proliferation at 250 μmol/L and 2.5 mmol/L (Figure 2B). t-BHP potently induced VSMC apoptosis that was inhibited by all concentrations of caffeine tested (Student t test P<0.05). Thus, caffeine slows DNA repair, prevents DNA damage–mediated growth arrest in VSMCs at low doses, inhibits proliferation at higher doses, but protects against apoptosis.

Caffeine reduces ROS and oxygen consumption.

To examine the ability of caffeine or its major metabolite 1-MUA to act as antioxidants, ROS (including superoxide and hydrogen peroxide) were assayed in cultured VSMCs using the redox-sensitive fluorescent dye CM-H2DCFDA. These nonlinear dyes are indirect measures of ROS and require an intracellular peroxidase and transition metal as a catalyst. t-BHP markedly induced ROS; caffeine and 1-MUA dose dependently attenuated t-BHP–induced ROS (Figure 3A) (Student t test, P<0.05). Interestingly, 100 μmol/L caffeine suppressed ROS to a similar level as 1 μmol/L 1-MUA, indicating that 1-MUA was ≈100-fold more potent than caffeine (Figure 3A).
ROS are generated as a by-product of oxidative phosphorylation in mitochondria, and ROS have also been implicated in regulating oxidative phosphorylation. We therefore examined the effect of caffeine on oxidative phosphorylation using a Seahorse XF24 bioanalyzer, which simultaneously measures oxygen consumption rate as a measure of aerobic respiration and pH as a measure of glycolysis. The respiratory chain is composed of 5 multimeric protein complexes. Energy released from nutrient oxidation is converted to ATP via the transfer of electrons through these respiratory complexes that also creates a proton gradient that can be used by complex V to convert ADP to ATP. The chain can be divided and tested into several component stages (Figure 3B). Basal respiration can be used to calculate the total oxygen-dependent energy consumption of the cell; the addition of the complex V inhibitor oligomycin can be subtracted from this to identify the ATP turnover and the proton leak. Porating the intermitochondrial membrane induces uncoupling of the respiratory chain and consequential futile pumping of protons to maintain a broken gradient. This induces maximal respiration and an oxygen consumption rate during which ROS generation is greatest. Subtracting the basal rate achieves the maximum spare respiratory capacity that could be used for ATP generation. Sequential addition of respiratory chain inhibitors rotenone and myxothiazol inhibits the respiratory chain oxygen consumption rate and confirms the specificity of the reaction, with the residual oxygen consumption being from nonmitochondrial respiratory activity. In confluent (nonproliferating) VSMCs, 2.5 mmol/L caffeine significantly reduced resting oxygen consumption by 2.5-fold (Figure 3C) (Mann-Whitney U test P<0.05). When challenged during uncoupling to examine maximum oxygen and by inference oxygen-dependent ROS generation, caffeine caused a 55.4%±10.5% reduction in spare respiratory capacity, indicating that the amount of oxygen consumed during uncoupling was significantly reduced by caffeine.

Caffeine Reduces the DDR and Atherosclerosis in ApoE−/− Mice

To examine the effect of caffeine on atherosclerosis, we first determined the dose of caffeine required to suppress ATM activation and ROS generation in vivo in nonfat-fed ApoE−/− mice. Mice were administered an escalating dose of caffeine (100–400 μg/g) in their drinking water; 400 μg/g resulted in a serum concentration of 2.4 μg/mL of caffeine, which compares closely with the minimum dose used in vitro. Tissues were removed at 14 weeks and processed for either histology or Western blotting. 400 μg/g caffeine reduced ATM/R subcellular fraction (100–400 μg/mL of caffeine, which compared closely with the minimum dose used in vitro. Tissues were removed at 14 weeks and processed for either histology or Western blotting. 400 μg/g caffeine reduced ATM/R subcellular fraction (100–400 μg/g caffeine in their drinking water. Caffeine resulted in a decrease in atherosclerotic plaque area (Figure 5A and 5B, and IIIB in the online-only Data Supplement), blood pressure, or weight gain (not shown). In contrast, caffeine significantly reduced atherosclerotic plaque area (Figure 5A and 5B, Table). We examined plaque composition using markers of DDR, collagen, and smooth muscle cell markers. The results showed that caffeine significantly reduced the area of atherosclerotic plaques and decreased the expression of DDR proteins (Figure 4A and 4B).

Figure 3. Caffeine reduces reactive oxygen species (ROS) and oxygen consumption in vitro. A, ROS assay by the redox-sensitive fluorescent dye CM-H2DCFDA in vascular smooth muscle cells (VSMCs) treated with increasing concentrations of caffeine or its metabolite 1-methyluric acid (1-MUA) (10 mmol/L–100 μmol/L in the presence of 50 μmol/L tert-butyl-hydroperoxide (t-BHP). Controls are untreated cells or after t-BHP (black bar) (n=3). As indicated 100 μmol/L caffeine gives approximately the same suppression of ROS as 1 μmol/L 1-MUA. B, Stages of mitochondrial respiration. Phases represent oxygen consumption rate (OCR) after inhibition of complex V, during uncoupling, and after the addition of inhibitors to complex II (Rotenone) or complex III (Myxiathiazol). C, OCR in control VSMCs or in the presence of 2.5 mmol/L caffeine (n=3). OCR is compared basally and after injection of inhibitors and respiratory uncoupler FCCP. (**P<0.05).
VSMCs (α-smooth muscle actin and macrophages [Mac3]). Caffeine inhibited atherogenesis with no change in VSMC or macrophage percentages of plaques (Figure 5A and 5B; Table). In contrast, caffeine reduced the number of proliferating cells in plaques, but there was no difference in apoptosis frequencies. Caffeine reduced the expression of ATM/R substrate expression and nitrotyrosine expression as a marker of DDR activity and reactive nitrogen species, respectively, in plaques. Caffeine also reduced serum levels of 8-oxo-G (Figure IV in the online-only Data Supplement) (Student t test, P<0.05).

Discussion

DNA damage is a component of normal cellular ageing that leads to cell cycle arrest, apoptosis, and senescence. The atherosclerotic plaque has increased DNA damage compared with normal vessels, which correlates to the extent of atherosclerotic disease. At present, it is unclear whether DNA damage directly promotes atherosclerosis or is just associated with advanced disease. Studies using inhibitors of DNA damage in models of atherosclerosis should, therefore, indicate whether a causal relationship exists. Here, we describe that the methylxanthine caffeine inhibits the DDR and has potent antioxidant effects in vitro and in vivo and reduces atherosclerosis.

We used t-BHP to stimulate the DDR in cultured mouse VSMCs. Caffeine reduced both phospho-ATM and γ-H2AX activation, foci formation, and downstream signaling, reduced DNA repair, and resulted in an accumulation of DNA damage observed as unrepaired DNA strands extruded from COMET nuclei. t-BHP induced growth arrest, which was blocked by caffeine at low concentrations (25 mol/L). However, 250 μmol/L and 2.5 mmol/L caffeine did not prevent t-BHP–induced inhibition of VSMC proliferation, suggesting that these doses of caffeine directly reduce cell proliferation. t-BHP also potently induced VSMC apoptosis, which was inhibited by all concentrations of caffeine, which has previously been described as mediated through adenosine blockade and attenuation of the cAMP response to cell death. In vitro, caffeine and its major metabolite 1-MUA reduced ROS, and caffeine also reduced basal and uncoupled oxygen consumption, a key source of oxygen free radicals, consistent with observations that RS produced by the respiratory chain can regulate its efficiency. In vivo caffeine reduced DDR activation and RS in the vessel wall and other organs and significantly reduced atherosclerotic plaque area in the aortic root. Caffeine also reduced cell proliferation, DDR activation, and RS in plaques and reduced serum 8-oxo-G.

Caffeine has a number of effects that can directly alter development of atherosclerosis. Early plaques are thought to initiate from endothelial dysfunction and monocyte migration that propagates a proinflammatory environment associated with increased ROS. The ability of caffeine and its metabolites to act as antioxidants and in limiting RS generation would be predicted to delay atherogenesis. In contrast, we and others...
have also shown that DNA damage associated with DDR activation and inhibition of ATM activation and downstream signaling promotes atherosclerosis; therefore, an agent that promotes DNA damage and reduces ATM downstream signaling would be predicted to promote atherosclerosis.

However, caffeine can also regulate cell proliferation and cell death independent of ATM and p53 that may also be predicted to protect against atherosclerosis. At 25 μmol/L, similar to the concentration we achieved in serum in vivo, caffeine inhibited DNA damage–induced cell cycle arrest. However, at higher concentrations a direct inhibition of proliferation was seen and reduced proliferation was also seen in plaques. Caffeine has been reported to induce a G₁ arrest and a DNA damage–induced G₂/M block at 2.5 mmol/L in other studies, although these effects are dose- and cell-type specific. Other reports suggest that caffeine may reverse S and G₁ checkpoints in response to DNA damage but may be less effective in G₁ in fibroblasts and aortic VSMCs at lower doses. It is thus difficult to know whether the potent effect of caffeine on atherogenesis in vivo relates to its ability to regulate cell proliferation. A similar problem is encountered with the effects of caffeine on apoptosis. We have shown that VSMC apoptosis promotes atherogenesis and the progression of established plaques. Here, we show that caffeine protects against DNA damage–induced apoptosis at all concentrations used, but there was no difference in rates of apoptosis seen in plaques between mice treated with caffeine and controls. This highlights the difficulties between in vitro and in vivo models to recapitulate the effects of caffeine. The mechanism by which caffeine is able to protect against cell cycle arrest and apoptosis also remains unclear. Caffeine has multiple actions, most likely reflecting the multiple targets of this agent within the cell. Indeed, recent cDNA microarray work suggests that caffeine is capable of altering expression profiles of multiple genes that regulate these processes, including upregulation of cytochrome p450 expression.

We demonstrate that caffeine inhibits ATM/R activation downstream signaling through p53. However, a number of alternative pathways have been recognized that may mediate cell cycle arrest and apoptosis in the absence of p53 activity, which include p14ARF, c-Jun N-terminal kinase, tumor necrosis factor, and other p53 family members, such as p73. In addition, the recent identification of p38 kinase–mediated cdc25b signal transduction via weel seemed to be a likely candidate in the context of our study. p38 is a mitogen-activated protein kinase that is responsive to stress-activated stimuli, including oxidative stress and DNA damage. Although p38 maintains a variety of housekeeping and homeostatic functions, in the absence of p53 it can regulate the cell’s response to DNA damage and oxidative stress and, ultimately, it is implicated in the induction of cellular senescence. In contrast to ATM, we find that caffeine does not reduce phosphorylation of either Akt or p38.

Although tea and coffee consumption have been extensively studied in human populations, relatively few controlled studies have investigated their effects on cardiovascular disease in humans. Tea and coffee consumption has been suggested to result in mild changes in lipid profiles and hypertension, but studies remain inconclusive that caffeine consumption either prevents or promotes atherosclerosis. Tea and coffee also contain many other biologically active compounds; for example, tea catechins reduce atherosclerosis in ApoE⁻/⁻ mice also without altering lipid profile. However, their effects were associated with reduced plasma lipid peroxides, suggesting that in vivo oxidative stress was reduced, which resulted in reduced atherosclerotic plaque area. In addition, human studies of caffeine intake on atherosclerosis fail to correlate with murine models, which may reflect differences in dose, pharmacokinetics, and species.

In summary, we show that caffeine is a potent inhibitor of DDR activation and RS generation in vitro in VSMCs and in vivo and markedly reduces atherogenesis. Caffeine affects multiple pathways regulating DNA repair, cell proliferation, apoptosis, RS generation, and oxygen consumption, and its effects are related to concentration. The precise mechanisms underlying the potent in vivo effects will require further study.

Sources of Funding
This study was supported by British Heart Foundation grant RG08/009/25841 and the Cambridge National Institute for Health Research Biomedical Research Centre.

Disclosures
None.

References

Table. Histological Analysis of Control ApoE⁻/⁻ Mice Treated With Caffeine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic root plaque area, mm²</td>
<td>3.60±0.08 (n=18)</td>
<td>0.07±0.01* (n=15)</td>
</tr>
<tr>
<td>α-SMA–positive area, %</td>
<td>3.39±0.95 (n=10)</td>
<td>3.38±1.06 (n=13)</td>
</tr>
<tr>
<td>MAC-positive area, %</td>
<td>38.49±4.18 (n=12)</td>
<td>46.87±10.02 (n=12)</td>
</tr>
<tr>
<td>Ki67–positive cells, %</td>
<td>1.36±0.31 (n=18)</td>
<td>0.38±0.32* (n=13)</td>
</tr>
<tr>
<td>Cleaved caspase 3–positive cells, %</td>
<td>0.69±0.20 (n=18)</td>
<td>0.68±0.53 (n=13)</td>
</tr>
<tr>
<td>p-ATM/R substrate–positive cells, %</td>
<td>3.48±1.14 (n=11)</td>
<td>1.57±0.68* (n=10)</td>
</tr>
<tr>
<td>Nitrotyrosine, %</td>
<td>7.42±1.26 (n=9)</td>
<td>2.21±0.43* (n=7)</td>
</tr>
</tbody>
</table>

SMA indicates smooth muscle actin; p-ATM, phosphorylation–ataxia telangiectasia–mutated protein. Morphometric and immunohistochemical analysis of aortic root plaques of control ApoE⁻/⁻ mice or those treated with 400 μg/g caffeine. Data are mean±SEM. *P<0.01.
Caffeine Inhibits Atherosclerosis in ApoE−/− Mice


The Methyl Xanthine Caffeine Inhibits DNA Damage Signaling and Reactive Species and Reduces Atherosclerosis in ApoE−/− Mice

John R. Mercer, Kelly Gray, Nichola Figg, Sheetal Kumar and Martin R. Bennett

Arterioscler Thromb Vasc Biol. 2012;32:2461-2467; originally published online August 2, 2012; doi: 10.1161/ATVBAHA.112.251322

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/10/2461

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/08/02/ATVBAHA.112.251322.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplement Material

<table>
<thead>
<tr>
<th></th>
<th>2.5 mM</th>
<th>250 μM</th>
<th>25 μM</th>
<th>2.5 μM</th>
<th>0.25 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BHP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure I**
Western blot for γ-H2AX in VSMCs treated with decreasing doses of caffeine ± 50 μM BHP.

<table>
<thead>
<tr>
<th></th>
<th>2.5 mM</th>
<th>250 μM</th>
<th>25 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BHP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P-Akt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-p38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure II**
Western blot for P-Akt and P-p38 in VSMCs treated with decreasing doses of caffeine ± 50 μM BHP.
Figure III
Serum lipid levels in control and caffeine-treated mice prior to fat feeding (A), or after 14w fat feeding (B).
Figure IV
Serum 8-oxo-G levels in control and caffeine-treated mice after 14w fat feeding.
* Student’s t-test (p<0.05)