Differential Activation of Mitogenic Signaling Pathways in Aortic Smooth Muscle Cells Deficient in Superoxide Dismutase Isoforms


Objective—Reactive oxygen species (ROS) integrate cellular signaling pathways involved in aortic smooth muscle cell (SMC) proliferation and migration associated with atherosclerosis. However, the effect of subcellular localization of ROS on SMC mitogenic signaling is not yet fully understood.

Methods and Results—We used superoxide dismutase (SOD)–deficient mouse aortic SMCs to address the role of subcellular ROS localization on SMC phenotype and mitogenic signaling. Compared with wild-type, a 54% decrease in total SOD activity (≏50% decrease in SOD1 protein levels) and a 42% reduction in SOD2 activity (≏50% decrease in SOD2 protein levels) were observed in SOD1+/− and SOD2+/− SMCs, respectively. Consistent with this, basal and thrombin-induced superoxide levels increased in these SMCs. SOD1+/− and SOD2+/− SMCs exhibit increased basal proliferation and enhanced [3H]-thymidine and [3H]-leucine incorporation in basal and thrombin-stimulated conditions. Our results indicate preferential activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinases in SOD1+/− and janus kinase/signal transducer and activator of transcriptase (JAK/STAT) pathway in SOD2+/− SMCs. Pharmacological inhibitors of ERK1/2 p38 and JAK2 confirm the SOD genotype-dependent SMC proliferation.

Conclusions—Our results suggest that SOD1 and SOD2 regulate SMC quiescence by suppressing divergent mitogenic signaling pathways, and dysregulation of these enzymes under pathophysiological conditions may lead to SMC hyperplasia and hypertrophy. (Arterioscler Thromb Vasc Biol. 2005;25:950-956.)

Key Words: ROS □ SMC □ thrombin □ SOD □ cell signaling

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Smooth muscle cells (SMCs) are characterized by marked plasticity in their proliferative potential and differentiation status. As the primary constituents of the arterial media, they provide mechanical support for blood vessels and participate in regulation of vasomotor tone. In addition, SMCs contribute to neointimal formation after vascular injury and during atherosclerotic lesion progression in humans. In advanced atherosclerotic lesions, SMCs are prone to apoptosis and release proteolytic enzymes such as matrix metalloproteinases that may contribute to plaque instability. The remarkable diversity of SMC phenotype and function is determined in large part through regulatory cues in the extracellular microenvironment and interactions among intracellular signaling pathways. In addition, SMC phenotypes are modified over time by the accumulation of injuries to cellular macromolecules.

Reactive oxygen species (ROS) are key components for integration of SMC signaling events, whereas at the same time, they are major contributors to the degradation of cellular function through their interactions with proteins and structural components of the cell. Exogenous ROS are potent stimuli for activation of SMC signaling and mitogenesis, although in high (but physiological) concentrations, they may induce DNA damage and mitochondrial dysfunction. SMCs have several intrinsic mechanisms for generating ROS, the major one (at least with respect to intracellular signaling) being the SMC NAD(P)H oxidase. This oxidase contains a catalytic component, Nox1, and the cytoplasmic component p47phox, which is recruited to the active enzyme by growth factor stimulation and activation of the small GTPase Rac. The importance of the vascular NAD(P)H oxidase in general, and its activating component p47phox in particular, has been demonstrated in genetic studies, indicating that this oxidase is required for superoxide production in aortic explants and cultured SMCs. Pathophysiologically, this oxidase is required for regulation of

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blood pressure and progression of atherosclerotic lesions in apolipoprotein E−/− (apoE−/−) mice. Because vascular cells exist in a delicate oxidative balance, the systems that quench oxidative species are of equal importance in modulating SMC behavior. Indeed, exogenous antioxidants can inhibit growth factor–dependent proliferative and signaling responses in SMCs. The role of endogenous antioxidant defenses is less clear. Members of the superoxide dismutase (SOD) family react with superoxide ion to produce hydrogen peroxide, which is subsequently converted to water by catalase. There are 3 members of this family, which are enzymatically similar but distinguished by their localization. SOD1 (copper-zinc SOD) is present in the cytoplasm and nucleus, SOD2 (manganese SOD) is localized to the extracellular space. We recently demonstrated that SOD2−/− mice develop arterial mitochondrial DNA damage. More importantly, a phenotype of accelerated atherogenesis at arterial branch points of apoE−/− mice is observed when they also lack a single copy of SOD2. These studies demonstrate the importance of oxidant scavenging in protection against oxidative injury and atherogenesis. On the basis of these observations, we examined the cellular phenotypes and signaling profiles of aortic SMCs derived from mice lacking a single copy of either the SOD1 or SOD2 gene. These studies provide further support for the importance of endogenous antioxidant systems in SMC function. Unexpectedly, divergent mitogenic signaling pathways are activated in response to deficiency of either SOD1 or SOD2 in SMCs, indicating that ROS-dependent signaling is compartmentalized and is regulated by ROS-scavenging enzymes in subcellular compartments.

Materials and Methods

Cell Culture

Mouse aortic SMCs were isolated from 4-month-old C57BL/6 SOD1−/− and SOD2−/− mice as described previously. SOD1−/− and SOD2−/− mice were back-crossed at least 8× into the C57BL/6J background. Experiments were performed on multiple primary cultures derived from several mice. Cells were grown in DMEM containing 10% FBS between passage numbers 4 and 11 that were growth-arrested by incubation in DMEM containing 0.1% FBS for 72 hours.

SOD Activity

Total and SOD2 activity were determined by measuring the ability of SOD to inhibit xanthine/xanthine oxidase–induced cytochrome c reduction. SOD2 activity was determined in the presence of 5 mmol/L potassium cyanide (KCN), which inactivates SOD1 and SOD3 activities.

Measurement of Superoxide Generation by SMCs

Intracellular O2− generation was measured by assaying the activity of aconitase. In situ O2− levels in SMCs were also detected by oxidative fluorescent probe dihydroethidium. Mitochondria were visualized with MitoTracker Green, whereas mitochondrial ROS production was visualized using reduced MitoTracker Red (see online supplement).

Cell Counts, [3H]-Thymidine, and [3H]-Leucine Incorporation Assays

Cell counts, [3H]-thymidine, and [3H]-leucine uptake experiments were performed in SMCs as described previously (see online supplement).

Results

Characterization of Primary SMCs With Haploinsufficiency of SOD Isoforms

To test the effects of impaired superoxide scavenging on SMC behavior, we characterized primary culture SMCs derived from SOD1−/− and SOD2−/− mice. Western blot analysis indicated that SOD1 protein levels were reduced by 50% in SOD1−/− mice without evidence of compensation by SOD2 (Figure 1A). Similarly, SOD2−/− mice had 50% less SOD2 protein, but SOD1 protein levels were not significantly different from wild-type SMCs (Figure 1B).

Total SOD activity was reduced by 54% in SOD1−/− SMCs (P<0.001 compared with wild-type SMCs; Figure 1C), consistent with the notion that this isoform accounts for the majority of cellular SOD activity. Total SOD activity was not significantly reduced in SOD2−/− SMCs. However, SOD2 activity in SOD2−/− mice was 42% of that in wild-type SMCs (P<0.01; Figure 1D), whereas SOD2 activity was unchanged in SOD1−/− SMCs. Decrease in the expression of SOD isoforms had no significant effect on catalase activity in SMCs (data not shown). Thus, our results show that deficiency in 1 SOD isoform does not lead to compensatory changes in protein levels or activity of the second SOD.
isofrom and suggest that in all likelihood, the effects observed are attributable specifically to the absence of each isofrom.

**ROS Generation in SOD-Deficient SMCs**

To correlate the haploinsufficiency of SOD isofroms with intracellular ROS production, we assayed O$_2^-$ generation indirectly by measuring aconitase activity. In conformity with our previous results, thrombin significantly induced O$_2^-$ generation in wild-type SMCs (Figure I, available online at http://atvb.ahajournals.org). In addition, basal- and thrombin-stimulated O$_2^-$ levels were increased in SOD1$^{-/-}$ and SOD2$^{+/-}$ SMCs. Superoxide levels were significantly higher in SOD2$^{+/-}$ SMCs compared with SOD1$^{-/-}$ SMCs under quiescent and thrombin-stimulated conditions ($P<0.05$), which suggests that SOD2 scavenges a greater proportion of O$_2^-$ despite its smaller proportion in total SOD activity. The reduction in aconitase activity was inhibited by pretreatment of cells with polyethylene-glycolated SOD (data not shown).

We also determined ROS levels directly in SMCs by staining mitochondria with MitoTracker Green and O$_2^-$ reduction in aconitase activity was inhibited by pretreatment of cells with polyethylene-glycolated SOD (data not shown).

**Proliferation and Hypertrophy of SOD-Deficient SMCs**

Based on the established role of ROS as signaling intermediaries for mitogenic and hypertrophic stimuli in SMCs, we determined the role of the intracellular SOD isofroms on these events. In wild-type SMCs, thymidine incorporation was very low after serum deprivation and increased by 51% after thrombin stimulation (Figure 3A), consistent with previous observations. Surprisingly, SOD1$^{-/-}$ and SOD2$^{+/-}$ SMCs had elevated thymidine uptake after serum deprivation compared with wild-type SMCs ($P<0.05$), indicating that these SMCs are capable of mitogen-independent proliferation. This is consistent with the notion that intracellular oxidant species can play a direct role in mitogenesis. After treatment with thrombin, thymidine uptake was increased markedly and to equivalent degrees in SOD1$^{-/-}$ and SOD2$^{+/-}$ cells (118% and 119%, respectively; $P<0.001$ compared with untreated SMCs for each genotype). To confirm that changes in thymidine incorporation accurately reflected cell cycle entry, we measured cell numbers 2, 4, and 6 days after thrombin treatment. At each time point, cell counts were increased in SOD1$^{-/-}$ and SOD2$^{+/-}$ SMCs (Figure 3B), indicating that rates of proliferation were indeed increased. Because ROS are also required for SMC hypertrophy, we measured leucine uptake. As with their proliferative responses, leucine incorporation was increased under serum-deprived and thrombin-stimulated conditions in SOD1$^{-/-}$ and SOD2$^{+/-}$ SMCs (Figure 3C). These results are consistent with previous observations indicating a critical role for ROS in SMC proliferation and hypertrophy and indicate that lack of a single allele of either SOD1 or SOD2 enhances ROS generation. Interestingly, there does not seem to be a direct correlation between either absolute superoxide levels or total SOD activity and proliferation/hypertrophy, which indicates the absence of a linear relationship between ROS levels above a threshold and mitogenesis.

**Mitogen-Activated Protein Kinase Activity in SOD1$^{-/-}$ and SOD2$^{+/-}$ SMCs**

Mitogen-activated protein (MAP) kinase family members are key proliferative signaling intermediaries for many mitogens, and ROS have been implicated in activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinase in SMCs. Thrombin induced phosphorylation of ERK1 and ERK2 in wild-type SMCs (2.8±0.5-fold increase at 5 minutes) and basal (2.1±0.3-fold increase versus wild-type SMCs), and thrombin-induced ERK phosphorylation (4.5±0.8-fold increase at 5 minutes) was consistently increased in SOD1$^{-/-}$ SMCs (Figure 4A). However, ERK1/2 phosphorylation was decreased in SOD2$^{+/-}$ SMCs under basal (0.7±0.1-fold) and thrombin-stimulated conditions (2.1±0.5-fold at 5 minutes) compared with wild-type SMCs. In concordance, the phosphorylated form of MEK1 (the upstream activator of ERK1/2) was thrombin inducible in wild-type SMCs (3.7±0.9-fold increase at 5 minutes), and phospho-MEK1 levels were much higher in SOD1$^{-/-}$ cells (2.7±0.3-fold increase at basal level and 5.0±0.5-fold increase at 5 minutes versus wild-type SMCs; Figure II, available online at http://atvb.ahajournals.org). Consistent with attenuated thrombin-inducible ERK1/2 activation in SOD2$^{+/-}$ cells, phospho-MEK1 levels were also lower under...
SMCs were treated with thrombin for 24 hours, and [3H]-leucine incorporation was measured during the last 4 hours (*P < 0.001 vs wild-type; **P < 0.01 vs SOD1−/−; ***P < 0.001 vs SOD2−/− SMCs). B, Wild-type, SOD1−/−, SOD2−/− SMCs were plated at equal density and cultured in standard growth medium for 6 days. Counts were performed in triplicate on days 2, 4, and 6. A significant change in cell proliferation was observed for cell type, time, and their interaction (2-way ANOVA; P < 0.0001). C, Growth-arrested SMCs were treated with thrombin for 24 hours, and [3H]-leucine incorporation was measured during the last 4 hours (P < 0.001 vs wild-type; **P < 0.01 vs SOD1−/−; ***P < 0.05 vs SOD2−/− SMCs). Data presented are mean ± SEM (n=3) and representative of 3 separate experiments.

basal (0.7 ± 0.1-fold) and stimulated (2.2 ± 0.3-fold increase at 5 minutes) conditions.

Similarly, basal p38 phosphorylation was markedly increased in SOD1−/− SMCs compared with wild-type cells (4.1 ± 0.2-fold increase) but was suppressed in SOD2−/− SMCs (Figure 4B). These results were paralleled by increased activation of MAP kinase kinase 3 (MKK3) (the direct upstream kinase of p38) in SOD1−/− SMCs (2.4 ± 0.1-fold increase) and a concomitant attenuation of phospho-MKK3 levels in SOD2−/− SMCs (Figure II). In contrast to ERK1/2 and p38, JNK phosphorylation was inducible by thrombin to similar levels in SMCs regardless of genotype (data not shown). These results indicate that constitutive and thrombin-inducible MAP kinase activation in SOD1−/− SMCs may account for the increased proliferative potential of these cells; however, SOD2−/− SMCs must use a different proliferative mechanism.

**Constitutive Activation of the JAK/STAT Pathway in SOD2−/− SMCs**

Examination of MAP kinase activation provides a possible explanation for the proliferative advantage of SOD1−/− SMCs but leaves unexplained the more robust behavior of SOD2−/− cells. We demonstrated previously thrombin-induced, ROS-dependent Janus kinase/signal transducer and activator of transcription (JAK/STAT) activation in SMCs, so we considered that this signaling pathway might be constitutively activated in either SOD1−/− or SOD2−/− SMCs. Phosphorylated JAK2 levels were barely detectable in either wild-type or SOD1−/− SMCs but were markedly elevated in SOD2−/− cells (Figure 5A). Quantitative analysis indicated that activated JAK2 levels were 2.7-fold greater in SOD2−/− SMCs (P < 0.05 compared with wild-type SMCs). Consistent with these observations, phospho-STAT3 levels were increased by 3.4-fold in SOD2−/− SMCs (P < 0.05 compared with wild-type cells; Figure 5B). Increased mitochondrial ROS production in SOD2−/− SMCs and enhanced nuclear STAT3 levels (increased tyrosine phosphorylation) in SOD-deficient SMCs were confirmed in confocal laser microscopy by staining the cells with reduced MitoTracker Red and phosphospecific STAT3 antibody and a secondary antibody conjugated to a green fluorescent probe (Figure 5C).

**Differential Effects of MAP Kinase and JAK2 Inhibitors on Cell Proliferation in SOD-Deficient SMCs**

The disparities in signaling pathway activation between SOD1−/− and SOD2−/− SMCs was unanticipated and suggested that increased ambient ROS levels in different cellular compartments have the ability to activate divergent mitogenic
pathways. We used specific inhibitors of ERK1/2 (UO126 and PD980589), p38 (SB203580), and JAK2 (AG490) to test the necessity of these enzymes in SOD genotype-dependent SMC proliferation. We performed these experiments under unstimulated conditions for 2 reasons. First, proliferation of SOD-deficient SMCs occurred in spite of serum deprivation (Figure 3A), indicating that the relevant signaling pathways are activated under these conditions. Second, multiple proliferative pathways are activated after mitogenic stimulation, and the consequent partial responses to inhibitors are less easily interpreted. As anticipated, thymidine incorporation in growth-arrested wild-type SMCs was unaffected by inhibition of MAP kinase or JAK/STAT signaling (Figure 6A) because activation of these pathways is minimal or absent under these conditions. In SOD1+/− SMCs, inhibitors of ERK1/2 (UO126 and PD980589) and p38 (SB203580) significantly inhibited thymidine uptake (P < 0.001 for each compared with untreated cells; Figure 6B), whereas AG490 had no effect in these cells. In contrast, SOD2−/− SMCs were unaffected by ERK1/2 or p38 inhibitors, but thymidine uptake was significantly reduced by inhibition of JAK/STAT signaling (P < 0.001 compared with untreated cells; Figure 6C). Thus, SOD1 and SOD2 deficiency results in elevated ambient O2− levels and increased proliferative capacity, but the signaling pathways activated are surprisingly distinct.

**Discussion**

In spite of the long-standing association of SMC proliferation and ROS derived from exogenous or endogenous sources, the role of endogenous ROS-scavenging systems in vascular function has received limited attention. In the present report, we characterize in detail the phenotypes of SMCs derived from mice lacking a single copy of the genes encoding the cytoplasmic or mitochondrial isoforms of SOD. Heterozygous mice are ideal to study the relative importance of these isoforms in vascular pathology because SOD2−/− mice die within weeks of birth. The consequences of SOD genetic modifications are much less subtle than might otherwise be expected and indicate that function of this enzyme within the cytoplasmic and mitochondrial compartments is crucial for tuning ambient ROS levels and modulating SMC proliferation. Whereas other studies have emphasized the importance of ROS generation (in particular, by the vascular NAD(P)H oxidase) in these processes,1,2,5 the present experiments reinforce the importance of ROS-scavenging systems in modulation of SMC phenotypes.

In SMC mitogenic cascades, several signaling pathways are downstream of ROS generated by the vascular NAD(P)H oxidase. ROS generation is a relatively proximal event in response to growth factors such as thrombin and angiotensin II, insofar as growth factor–induced MAP kinase activity and JAK/STAT signaling are ROS dependent.14,17,18 Although absolute ROS levels have been considered a major determinant of kinase cascade activation, the studies presented here indicate that mitogen-dependent signaling must have other determinants. In particular, our studies indicate that impaired cytosolic SOD activity preferentially activates the ERK1/2 and p38 MAP kinases, whereas JAK/STAT activation occurs when mitochondrial SOD activity is decreased (Figures 4 and 5). We also infer from these studies that suppression of MAP kinases and the JAK/STAT pathway by SOD1 and SOD2, respectively, may keep SMCs in quiescent state in normal conditions, and that deficiency/overwhelming of these en-
zymes under pathophysiological conditions may be linked mechanistically to SMC hyperplasia and hypertrophy.

We consider at least 2 explanations for this divergence in signaling pathway activation. One possibility is that activation of these 2 pathways is dose dependent; we observe higher superoxide levels in SOD2+/− than in SOD1+/− SMCs (Figure 2), so it is possible that the JAK/STAT pathway has a higher threshold for activation. However, this would not explain either the suppression of ERK1/2 and p38 MAP kinase phosphorylation in SOD2+/− SMCs (Figure 4) or the absence of a dose response in signaling pathway activation. It is also possible that SOD1 and SOD2 have functions other than O$_2^-$ scavenging that account for divergent signaling cascade activation.

A second possibility, and the one we favor, is that the location of ROS is critical for activation of specific mitogenic signaling events. To our knowledge, such compartmentalization of ROS-dependent signaling has not been reported. Our linkage of SOD1 and MAP kinase activity is supported by the observation that overexpression of SOD1 decreases phospho-ERK1/2 levels and attenuates neuronal cell death after transient focal cerebral ischemia. Although we have not tested this possibility, SOD1 deficiency can alter the redox status of the cell and activate apoptosis signal-regulated kinase 1 and its downstream effector p38 MAP kinase. An inverse relationship between phospho-ERK1/2 and p38 MAP kinase levels and SOD1 protein expression in endothelial cells also complements our observation.

In contrast, a specific linkage between SOD2 function and JAK/STAT activation has not been reported previously, although STAT3 activation by mitochondrial ROS generation was reported. Alternatively, overexpression of constitutively active STAT3 (caSTAT3) significantly suppresses ROS generation and augments SOD2 activity, and dominant-negative STAT3 suppresses SOD2 expression, suggesting that increased STAT3 activation in SOD2+/− SMCs in the present investigation may reflect a compensatory response to decreased SOD2 levels.

The molecular events that determine the divergence between JAK/STAT activation by mitochondrial ROS and MAP kinase activation by cytosolic ROS are unclear, but it is interesting to note that apoptotic signaling can similarly occur under pathophysiological conditions may be linked mechanistically to SMC hyperplasia and hypertrophy.

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I. Expansion of Materials and Methods

SOD activity

Total and SOD2 activity were determined by measuring the ability of SOD to inhibit xanthine-xanthine oxidase-induced cytochrome c reduction. Cells were harvested in phosphate-buffered saline, homogenized and centrifuged. Cell homogenate (16.5 µl) was added to 967 µl of reaction buffer containing 50 µM xanthine, 20 µM cytochrome c, 25 µM KH₂PO₄, 25 µM K₂HPO₄, and 0.1 mM EDTA in a cuvette. To this solution, 16.5 µl of xanthine oxidase (0.2 U/ml in 0.1 mM EDTA) were added and increase in absorbance was read at 550 nm at 1 min intervals for 10 min. Results were measured against a standard curve and SOD activity was expressed as units per microgram protein. SOD2 activity was determined in the presence of 5 mM potassium cyanide (KCN), which inhibits SOD1 and SOD3 activities.

Measurement of superoxide generation by SMC

Intracellular O₂⁻ generation was measured by assaying the activity of aconitase, a tricarboxylic acid cycle enzyme that is highly sensitive to inactivation by O₂⁻. Quiesced SMCs were either untreated or treated with thrombin (1.0 U/ml) for 10 min. Cells were rinsed with ice-cold PBS, scraped, pelleted and transferred to 500 µl cold homogenization buffer consisting of 50 mM Tris-HCl (pH 7.6), 0.2% Triton-X-100, 1 mM cysteine, 5 mM citrate and 100 mmol/L DTPA. The pellet was dispersed by vortexing and lysed by sonication. Fifty µl of lysate was added to 200 µl of assay buffer [50 mM Tris-HCl (pH 7.6), 30 mM citrate, 0.6 mM MnCl₂, 0.2 mM NADP⁺ and 2U/ml isocitrate dehydrogenase] in a cuvette. Production of NADPH was determined by reading absorbance at 340 nm every 10 seconds for 2 minutes (Beckman Coulter DU 7500), and normalized to protein per sample.
**Immunocytochemistry**

*In situ* O$_2^-$ levels in SMCs were detected by oxidative fluorescent probe dihydroethidium (DHE) and mitochondria were visualized with MitoTracker Green (MTG; M-7514, Molecular Probes). Quiescent SMCs were incubated with MitoTracker Green (50 ng/ml) and DHE (10 µM) for 20 min at 37°C in dark. Fluorescence was monitored using a Zeiss LSM-510 laser scanning confocal microscope. Red fluorescence of DHE was excited at 488 nm using the argon laser and images were collected at 590 nm using a long-pass filter. MTG was also excited at 488 nm and the emitted green fluorescence was detected using a 505-530-nm band pass emission filter. Mitochondrial ROS production was visualized in a separate experiment using nonfluorescent reduced MitoTracker Red (MTR; M-7513, Molecular Probes) which becomes fluorescent upon oxidation. Growth-arrested SMCs were incubated with 500 nM MTR for 15 min at 37°C in the dark. The cells were washed with serum-free medium and images were captured at 586 nm emission through a long-pass filter after initial excitation at 543 nm. Enhanced STAT3 phosphorylation in SOD-deficient SMCs were visualized in confocal laser microscopy using phosho-specific STAT3 antibody and a secondary antibody conjugated to Alexa 488.

**Cell counts, [3H]-thymidine and [3H]-leucine incorporation assays**

For cell counts, SMC were plated at equivalent densities (30 X 10$^3$) in 6-well plates. At defined intervals, cells were trypsinized and cell counts were done using a Coulter Counter. $[^3]$H-thymidine-uptake experiments in cells treated with thrombin were done as described previously. $[^3]$H-thymidine-uptake experiments with various kinase inhibitors were performed in growth-arrested SMCs. For analysis of protein synthesis, growth-arrested SMC were either treated or untreated with thrombin (1 unit/ml) for 24 h and incubated with $[^3]$H-leucine (1 µCi/ml) during the final 4 h of treatment. $[^3]$H-thymidine or $[^3]$H-leucine treated cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cells were lysed by vortexing vigorously in cold 10% (w/v) trichloroacetic acid. After keeping on ice for 20 min, the lysate was passed through a glass fiber filter (GF/C, Whatman). The filter was washed once with cold 5%
trichloroacetic acid and once with cold 70% (v/v) ethanol. The filter was dried and the radioactivity measured in a liquid scintillation counter (LS 3801 Beckman).

**Western blotting**

Preparation of protein extracts and Western blotting were performed as described. The antibodies used were anti-SOD1, anti-SOD2 (Upstate Biotechnology), anti-GAPDH (Chemicon International), anti-phospho ERK1/2 MAP kinase (Thr202/Tyr204), antiphospho-SAPK/JNK (Thr183/Tyr185), antiphospho-p38 MAP kinase (Thr180/Tyr182), anti-ERK1/2 MAP kinase, anti-p38 MAP kinase, anti-phospho MEK1/2 (Ser217/Ser221), anti-MEK1/2, anti-phospho MKK3/MKK6 (Ser 189/209), anti-MKK3, anti-phospho STAT3 (Tyr705), anti-STAT3 (Cell Signaling Technology), anti-phospho JAK2 (Tyr1007/1008), anti-JAK2 (BIOSOURCE International), and anti-JNK1 (Santa Cruz Biotechnology).

**Statistical analysis**

Results were analyzed with one-way and two-way analysis of variance (ANOVA) and in case of one-way ANOVA, post-hoc analysis was performed using Newman-Keuls test. The statistical analysis was performed using Graphpad Prism version 3.0 (GraphPad software, San Diego, CA).

**References**


**Figure I. SOD-deficient SMCs generate higher \( \text{O}_2^- \) constitutively and in response to thrombin treatment.** A significant increase in intracellular \( \text{O}_2^- \) production, as reflected by decrease in aconitase activity, is observed in wild-type SMCs treated with thrombin (\( * p < 0.001 \) vs. untreated SMCs). SOD1 and SOD2 deficient SMCs produce constitutively higher \( \text{O}_2^- \) levels (\( * p < 0.001 \) vs. untreated wild-type SMCs) and in response to thrombin treatment (\( ** p < 0.05 \) vs. untreated SOD1\(^{+/−} \) SMCs; \( *** p < 0.05 \) vs. untreated SOD2\(^{+/−} \) SMCs).

**Figure II. Differential regulation of MAP kinases in SMC cell types.** Growth-arrested SMCs were treated with thrombin (1.0 unit/ml) and cell lysates were prepared. (A) Western blot analysis of cell lysates was performed with either phosphospecific MEK1/2 (top) or MEK1/2 antibody (bottom). (B) Cell lysates were analyzed by Western blotting with either phosphospecific MKK3 (top) or MKK3 (bottom) antibody. Data presented are mean ± SEM of three separate experiments.
Wild-type
SOD1^{+/-}
SOD2^{+/-}

Aconitase activity (µmoles NADPH/min/mg protein)

Supplemental Figure I
Supplemental Figure II

A

<table>
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<th>Thrombin (min)</th>
<th>Wild-type</th>
<th>SOD1(^{+/-})</th>
<th>SOD2(^{+/-})</th>
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Fold activation (mean) (SEM)

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Fold activation (mean) (SEM)

B

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Fold activation (mean) (SEM)
ONLINE DATA SUPPLEMENT

I. Expansion of Materials and Methods

SOD activity

Total and SOD2 activity were determined by measuring the ability of SOD to inhibit xanthine-xanthine oxidase-induced cytochrome c reduction.\(^1\) Cells were harvested in phosphate-buffered saline, homogenized and centrifuged. Cell homogenate (16.5 µl) was added to 967 µl of reaction buffer containing 50 µM xanthine, 20 µM cytochrome c, 25 µM KH\(_2\)PO\(_4\), 25 µM K\(_2\)HPO\(_4\), and 0.1 mM EDTA in a cuvette. To this solution, 16.5 µl of xanthine oxidase (0.2 U/ml in 0.1 mM EDTA) were added and increase in absorbance was read at 550 nm at 1 min intervals for 10 min. Results were measured against a standard curve and SOD activity was expressed as units per microgram protein. SOD2 activity was determined in the presence of 5 mM potassium cyanide (KCN), which inhibits SOD1 and SOD3 activities.

Measurement of superoxide generation by SMC

Intracellular O\(_2^-\) generation was measured by assaying the activity of aconitase, a tricarboxylic acid cycle enzyme that is highly sensitive to inactivation by O\(_2^-\).\(^2\) Quiesced SMCs were either untreated or treated with thrombin (1.0 U/ml) for 10 min. Cells were rinsed with ice-cold PBS, scraped, pelleted and transferred to 500 µl cold homogenization buffer consisting of 50 mM Tris-HCl (pH 7.6), 0.2% Triton-X-100, 1 mM cysteine, 5 mM citrate and 100 mmol/L DTPA. The pellet was dispersed by vortexing and lysed by sonication. Fifty µl of lysate was added to 200 µl of assay buffer [50 mM Tris-HCl (pH 7.6), 30 mM citrate, 0.6 mM MnCl\(_2\), 0.2 mM NADP\(^+\) and 2U/ml isocitrate dehydrogenase] in a cuvette. Production of NADPH was determined by reading absorbance at 340 nm every 10 seconds for 2 minutes (Beckman Coulter DU 7500), and normalized to protein per sample.
Immunocytochemistry

In situ $O_2^-$ levels in SMCs were detected by oxidative fluorescent probe dihydroethidium (DHE) and mitochondria were visualized with MitoTracker Green (MTG; M-7514, Molecular Probes). Quiescent SMCs were incubated with MitoTracker Green (50 ng/ml) and DHE (10 µM) for 20 min at 37°C in dark. Fluorescence was monitored using a Zeiss LSM-510 laser scanning confocal microscope. Red fluorescence of DHE was excited at 488 nm using the argon laser and images were collected at 590 nm using a long-pass filter. MTG was also excited at 488 nm and the emitted green fluorescence was detected using a 505-530-nm band pass emission filter. Mitochondrial ROS production was visualized in a separate experiment using nonfluorescent reduced MitoTracker Red (MTR; M-7513, Molecular Probes) which becomes fluorescent upon oxidation. Growth-arrested SMCs were incubated with 500 nM MTR for 15 min at 37°C in the dark. The cells were washed with serum-free medium and images were captured at 586 nm emission through a long-pass filter after initial excitation at 543 nm. Enhanced STAT3 phosphorylation in SOD-deficient SMCs were visualized in confocal laser microscopy using phospho-specific STAT3 antibody and a secondary antibody conjugated to Alexa 488.


For cell counts, SMC were plated at equivalent densities (30 X 10^3) in 6-well plates. At defined intervals, cells were trypsinized and cell counts were done using a Coulter Counter. $[^3]$H-thymidine-uptake experiments in cells treated with thrombin were done as described previously. $[^3]$H-thymidine-uptake experiments with various kinase inhibitors were performed in growth-arrested SMCs. For analysis of protein synthesis, growth-arrested SMC were either treated or untreated with thrombin (1 unit/ml) for 24 h and incubated with $[^3]$H-leucine (1 μCi/ml) during the final 4 h of treatment. $[^3]$H-thymidine or $[^3]$H-leucine treated cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cells were lysed by vortexing vigorously in cold 10% (w/v) trichloroacetic acid. After keeping on ice for 20 min, the lysate was passed through a glass fiber filter (GF/C, Whatman). The filter was washed once with cold 5%
trichloroacetic acid and once with cold 70% (v/v) ethanol. The filter was dried and the radioactivity measured in a liquid scintillation counter (LS 3801 Beckman).

**Western blotting**

Preparation of protein extracts and Western blotting were performed as described. The antibodies used were anti-SOD1, anti-SOD2 (Upstate Biotechnology), anti-GAPDH (Chemicon International), anti-phospho ERK1/2MAP kinase (Thr202/Tyr204), antiphospho-SAPK/JNK (Thr183/Tyr185), antiphospho-p38 MAP kinase (Thr180/Tyr182), anti-ERK1/2 MAP kinase, anti-p38 MAP kinase, anti-phospho MEK1/2 (Ser217/Ser221), anti-MEK1/2, anti-phospho MKK3/MKK6 (Ser 189/209), anti-MKK3, anti-phospho STAT3 (Tyr705), anti-STAT3 (Cell Signaling Technology), anti-phospho JAK2 (Tyr1007/1008), anti-JAK2 (BIOSOURCE International), and anti-JNK1 (Santa Cruz Biotechnology).

**Statistical analysis**

Results were analyzed with one-way and two-way analysis of variance (ANOVA) and in case of one-way ANOVA, post-hoc analysis was performed using Newman-Keuls test. The statistical analysis was performed using Graphpad Prism version 3.0 (GraphPad software, San Diego, CA).

**References**


Figure I. SOD-deficient SMCs generate higher $O_2^-$ constitutively and in response to thrombin treatment. A significant increase in intracellular $O_2^-$ production, as reflected by decrease in aconitase activity, is observed in wild-type SMCs treated with thrombin (* p < 0.001 vs. untreated SMCs). SOD1 and SOD2 deficient SMCs produce constitutively higher $O_2^-$ levels (* p < 0.001 vs. untreated wild-type SMCs; *** p < 0.05 vs. untreated SOD1$^{+/−}$ SMCs) and in response to thrombin treatment (** p < 0.05 vs. untreated SOD1$^{+/−}$ SMCs; # p < 0.05 vs. untreated SOD2$^{+/−}$ SMCs).
## Figure II. Differential regulation of MAP kinases in SMC cell types.

Growth-arrested SMCs were treated with thrombin (1.0 unit/ml) and cell lysates were prepared. (A) Western blot analysis of cell lysates was performed with either phosphospecific MEK1/2 (top) or MEK1/2 antibody (bottom). (B) Cell lysates were analyzed by Western blotting with either phosphospecific MKK3 (top) or MKK3 (bottom) antibody. Data presented are mean ± SEM of three separate experiments.