Reactive Oxygen Intermediates Induce Regulated Secretion of von Willebrand Factor From Cultured Human Vascular Endothelial Cells

By Ulrich M. Vischer, Lan Jornot, Claes Wollheim, and Jean-Marc Theler

Exocytosis from Weibel-Palade bodies, the secretory granules of vascular endothelial cells, causes the rapid release of von Willebrand factor (vWF), an adhesive glycoprotein involved in primary hemostasis, and subendothelial vWF deposits typically seen after exposure to Weibel-Palade bodies were observed after exposure to XO. XO caused a rapid, sustained increase in intracellular free calcium concentration ([Ca^{2+}]_i). vWF secretion was markedly inhibited by BAPTA-AM, a calcium chelator. Removal of extracellular calcium did not inhibit vWF secretion, although the sustained phase of the [Ca^{2+}]_i increase was suppressed. These results suggest that XO-induced vWF release is mediated by the initial increase in [Ca^{2+}]_i, which is caused by calcium mobilization from intracellular stores rather than by calcium influx. Exocytosis from Weibel-Palade bodies may contribute to the pathogenic effect of ROIs in atherosclerosis and inflammation.

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MATERIALS AND METHODS

Materials. RPMI1640 was from Biological Industries (Beth Haemek, Israel). Fetal calf serum (FCS) from Seromed (Berlin, Germany), endothelial cell growth factor (ECGF) from Upstate Biotechnology Inc (Lake Placid, NY). Anti-vWF antibodies were from Stago (Asnières, France) or Dako (Glostrup, Denmark). Secondary antibodies were from Cappel (Turnhout, Belgium). XO, hypoxanthine, and H2O2 were from Sigma or Fluka (Buchs, Switzerland).

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**Cell culture.** Primary cultures of endothelial cells were obtained from individual human umbilical veins by collagenase digestion as previously described and grown in medium RPMI 1640 supplemented with 10% FCS, 90 μg/mL heparin, and 15 μg/mL ECGF. All cells used in these experiments were passaged no more than twice from primary cultures. Tissue culture dishes, as well as the 12-well plates (Costar, Cambridge, MA) and the glass coverslips used for experiments were coated with 0.1% gelatin before plating.

**Stimulation with ROIs.** Confluent monolayers of HUVECs were washed four times, and incubated in Krebs-Ringer bicarbonate buffer (120 mmol/L NaCl, 4.75 mmol/L KCl, 1.2 mmol/L KH2PO4, 1.2 mmol/L MgSO4, 2.5 mmol/L CaCl2, 25 mmol/L NaHCO3, 5 mmol/L glucose) supplemented with 25 mmol/L HEPES, pH 7.4 (KRBH). Various concentrations of XO or H2O2 were added as indicated. XO was always added to KRBH containing 2 mmol/L hypoxanthine. At the end of the incubations, the supernatants were cleared of cell debris by centrifugation and stored at −20°C until the time of assay.

XO activity was verified by measurement of the reduction of ferricytochrome-c in the presence of 2 mmol/L hypoxanthine, as described. One milliunit is defined as the production of 1 mol O2/min at 37°C. Cell loading with BAPTA-AM and fura-2 AM was achieved by preincubation for 30 minutes at 37°C with these substances dissolved in KRBH from dimethylsulfoxide (DMSO) stock solutions. At the final concentrations used, DMSO (less than 0.1%) neither affected vWF secretion nor decreased superoxide production by XO in the ferricytochrome-c assay.

Lactate dehydrogenase (LDH) enzymatic activity was measured using a fluorometric method as described. vWF was measured quantitatively by enzyme-linked immunosorbent assay (ELISA), essentially as described previously. Microtiter plates were coated with 200 μL purified rabbit antihuman vWF antibodies (3 μg/mL in 50 mmol/L Na carbonate, pH 9.6). After washing, samples were added after mixing with bovine serum albumin to a final concentration of 1.5%. Bound antigen was detected by a second anti-vWF antibody conjugated to horseradish peroxidase. A standard curve was constructed from serial dilutions of normal pooled plasma, assuming a vWF concentration of LDH, a cytosolic enzyme, was minimally affected by cell lysis. As shown in control experiments, preincubation of diluted plasma samples with XO or H2O2 did not affect the quantitation of vWF. vWF content and release may vary between individual cultures. Therefore, when necessary, results were expressed in relative values, with the highest value measured in the experiment taken as 100%.

**Assessment of XO-induced secretion by immunofluorescence and confocal microscopy.** vWF released from WP bodies remains in part associated with the external surface of the cell, presumably because of its highly multimerized structure. Exocytosis results in a depletion of Weibel-Palade bodies and the appearance of extracellular vWF observed as large patches of fluorescence. We used immunofluorescence to clarify the role of exocytosis from WP bodies in XO-induced vWF release. Confocal microscopy was used to study the three-dimensional (3D) distribution of the secreted product. Endothelial cells grown on glass coverslips were exposed to XO (5 to 10 μM/mL), A23187 (10 μmol/L) or control buffer for 10 minutes, processed for indirect immunofluorescence and examined by confocal microscopy. Twenty consecutive optical sections through the cells were taken, parallel to the coverslip, 0.3 to 0.4 μm apart. These sections were then superimposed to create a single image, and pseudo-color coding was used to display the relative depth of each point. In control cells, WP bodies were visualized as typical rod-shaped granules spread throughout the thickness of the cytoplasm (Fig 3A). In contrast, after incubation with either XO (Fig 3B) or A23187 (Fig 3C), there was a striking depletion in WP bodies, and the appearance of irregular patches typical of extracellular vWF. As observed by 3D analysis, these patches were closer to the glass coverslip than most residual Weibel-Palade bodies, implying that they are located on the baso-lateral side of the cell. This was particularly clear when the patches were superimposed on the nuclear area, where the distance between the baso-lateral and the luminal side of the cells is greatest. These

**RESULTS**

**Effect of ROIs on vWF release.** To assess the effect of ROIs on vWF secretion, HUVECs were incubated for 30 minutes in the presence of a superoxide generating system consisting of increasing amounts of XO (1.25 to 10 μM/mL) and 2 mmol/L hypoxanthine. Alternatively, the cells were exposed to H2O2 (0.125 to 1 mmol/L). XO induced a dose-dependent release of vWF, up to an enzyme activity of 10 μM/mL reaching a fourfold increase in secretion (Fig 1A). This response was 80% of that obtained with a maximal dose (10 μmol/L) of the calcium ionophore A23187, a well-established, potent secretagogue (Fig 1A). Higher concentrations of XO were not tested, as they may cause morphologic changes of the monolayer or cell detachment. In contrast with XO, incubation with H2O2 elicited a much weaker secretory response, which did not reach statistical significance (Fig 1B).

We next performed a time course study of XO-induced vWF release (Fig 2). Release from cells stimulated with XO (5 μM/mL) was significantly different from controls after only 5 minutes incubation; this continued to increase during the 30 minutes of the study. The rapid response is suggestive of vWF release from preformed storage granules. Cell lysis is unlikely to account for vWF release. Indeed, the release of LDH, a cytosolic enzyme, was minimally affected by exposure to XO (10 μM/mL for 30 minutes), increasing from 2.7% ± 0.44% to 3.5% ± 0.26% of cellular LDH content (mean ± SD, n = 3).

**Assessment of XO-induced secretion by immunofluorescence and confocal microscopy.** vWF released from WP bodies remains in part associated with the external surface of the cell, presumably because of its highly multimerized structure. Exocytosis results in a depletion of Weibel-Palade bodies and the appearance of extracellular vWF observed as large patches of fluorescence. We used immunofluorescence to clarify the role of exocytosis from WP bodies in XO-induced vWF release. Confocal microscopy was used to study the three-dimensional (3D) distribution of the secreted product. Endothelial cells grown on glass coverslips were exposed to XO (5 to 10 μM/mL), A23187 (10 μmol/L) or control buffer for 10 minutes, processed for indirect immunofluorescence and examined by confocal microscopy. Twenty consecutive optical sections through the cells were taken, parallel to the coverslip, 0.3 to 0.4 μm apart. These sections were then superimposed to create a single image, and pseudo-color coding was used to display the relative depth of each point. In control cells, WP bodies were visualized as typical rod-shaped granules spread throughout the thickness of the cytoplasm (Fig 3A). In contrast, after incubation with either XO (Fig 3B) or A23187 (Fig 3C), there was a striking depletion in WP bodies, and the appearance of irregular patches typical of extracellular vWF. As observed by 3D analysis, these patches were closer to the glass coverslip than most residual Weibel-Palade bodies, implying that they are located on the baso-lateral side of the cell. This was particularly clear when the patches were superimposed on the nuclear area, where the distance between the baso-lateral and the luminal side of the cells is greatest. These
experiments further suggest that XO-derived ROIs induce vWF release by exocytosis from WP bodies, since XO causes vWF release and deposition in the extracellular space in a pattern similar to that obtained with A23187.

The extracellular patches observed after exposure to XO were sometimes smaller and less abundant than after exposure to A23187, although depletion of WP bodies was very pronounced with both agents. We cannot exclude the possibility that XO-induced ROIs alter vWF binding sites in the subendothelium, thus reducing vWF trapping.

Effect of ROIs on cytosolic free calcium ([Ca$^{2+}$]). To determine the role of intracellular calcium in XO-induced vWF release, we measured [Ca$^{2+}$], at the single cell level. Endothelial cells grown on glass coverslips were loaded with the fluorescent calcium indicator fura-2 AM and [Ca$^{2+}$] was recorded by real-time, digital video imaging. The traces obtained from single cells after exposure to ROIs showed strikingly heterogeneous patterns of [Ca$^{2+}$]. In greater than 90% of the cells analyzed, XO (10 μU/mL) caused a rapid increase in [Ca$^{2+}$], of variable amplitude, followed either by a sustained plateau or by oscillations (Fig 4, A, C, and D). In contrast, application of 1 mmol/L H$_2$O$_2$ caused a smaller and slower increase in [Ca$^{2+}$],; biphasic responses were observed (Fig 4B). Averages from individual cells confirmed that the peak [Ca$^{2+}$], level was much higher after exposure to superoxides (from 74 ±10 nmol/L to 249 ±32 nmol/L, n = 72 cells) than to H$_2$O$_2$ (79 ±12 nmol/L to 130 ±17 nmol/L, n = 54 cells) (Fig 4G). The time required to reach the peak [Ca$^{2+}$], value was also much shorter after exposure to XO than to H$_2$O$_2$ (6 ±1 s vs 37 ±14 s, n = 6 cells). Thus, XO-derived ROIs induce a rapid and strong calcium response, whereas H$_2$O$_2$ causes only a small and slow increase in [Ca$^{2+}$].

When XO was removed, [Ca$^{2+}$], quickly returned to baseline values, which were maintained for at least 30 minutes (Fig 4, E and F). Prior exposure to XO did not prevent a second increase in [Ca$^{2+}$], after exposure to H$_2$O$_2$. Thus the XO-induced increase in [Ca$^{2+}$], is a finely regulated, rapidly reversible phenomenon rather than a generalized disruption of cellular calcium homeostasis.

Effect of calcium chelators on XO-induced vWF release and on [Ca$^{2+}$]. To better define the role of the XO-induced [Ca$^{2+}$], increase in vWF secretion, we performed studies with
Fig 3. Demonstration of XO-induced vWF release by immunofluorescence. Preconfluent HUVECs were stimulated with control buffer (A), 10 μM/mL XO (B), or 10 μM/L A23187 (C) for 10 minutes. After fixation and permeabilization, the cells were stained by sequential incubation with rabbit anti-vWF antibodies and fluorescein-conjugated antirabbit antibodies. The image shown is a 3D reconstruction obtained by confocal microscopy, with the relative depth expressed by pseudo-color coding. In the color scale shown in the upper-left corners, blue-green represents the baso-lateral side of the reconstructed volume. The rod-shaped WP bodies (arrowheads) are found throughout the thickness of the cell, whereas the extracellular patches (arrows) are always on the baso-lateral side. Bar, 25 μm.

calcium chelators. Endothelial cells were preincubated for 30 minutes in KRBH containing BAPTA-AM (20 μmol/L), which is a cell-permeant calcium chelator, or Ca²⁺-free medium supplemented with 1 mmol/L EGTA (1 mmol/L), or both conditions combined. After a change in buffer, the cells were then incubated with XO for 10 minutes, and released vWF was measured by ELISA (Fig 5). Incubation with EGTA caused a 26% inhibition of XO-induced release (P = .006 by Student’s paired t-test). EGTA also caused a small decrease in basal secretion. When this was taken into account, the inhibition of XO-induced vWF release was no longer significant (~12%, P > .05). In contrast, secretion was inhibited by 65% (P = .008) in cells loaded with BAPTA-AM in the presence of extracellular calcium (Ca²⁺). The inhibition was slightly larger with BAPTA-AM added together with EGTA (in the absence of extracellular Ca²⁺), but XO-induced vWF release could not be totally suppressed by the combination of the two chelators. BAPTA-AM caused only a 56% inhibition of XO-induced vWF release when compared with EGTA alone.

To correlate XO-induced vWF secretion with changes in intracellular calcium, we next measured the effect of the calcium chelators on XO-induced [Ca²⁺], (Fig 6). EGTA caused a small decrease of the maximal increase in [Ca²⁺], reached in response to 16 mM/mL XO (from 64 ± 7 to 186 ± 8 nmol/L; 63 ± 16 to 217 ± 22 nmol/L, n = 27 in the presence of calcium) (Fig 6E). At the single cell level, the rapidity of the initial increase was not affected. After the initial peak, brief plateaus or a few oscillations could be observed, but the average [Ca²⁺], decreased rapidly. At 3 minutes, the mean [Ca²⁺], was 70 ± 11 nmol/L, compared with 153 ± 13 nmol/L (n = 20) in the presence of 2.5 mmol/L CaCl₂. When BAPTA-AM was added together with EGTA, the effect of XO on [Ca²⁺], was strongly blunted; [Ca²⁺], increased from 58 ± 13 nmol/L to a maximum of 92 ± 16 nmol/L (n = 27) (Fig 6E), and the peak [Ca²⁺], level was reached only very slowly, over a period of several minutes (Fig 6, C and D). Thus, there was a striking correlation between the effect of the chelators on vWF secretion and their effect on intracellular calcium.

DISCUSSION

Our results show that XO-derived ROIs, but not H₂O₂, are potent agonists for the release of vWF from HUVECs. The increase in vWF secretion rate in response to XO occurred within minutes, suggesting release from preformed stores. Indeed, an increase in biosynthesis and in constitutive release would be expected to occur much more slowly (1 to 2 hours). As observed by immunofluorescence, XO caused cell depletion of WP bodies and the appearance of extracellular vWF deposits. vWF is released from WP bodies in a polarized fashion to the baso-lateral side of cultured HUVECs. Our finding that, after stimulation with either XO or A23187, extracellular vWF is located baso-laterally is in keeping with this observation, and further indicates that XO induces exocytosis from WP bodies. It is highly unlikely that vWF release results from cell lysis. Indeed, the cells appear morphologically unaltered after XO stimulation. [Ca²⁺], returns to basal levels after removal of XO, indicating that the cells remain viable after exposure to XO. Finally, XO had only minimal effect on LDH release, a marker of cell lysis.

We also observed a striking correlation between the ef-
Effects of ROIs on vWF release and on \([\text{Ca}^{2+}]_{i}\). XO-induced vWF release was accompanied in most cells by large, rapid increases in \([\text{Ca}^{2+}]_{i}\), followed either by a sustained plateau or by oscillations. In contrast, \(\text{H}_{2}\text{O}_{2}\) caused only a modest, slow increase in \([\text{Ca}^{2+}]_{i}\), and failed to induce vWF secretion. Removal of extracellular calcium and addition of EGTA caused a small inhibition of XO-induced vWF release. The initial \([\text{Ca}^{2+}]_{i}\) peak was minimally affected, but the subsequent plateaus or oscillations were of much shorter duration, suggesting that calcium influx induced by XO is not required for vWF release. In contrast, chelation of intracellular calcium by BAPTA-AM prevented the increase in \([\text{Ca}^{2+}]_{i}\), and caused a marked inhibition of XO-induced vWF release, whether in the presence or absence of EGTA. Together, our observations suggest that XO induces vWF release in a process largely dependent on an increase in cyto-

Fig 4. Effect of ROIs on \([\text{Ca}^{2+}]_{i}\). \([\text{Ca}^{2+}]_{i}\) was measured in fura-2-loaded HUVECs grown on glass coverslips by real-time digital video imaging. (A through F) Tracings of the average \([\text{Ca}^{2+}]_{i}\) from individual cells, stimulated with XO (10 mU/mL) or \(\text{H}_{2}\text{O}_{2}\) (1 mmol/L) as indicated. (E through G) After sequential stimulation with XO and \(\text{H}_{2}\text{O}_{2}\), \([\text{Ca}^{2+}]_{i}\) returned to basal levels and was maintained for more than 30 minutes, until membrane permeabilization by the addition of digitonin (dig) at the end of the study caused a massive calcium influx. These traces indicate that the cells remain viable after stimulation with the ROIs. (G) Average peak \([\text{Ca}^{2+}]_{i}\) reached after stimulation with XO or \(\text{H}_{2}\text{O}_{2}\), \([\text{Ca}^{2+}]_{i}\), compared with prestimulation levels (BAS), expressed as means ± SEM of 72 (XO) or 54 (\(\text{H}_{2}\text{O}_{2}\) cells. *P < .05 compared with prestimulation values by paired Student’s t-test.
ROIs INDUCE vWF SECRETION

Therefore, the heterogeneous pattern we observed may represent a diversity in sensitivity to ROIs. Although the correlation between the calcium pattern and vWF release at the single cell level remains to be studied, the heterogeneous [Ca^{2+}], patterns we observed are unlikely to determine large differences in secretion, since EGTA abolished both plateaus and oscillations without a significant inhibition of the secretory response.

Although the large increases in [Ca^{2+}], induced by calcium ionophores or exogenous calcium added to permeabilized cells are sufficient to induce WP body exocytosis, the smaller increase in [Ca^{2+}], caused by XO or other secretagogues may not be sufficient to mediate exocytosis. For instance, extracellular ATP fails to induce vWF release in spite of marked increases in [Ca^{2+}].\(^{30,32}\) A role for additional, calcium-independent messenger systems is suggested by our observation that XO-induced vWF release could not be completely inhibited even when the [Ca^{2+}], increase was blocked by EGTA and BAPTA-AM added together. This residual secretion is unlikely to be caused by cell damage or incomplete chelation of intracellular calcium. It is conceivable that XO causes the release of vWF trapped in the extracellular matrix. However, the fraction of secreted vWF that is retained in the extracellular matrix is thought to be very small.\(^{33}\) Although thrombin-induced vWF secretion is not prevented by protein kinase C inhibitors, phorbol esters cause vWF release without any changes in [Ca^{2+}], suggesting the presence of a calcium-independent signaling pathway.\(^ {16}\) Oxidative stress has been reported to increase the production of diacylglycerol derived from phosphatidyl inositol or other membrane phospholipids, which may activate protein kinase C.\(^ {34}\) Likewise, oxidative stress induces the production of platelet-activating factor (PAF),\(^ {35}\) which has been reported to cause vWF release.\(^ {36}\) However, PAF activity was induced only by high concentrations of H_{2}O_{2} (>2.5 mmol/L); in our hands, PAF failed to induce vWF secretion over a large range of concentrations (not shown).

The XO-derived ROIs that induce vWF release may be superoxide anions, the primary reaction product. H_{2}O_{2} is generated from superoxide anions (either by spontaneous reduction or via the activity of cytosolic superoxide dismutase), but exogenous H_{2}O_{2} fails to induce rapid vWF release (Fig 1B). Patel et al\(^ {17}\) observed exocytosis of WP bodies in response to H_{2}O_{2} (as measured by surface expression of P-selectin), but only after prolonged exposure (>1 hour). Another possibility is that superoxide anions and H_{2}O_{2} react to form the highly reactive hydroxyl radicals in the Haber-Weiss reaction, which is dependent on the presence of reduced iron. This possibility is supported by previous studies, using the same cell system, showing that the effect of XO on [Ca^{2+}], is inhibited by the iron chelator o-phenanthroline.\(^ {31}\) HUVECs are thought to express XO at low levels. However, this enzyme may be expressed at much higher levels in arterial or arteriolar endothelial cells.\(^ {29}\) Therefore, it is possible that in such cells, a wider range of free radicals, in particular hydrogen peroxide, induce WP body exocytosis by generating hydroxyl radicals through their reaction with endogenous superoxide anions.

Our observations may be relevant to a number of pathophysiologic situations. vWF-mediated platelet adhesion to the subendothelium may be critical for thrombogenesis as well as the initiation or the progression of atherosclerosis. Indeed, vWF-deficient pigs are protected from the athero-

![Fig 5. Effect of calcium chelators on XO-induced vWF release.](image)

Confluent HUVECs were praincubated either in KRBH containing 2.5 mmol/L CaCl_{2} (Ca) or 1 mmol/L EGTA without extracellular calcium (EGTA), with or without 20 μmol/L BAPTA-AM (BAP) for 30 minutes. After a buffer change, the cells were then incubated for 10 minutes with XO (5μU/mL). vWF release was measured by ELISA. Results are expressed as means ± SEM of four experiments. For statistical analysis, see text.
genic effects of a high-fat diet. The vWF trapped in the subendothelium after baso-lateral secretion from overlying endothelial cells could be particularly active in this process. A high-fat diet has been shown to induce endothelial activity in rabbits. The superoxide anions thus generated could enhance vWF secretion and platelet adhesion, offering one more possible link between hypercholesterolemia and atherosclerosis. Similarly, cigarette smoke has been associated with acute elevation of plasma vWF as well as enhanced neutrophil adhesion to the vascular endothelium. These observations could be explained by the secretion of vWF and the activation of P-selectin induced by free radicals, which could underlie the link between cigarette smoke and accelerated atherosclerosis or neutrophil infiltration of the lungs.

ROIs are key mediators in acute lung injury due to complement activation by cobra venom factor. This inflammation model is characterized by increased vascular permeability, neutrophil accumulation and hemorrhage into the lungs within a few minutes, which are partly inhibited by the radical scavenging enzymes catalase and SOD. A key role for P-selectin has been shown by inhibition studies with anti-P-selectin antibodies. Exposure to H2O2 has been found to induce endothelial cell surface expression of P-selectin, although with a slow time course (>1 hour) suggestive of defective endocytosis. Exocytosis from WP bodies induced by XO-derived ROIs as observed in our study could cause
activation of P-selectin with the rapid time course typical of the neutrophil accumulation seen in this model.

In summary, we have shown that XO-derived ROIs induce exocytosis from WP bodies, in a process dependent on an increase in [Ca^{2+}]. This mechanism may underlie VWF release and P-selectin activation, thus contributing to conditions such as inflammation and atherosclerosis.

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