Trans-Cellular Proliferating Cell Nuclear Antigen Gene Activation in Cerebral Vascular Smooth Muscle by Endothelial Oxidative Injury In Vivo

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Objective—This study was undertaken to assess the role of vascular smooth muscle cell (VSMC) Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) in gene regulation after oxidative endothelial injury (OEI).

Methods and Results—OEI was produced by infusion of Na fluorescein (NaFluo) photoactivated by UV light immediately before intravenous injection. Posterior cerebral arteries were studied using immunofluorescence imaging, Western blotting, or patch clamping of isolated cells. After infusion of photoactivated NaFluo, but not NaFluo, (1) superoxide dismutase-1 (SOD-1) was upregulated in endothelium, consistent with oxidant stress; (2) the fraction of VSMC nuclei labeled for proliferating cell nuclear antigen (PCNA) increased 7-fold at 6 hours, preceded by a several-fold increase in nuclear phospho-cAMP-response element binding protein, with PCNA upregulation prevented by pretreatment with polyethylene glycol (PEG)-SOD; (3) in VSMCs, phospho-CaMKII increased 20-fold 5 minutes after OEI, with a 2-fold increase in peak Ca$^{2+}$ channel currents; and (4) changes in cAMP-response element binding protein and PCNA were blocked by systemic administration of lipophilic (nifedipine) or hydrophilic (amlodipine) 1,4-dihydropyridine Ca$^{2+}$ channel blockers, the calmodulin inhibitor trifluoperazine, or the CaMKII inhibitor KN-93, with none of these agents preventing SOD-1 upregulation in endothelium.

Conclusions—Activation of VSMC Ca$^{2+}$ channels and CaMKII is a key early signaling event required for upregulation of PCNA gene expression in VSMCs after oxidative injury to endothelium. (Arterioscler Thromb Vasc Biol. 2003;23: 2048-2054.)

Key Words: endothelium ■ vascular smooth muscle ■ oxidant stress ■ calcium channel ■ Ca$^{2+}$/calmodulin-dependent protein kinase II

Oxidant stress is a common mechanism of injury in many insults that affects the walls of blood vessels. Oxidant stress is implicated in the pathogenesis of hypertension, postischemic reperfusion injury, arteriosclerosis, and formation of atheromatous plaque and is an important initiator of vessel wall damage leading to stroke. In the vessel wall, reactive oxygen species (ROS) can be formed by macrophages or neutrophils within an atherosclerotic plaque, or they can be synthesized by endothelial and vascular smooth muscle cells (VSMCs) by NAD(P)H oxidase, xanthine oxidase, and nitric oxide synthase. Of the ROS involved, superoxide is of particular importance because of the prominent role it plays in many forms of hypertension, including renovascular hypertension, obesity-related hypertension, the deoxycorticosterone acetate-salt model, the spontaneously hypertensive rat, and the angiotensin II–infused rat.

The 2 primary manifestations of oxidant stress in the vessel wall are altered vasomotor tone and increased proliferation of VSMC (neointima formation). Altered tone is ascribed to altered Ca$^{2+}$ signaling, but the mechanism by which oxidant stress in the vessel wall triggers a proliferative response of VSMC in vivo has not been determined. A direct action of ROS on VSMC is possible, but an indirect effect on VSMCs attributable to ROS-mediated endothelial injury can also be postulated. The latter is suggested by findings with mechanical injury from intravascular balloon inflation, the prototypical endothelial injury model in vivo, in which antioxidants are found to reduce neointimal formation. In addition, the proliferative response of VSMCs to a variety of injurious stimuli in vivo, including balloon injury to endothelium, likely involves augmented Ca$^{2+}$ signaling, as suggested by the finding that Ca$^{2+}$ channel blockers are highly effective in preventing neointimal formation in these settings. Thus, data from balloon and other injury models suggest a potentially important connection between endothelial injury and ROS on the one hand and altered Ca$^{2+}$ signaling and a proliferative response in VSMCs on the other hand, although a direct link between them has not been shown.
We hypothesized that intravascular oxidant stress sufficient to cause oxidative endothelial injury (OEI) would cause activation of Ca\(^{2+}\) channels and initiate a proliferative response in VSMCs. We examined this hypothesis in cerebral vessels, reasoning that tight junctions between endothelial cells (blood brain barrier [BBB]) would make it less likely that ROS generated intravascularly would act directly on VSMCs. To produce OEI, we used Na fluorescein (NaFluo) exposed to light immediately before intravenous injection, thus precluding the need for direct exposure of vessels to light. Oxidant stress in endothelium was confirmed by showing upregulation of superoxide dismutase (SOD-1).\(^9\) Maintained integrity of the BBB was confirmed by showing absence of NaFluo extravasation. We found that (1) OEI caused a large increase in activation of cAMP-response element binding protein (CREB) and in expression of proliferating cell nuclear antigen (PCNA) in VSMC nuclei; (2) nuclear events were preceded by activation of Ca\(^{2+}\) channels and of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in VSMCs; and (3) nuclear events were blocked by inhibitors of calmodulin, CaMKII, and Ca\(^{2+}\) channels. These findings provide the first direct evidence of a causal link between oxidant stress in endothelium and PCNA expression in VSMCs and demonstrate the critical role of L-type Ca\(^{2+}\) channels and CaMKII in VSMCs in this proliferative response.

**Methods**

**Animal Model**

Animal protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Under general anesthesia (ketamine 60 mg/kg plus xylazine 7.5 mg/kg), a femoral vein was catheterized and used for intravenous injection of control saline, NaFluo, or photoactivated NaFluo (PA-NaFluo), both 64 mg/mL, 150 mg/kg.\(^{10}\) PA-NaFluo was activated immediately before infusion by exposure to UV light (Hg lamp, 95 000 lux) for 2 minutes. For some experiments, drugs were delivered via osmotic minipumps implanted subcutaneously. Control experiments included study of isolated basilar and posterior cerebral arteries (PCAs) perfused at a pressure of 100 cm H\(_2\)O with Kreb’s solution, to which was added NaFluo or PA-NaFluo (150 mg/L).

**Fluorescence Imaging**

After administration of NaFluo or PA-NaFluo, the vascular injury response was terminated at precise times by perfusion fixation using 4% paraformaldehyde in PBS at room temperature. Brains were processed for paraffin sectioning (4 μm) as described.\(^{11}\) Primary antibodies used were directed against PCNA (1:400; Santa Cruz Biotechnology), activated CREB (phospho-CREB; 1:500; Upstate Biotechnology), SOD-1 (1:100; BD Biosciences), and activated CaMKII (1:100; Affinity BioReagents). Nuclei were labeled with 4',6-diamidino-2-phenylindol (DAPI).

For quantitative immunofluorescence,\(^{12}\) tissue sections from all animals in all experimental groups were immunolabeled as a single batch. Images were collected with IPLab software (version 3.06, Scanalytics) using uniform parameters of magnification and exposure. Images were then deconvoluted (Huygens Essential, SVI), and the number of bright particles in equal-sized regions of interest were counted using IPLab software.

**Western Blotting**

Isolated vessels were flash-frozen for immunoblotting\(^{14}\) using antibodies directed against phospho-CaMKII.

**Nystatin-Perforated Patch Clamping**

PCAs were processed by enzymatic digestion.\(^{13}\) For macroscopic Ca\(^{2+}\) channel recordings, the bath solution contained (in mmol/L) TEA - Cl 130, MgCl\(_2\) 1, BaCl\(_2\) 10, HEPES 10, glucose 12.5, and 4-aminopyridine 2, pH 7.2 with TEA - OH, and the pipette solution contained (in mmol/L) CsCl 130, MgCl\(_2\) 8, and HEPES 10, pH 7.35 with CsOH plus nystatin.

**Data Analysis**

Statistical comparisons were evaluated using either ANOVA with SNK comparisons or Student’s t test, as appropriate. Data are given as mean±SE.

**Results**

**PA-NaFluo Induces Oxidative Endothelial Injury**

Infusion of PA-NaFluo resulted in OEI, as suggested by upregulation of endothelial SOD-1. This was seen first at 1 hour as small regions of labeling on the luminal side of endothelial cell membranes (Figure 1A versus 1B) and later as more diffusely distributed labeling in endothelium (Figure 1C). Infusion of NaFluo that had not been activated by UV light failed to upregulate endothelial SOD-1 (Figure 1D), suggesting that NaFluo itself was not responsible for SOD-1 upregulation observed with PA-NaFluo. Quantification of deconvoluted images corroborated a progressive increase in SOD-1 over time that was absent without photoactivation (Figure 1E).

Infusion of PA-NaFluo caused no physical damage to endothelium and no apparent disruption of the continuous endothelial layer that forms the BBB (Figure 1F versus 1G). Labeling for SOD-1 was minimal in smooth muscle layers at 1 and 3 hours (Figures 1B and 1C), suggesting that an intact BBB had protected VSMCs from direct action of PA-NaFluo. To additionally assess permeability of the BBB, we examined the fluorescence signal attributable to NaFluo itself in vessel walls after infusion of PA-NaFluo. We compared the fluorescence signal in PCA, which should have an intact BBB, and femoral arteries, which should not. In both control animals that received no NaFluo (Figure 1H) and in animals that received PA-NaFluo 1 hour earlier (Figure 1I), imaging of PCA showed no green fluorescence signal in vessel wall tissues except for the autofluorescent internal elastic lamina and DAPI-labeled nuclei. By contrast, in femoral arteries from the same animals, green fluorescence signal was very prominent in tissues from animals receiving PA-NaFluo (Figure 1K versus 1L). Together, these data showed that infusion of PA-NaFluo had not disrupted endothelial function sufficiently to alter permeability of the BBB in PCA.

**OEI Increases Expression of PCNA**

We used PCNA immunolabeling to examine the proliferative response of VSMCs after OEI induced by PA-NaFluo. At 1 hour, when oxidant stress was clearly evident in endothelium, PCNA labeling showed no change (Figure 2A versus 2B). However, by 6 hours, a large increase was evident in the proportion of VSMC nuclei labeled with PCNA (Figure 2C), with the increase in labeling persisting for more than 7 days (Figure 2D). Animals pretreated with polyethylene glycol (PEG)-SOD 20 minutes before infusion of PA-NaFluo...
showed no PCNA upregulation (Figure 2E), consistent with involvement of superoxide.

Using DAPI to label nuclei (Figures 2F through 2J) allowed computation of the PCNA index, the fraction of nuclei with positive label for PCNA. The PCNA index was significantly (by ANOVA, \( P < 0.05 \)) elevated 3 hours after OEI, reached a peak 6 hours after OEI, and remained significantly elevated for more than 1 week (Figure 2K, empty bars). By contrast, the PCNA index remained within normal levels at 6 and 24 hours when animals were infused with NaFluo not exposed to light (Figure 2K, shaded bars) or when pretreated with PEG-SOD (Figure 2K, black bar).

OEI Causes Activation of Transcription Factor CREB

We assessed for potential involvement of CREB in the response of VSMCs to OEI.\(^\text{14,15}\) When examined at the peak time of 6 hours, activated CREB, phospho-CREB, was found to be elevated in regions (Figure 3A versus 3B) coincident with DAPI labeling (Figure 3C versus 3D). We computed a phospho-CREB index, the fraction of DAPI-positive nuclei with positive label for phospho-CREB. The phospho-CREB index was significantly (by ANOVA, \( P < 0.05 \)) elevated as early as 30 minutes after OEI and continued to rise for up to 6 hours (Figure 3E). Thus, activation of CREB, which was significantly elevated at 30 and 60 minutes, preceded upregu-
lation of PCNA expression, which was the same as control at 60 minutes and required 3 hours for significant elevation.

Involvement of CaMKII

An important upstream regulator of CREB activation is CaMKII.14,16 We evaluated activation of CaMKII using immunofluorescence examination for phosphorylated CaMKII (phospho-CaMKII). Initial experiments with measurements obtained 1 hour or more after injury were unrevealing (not shown). However, when vessels were examined within the first few minutes after PA-NaFluo infusion, marked increases in phospho-CaMKII were detected in VSMCs (Figures 4A through 4C). High-resolution images indicated that phospho-CaMKII was predominantly located at the plasmalemmal membrane of VSMCs (Figure 4D). Data from deconvoluted images were quantified.12 From this analysis, we found that levels of phospho-CaMKII in VSMCs were maximally elevated 5 minutes after injury and then tapered off gradually toward normal (Figure 4E).

We sought to confirm our findings on CaMKII using Western blots. The rapidity of the response after the injury stimulus, coupled with its transient nature, precluded use of our animal model, in which cerebral vessels cannot be harvested at such early times. We thus performed experiments using isolated pressurized basilar arteries, duplicating the essential features of in vivo PA-NaFluo infusion. Western blots of arteries exposed to PA-NaFluo and flash-frozen 5 minutes later confirmed the early increase in CaMKII (Figure 4F). This experiment corroborated the specificity of the anti-phospho-CaMKII antibody and validated our use of quantitative immunofluorescence to study vessels from the animal model. In addition, this experiment precluded possible involvement of circulating blood elements in the early response of CaMKII with in vivo administration of PA-NaFluo.

To provide additional evidence for involvement of CaMKII in the response of VSMC to OEI, we evaluated the
effects of the calmodulin inhibitor trifluoperazine (25 mg/kg IP, 30 minutes before and 30 minutes after infusion of PA-NaFluo; 3 animals) and of the CaMKII inhibitor KN-93 (120 μg/kg per h for 24 hours before OEI via osmotic minipump; 3 animals). When examined at the peak response time of 6 hours after PA-NaFluo infusion, both agents fully prevented activation of CREB (Figures 5A and 5B) and expression of PCNA (Figures 5C and 5D) when evaluated against DAPI-labeled nuclei (Figures 5E through 5H). However, neither agent prevented upregulation of SOD-1 in endothelium of the same animals (Figures 5I and 5J), indicating that Ca2+ channel blockers had not prevented oxidant stress in endothelium.

We measured Ca2+ channel currents in freshly isolated VSMCs from PCA of controls and OEI-animals within 1 hour after infusion of PA-NaFluo, the earliest time that we could obtain isolated cells. The recording protocol yielded currents attributable exclusively to 1,4-dihydropyridine-sensitive L-type Ca2+ channels.11 Currents from OEI cells exhibited normal kinetics (Figures 6F and 6G), normal voltage dependence (Figure 6H), and normal pharmacological response to nifedipine (not shown). However, compared with controls, peak currents recorded from OEI cells were significantly larger (by t test, P<0.01) by a factor of 2.1 (Figure 6H), although cell size measured as cell capacitance was the same.

We also examined effects of NaFluo and of PA-NaFluo when added directly to the bath while recording Ca2+ channel currents in control cells (5 cells with each agent). Currents were reduced with both NaFluo (Figure 6I) and PA-NaFluo (not shown), with both showing decreases to approximately half of control values. A comparable reduction in current with both agents suggested a nonspecific block by NaFluo rather than oxidation of the channel. Overall, these data indicated that the increase in Ca2+ channel currents observed in OEI cells could not be attributed to a direct action of NaFluo or PA-NaFluo on the channel and additionally substantiated the previous experiment, suggesting that the BBB had remained intact, protecting VSMCs from circulating factors.

**Discussion**

We report here that oxidant stress to endothelium in vivo caused significant activation of Ca2+ channels and of CaMKII and caused a significant proliferative response characterized by activation of CREB and upregulation of PCNA expression in VSMCs and that the proliferative response was completely blocked by inhibitors of Ca2+ channels, calmodulin, or CaMKII.

We used intravenous injection of PA-NaFluo to induce OEI in vivo. This technique, which is a novel adaptation of an otherwise standard method for photodynamic vessel wall injury, has several advantages. On infusion, PA-NaFluo causes generation of singlet oxygen molecules that interact with NADP, resulting in a rapid burst of production of...
superoxide, giving a well-defined time-of-onset of injury. Studies on effects of superoxide have generally required in situ production by direct illumination of infused photosensitive agent or use of endogenous mechanisms activated by angiotensin II, both of which may have inherent disadvantages. Our procedure allowed use of a very convenient control, NaFluo not exposed to light, which caused no vessel wall response. Unlike conventional photodynamic injury or mechanical balloon injury, PA-NaFluo showed no evidence of physical disruption of endothelium and did not disrupt the integrity of the BBB, thus precluding potentially confounding effects on VSMC attributable to direct trauma from stretch or from serum growth factors and cytokines or from PA-NaFluo itself. The effect of PA-NaFluo on the vessel wall seemed to be attributable directly to oxidant stress of endothelium, which we documented as an increase in expression of SOD-1 and which was prevented by pretreatment with PEG-SOD. Effects on the vessel wall could not be attributed to oxidant stress of any circulating blood elements, because the same early effects on CaMKII activation were observed with isolated perfused vessels in vitro.

1,4-Dihydropyridines exert complex effects on various molecular processes. Apart from their well-known effect on \( \text{Ca}^{2+} \) channels, these agents may also exert vascular protective effects by suppressing free radical generation. Antioxidant properties are observed principally with the lipophilic 1,4-dihydropyridines, not with hydrophilic agents such as amlodipine. However, the 1,4-dihydropyridines did not prevent upregulation of SOD-1 in endothelium after administration of PA-NaFluo, and both calmodulin and CaMKII inhibitors, which are not antioxidants, also blocked PCNA upregulation after OEI. The strongest evidence indicating that the effect of the 1,4-dihydropyridines was on L-type \( \text{Ca}^{2+} \) channels and not attributable to an antioxidant effect came from the patch-clamp data showing that these channels were significantly activated after OEI.

Activation of L-type \( \text{Ca}^{2+} \) channels and CaMKII was significantly increased after OEI, and PCNA gene expression after OEI was completely blocked by inhibitors of calmodulin, CaMKII, and \( \text{Ca}^{2+} \) channels. It is evident that the \( \text{Ca}^{2+} \) channels involved reside in VSMCs, but CaMKII is present in both VSMCs and endothelium. Use of quantitative immunofluorescence rather than Western blots to assess CaMKII allowed not only precise timing of early changes after the injury stimulus but also gave precise localization of changes, indicating that VSMCs and not endothelium was involved. Also suggesting involvement of CaMKII in VSMCs and not in endothelium is that in endothelium, CaMKII activates eNOS, resulting in generation of NO, which would downregulate \( \text{Ca}^{2+} \) channel activity in VSMCs, not upregulate activity as we observed.

\( \text{Ca}^{2+} \) channel blockers have long been known to effectively block intimal hyperplasia and proliferation in various models of vascular disease as well as in atherosclerosis in humans. This finding has generally been taken as evidence that the pathophysiological mechanism of these conditions involves an increase in activity of \( \text{Ca}^{2+} \) channels, although direct evidence for an increase in \( \text{Ca}^{2+} \) channel activity has generally been lacking. In the sparse reports available on this topic, \( \text{Ca}^{2+} \) channel activity in VSMCs was found to be diminished after balloon injury, a finding that seems paradoxical. However, our data suggest that the initial injury response entails an increase in \( \text{Ca}^{2+} \) channel availability that occurs very early—within a few minutes of the injury stimulus, causing a large influx of \( \text{Ca}^{2+} \) and activation of CaMKII, leading subsequently to gene upregulation. VSMCs may then lose functional \( \text{Ca}^{2+} \) channels as they respond to injury-induced gene upregulation and transition from a contractile to a synthetic phenotype. Accurately assessing early
events involving increased Ca\(^{2+}\) channel activity is difficult in most injury models, when the endothelial injury itself may be dispersed in time, as in atherosclerosis, or may be complicated by direct trauma to VSMC, as in the stretch induced by balloon inflation. In the model we used, the endothelial injury was synchronized and did not directly traumatize VSMC, fortuitously allowing early events in VSMC to be studied.

A rise in intracellular [Ca\(^{2+}\)] attributable to depolarization-induced Ca\(^{2+}\) channel activation has previously been reported to cause CREB activation and c-fos gene expression in VSMCs, with both being blocked by Ca\(^{2+}\) channel and CaMKII inhibitors.\(^{14,15}\) The present report, however, is the first to link OEI with a pathological response that involves PCNA gene expression in VSMCs. The specific mechanism by which OEI led to activation of Ca\(^{2+}\) channels and ultimately to upregulation of PCNA expression in VSMCs remains to be determined. Reduced bioavailability of NO attributable to endothelial injury,\(^{11,13}\) release of endothelin,\(^{21}\) or perhaps other mechanisms could act as endothelium-derived signals to cause activation of VSMC Ca\(^{2+}\) channels. Exploration of these possibilities and elucidation of this mechanism, however, will require additional experiments.

In summary, the present work builds on the seminal findings from Nelson’s group\(^{14,15}\) of a critical role for Ca\(^{2+}\) channels in upregulation of gene expression in smooth muscle. The novel aspect of our work is that this cascade in VSMCs, from Ca\(^{2+}\) channel to nuclear PCNA, can be initiated not only by direct manipulation of the channel, as with VSMC depolarization, but by manipulation of the endothelial cells that form the BBB and that shield VSMCs. Experiments of the sort reported here, in which the injury stimulus is delivered in vivo under physiological conditions, are inevitably difficult to perform and interpret but are crucial to understanding pathophysiological processes as they occur in life. The findings reported here provide strong evidence that a pathological disturbance of endothelium that is not morphologically evident can propagate to and affect smooth muscle cell function, resulting essentially in transcriptional regulation of gene expression. The identity of the signal that is transmitted from endothelium to smooth muscle Ca\(^{2+}\) channels and the mode of its delivery to smooth muscle, be it extracellular or via myoendothelial gap junctions, remain as unsolved challenges to be elucidated by future experiments.

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References
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