

Regulation of Xanthine Oxidoreductase Protein Expression by Hydrogen Peroxide and Calcium

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Objective—We have previously demonstrated that endothelial xanthine oxidase (XO) levels are dependent on the NADPH oxidase. We postulated that H₂O₂ may modulate the irreversible conversion of xanthine dehydrogenase (XDH) to XO and sought to examine mechanisms involved.

Methods and Results—H₂O₂ (100 μmol/L) decreased bovine aortic endothelial cell (BAEC) XDH protein expression, and metabolic labeling studies indicated that H₂O₂ stimulated conversion of XDH to XO. The decline in XDH was mimicked by the reactive oxygen species (ROS) generating compounds SIN-1 and Menadione, as well as by stimulating BAECs with angiotensin II (200 nmol/L). BAPTA-AM prevented the decline in XDH by H₂O₂, indicating that it was calcium-dependent. In keeping with calcium acting downstream of H₂O₂, the calcium ionophore A23187 (1 μmol/L) caused XDH-to-XO conversion, and this was not prevented by the antioxidants. In addition, XDH-to-XO conversion was blocked by 2-APB and NO donors and induced by thapsigargin and M-3M3FBS, implicating phospholipase C and endoplasmic reticulum calcium stores in this process.

Conclusions—Endothelial XO and XDH expression are strongly dependent on H₂O₂ and calcium. Stimulation of XDH conversion to XO may represent a feed-forward mechanism whereby H₂O₂ can stimulate further production of ROS. (*Arterioscler Thromb Vasc Biol.* 2005;25:1623-1628.)

Key Words: calcium ■ hydrogen peroxide ■ reactive oxygen species ■ vascular endothelium ■ xanthine oxidoreductase

In mammalian cells, a major source of reactive oxygen species (ROS) is xanthine oxidase (XO), an enzyme that uses xanthine and hypoxanthine as reducing substrates and yields both superoxide (O₂⁻) and H₂O₂ via 1- and 2-electron reductions of molecular oxygen.¹ XO has been implicated in a variety of pathophysiological states in the cardiovascular system, including ischemia/reperfusion injury, endothelial dysfunction associated with congestive heart failure, atherosclerosis, and left ventricular dysfunction after myocardial infarction.²⁻⁵ In humans, the levels of endothelial-bound XO correlate with the presence of coronary artery disease and are inversely proportional to flow-mediated brachial artery vasodilatation.⁶

XO and xanthine dehydrogenase (XDH) are often collectively referred to as xanthine oxidoreductase (XOR). XDH is initially synthesized as the 150-kDa protein from which XO is derived, either reversibly by conformational changes or irreversibly by proteolysis.⁷ The mechanisms responsible for either the irreversible or the reversible conversions of XDH to XO are incompletely understood. Under baseline conditions, the majority of 150-kDa protein contains XDH activity. Reversible formation of XO from XDH is achieved by the oxidation of cysteines 535 and 992, a process that changes enzymatic function but not molecular weight.⁸ The irrevers-

ible proteolysis of XDH occurs at a specific site, leading to formation of the 130-kDa form of XO, which can be further proteolyzed into smaller proteins with XO activity. Importantly, the absolute amount of XO is important and the ratio of XO to XDH is critical in modulating cellular ROS generation. Recently, we found that oscillatory shear stress causes a reduction in endothelial cell XDH without changing XO levels. This was associated with an increase in endothelial cell O₂⁻ production, which could be inhibited by oxypurinol, an XO antagonist, or prolonged exposure to tungsten, which replaces molybdenum and renders the catalytic site of XO inactive.⁹ In addition, xanthine-driven O₂⁻ production, as measured by electron spin resonance (ESR), was increased in homogenates of endothelial cells previously exposed to oscillatory shear stress. In this previous study, the irreversible formation of XO from XDH seemed caused by oxidative mechanisms. In cells from mice lacking p47^{phox}, a critical component of the NADPH oxidase, XOR existed principally as XDH with minimal amounts of XO being present. Likewise, treatment of bovine aortic endothelial cells (BAECs) with apocynin, an inhibitor of the NADPH oxidase, led to a loss of XO, whereas it did not affect XDH, again supporting the concept that the NADPH oxidase governs XO expression.¹⁰ This previous study strongly suggested that ROS

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derived from the NADPH oxidase may participate in formation of XO from XDH. In the present study, we sought to understand the mechanisms involved in this regulation. We demonstrate that H₂O₂ markedly enhances the irreversible conversion of XDH to XO, leading to ROS production, and that this is mediated by increased calcium released from the endoplasmic reticulum (ER).

Methods

Materials and Reagents

The bicarbonate-buffered saline used for calcium measurements contained (in mol/L) 116.4 NaCl, 5.4 KCl, 1.6 MgSO₄, 1.5 CaCl₂, 1.0 NaH₂PO₄, 5.6 D-glucose, and 26.2 NaHCO₃ (pH=7.4). FURA 2-AM and Pluronic F-127 were obtained from Molecular Probes. Reagents A23187, BAPTA/AM, thapsigargin, 2-aminoethoxy-diphenylborate (2-APB), and *m*-3M3FBS were obtained from Calbiochem and were dissolved in DMSO. DPTA-NONOate and DETA-NONOate were obtained from Cayman. Redivue L-[³⁵S]-Methionine was obtained from Amersham. Met/Cys-free DMEM was purchased from Gibco. Autofluor was from National Diagnostics. Protein A/G beads were obtained from Santa Cruz. All other reagents were obtained from Sigma. Polyethylene-glycolated catalase (polyethylene glycol [PEG]-CAT, 200 U/mL) was added 24 hours before cells were studied to assure an increase in intracellular concentration of catalase.¹¹

Cell Culture

BAECs (Cell Systems, Kirkland, Wash) were cultured in Media 199 (M199; Fischer) containing 10% fetal calf serum (Hyclone Laboratories, Logan, Utah) as previously described.¹⁰ The purine content of M199 medium is xanthine, Na (0.34 mg/mL), and hypoxanthine Na (0.354 mg/mL). Postconfluent BAECs between passages 4 through 9 were used for experiments.

Western Analysis of Xanthine Oxidoreductase

Western analysis on XOR was performed as previously described.¹⁰ Immunoreactive bands for XO and XDH were visualized by Enhanced Chemiluminescence (Amersham) and were quantified using densitometry.

Metabolic Labeling and Immunoprecipitation of XOR

BAECs in p100 dishes were washed with Met/Cys-free DMEM and then incubated for 2 hours in the same medium; 250 μCi of Redivue [³⁵S]-Met was added to cells for 24 hours (pulse). Cells were washed 3 times with M199 containing 10% FBS and then incubated for 24 hours (chase). Protein was harvested as described; 500 μg of protein was immunoprecipitated with α-bXOR (1:10) for 1 hour at 4°C. Immunocomplexes were captured with protein A/G beads and centrifuged for 1 minute at 14 000 rpm. Complexes were washed with phosphate-buffered saline and resuspended in sample buffer. The immunoprecipitate was boiled and proteins electrophoresed at 110 V for 1.5 hours on a 7.5% SDS polyacrylamide gel. The gel was immersed in Autofluor for 1 hour, dried, and autoradiographed.

ESR Measurement of Endothelial Cell Superoxide Production

O₂⁻ production was measured in intact BAECs using ESR as described previously.¹⁰ Briefly, a chelexed phosphate buffer containing 2.5 × 10⁶ cells and the spin label 1-hydroxy-3-carboxyl-2,2,5-tetramethyl-pyrrolidine hydrochloride (CPH) (5 mmol/L) was loaded into a 50-μL capillary tube and placed in the ESR cavity. We have previously shown that oxidation of CPH is predominantly mediated by O₂⁻ and is inhibited by PEG-SOD. ESR measurements were performed at room temperature using an EMX ESR spectrometer (Bruker).

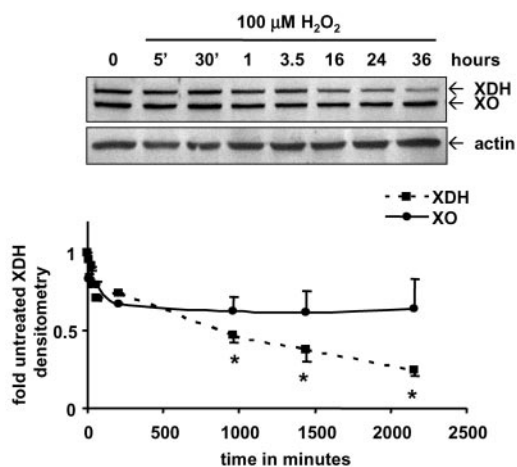


Figure 1. Western analysis of endothelial XO and XDH protein expression after exposure to H₂O₂. Time course of XO and XDH expression in BAECs treated with 100 μmol/L H₂O₂ (n=4).

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured as previously described.¹² Cells were incubated with FURA 2-AM (2 μmol/L) and cell dispersion agent Pluronic F-127 (0.04%) at 37°C in the dark for 30 minutes. Values of [Ca²⁺]_i were obtained by determining maximum FURA 2 fluorescence ratio (R_{max}) and minimum (R_{min}) using 10 μmol/L digitonin followed by 30 mmol/L EGTA as described previously.¹³ K_d was determined to be 0.2 μmol/L using the calcium calibration kit 1 with magnesium (Molecular Probes).

Statistical Analysis

Data in this article are presented as mean ± standard error of the mean. Differences between groups and control were analyzed using 1-sided *t* tests or ANOVA. A Dunnett's post hoc test was used when ANOVA indicated statistical significance was present. Differences were considered significant when *P* < 0.05.

Results

Determination of the Role of H₂O₂ in Endothelial Levels of XDH to XO

We have previously shown that endothelial XO expression is dependent on the presence of a functional NADPH oxidase, which predominantly produces H₂O₂.¹⁰ We therefore postulated that H₂O₂ may modulate the relative protein levels of XO and XDH. After 24 to 36 hours of exposure to H₂O₂ (100 μmol/L), BAEC XDH levels decreased, whereas XO levels remained stable (Figure 1). The decrease in XDH protein expression was completely prevented by incubating cells with the specific H₂O₂ scavenger PEG-CAT (200 U/mL) as well as compounds that increase intracellular-reduced glutathione levels of N-acetylcysteine (10 mmol/L) and oxathiazolidine carboxylate (10 mmol/L) (Figure I, available online at <http://atvb.ahajournals.org>). Preliminary data showed that N-acetylcysteine and oxathiazolidine carboxylate increased glutathione levels ≈10-fold after 24 hours. Interestingly, PEG-CAT increased XDH expression in untreated cells, suggesting that baseline regulation of the XOR system by H₂O₂ was present at a low level.

To determine whether oxidants other than H₂O₂ might regulate XDH protein levels, we treated cells with the ROS-generating compounds SIN-1 and Menadione. SIN-1 generates nitric oxide and superoxide, which combine to form

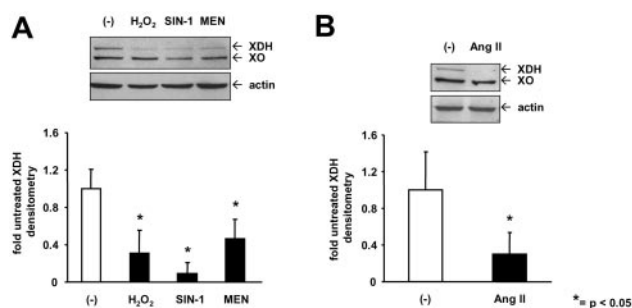


Figure 2. Effect of oxidants and angiotensin II on endothelial XDH expression. A, Western data showing the effect of SIN-1 (0.5 mmol/L) and Menadione (MEN) (5 μmol/L) (n=4). B) Western analysis of XDH expression in response to 200 nmol/L angiotensin II (n=4). Cells were treated for 36 hours with SIN-1, MEN, or angiotensin II and harvested for Western analysis.

peroxynitrite, whereas Menadione steadily produces superoxide and thus H₂O₂ by spontaneous dismutation.¹⁴ Western analysis showed that both SIN-1 and Menadione caused a decrease in XDH protein expression, much like H₂O₂ (Figure 2A). We also examined the effect of angiotensin II, a known stimulus for endothelial ROS generation, in the regulation of XDH protein expression. Low passage (p2-4) BAECs were treated with angiotensin II (200 nmol/L) for 36 hours. Western analysis revealed that angiotensin II treatment mimicked the effect of ROS on the endothelial expression of XO and XDH (Figure 2B).

One explanation for these results is that H₂O₂ enhanced transformation of XDH to XO, and that XO levels remained relatively constant because of further degradation to proteins not recognized by Western analysis. To test this hypothesis, BAECs were metabolically labeled with [³⁵S]-Methionine for 24 hours and subsequently treated with H₂O₂. Immunoprecipitation was performed to monitor levels of labeled XDH and XO. After 24 hours of incubation (0 hours chase), the [³⁵S]-Methionine label is located in both the XDH and XO forms of XOR (Figure 3A). In the absence of H₂O₂, the percent of labeled XOR in the XO form increased slightly during the 24-hour chase, suggesting that proteolytic conversion occurs at a low rate under basal conditions (Figure 3B). In contrast, H₂O₂ dramatically accelerated conversion of XDH to XO as evidenced by the increased percent of XOR labeled as XO (Figure 3A and 3B). These data show that conversion of XDH to XO is facilitated by H₂O₂.

Effect of H₂O₂ on Endothelial Cell Superoxide Production

We have previously demonstrated that endothelial cell O₂⁻ production is increased by oscillatory shear stress, and that this is largely inhibited by oxypurinol. In these experiments, we found that oscillatory shear stress resulted in a decrease in XDH without changing XO production, a situation identical to that observed in the present study with H₂O₂ treatment. We therefore reasoned that H₂O₂ would increase endothelial cell O₂⁻ production in part via XO. BAECs were exposed to H₂O₂ for 36 hours and the rate of endothelial cell O₂⁻ production was analyzed by ESR as previously described.¹⁰ H₂O₂ exposure doubled O₂⁻ production, and the increase was predominantly caused by the oxypurinol-inhibitable fraction, which indicates the O₂⁻ production rate by XO (Figure 3C).

Determination of the Role of Calcium in XO and XDH Protein Expression

H₂O₂ has been shown to increase [Ca²⁺]_i in certain endothelial cell types and calcium plays a crucial role in many signal cascades involving H₂O₂, including endothelial nitric oxide synthase (eNOS) activation, eNOS gene induction, membrane permeabilization, and induction of membrane blebbing.¹⁵⁻¹⁹ Further, several proteolytic enzymes that might participate in cleavage of XDH to XO are calcium-dependent. To determine whether H₂O₂ increases [Ca²⁺]_i in BAECs, fluorometric analysis with FURA 2-AM was used. The fluorescence measurements of BAECs showed an increase in the FURA 2 fluorescence ratio in response to 100 μmol/L H₂O₂ (Figure IIA, available online at <http://atvb.ahajournals.org>). [Ca²⁺]_i increased ≈2-fold after exposure to H₂O₂ (Figure IIB). In preliminary studies, we found that this was prolonged, with a sustained increase in [Ca²⁺]_i lasting up to 4 hours.

We next sought to determine the role of [Ca²⁺]_i in regulation of XDH protein expression by H₂O₂. Pretreatment of cells with the cell-permeable calcium chelator BAPTA-AM (10 μmol/L) completely prevented the decrease in XDH protein expression (Figure 4A). This experiment indicated that calcium is necessary for the regulation of the XOR system by H₂O₂. To determine whether calcium alone is sufficient to modulate XDH protein expression, BAECs were treated with calcium ionophore (A23187, 1 μmol/L). Mimicking the effect of H₂O₂, A23187 caused a time-dependent decrease in XDH expression whereas XO remained stable (Figure 4B). In contrast to H₂O₂, none of the

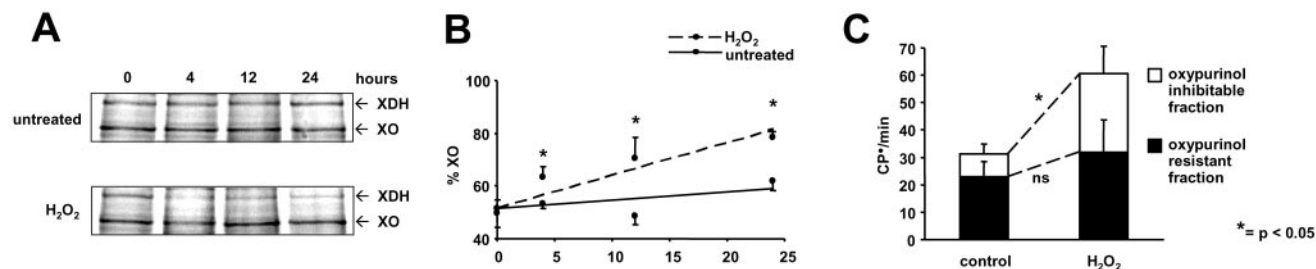


Figure 3. Analysis of XDH to XO conversion in response to H₂O₂. Representative [³⁵S]-Methionine pulse-chase in the absence (A, top) and presence of H₂O₂ (A, bottom). B, Percentages of XO compared with total labeled XO plus XDH (n=4). C, Total and oxypurinol-inhibitable ROS production as determined by ESR (n=6). Whole-cell homogenates from cells either treated or not treated with H₂O₂ were exposed to xanthine and superoxide production monitored by ESR using the spin label CPH.

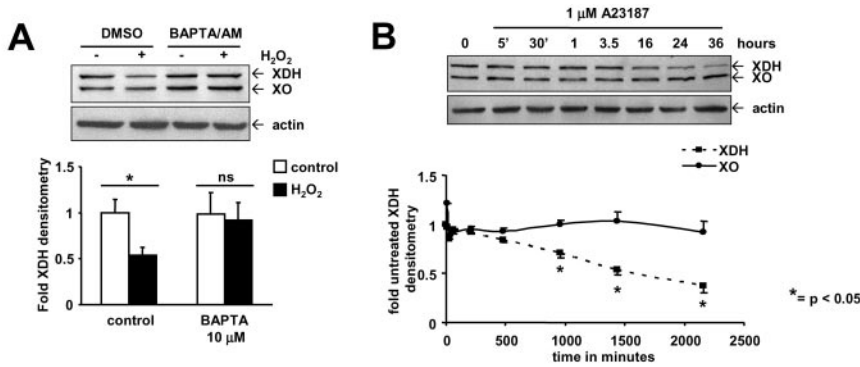


Figure 4. Modulation of endothelial cell XDH levels by $[Ca^{2+}]_i$. A, Effect of BAPTA-AM (10 $\mu\text{mol/L}$) on XDH levels after 36-hour H_2O_2 treatment. Representative Western blot is shown above and mean data are shown below ($n=5$). B, Effect of calcium ionophore (A23187) on endothelial XDH and XO protein levels. BAECs were treated with A23187 (1 $\mu\text{mol/L}$) and XDH and XO levels were detected by Western ($n=4$).

scavengers prevented the decreased XDH levels caused by calcium ionophore (Figure III, available online at <http://atvb.ahajournals.org>). These data suggest that calcium acts downstream of H_2O_2 in regulating the relative protein expression of XDH and XO.

Determination of the Role of Intracellular Calcium Stores in XOR Protein Modulation

Recent research suggests that H_2O_2 can release calcium from stores in the ER.¹⁵ To determine the role of ER calcium stores, cells were treated with thapsigargin and 2-APB, antagonists of the calcium-ATPase and inositol triphosphate (IP_3) receptor, respectively. As shown by FURA 2 analysis, thapsigargin increased $[Ca^{2+}]_i$ in untreated cells, and H_2O_2 did not further increase this signal (Figure IVA, available online at <http://atvb.ahajournals.org>). This suggested that intact ER calcium stores are essential for the increased $[Ca^{2+}]_i$ in response to H_2O_2 . In addition, 2-APB prevented the increase in $[Ca^{2+}]_i$ in response to H_2O_2 (Figure IVA). These data implicate the ER as the source of calcium in response to H_2O_2 .

We next used Western analysis to determine the role of ER calcium stores in the regulation of XOR by H_2O_2 . Consistent with the FURA 2 data, thapsigargin decreased XDH protein levels even in the absence of H_2O_2 , and this was not decreased further by additional H_2O_2 (Figure 5A). Furthermore, 2-APB completely prevented the decrease in XDH protein expression in response to H_2O_2 (Figure 5A). These data show that calcium released from the ER is required for the regulation of XOR protein expression in response to H_2O_2 .

Determination of the Role of Phospholipase C in XOR Expression

Phospholipid cleavage by phospholipase C (PLC) is known to increase IP_3 , which in turn stimulates calcium release from

the ER.²⁰ To determine whether PLC is responsible for increased $[Ca^{2+}]_i$, BAECs were treated with the specific PLC activator *m*-3M3FBS. Activation of PLC increased $[Ca^{2+}]_i$ and selectively decreased XDH protein levels, whereas XO expression remained constant (Figures IVB and 5B). Thus, activation of PLC is sufficient to regulate XOR protein expression in a manner identical to H_2O_2 exposure.

The Role of Nitric Oxide in XDH Expression

Recently, nitric oxide (NO) was found to inhibit calcium entry into the cell and enhance uptake of calcium into the endothelial cell ER.^{21,22} To determine whether NO can attenuate $[Ca^{2+}]_i$ levels in response to H_2O_2 , cells were treated with the NO donor DPTA-NONOate (half life=5 hours). FURA 2 analysis showed that DPTA-NONOate blunted the increase in $[Ca^{2+}]_i$ in response to H_2O_2 (Figure 6A). To determine whether NO can prevent the decrease in XDH protein expression by H_2O_2 , cells were treated with the long-acting NO donor DETA-NONOate (half life=20 hours). Western analysis revealed that DETA-NONOate completely prevented the decrease in XDH by H_2O_2 (Figure 6B).

Discussion

In the present study, we demonstrated that H_2O_2 modulates the relative levels of XO and XDH in endothelial cells via a pathway requiring ER calcium release. We also showed that ROS slowly released from Menadione and SIN-1 and stimulation of endogenous ROS using angiotensin II decreased the XDH content of endothelial cells in a manner similar to bolus H_2O_2 . H_2O_2 evoked a progressive decline in XDH, and our metabolic labeling studies demonstrated that this was caused by conversion of XDH to XO. This conversion resulted in increased ROS production by XO as shown by

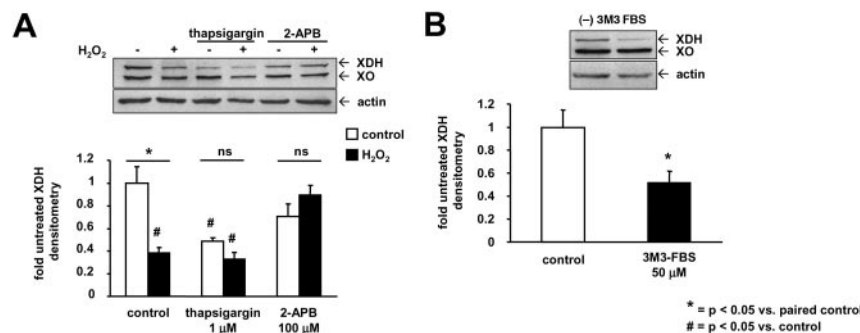


Figure 5. Contribution of ER and PLC/ IP_3 pathway on XDH expression in response to H_2O_2 . A, Western blot showing the effect of thapsigargin (1 $\mu\text{mol/L}$) and aminoethoxy-diphenylborate (2-APB) (100 $\mu\text{mol/L}$) on XDH and XO expression is shown above and mean data are presented below ($n=4$). B, Effect of *m*-3M3FBS (50 $\mu\text{mol/L}$) on XDH and XO expression. A representative Western blot is shown above and mean data are given below ($n=5$).

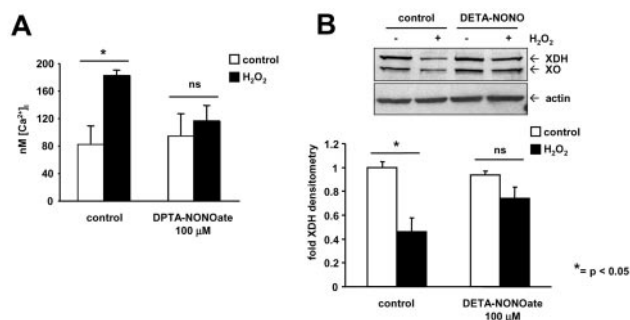


Figure 6. Western analysis demonstrating the role of nitric oxide in the expression of XDH in response to H₂O₂. Mean [Ca²⁺]_i measurements (A) and Western blots of XDH (B) in response to H₂O₂ in the presence of NO donors (n=4).

ESR analysis. The effect of H₂O₂ was mediated by [Ca²⁺]_i, because it was prevented by the calcium chelator BAPTA-AM and mimicked by the calcium ionophore A23187. Further, we demonstrated that H₂O₂ stimulated release of calcium from the ER, and that this was prevented by thapsigargin and the IP₃ receptor antagonist 2-APB. The PLC activator m-3M3FBS mimicked the effect of H₂O₂, in keeping with the concept that PLC activation may be a proximal event after exposure of cells to H₂O₂, leading to an increase in [Ca²⁺]_i. Finally, we demonstrated that NO prevented the decrease in XDH protein levels, likely via inhibition of calcium release from the ER.

The present findings are consistent with our previous studies showing the NADPH oxidase importantly modulates the relative levels of XDH and XO in endothelial cells.¹⁰ In this previous study, we found that cells lacking the NADPH oxidase subunit p47^{phox} had very low levels of XO and low XO activity and that re-introduction of p47^{phox} into these cells restored their XO expression and activity. Paradoxically, even though our metabolic labeling studies proved that H₂O₂ enhanced conversion of XDH to XO, we did not observe an increase in the 130-kDa XO by Western blot after H₂O₂ exposure. This is likely caused by further degradation of XO to species not detected by the antibody used in these studies. Importantly, 87-kDa degradation product of XOR has been identified in human liver.²³ In addition, the relative loss of XDH would lead to a condition in which xanthine and hypoxanthine are preferentially oxidized by XO, leading to excessive production of O₂^{•-} and H₂O₂. Of note, some of the 150-kDa protein may contain reversible XO activity that cannot be discerned by Western analysis. We believe this reversible component of XO activity is negligible in endothelial cells, because we previously found that XO/XDH pterine conversion ratios are comparable to the 130/150 kDa ratio as identified by Western.¹⁰

Our findings indicate that calcium plays an important role in signaling the conversion of XDH to XO. This finding is in keeping with several previous studies showing that an as-yet-undefined calcium-dependent protease is involved in cleavage of XDH to XO and that the conversion is sensitive to calmodulin inhibitors.^{24–27} Recently, a novel protease that cleaves XDH to XO has been found in the mitochondrial intermembrane space.²⁸ This protease has not been identified

and no specific antagonists are currently available; however, it is possible that changes in mitochondrial function and permeability caused by increased [Ca²⁺]_i may lead to its release. We further demonstrate that H₂O₂ likely mediates increased [Ca²⁺]_i via a PLC-, IP₃-, and ER-dependent pathway. It has previously been demonstrated that H₂O₂ can stimulate an increase in PLC-γ activity via tyrosine phosphorylation by Src, which could represent an early event in this pathway.²⁹ It is of interest that although H₂O₂ is only transiently present, it stimulates a prolonged increase in [Ca²⁺]_i. In preliminary studies, we found [Ca²⁺]_i was increased 5-fold 4 hours after H₂O₂ addition.

In the present study, we also found that the expression of XDH was modulated by NO. An exogenous NO donor prevented the decline in XDH. These results were mirrored by changes in [Ca²⁺]_i and are consistent with previous observations that NO facilitates uptake of calcium into the ER of endothelial cells.²¹ NO is known to act as an antioxidant by directly reacting with O₂^{•-} and lipid peroxy and alkoxy radicals.^{30,31} NO is also known to inhibit XO by reactions within the catalytic molybdenum center.³² Our findings provide yet another mechanism whereby NO can have antioxidant effects via inhibition of XDH-to-XO conversion.

We also found that lower level ROS could directly modulate the expression of XDH. Menadione, a superoxide/H₂O₂-generating compound, mirrored the effect of bolus H₂O₂ on XDH protein levels. In addition, the potent peroxynitrite-generating compound SIN-1 resulted in a decrease in XDH protein expression similar to that of H₂O₂ stimulation. These findings suggest that both bolus and low-level ROS regulate the XO/XDH protein ratio. Also, these results were mirrored by treating endothelial cells with angiotensin II, a potent stimulator of the endothelial NADPH oxidase. Together, these data elucidate a pathway by which the NADPH oxidase and ROS modulate the ratio of XO/XDH protein in endothelial cells.

In mammalian cells, and in particular in vascular cells, there appear to be a variety of mechanisms whereby ROS produced by one enzyme can stimulate production of ROS by other enzymes in a “feed-forward” fashion. As an example, oxidation of tetrahydrobiopterin by peroxynitrite and other strong oxidants can lead to eNOS uncoupling, a situation in which eNOS produces O₂^{•-} rather than NO.³³ ROS have also been shown to damage mitochondrial DNA, leading to perturbations in mitochondrial transport and increased electron leak from this pathway.³⁴ Likewise, H₂O₂ and lipid peroxides have been shown to stimulate the NADPH oxidase in a Src-dependent fashion.³⁵ In the present studies, we observed an increase in XO production of O₂^{•-} 36 hours after exposure of cells to hydrogen peroxide. It is therefore conceivable that H₂O₂ not only directly stimulated conversion of XDH to XO but also activated the endothelial cell NADPH oxidase, leading to prolonged H₂O₂ production and sustained XDH degradation. In this fashion, brief exposure of cells to an oxidant such as H₂O₂ may lead to prolonged ROS generation. Of note, both NADPH oxidase and xanthine oxidase activity have been found to be increased in human atherosclerotic coronary arteries. Further, both the NADPH oxidase and XO have been implicated in ischemia reperfusion

injury. Our current findings indicate that the coexistence of these 2 ROS-generating enzyme systems may be closely linked, and that other ROS-producing enzymes may stimulate endothelial XO activity.

Acknowledgments

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References

- Harrison R. Structure and function of xanthine oxidoreductase: where are we now? *Free Radic Biol Med.* 2002;33:774–797.
- Kinugasa Y, Ogino K, Furuse Y, Shiomi T, Tsutsui H, Yamamoto T, Igawa O, Hisatome I, Shigemasa C. Allopurinol improves cardiac dysfunction after ischemia-reperfusion via reduction of oxidative stress in isolated perfused rat hearts. *Circ J.* 2003;67:781–787.
- Farquharson CA, Butler R, Hill A, Belch JJ, Struthers AD. Allopurinol improves endothelial dysfunction in chronic heart failure. *Circulation.* 2002;106:221–226.
- Patetsios P, Song M, Shutze WP, Pappas C, Rodino W, Ramirez JA, Panetta TF. Identification of uric acid and xanthine oxidase in atherosclerotic plaque. *Am J Cardiol.* 2001;88:188–191, A186.
- Engberding N, Spiekermann S, Schaefer A, Heineke A, Wiencke A, Muller M, Fuchs M, Hilfiker-Kleiner D, Hornig B, Drexler H, Landmesser U. Allopurinol attenuates left ventricular remodeling and dysfunction after experimental myocardial infarction: a new action for an old drug? *Circulation.* 2004;110:2175–2179.
- Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H. Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine oxidase and extracellular superoxide dismutase. *Circulation.* 2002;106:3073–3078.
- Waud WR, Rajagopalan KV. The mechanism of conversion of rat liver xanthine dehydrogenase from an NAD⁺-dependent form (type D) to an O₂-dependent form (type O). *Arch Biochem Biophys.* 1976;172:365–379.
- Rasmussen JT, Rasmussen MS, Petersen TE. Cysteines involved in the interconversion between dehydrogenase and oxidase forms of bovine xanthine oxidoreductase. *J Dairy Sci.* 2000;83:499–506.
- Paler-Martinez A, Panus PC, Chumley PH, Ryan A, Hardy MM, Freeman BA. Endogenous xanthine oxidase does not significantly contribute to vascular endothelial production of reactive oxygen species. *Arch Biochem Biophys.* 1994;311:79–85.
- McNally JS, Davis ME, Giddens DP, Saha A, Hwang J, Dikalov S, Jo H, Harrison DG. Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. *Am J Physiol Heart Circ Physiol.* 2003;285:H2290–H2297.
- Beckman JS, Minor RL, Jr., White CW, Repine JE, Rosen GM, Freeman BA. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *J Biol Chem.* 1988;263:6884–6892.
- Tong DC, Buck SM, Roberts BR, Klein JD, Tumlin JA. Calcineurin phosphatase activity: activation by glucocorticoids and role of intracellular calcium. *Transplantation.* 2004;77:259–267.
- Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985;260:3440–3450.
- Fink B, Laude K, McCann L, Doughan A, Harrison DG, Dikalov S. Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. *Am J Physiol Cell Physiol.* 2004;287:C895–C902.
- Volk T, Hensel M, Kox WJ. Transient Ca²⁺ changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. *Mol Cell Biochem.* 1997;171:11–21.
- Cai H, Li Z, Davis ME, Kanner W, Harrison DG, Dudley SC Jr. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Mol Pharmacol.* 2003;63:325–331.
- Cai H, Davis ME, Drummond GR, Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. *Arterioscler Thromb Vasc Biol.* 2001;21:1571–1576.
- Niwa K, Inanami O, Ohta T, Ito S, Karino T, Kuwabara M. p38 MAPK and Ca²⁺ contribute to hydrogen peroxide-induced increase of permeability in vascular endothelial cells but ERK does not. *Free Radic Res.* 2001;35:519–527.
- van Gorp RM, Heeneman S, Broers JL, Bronnberg NM, van Dam-Mieras MC, Heemskerk JW. Glutathione oxidation in calcium- and p38 MAPK-dependent membrane blebbing of endothelial cells. *Biochim Biophys Acta.* 2002;1591:129–138.
- Nilius B, Droogmans G, Wondergem R. Transient receptor potential channels in endothelium: solving the calcium entry puzzle? *Endothelium.* 2003;10:5–15.
- Dedkova EN, Blatter LA. Nitric oxide inhibits capacitative Ca²⁺ entry and enhances endoplasmic reticulum Ca²⁺ uptake in bovine vascular endothelial cells. *J Physiol.* 2002;539:77–91.
- Takeuchi K, Watanabe H, Tran QK, Ozeki M, Sumi D, Hayashi T, Iguchi A, Ignarro LJ, Ohashi K, Hayashi H. Nitric oxide: inhibitory effects on endothelial cell calcium signaling, prostaglandin I₂ production and nitric oxide synthase expression. *Cardiovasc Res.* 2004;62:194–201.
- Sarnesto A, Linder N, Raivio KO. Organ distribution and molecular forms of human xanthine dehydrogenase/xanthine oxidase protein. *Lab Invest.* 1996;74:48–56.
- McKelvey TG, Hollwarth ME, Granger DN, Engerson TD, Landler U, Jones HP. Mechanisms of conversion of xanthine dehydrogenase to xanthine oxidase in ischemic rat liver and kidney. *Am J Physiol.* 1988;254:G753–G760.
- McCord JM, Roy RS. The pathophysiology of superoxide: roles in inflammation and ischemia. *Can J Physiol Pharmacol.* 1982;60:1346–1352.
- Hirata Y, Ishii K, Taguchi T, Suita S, Takeshige K. Conversion of xanthine dehydrogenase to xanthine oxidase during ischemia of the rat small intestine and the effect of trifluoperazine on the conversion. *J Pediatr Surg.* 1993;28:597–600.
- Greene EL, Paller MS. Calcium and free radicals in hypoxia/reoxygenation injury of renal epithelial cells. *Am J Physiol.* 1994;266:F13–F20.
- Saksela M, Lapatto R, Raivio KO. Irreversible conversion of xanthine dehydrogenase into xanthine oxidase by a mitochondrial protease. *FEBS Lett.* 1999;443:117–120.
- Tokmakov AA, Sato KI, Iwasaki T, Fukami Y. Src kinase induces calcium release in *Xenopus* egg extracts via PLCγ and IP₃-dependent mechanism. *Cell Calcium.* 2002;32:11–20.
- Thomson L, Trujillo M, Telleri R, Radi R. Kinetics of cytochrome c₂ oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems. *Arch Biochem Biophys.* 1995;319:491–497.
- O'Donnell VB, Chumley PH, Hogg N, Bloodworth A, Darley-Usmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with α-tocopherol. *Biochemistry.* 1997;36:15216–15223.
- Ichimori K, Fukahori M, Nakazawa H, Okamoto K, Nishino T. Inhibition of xanthine oxidase and xanthine dehydrogenase by nitric oxide. Nitric oxide converts reduced xanthine-oxidizing enzymes into the desulfo-type inactive form. *J Biol Chem.* 1999;274:7763–7768.
- Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem.* 2003;278:22546–22554.
- Wei YH. Oxidative stress and mitochondrial DNA mutations in human aging. *Proc Soc Exp Biol Med.* 1998;217:53–63.
- Cifuentes ME, Pagano PJ. c-Src and smooth muscle NAD(P)H oxidase: assembling a path to hypertrophy. *Arterioscler Thromb Vasc Biol.* 2003;23:919–921.