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Cigarette Smoke Initiates Oxidative Stress-Induced Cellular Phenotypic Modulation Leading to Cerebral Aneurysm Pathogenesis

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Abstract

Objective—Cigarette smoke exposure (CSE) is a risk factor for cerebral aneurysm (CA) formation, but the molecular mechanisms are unclear. Although CSE is known to contribute to excess reactive oxygen species (ROS) generation, the role of oxidative stress on vascular smooth muscle cell (VSMC) phenotypic modulation and pathogenesis of CAs is unknown. The goal of this study was to investigate if CSE activates a NAPDH oxidase (NOX) dependent pathway leading to VSMC phenotypic modulation and CA formation and rupture.

Approach and Results—In cultured cerebral VSMCs, CSE increased expression of NOX1 and ROS which preceded upregulation of pro-inflammatory/matrix remodeling genes (MCP-1, MMPs, TNF-α, IL-1β, NF-κB, KLF4) and down-regulation of contractile genes (SM-α-actin, SM-22α, SM-MHC) and myocardin. Inhibition of ROS production and knock-down of NOX1 with siRNA or antisense decreased CSE-induced upregulation of NOX1 and inflammatory genes and

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downregulation of VSMC contractile genes and myocardin. p47phox−/−, NOX knockout mice or pre-treatment with NOX inhibitor, apocynin, significantly decreased CA formation and rupture compared to controls. NOX1 protein and mRNA expression were similar in p47phox−/− mice or those pre-treated with apocynin, but was elevated in unruptured and ruptured CAs. CSE increased CA formation and rupture, which was diminished with apocynin pre-treatment. Similarly, NOX1 protein and mRNA, and ROS were elevated by CSE, and in unruptured and ruptured CAs.

Conclusions—CSE initiates oxidative stress-induced phenotypic modulation of VSMCs and CA formation and rupture. These molecular changes implicate oxidative stress in the pathogenesis of CAs and may provide a potential target for future therapeutic strategies.

Keywords

Smooth muscle; phenotypic modulation; cigarette; smoke; cerebrovascular; atherosclerosis; stroke; aneurysm; subarachnoid hemorrhage; rupture; reactive; oxygen; species; oxidative; stress

Subject Codes

Cerebral Aneurysms; Intracranial Hemorrhage; Ischemic Stroke; Vascular Biology; Hypertension; Inflammation; Cerebrovascular Disease/Stroke

INTRODUCTION

Cigarette smoking exposure (CSE) is a major risk factor for cerebral vascular injury, including atherosclerosis,[1–5] a key process underlying cerebral aneurysm (CA) formation.[6–10] Additionally, CSE is the most significant modifiable risk factor associated with CA formation, progression, and rupture.[11–24] Although CSE has been demonstrated to cause significant alterations in the cerebral immune system and inflammatory response,[25–35] the means by which CSE contributes to the pathogenesis of CAs has not been elucidated.

In vitro and *in vivo* studies have shown that CSE results in endothelial dysfunction in nearly every vascular territory.[27–32] This may be secondary to activation of immune cells, upregulation of pro-inflammatory mediators, and production of reactive oxygen species (ROS).[27] The key mechanisms which lead to CSE induced vascular dysfunction remain unclear.

Unlike terminally differentiated cardiac or skeletal muscle cells, vascular smooth muscle cells (VSMC) retain remarkable plasticity.[36,37] In response to environmental stimuli, VSMCs can undergo changes from cells principally concerned with contraction to cells that are primarily involved in inflammation and matrix remodeling.[36] This phenotypic modulation is defined by decreased expression of key VSMC contractile proteins (smooth muscle myosin heavy chain [SM-MHC], SM-α-actin, and SM-22α) with concomitant increased expression of inflammatory mediators.[36,38–43] VSMC phenotypic modulation is known to be important in the response to vascular injury[41] and the development and progression of atherosclerosis[42], both critical processes behind CA pathogenesis.[43] Although a role has been postulated for the significant pathological effects of CSE, studies

have not assessed its role in VSMC phenotypic modulation or the mechanisms directly contributing to CA formation, progression, or rupture.

Therefore, the aims of the present study are: (1) To evaluate the effect of CSE in inducing phenotypic modulation of cultured cerebral VSMCs through a NAPDH oxidase (NOX) dependent pathway; (2) To assess *in vivo* whether phenotypic modulation of cerebral VSMCs during CA formation is accompanied by increased free radical generation and oxidative stress; (3) To determine if CSE further increases oxidative stress along with downregulation of VSMC marker genes and upregulation of inflammatory genes during CA formation and rupture. 4) To assess whether CSE-induced oxidative stress leads to increased CA formation and rupture and whether genetic and pharmacological inhibition of NOX reverses CSE initiation of oxidative stress, induction of phenotypic modulation, and CA formation and rupture.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

Statistical Analysis

Please see supplemental methods for power analysis. All experiments were performed with a minimum of triplicate samples and were conducted in 3 to 6 independent experiments unless otherwise indicated. Data are presented as mean and range for continuous variables, and as frequency for categorical variables. Analysis was carried out using Chi-square, Fisher's exact, Wilcoxon rank sum, and Kruskal-Wallis test with Wilcoxon rank sum post hoc test as appropriate. Secondary assessment of risk of rupture was carried out by Kaplan-Meier survival analysis with Cox regression analysis to assess hazard ratios (HR). Error bars represent standard error of the mean (SEM). Statistical significance was considered defined as $P<0.05$.

RESULTS

CSE-Induced Expression of NOX and Oxidative Stress in VSMC

As cigarette smoke has been shown to increase inflammation and ROS levels in VSMCs, [27] we sought to determine the role of CSE on NOX expression and on the phenotypic modulation of cultured cerebral VSMCs. VSMC were treated with either 10 or 40 μg/ml CSE for up to 48 h. CSE exposure increased expression of NOX1 mRNA in a dose and time dependent fashion with maximal expression occurring 4 h after exposure followed by a gradual decrease in expression over time (Figure 1A). In contrast, NOX2, NOX3, and NOX4 expression were not significantly changed by CSE (Figure 1 B–D).

To assess if CSE was inducing oxidative stress, we exposed VSMC to 10 or 40 μg/ml CSE and ROS levels were measured for up to 48 h following CSE using lucigenin chemiluminescence as described in the methods. As shown in Figure 1E, ROS levels were increased significantly 30 min following CSE exposure and they continued to increase reaching maximal levels of approximately 5 fold of controls by 4 h which was followed by a gradual decease in ROS levels reaching approximately 2 fold of controls at 48 h (Figure 1E).

Similarly, in situ superoxide imaging of VSMC with dihydroethidium demonstrated an increase in free radical expression following CSE exposure (Figure 1F).

CSE-Induces a Pro-inflammatory & Matrix Remodeling Phenotypic Modulation of VSMCs

To determine if CSE altered the gene expression profile of VSMC, we exposed VSMC to CSE for 24 h and measured gene expression by RT-PCR. CSE significantly increased expression of the pro-inflammatory and matrix remodeling genes MCP-1, MMP-3, MMP-9, TNF-α, IL-1β, iNOS, NF-κB, and KLF-4 (Figure 2). Gene expression was generally maximal at 24 h, at a time point after maximal NOX1 expression. Additionally, pretreatment with the antioxidant enzyme, SOD, abrogated CSE-induced upregulation of pro-inflammatory and matrix remodeling genes (Figure 2A).

CSE Exposure Repressed Myocardin & Phenotypic Marker Gene Expression in VSMC

Although a number of factors control VSMC differentiation, myocardin has been shown to be a key transcriptional regulator of SMC differentiation in peripheral vascular beds.[36] To assess the effects of CSE on phenotypic modulation, we exposed VSMC to CSE and analyzed expression of VSMC marker genes by RT-PCR. CSE exposure significantly reduced expression of myocardin mRNA with a maximal suppression at 24 h (Figure 2B). Similarly, CSE significantly downregulated SM-α-actin, SM-MHC and SM-22α mRNA expression (Figure 2B). CSE-dependent suppression of myocardin and SMC marker gene expression was prevented by pretreatment with SOD (Figure 2B).

NOX1 Decreases Promoter Activity and Expression of VSMC Marker Genes

Our data indicates that the phenotypic modulation of VSMC by CSE is dependent upon oxidative stress. Since we show in Figure 1 that NOX1, an inducer of oxidative stress, is upregulated early following CSE, we were interested in determining if NOX1 regulates VSMC gene expression. To assess the role of NOX1 on the expression of VSMC marker genes, VSMCs were co-transfected with VSMC promoter-reporter constructs and with a NOX1 expression vector. Overexpression of NOX1 significantly decreased SM-α-actin and SM-MHC promoter activity (Figure 2C). The reduced promotor activity in NOX1 overexpressing VSMC was paralleled with a significant reduction in endogenous gene expression of SM- α-actin, SM-22α, SM-MHC, and myocardin levels (Figure 2D). Similarly overexpression of NOX1 significantly increased expression of pro-inflammatory and matrix remodeling genes (KLF-4, MCP-1 MMP2, MMP3, MMP9, IL-1β, NF-κB, iNOS; Figure 2E). To determine if NOX1 is involved in CSE mediated VSMC gene expression, we knocked-down NOX1 protein expression by adenovirus delivered anti-sense NOX1 and analysed CSE changes in gene expression. As demonstrated in Figure 2F, when cells were infected with control anti-sense and CSE, there was a significant decrease in myocardin and SM-MHC and an increase in MCP-1 and KLF-4 expression. In contrast, knock-down of NOX1 reversed CSE-induced decrease of myocardin and SMC-MHC and increase of MCP-1 and KLF-4 (Figure 2F).

To confirm a role of NOX1 in CSE gene regulation, cultured VSMC were transfected with siRNA specific to NOX1 or GFP for 24 hours and treated with CSE (40 μg/ml) for 24 hours. CSE treatment significantly increased the expression of NOX1, KLF-4, IL-1β and VCAM

when treated with control (siGFP), but not when cells were treated with CSE and NOX1spedific siRNA (Figure 2G).

Cerebral Aneurysm Formation

CA formation was carried out as previously described.[44] Based upon dose assessment, experimental planning, and power analysis, mice were injected with vehicle (N=10) or a single stereotactic elastase injection (3.5 mU; N=10, 17.5 mU; N=10, and 35 mU; N=10) and aneurysm formation, defined as 1.5 times the diameter of the parent vessel, determined 28 days later. Cerebral aneurysms were not observed in sham treated animals but were formed in a dose dependent fashion with 10%, 55%, and 80% of animals developing aneurysm formation, respectively (Figure 3A).

Role of NOX in the Formation of Cerebral Aneurysms

Based on our results and other published data,[45] we sought to assess the role of NOX in cerebral aneurysm formation *in vivo*. To test the role of NOX in CA formation, animals began treatment with the NOX inhibitor apocynin three days prior to elastase injection and were compared to CA formation in vehicle treated mice and p47phox−/− mice, which lack NOX activity. Mice pre-treated with apocynin, p47phox−/− mice, and those treated only with vehicle undergoing CA induction had significant increases in systolic blood pressure 7 days after elastase injection (35 mU) that was sustained until 28 days, but was not significantly different between the 3 cohorts at any time point (Figure 3B).

Analysis of CA formation reveled aneurysm formation in 21 of 25 animals (84%) receiving 35 mU of elastase and vehicle as compared to 8 of 25 animals (32%, $p<0.001$) treated with apocynin (Figure 3C). The incidence of ruptured aneurysm was also significantly increased in vehicle treated mice, 15 of 25 (60%), compared to those receiving apocynin (3 of 25, 12%, p=0.001; Figure 3C). CA formation was associated with the development of neurological deficits (score 1–5) and aneurysmal SAH between day 7 and 21. Survival analysis demonstrated that animals treated with apocynin were 7.2 times (95% CI 2.1–25.3, p=0.002) less likely to have CA rupture compared to those receiving vehicle. Next we induced CA formation in p47phox−/− mice, which lack NOX activity. As compared to wild-type mice, p47phox−/− mice were significantly less likely to form CAs (2 of 12; 16.7%; p=0.001; Figure 3C), and had a lower incidence of CA rupture (1 of 12; 8.3%; p=0.004; Figure 3C). Similarly, survival analysis demonstrated that p47phox−/− mice were also less likely to experience CA rupture (HR=10.3; 95% CI 1.4–78.2, p=0.024). As the incidence of CA formation and rupture was low in p47phox−/− mice, these experiments were aborted after the first cohort of 12 mice. These results suggest that NOX plays an important role in CA formation.

NOX Expression in Unruptured and Ruptured Intracranial Aneurysms

Histological assessment of the CAs demonstrated structural changes that were similar to those found in human CAs (Figure 4, an enlarged image is shown in supplemental Figure I).[46,47] Hematoxylin and Eosin and trichrome staining of cerebral vessels from sham operated mice demonstrated two to three layers of smooth muscle cells and a single layer of continuous endothelial cells (Figure 4; A1–2). Trichrome staining demonstrated one layer of

elastic lamina (A2).[47] In CA, there were layers of discontinuous endothelial cells and scattered VSMCs (Figure 4; B1–2, unruptured and C1–2; ruptured). Trichrome staining revealed disorganized elastic lamina (B2 and C2).

As compared to sham mice, there was no difference in immunofluorescence reactivity for NOX1 when comparing p47phox−/− mice or those treated with apocynin (not shown). NOX1 immunoreactivity was significantly higher in unruptured CAs, and highest in ruptured CAs (Figure 4; A–C3 and A–C4). Next we sought to determine the cellular source of NOX1. To localize NOX1 expression, samples were co-stained with either SMC-22α for SMCs or CD68 for macrophages and NOX1 (Figure 4). NOX1 expression was observed to co-localize with both SMCs and macrophages in both unruptured (Figure 4; B3–4) and ruptured CAs (Figure 4; C3–4). SM-22α but not with CD68 was observed in normal-sized cerebral blood vessels adjacent to CAs (C3). There were more macrophages observed in ruptured CAs (C4) as compared to unruptured CAs (B4), and an absence of macrophages in sham operated mice (A4).

To assess whether an increase in NOX1 expression leads to increased oxidative stress during CA formation, we measured superoxide production with dihydroethidium as described in methods. As shown in Figure 4 (panel A5) there was a low level of free radical formation observed in sham operated mice. In contrast free radical expression was increased in unruptured CAs (panel B5) and further increased in ruptured CAs (panel C5) as compared to sham operated mice (A5). There was limited expression of free radicals in p47phox−/− mice or those treated with apocynin (not shown), suggesting a possible connection between NOX1 expression and free radical production in CA formation.

To confirm that NOX1 is expressed during CA formation, we analyzed NOX1 expression by RT-PCR. As compared with sham operated mice, RT- PCR analysis demonstrated no significant difference in NOX1 mRNA expression in cerebral blood vessels from p47phox −/− mice or those treated with apocynin (Figure 4D). NOX1 mRNA extracted from unruptured, and even more so from ruptured CAs, was significantly elevated as compared with sham operated mice. NOX2, NOX3, and NOX4 expression were not significantly changed in the various cohorts (Figure 4E–G). As a secondary control, β-actin expression was assessed and found to be not significantly different among sham operated mice, p47phox −/− mice, or those treated with apocynin, unruptured aneurysm and ruptured CAs (data not shown).

NOX Regulates SMC Phenotypic Modulation in Unruptured and Ruptured Cerebral Aneurysms

Our *in vitro* VSMC studies reveal a NOX1 dependent regulation of VSMC phenotypic gene expression. Therefore we were interested in determining if NOX also regulated VSMC gene expression, *in vivo*. To test this possibility we first assessed whether VSMC phenotypic modulations occur in our CA model system, by examining alterations in expression of VSMC marker genes and pro-inflammatory, and matrix remodeling genes. As shown in Figure 4H, SM-α-actin, SM-MHC, SM-22α mRNA levels where decreased in ruptured CAs as compared to unruptured CAs. As compared to both unruptured and ruptured CA, sham operated mice, p47phox −/− mice, and those treated with apocynin had significantly higher

levels of the VSMC marker genes (Figure 4H). Additionally, myocardin was decreased in unruptured CAs, and further decreased in ruptured CAs (Figure 4H).

Similar to our *in vitro* gene expression studies we found that the pro-inflammatory and matrix remodeling genes, MCP-1, MMP-3, MMP-9, TNF-α, IL-1β, iNOS, TNF-α, NF-κB, VCAM and KLF-4 were increased in unruptured CAs, and further increased in ruptured CAs (Figure 4I).

The Role of CSE in Cerebral Aneurysm Formation and Rupture

To assess the effects of CSE in CA development and rupture, mice underwent daily CSE for 7 days prior to nephrectomy. Mice then underwent CA induction surgery (3.5 mU elastase) as described in the Methods. CSE control mice and mice exposed to CSE, and CSE + apocynin pre-treatment had significant increases in systolic blood pressure 7 days after elastase injection that was sustained until day 28, but was not significantly different between the 3 cohorts at any time point (Figure 5A).

Analysis of CA formation revealed that the incidence of CA formation was significantly higher in mice receiving CSE (13 of 30; 43.3%) when compared to mice receiving CSE with pre-treatment of apocynin (6 of 30; 20%), or control mice not receiving CSE (3 of 30; 10%; p=0.011, Figure 5B). Similarly, the rate of CA rupture was also significantly increased in mice receiving CSE alone (9 of 30; 30%) versus those receiving CSE, but pre-treated with apocynin (2 of 30; 6.7%) and control mice that did not receive CSE (0 of 30; 0%; p=0.001; Figure 5B). CA formation was not observed in animals receiving CSE and undergoing sham surgery (data not shown).

Since CSE enhances both CA formation and rupture we were then interested in determining if CSE altered NOX1 expression in both unruptured and ruptured CAs. Therefore, we assessed NOX1 mRNA levels following CA induction. As demonstrated in Figure 5C, RT-PCR analysis revealed that CSE increased expression of NOX1 in unruptured CAs, which was further increased in ruptured CA. In contrast there was no change in NOX2, NOX3, or NOX4 expression in any of the cohorts (Figure 5D–F). The elevated expression of NOX1 in CA of CSE treated mice was also increased over animals that underwent sham surgery but received CSE and furthermore over a control cohort that underwent sham surgery but did not receive CSE or a cohort undergoing surgery with CSE but pretreated with apocynin. The increase in NOX1 expression in CA following CSE was paralleled with a similar increase in superoxide production, as assessed by dihydroethidium imaging. Exposing mice to CSE alone did not substantially increase dihydroethidium fluorescence when compared to controls, whereas pretreatment with apocynin, which inhibits NOX activity, reduced the dihydroethidium fluorescence. (Figure 5G). Superoxide production was found to be increased in unruptured and furthermore in ruptured aneurysms (Figure 5G. an enlarged image is shown in supplemental Figure II).

CSE Initiates NOX-Dependent VSMC Phenotypic Modulation in Cerebral Aneurysm Formation and Rupture

To assess whether VSMC phenotypic modulation occurs in the formation and rupture of CAs following CSE, we assessed alterations in mRNA expression of VSMC marker genes

and pro-inflammatory and matrix remodeling genes. SM-α-actin, SM-MHC, SM-22α mRNA, and myocardin expression where found to be decreased in ruptured CAs as compared to unruptured CAs, and as compared to those pre-treated with apocynin (Figure 5H). Additionally, animals receiving CSE that underwent sham surgery, had decreased levels of SM-α-actin, SM-MHC, SM-22α mRNA as compared to those pre-treated with apocynin or a sham surgery group that was not exposed to cigarette smoke (Figure 5H).

Analysis of inflammatory and matrix remodeling genes revealed increased expression of MCP-1, MMP-3, MMP-9, TNF-α, IL-1β, iNOS, TNF-α, NF-κB, VCAM and KLF4 in unruptured CAs, which were further increased in ruptured CAs receiving CSE (Figure 5I). This was also increased over animals that underwent sham surgery, but received CSE and furthermore over cohorts either pretreated with apocynin or those only receiving sham surgery.

Direct Application of CSE Alters NOX1 Expression and VSMC Phenotype

To access if CSE directly affects NOX1 expression and modulates phenotypic gene expression, *in vivo*, or whether other systemic factors are required, we directly applied CSE to the adventitial surface of rat carotid arteries using a pluronic gel as detailed in Methods. Consistent with our previous findings NOX1 mRNA expression was significantly increased 6 h following CSE application when compared to application of pluronic gel alone (Figure 6A). The increase in NOX1 was followed with a significant decrease in mRNA expression of the VSMC marker genes (SM-22α, SM-αActin, and SM-MHC) and myocardin and with a parallel increase in pro-inflammatory and matrix remodeling genes, KLF4, MCP1, MMP3, MMP9, TNF-α, and IL-1β (Figure 6B–C) 12 h following CSE application.

DISCUSSION

Smoking is a key modifiable risk factor for CA formation and rupture. However the precise mechanisms of CSE induced vascular alteration and injury in the cerebral circulation remain unclear.[11–24] CSE can cause significant alterations in the cerebral immune system and inflammatory pathways, resulting in direct vascular injury and production of ROS.[25–35] Additionally, prior studies have found that CSE results in endothelial dysfunction, leading to attraction of pro-inflammatory mediators and immune cells, resulting in further SMC injury.[27–32] As these are key mechanisms underlying CA pathogenesis, it has been hypothesized that CSE [8], oxidative stress [48], and SMC phenotypic modulation [49] contribute to a pro-inflammatory environment [7], which promotes CA formation and rupture. In this study, we have found that NOX and oxidative stress contributes to CA formation and rupture. Additionally, CSE leads to upregulation of NOX1 and free radical oxidative stress both *in vitro* in cultured cerebral VSMCs and *in vivo* in a mouse model of CA formation and rupture. Excess free radical production leads to VSMC phenotypic modulation, with alteration of VSMC function from a role primarily concerned with contraction to that of inflammation and matrix remodeling. Both pharmacological and genetic inhibition of NOX-induced oxidative stress prevents VSMC phenotypic modulation and decreases both CA formation and rupture. CSE increases oxidative stress and CA formation and rupture, and

pharmacological inhibition of NOX is protective against CSE-induced oxidative stress, VSMC phenotypic modulation, and CA formation and rupture.

Increased ROS have been previously demonstrated in human CAs, which may arise from a number of potential sources.[50] In this study, we found upregulation of NOX1 and oxidative stress in an *in vivo* model of CA formation and rupture that involves induced hypertension. We, and others, have also found that NOX expression and ROS formation is increased in an *in vivo* model of CA formation that involves induced hypertension and hemodynamic stress prior to CA formation.[50,51] We also demonstrate that CSE further incites NOX1 expression and induces oxidative stress, and further contributes to CA formation and rupture. Hypertension, hemodynamic stress, and CSE are significant risk factors for CA formation and rupture, and likely all contribute to ROS formation [7,8,48]. CSE is a major potential cause of CA induction, which exert its effects through activation of NOX and production of ROS [52,53]. The observed effects of CSE may arise directly from the gas/tar phase, activated inflammatory cells, or endogenous sources of ROS, such as xanthine oxidase, uncoupled nitric oxide, and the mitochondrial electron transport chain.[32,53] Once activated, VSMCs and activated macrophages can further produce ROS within CAs.

In this study, we found that CSE results in NOX-induced VSMC phenotypic modulation both *in vivo* and *in vitro*. CSE-induced NOX1 upregulation and ROS formation was preceded by downregulation of VSMC marker genes. Additionally, inhibition of free radical formation reversed NOX1-induced VSMC phenotypic modulation. Similarly, NOX-induced VSMC phenotypic modulation occurred in CA formation and rupture, and was similarly abrogated by both genetic and pharmacological inhibition of NOX. In the peripheral circulation, myocardin is a key transcriptional promotor of SMC differentiation through transcriptional upregulation of SMC marker genes [36,38–40]. Myocardin was found to be decreased both in cultured cerebral VSMCs and CAs. Results from the present experiments support that peripheral phenotypic modulation in VSMC pathology occurs through similar mechanisms within CAs.

Increased NOX1 mRNA expression was observed in both isolated VSMC and in CA tissue following CSE and was paralleled with a robust increase in NOX1 protein expression in unruptured and ruptured CA tissue. This increased in NOX1 protein levels following CA induction was primarily observed in VSMC, and to a degree, in macrophages. This is in contrast to NOX2, NOX3, and NOX4 which were not significantly increased by CSE. Our in vitro results demonstrate a link between NOX1 expression and VSM gene regulation. However because apocynin and p47phox null mice inhibit other NOX family members besides NOX1 we cannot fully rule out the possibility that altered NOX2, NOX3, or NOX4 protein activity, and not gene expression, also contributed to the observed effects of CSE on aneurysm pathogenesis.

Immune cells and inflammatory markers have been found to be systemically increased in cigarette smokers.[33–35] Studies in cell culture and animal models have demonstrated increased immune modulating cells and cytokines following CSE.[27–32] In the present study CSE resulted in a pro-inflammatory, matrix remodeling phenotype in a NOX-dependent fashion. Both *in vivo* and *in vitro* CSE resulted in downregulation of myocardin, SM-α-

actin, SM-MHC and SM-22α, and upregulation of MCP-1, MMPs, TNF-α, IL-1β, and NF-κB. These alterations have been found to be critical steps in the initiation of atherosclerosis, vascular disease, and CA pathogenesis. We previously found that TNF-α expression is increased in CA formation and rupture, and that genetic and pharmacological inhibition of TNF-α decreases formation and rupture of CAs. ROS have been shown to activate TNF-α which, as an immune modulator, can activate further pro-inflammatory cascades in macrophages as well as phenotypic modulation in VSMCs. MCP-1 has been found to be increased in CA walls, and genetic inhibition of MCP-1 decreases MMP production and the incidence of CA formation. This may work through a variety of mechanisms, including chemoattraction of macrophages into CAs leading to further production of ROS, TNF-α and MMPs.[54] MMPs degrade the extracellular matrix[55] and have been found to be increased in human CAs.[56] Inhibition of MMPs has also been shown to decrease the incidence of CA formation and progression in animals.[47,57] NF-κB has also been shown to help produce ROS, and prior studies have demonstrated that it is necessary for CA formation. Additionally, IL-1β promotes SMC phenotypic modulation through increased expression of pro-inflammatory genes and has been found to be a key mediator of atherosclerosis and vascular injury.

It currently remains unclear whether CA rupture is the end result of CA formation, or if molecular mediators are activated after CA formation and are required for CA progression and eventual rupture. In the current study, CSE-initiated oxidative stress induced VSMC phenotypic modulation leading to both CA formation and rupture. Oxidative stress can result from increased production and/or decreased removal of free radicals, and can result in DNA damage, cellular toxicity, and apoptosis [58]. Other key inflammatory mediators, such as TNF-α, help regulate apoptosis and phagocytosis of VSMCs and may lead to CA progression and rupture.[44,59–61] Following exposure to CSE, VSMCs change from a contractile phenotype to one characterized by pro-inflammatory characteristics and matrix remodeling, which may eventually lead to apoptosis, with loss of both phenotypes and eventual CA rupture.

Further assessment of the molecular mechanisms underlying both CA formation and progression is indicated. We have previously found that epigenetic control mechanisms, including alterations in histone modifications characteristic of transcriptional suppression, [62–64] provided evidence that KLF4 regulates cerebral VSMC phenotypic modulation through inhibition of myocardin-mediated activation of SMC genes.[41,65] KLF4 is a pluripotency factor involved in the reprogramming of somatic cells.[66] Prior studies have also shown that KLF4 is required for SMC phenotypic modulation following vascular injury[41] and experimental atherosclerosis.[42] The SMC MHC gene[39] and myocardin promoter[38] also possess KLF4 binding sites, and are inhibited during SMC phenotypic switching to an inflammatory phenotype. KLF4 has also been found to be integral to the activation of macrophages, an inflammatory cell necessary for CA formation.[54,67] In the present study, KLF4 was increased and myocardin was decreased following CSE, but after NOX1 upregulation and formation of ROS.

Although oxidative stress and VSMC phenotypic modulation are likely key elements for CA pathogenesis, it is probably a multifactorial process that leads to CA pathogenesis. In this study we focused upon the effects of CSE on VSM phenotypic modulation, a characteristic

of aneurysm pathophysiology, and on CA pathogenesis. However, CSE would have a global effect on the cellular vascular environment, affecting not only VSM but also endothelial cell, and infiltrating macrophages which would all culminate in CA formation, progression and rupture. Interestingly CSE increases intracellular communication between VSM, and endothelial cells, and macrophages leading to altered modulation of signaling molecules associated with abdominal aortic aneurysm formation[68]. The ability of CSE to induce endothelial dysfunction in cerebral arteries is well documented [69]. Therefore it is likely that the CSE induced aneurysm formation and rupture observed in this study is the result of the accumulative effects of VSM, endothelial cells and macrophages dysfunction by CSE.

In conclusion, this is the first study to assess how CSE induces oxidative stress and cerebral VSMC phenotypic modulation in CA pathogenesis. It provides novel *in vitro* and *in vivo* evidence that CSE profoundly suppresses expression of mature cerebral VSMC marker genes and promotes matrix remodeling/inflammatory gene expression in CA formation and rupture. These processes are, at least in part, regulated by CSE induction of NOX1-associated oxidative stress. This provides important mechanisms whereby CSE may contribute to CA pathogenesis and provide pathways for protective therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

BAPN	Beta-aminoproprionitrile
CA	Cerebral aneurysm
CSE	Cigarette smoking exposure
DOCA	deoxycorticosterone acetate
GFP	Green fluorescent protein
FBS	Fetal bovine serum
HBSS	Hank’s Balanced Salt Solution
KLF4	Kruppel-like factor four
MOI	Multiplicity of infection

NOX	NADP
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SEM	Standard error of the mean
SM22α	Smooth muscle 22 alpha
SM-MHC	Smooth muscle myosin heavy chain
SM-α-actin	Smooth muscle alpha actin
SMC	Smooth Muscle Cells
VSMC	vascular smooth muscle cells

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Highlights

- Cigarette smoke exposure increases expression of NOX1 and ROS in vascular smooth muscle cells.
- Cigarette smoke exposure induces NOX1 and ROS dependent vascular smooth muscle phenotypic modulation.
- NOX1 and ROS are increased in aneurysms and even further in ruptured aneurysms.
- Inhibition of NOX reduces aneurysm formation and rupture.
- Cigarette smoke exposure increases cerebral aneurysm formation and rupture.

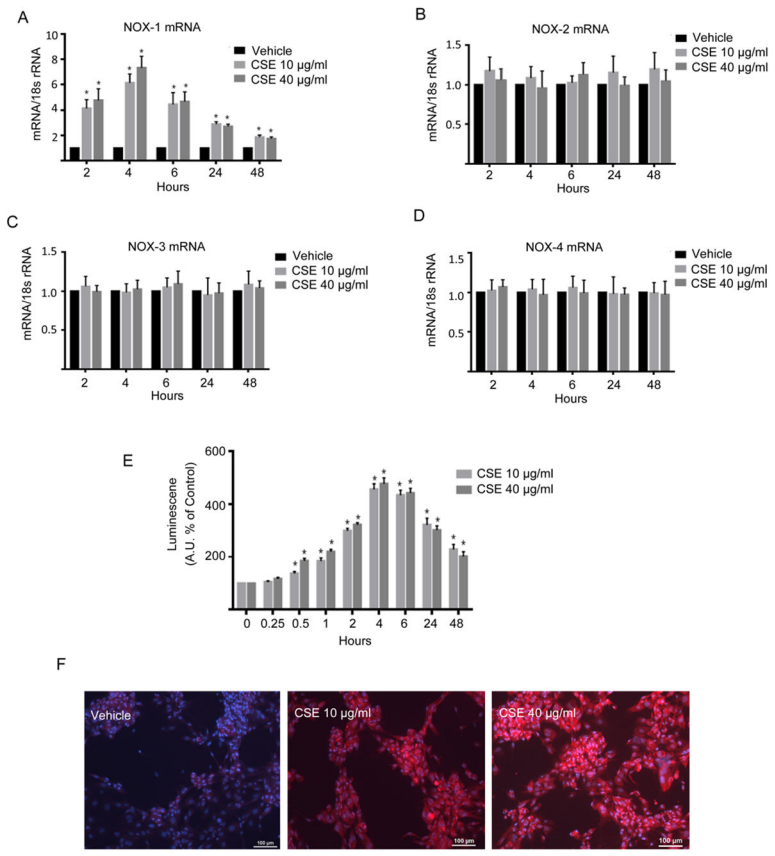

Figure 1. CSE-Induced expression of NOX1 and ROS in VSMCs

Cultured cerebral VSMCs were treated with the indicated concentrations of CSE. NOX1, NOX2, NOX3 and NOX4 mRNA expression was quantified using RT-PCR and normalized to 18S rRNA, and expressed as fold increase over vehicle A–D). Values represent mean ± SEM of n = 3–6. **P*<0.05 vs control (vehicle) using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. E) Differential time-course for CSE-induced ROS production in cerebral VSMCs assessed by lucigenin-enhanced chemiluminescence. Data represent the mean ± SEM of n=4 to 6 experiments; **P*<0.05 vs time 0 using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. F) Following stimulation with vehicle or CSE (10 or 40 μg/ml) cerebral VSMCs were incubated with dihydroethidium and then imaged by fluorescence microscopy.

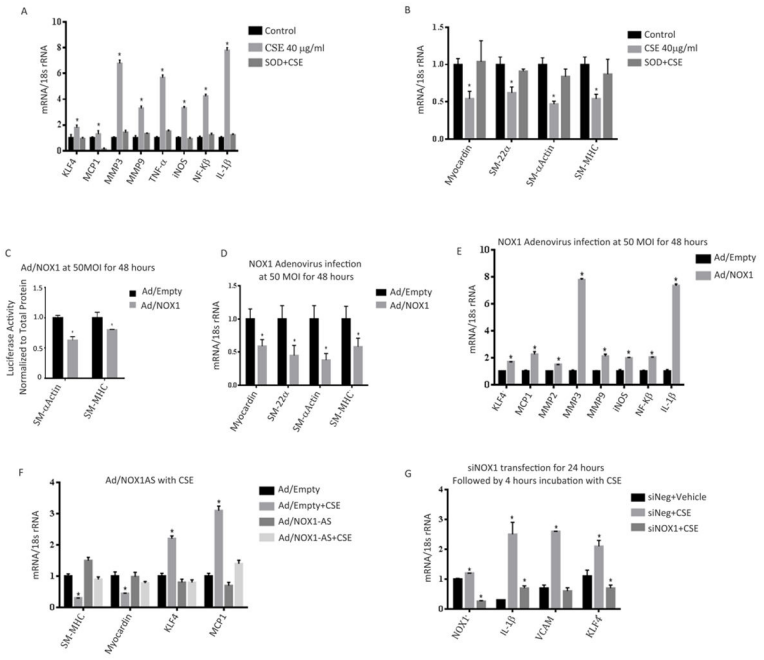

Figure 2. CSE Induces pro-inflammatory & matrix remodeling phenotypic modulation of VSMCs and represses myocardin and SMC marker gene expression in cultured cerebral VSMC

Cultured cerebral VSMCs were treated for 24 h with the indicated concentration of CSE with or without SOD pre-treatment. RT-PCR was performed, normalized to 18S rRNA, and expressed as fold increase over vehicle. Pro-inflammatory and matrix remodeling gene expression is shown in A) with myocardin and SMC marker gene expression shown in B). **P*<0.05 vs control (vehicle) using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. C) SM-MHC and SM- α-actin promoter-luciferase constructs were transiently transfected into cerebral VSMCs for 24 hours followed by overexpression of NOX1 with adenovirus for 24 hours. Luciferase activity was measured and normalized to total protein content and then expressed as fold increase over control (empty adenovirus). **P*<0.05 vs control using Wilcoxon rank sum. The effects of NOX1 overexpression on SMC marker genes (Myocardin, SM-22α, SM-α-actin and SM-MHC levels is shown in D) with pro-inflammatory and matrix remodeling genes expression levels shown in E). All RT-PCR data was normalized to 18S rRNA and expressed as fold increase over control (empty adenovirus). **P*<0.05 vs control using Wilcoxon rank sum. F) Cultured VSMC were treated with CSE (40 μg/ml) and infected with NOX1 adenovirus (AD) or anti-sense NOX1 adenovirus (AS) for 24 h. RT-PCR was performed to measure the mRNA expression levels of myocardin and the SMC marker gene, SM-MHC and the pro-inflammatory and matrix remodeling genes KLF4 and MCP1 which were normalized to 18S rRNA and expressed as fold increase over control (empty adenovirus). **P*<0.05 vs control using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. G) Cultured VSMC were transfected with siRNA specific to NOX1 or siNeg (GFP) and treated with CSE (40 μg/ml) for 24 h. mRNA expression of indicated genes were analyzed with RT-PCR and normalized to 18S rRNA. **P*<0.05 vs siNeg+ Vehicle using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. For all experiments values represent mean ± SEM of n = 3 to 6.

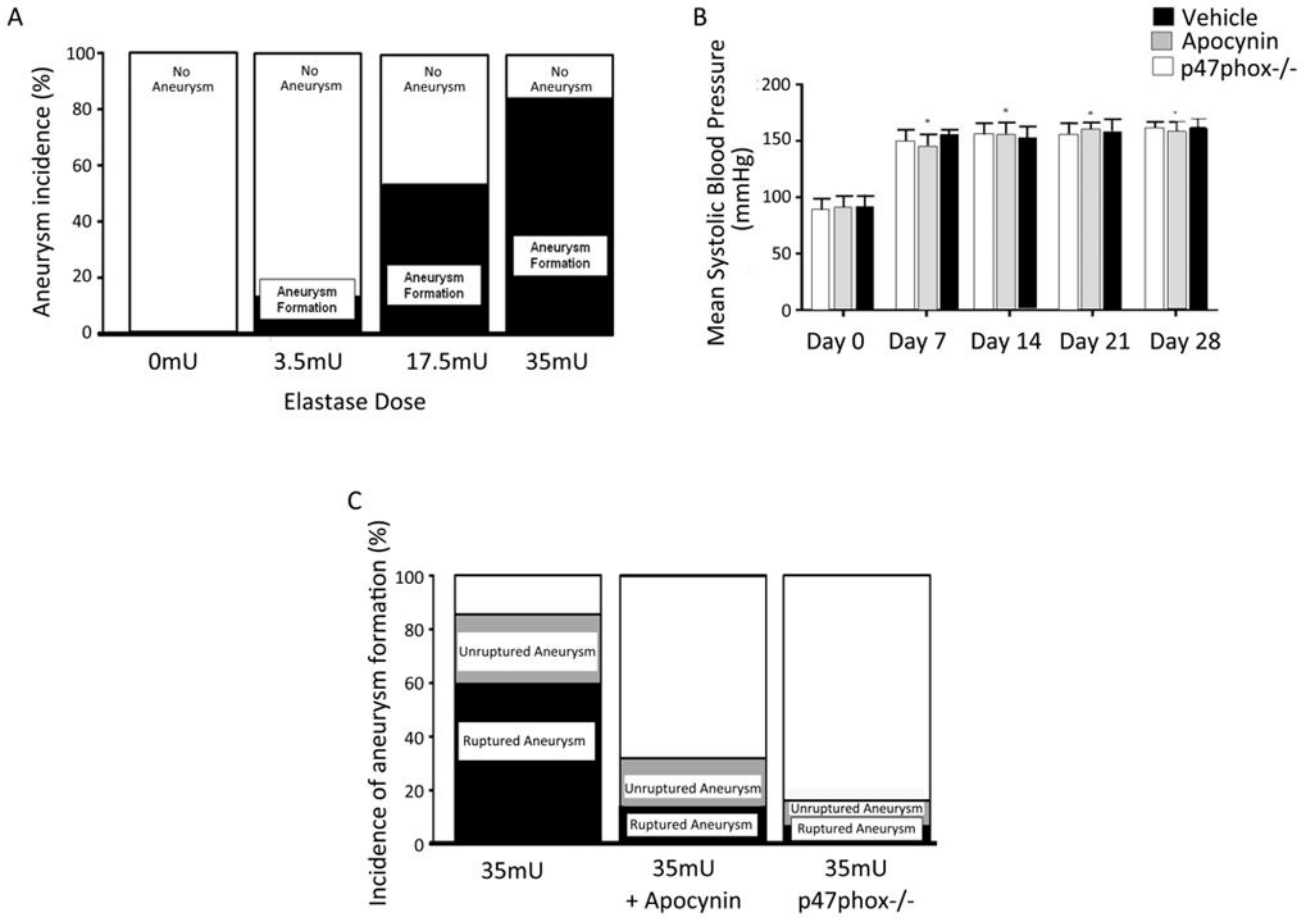

Figure 3. Cerebral aneurysm formation in mice varies with elastase dose, pretreatment with apocynin, and genetic inactivation of NOX1

Cerebral aneurysms were induced in mice and aneurysm formation determined 28 days later. Cerebral aneurysms formation occurred in 0 mice receiving vehicle (n=10), 10% receiving 3.5 mU of elastase (n=10), 55% receiving 17.5 mU of elastase (n=10), 80% receiving 35 mU (n=10) A). B) Blood pressure was elevated one week after aneurysm induction, but was not significantly different between vehicle, apocynin treated or p47phox−/− mice at any single time period. C) CA formation occurred in 21 of 25 animal (84%) controls, 8 of 25 animals (32.0%) treated with apocynin, and 2 of 12 (16.7%) p47phox −/− mice (*P<0.05*, apocynin using Chi-square analysis and p47phox−/− using Fisher's exact test versus control). Aneurysm rupture occurred in 15 of 25 (60.0%) controls, 3 of 25 (12%) animals treated with apocynin, and 1 of 12 (8.3%) of p47phox −/− mice (*P<0.05,* apocynin and p47phox−/− using Fisher's exact test versus control)..

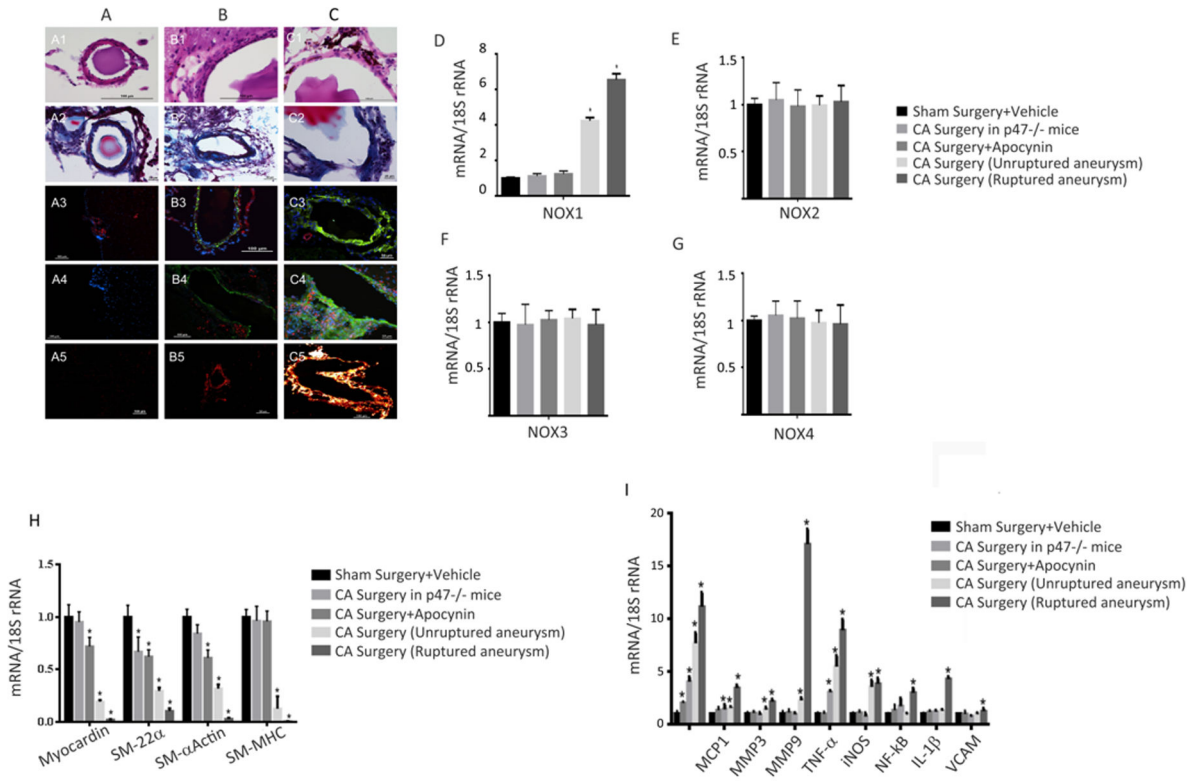

Figure 4. Histological and mRNA expression of cerebral aneurysms demonstrates NOX-Induced VSMC Phenotypic Modulation in Unruptured and Furthermore Ruptured Cerebral Aneurysms

Hematoxylin and Eosin (panel 1), and trichrome staining (panel 2) of cerebral vessels from sham operated mice (A1–2), CA unruptured (B1–2), and CA ruptured (C1–2). NOX1 immunostaining (green) is show in in panel 3 and 4 (A-sham, B-unruptured CA; C-ruptured CA), samples were co-stained with either SMC-22α for SMCs (red) in panel 3 or CD68 for macrophages (red) in panel 4. *In situ* superoxide imaging, as carried out with dihydroethidium is shown in panel 5 (A-sham, B-unruptured CA; C-ruptured CA). NOX1, NOX2, NOX3, and NOX4 mRNA expression was quantified using RT-PCR and normalized to 18S rRNA, and expressed as fold increase over vehicle (D–G). **P*<0.05 versus sham surgery+vehicle controls using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. RT-PCR analysis of VSMC marker gene expression in (H) and pro-inflammatory and matrix remodeling genes in (I) in sham, p47phox−/− mice, apocynin treated mice and in unruptured and ruptured CA. **P*<0.05 versus sham surgery+vehicle controls using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. For all experiments values represent mean ± SEM of n = 3 to 6.

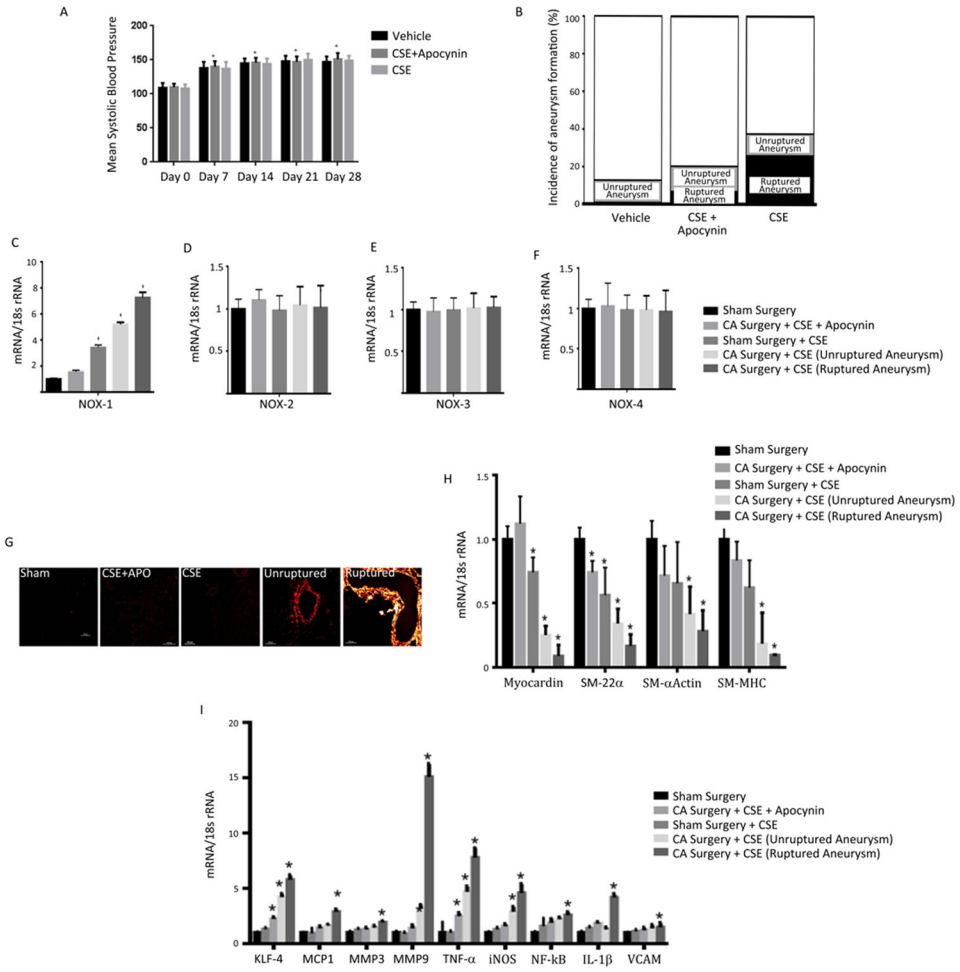

Figure 5. CSE increases the incidence of CA formation and rupture, and NOX1 mRNA expression *in vivo* and induces phenotypic modulation

To assess the role of CSE in CA development and rupture, mice underwent daily CSE starting 7 days prior to nephrectomy followed by CA induction surgery (3.5 mU elastase) as described in methods. The effect of CA induction on blood pressure is shown in A) **P*<0.05 versus Day 0 using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. The incidence of CA formation and rupture in animals treated with vehicle, CSE but pre-treated with apocynin, and CSE is shown in B). The effects of CSE, apocynin, CA formation and rupture on NOX1, NOX2, NOX3, and NOX4 mRNA expression are shown in C–F) and free radical expression is shown in G). **P*<0.05 versus sham surgery controls using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. mRNA expression of pro-inflammatory, matrix remodeling and VSMC phenotypic genes are shown in H) and I) respectively following indicated treatments. **P*<0.05 versus sham surgery controls using Kruskal-Wallis analysis with Wilcoxon rank sum post hoc test. For all experiments values represent mean ± SEM of n = 3 to 6.

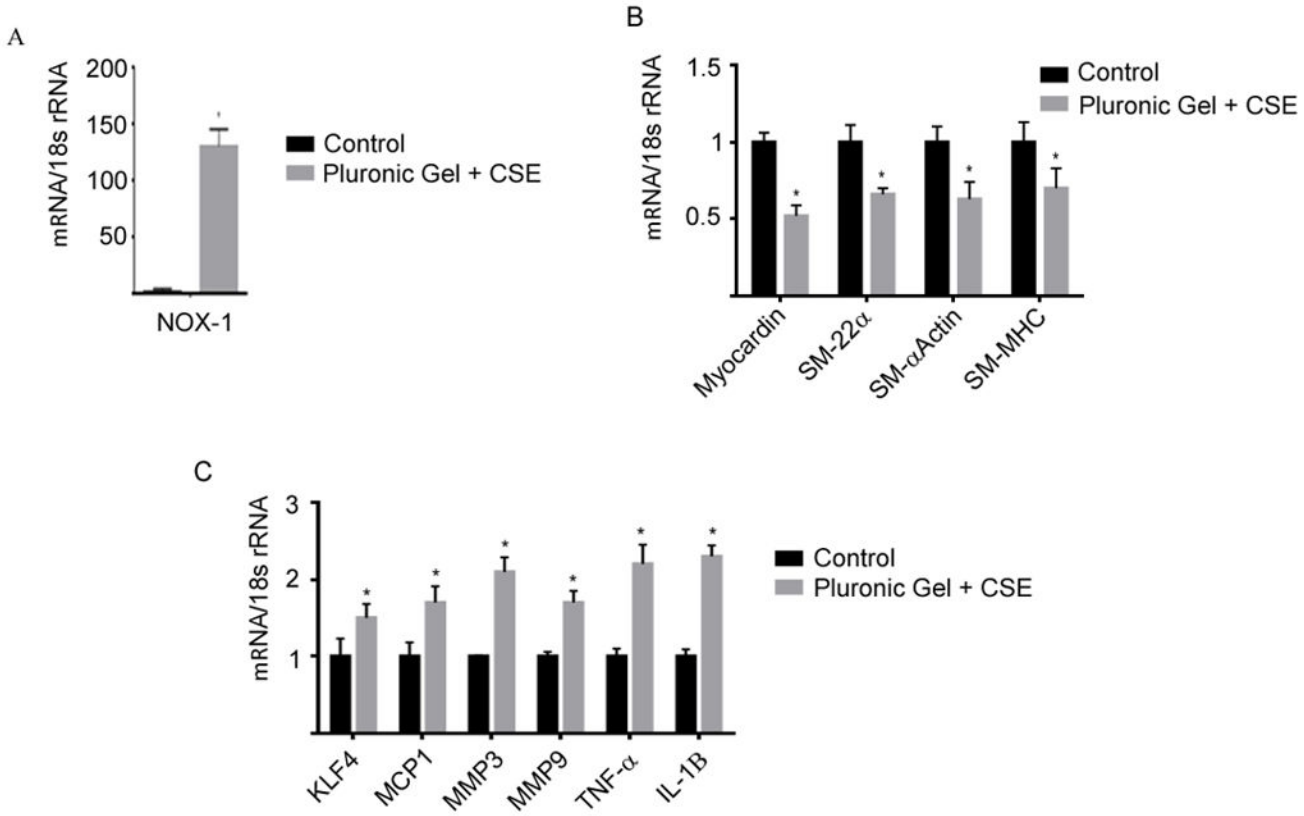

Figure 6. Direct effects of CSE on NOX1 mRNA expression and phenotypic modulation

CSE was directly applied to the adventitial surface of rat carotid arteries using a pluronic gel containing 0.8 mg/ml CSE. Changes in NOX1 mRNA expression 6–8 h following direct application of CSE is shown in A). mRNA expression of VSMC phenotypic genes, and pro-inflammatory and matrix remodeling genes are shown in B) and C) respectively following 12 h of direct CSE application. All values represent mean ± SEM of n =6. **P*<0.05 versus control using Wilcoxon rank sum.

Table 1

Real-time PCR Primer Sequences

	Forward Primer (Rat)	Reverse Primer (Rat)
1	**SM-α-Actin**	
	AGTCGCCATCAGGAACCTCGAG	ATCTTTTCGATGTCGTCCCAGTTG
2	**SM-MHC**	
	CAGTTGGACACTATGTCAGGGAAA	ATGGAGACAAATGCTAATCAGCC
3	**SM-22α**	
	GCATAAGAGGGAGTTCACAGACA	GCCTTCCCTTTCTAACTGATGATC
4	**Myocardin**	
	CGGATTCGAAGCTGTTGTCTT	AAACCAGGCCCCCTTCC
5	**KLF4**	
	CTTTCCTGCCAGACCAGATG	GGTTTCTCGCCTGTGTGAGT
6	**18S**	
	CGGCTACCACATCCAAGGAA	AGCTGGAATTACCGCGGC
7	**MMP3**	
	GCCAATGCTGAAGCTTTGATGTAC	GGGAGGTCCATAGAGGGATTGAAT
8	**IL-1β**	
	TTGTGCAAGTGTCTGAAGCA	TGTCAGCCTCAAAGAACAGG
9	**MCP1**	
	CTCAGCCAGATGCAGTTAATGC	TCTCCAGCCGACTCATTG G
10	**TNF-α**	
	AAAGCATGATCCGAGATGT	AGCAGGAATGAGAAGAGG C
11	**NF-κB (IκB-α)**	
	GCTGAAGAAGGAGCGCTACT	TCGTACTCCTCGTCTTTCATGGA
12	**MMP9**	
	AAGCCTTGGTGTGGCACGAC	TGGAAATACGCAGGGTTTGC
	NOX1	
	TTCCCTGGAACAAGAGATGG	GACGTCAGTGGCTCTGTCAA
	NOX2	
	AGCTATGAGGTGGTGATGTTAGTGG	CACAATATTTGTACCAGACAGACTTGAG
	NOX3	
	GCTGGCTGCACTTTCCAAAC	AAGGTGCGGACTGGATTGAG
	NOX4	
	CTGGAAGAACCCAAGTTCCA	CGGATGCATCGGTAAAGTCT