Tyrosine Phosphorylation of Caveolin 1 by Oxidative Stress Is Reversible and Dependent on the c-src Tyrosine Kinase but Not Mitogen-Activated Protein Kinase Pathways in Placental Artery Endothelial Cells

Dong-bao Chen,² Su-min Li,⁵ Xiao-Xian Qian,³,⁵ ChongSoo Moon,⁴,⁵ and Jing Zheng⁶

Department of Reproductive Medicine,¹ University of California San Diego, La Jolla, California 92093
Perinatal Research Laboratories,⁶ Department of Obstetrics and Gynecology, University of Wisconsin-Madison, Madison, WI 53715

ABSTRACT

Acute H₂O₂ exposure to placental artery endothelial cells induced an array of tyrosine-phosphorylated proteins, including caveolin 1 (CAV1) rapid and transient tyr⁴⁴ phosphorylated in a time- and concentration-dependent manner. Basal tyr⁴⁴ phosphorylated CAV1 was primarily located at the edges of cells and associated with actin filaments. Phosphorylated CAV1 was markedly increased and diffused with the disorganization of actin filaments at 20 min, disappeared at 120 min treatment with 0.2 mM H₂O₂. Treatment with H₂O₂ also disorganized actin filaments and changed cell shape in a time-dependent manner. Pretreatment with antioxidants catalase completely, whereas the other tested superoxide dismutase, N-acetyl-L-cysteine and sodium formate partially attenuated H₂O₂-induced CAV1 phosphorylation in a concentration-dependent manner. Acute treatment with H₂O₂ activated multiple signaling pathways, including the mitogen-activated protein kinases (MAPK) members (MAPK3/1-ERK2/1, MAPK8/9-JNK1/2, and MAPK11-p38 MAPK) and the c-src tyrosine kinase (CSK). Pharmacological studies demonstrated that, among these pathways, only the blockade of CSK activation abolished H₂O₂-induced CAV1 phosphorylation. Additionally, H₂O₂-induced CAV1 phosphorylation was reversible rapidly (<10 min) upon H₂O₂ withdrawal. Because maternal and fetal endothelia must make dynamic adaptations to oxidative stress resulting from enhanced pregnancy-specific oxygen metabolism favoring prooxidant production, which is emerging as one of the leading causes of the dysfunctional activated endothelium during pregnancy, these unique features of CAV1 phosphorylation on oxidative stress observed implicate an important role of CAV1 in placental endothelial cell biology during pregnancy.

caveolin 1, kinases, oxidative stress, placenta, placental artery

INTRODUCTION

Caveolin 1 (CAV1) is the product of the caveolin 1 gene that belongs to the caveolin gene family composed of three different genes, i.e., CAV1, CAV2, and CAV3 [1]. CAV1 protein is the principal residual protein component of the plasma membrane invaginations termed as caveolae [2, 3]. CAV1 is highly expressed and caveolae are extremely abundant in endothelial cells [4]. CAV1 and caveolae play a pivotal role in the regulation of various aspects of endothelial cell functions, including transcytosis [5], secretory activity [6], signal transduction [1, 3], proliferation and differentiation, as well as vascular permeability [7, 8]. Most, if not all, of these endothelial cell biologies are directly or indirectly mediated by CAV1 interactions with various membrane-associated molecules, such as receptors, neutral lipids, and protein kinases, etc. [1, 3, 7]. Recent data show that a tyrosine residue (Tyr⁴⁴) located at the NH2-terminus of CAV1 protein can be rapidly phosphorylated in response to a number of cellular stresses, including oxidative stress [9–11] and polypeptide growth factors, including insulin/insulin-like growth factor-1 [12, 13], platelet-derived growth factor [14], epidermal growth factor [15], and vascular endothelial growth factor [16]. Although the functional consequences of Tyr⁴⁴ phosphorylation of CAV1 are very much unknown, a few recent studies have suggested that Tyr⁴⁴ phosphorylated CAV1 is involved in regulating endothelial cell activation and migration [7, 17].

Reactive oxygen species (ROS), including superoxide (O₂⁻), hydroxyl radical (OH), and hydrogen peroxide (H₂O₂) are biologically important O₂ derivatives formed from univalent oxygen reduction in the presence of free electrons during the reduction-oxidation (redox) cycle of O₂ metabolism [18]. Under physiological conditions, ROS are produced at low concentrations controlled tightly by a balance between pro-oxidant production and antioxidant capacity. They participate in cell signaling events mediating almost all aspects of cellular activities and reactivities [19]. In living cells, the most common ROS is the unstable O₂⁻, which is rapidly reduced to the relatively stable H₂O₂ via the dismutation reaction catalyzed by superoxide dismutase (SOD) under physiological conditions [18]. A large body of evidence has shown that ROS are important in the maintenance of vascular biology because of their redox potential [20]. However, under pathophysiologic conditions, the balanced intracellular redox state shifts to the left, overwhelming pro-oxidant production, thus leading to the generation of excess ROS, which is referred to as oxidative...
stress [21]. Of interest to vascular biology is that, when excess $O_2^-$ forms, a significant portion of $O_2^-$-formed re-acts with nitric oxide (NO) to produce peroxynitrite (ONOO$^-$) [22], which may cause vasoconstriction and vas-cular damage in the placenta [23, 24].

ROS are ubiquitous reactive molecules found in the en-vIRONMENT and in all biological systems. Thus, all living aerobic organisms are subjected to continuous threats origin-atated from not only exogenous but also endogenous ROS-generated oxidative stress. A perfect example of this phe-nomenon is the mammalian pregnancy process, in which the mother and the developing fetus must make gestational age-dependent cellular changes adaptive to the dynamically changing redox state due to the unavoidable generation of excess ROS originated from enhanced pregnancy-specific $O_2$ metabolism. For example, in comparison with nonpreg-nant women, the maternal blood concentrations of lipid pero-xides, a marker of oxidative stress derived from ROS re-action with polyunsaturated fatty acids in cellular mem-branes, are much greater in normal pregnant women [25]. From this standpoint, normal pregnancy is a physiological oxidative stress condition that the mother and fetus can tol-erate. However, pregnant women with hypertension [23, 26], preeclampsia [27–29], and gestational diabetes [30] have further elevated levels of circulating lipid pero-xides [28, 31], which indicate the mother and fetus are even more subjected to oxidative stress.

Although the origin of maternal and fetal ROS is poorly defined, available evidence suggests that maternal blood lipid pero-xides and other oxidative stress markers are pri-marily originated from the placenta during pregnancy [26, 29, 32]. To this end, fetoplacental and possibly uteropla-cental endothelial cells are direct targets of placenta-derived ROS during vascular endothelial adaptations to normal pregnancy and, in particular, during dysfunctional endothelial activa-tion resulting from complicated pregnancies. Fur-thermore, it is also noteworthy that endothelial adaptation to normal pregnancy and, to a great extent, dysfunctional endothelial activation during complicated pregnancies are reversible postpartum [27, 28]. Although significant atten-tion has been paid to the vascular modifications during nor-mal and complicated pregnancies, our knowledge regarding vascular endothelial cell adaptations to pregnancy-specific oxidative stress is still very limited. In this study, we ex-posed placental artery endothelial cells to exogenous oxy-gen stress $H_2O_2$ to test a hypothesis that exogenous oxy-genative stress stimulates tyrosine phosphorylation of CAV1. In addition, we hypothesize that $H_2O_2$-induced tyrosine phosphorylation of CAV1 is reversible upon oxidative stress withdrawal and can be diminished by the addition of antioxidants. Moreover, the $H_2O_2$-initiated signaling path-ways were also investigated and their roles in $H_2O_2$-in-duced CAV1 phosphorylation were explored.

**MATERIALS AND METHODS**

**Materials**

Anti-CAV1, anti-mitogen-activated protein kinase (MAPK) 8/9 (also termed Jun-NH2-terminal kinase JNK1/2), anti-MAPK11 (also termed p38mapk), and anti-c-src tyrosine kinase (CSK) rabbit polyclonal antibodies (pAb) and anti-phosphotyrosine (PY99) monoclonal antibody (mAb) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-rabbit and anti-mouse peroxidase-conjugated IgGs were from Arshammers (Arlington Heights, IL). Tissue culture plas-ticware was from Corning (Corning, NY). Fetal calf serum (FCS), bovine serum albumin (BSA), medium-199 (M-199) and Dulbecco modified Eagle medium (DMEM) were from Life Technologies, Inc. (Grand Island, NY). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). Immobilon-P membrane was from Millipore (Bedford, MA). P9D0859, SB203580, SP600125, and 4-Amino-5-(4-chlorophenyl)-7-(t-butyloxy)pyrazolo-(3,4-d) pyrimidine (PP2) were purchased from Calbiochem (La Jolla, CA). $H_2O_2$ (30%), catalase, superoxide dismutase (SOD), N-acetyl-cysteine (NAC), and sodium formate (NaFM), TRITC-labeled phalloidin, and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

**Cell Culture, Experimental Conditions, and Preparation of Total-Cell Extracts**

Ovine fetoplacental artery endothelial cell (oFPAEC) line originated from primary or secondary fetoplacental arteries of pregnant (Day 120– 130) sheep. Cell culture collagenase digestion were cultured in growth media (DMEM with 20% FCS, 100 units/ml penicillin, and 100 $\mu$g/ml strepto-mycin) and propagated as previously described [33]. The University of California at San Diego Animal Subjects Committee approved these studies and the National Research Council’s Guide for the Care and Use of Laboratory Animals were followed.

The oFPAEC were plated in 100-mm dishes to grow to ~90% conflu-ence in growth media and were used at passages 9–11. Prior to experi-ments, cells were serum starved in treatment media (Phenol red-free M-199 containing 0.1% BSA, 25 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid—Hepes) for 16–20 h. The media were then replaced with treatment media and the cultures were allowed to equilibrate for 1 h. Ag-onists and/or antagonists were added for the time period as described in the figure legends. Cell stimulation was terminated by aspiration of the media. After rinsing twice with ice-cold phosphate-buffered saline (PBS), the cells were lysed with a nondenaturing lysis buffer A [34] on ice with continuous shaking for 30 min. The total cell extracts were collected using a disposable cell scraper, vortexed vigorously, and clarified by centrifugation (13,000 rpm, 5 min). The protein content of the samples was mea-sured by a Bio-Rad procedure using BSA as the standard. Aliquots of the extracts were used for immunoprecipitation or boiled in Laemmli buffer for 10 min and stored at $-20^\circ$C until immunoblot analysis could be per-formed.

**Immunoprecipitation**

Serum-starved cells (100-mm dishes) were treated with M-199- 0.1%BSA plus or minus $H_2O_2$ (0.2 mM) for up to 60 min. The cells were lysed in 0.5 ml nondenaturing buffer for immunoprecipitation as described previously [35]. The lysates were centrifuged at 4°C for 10 min at 13,400 rpm and protein contents were measured. The lysates (>200 $\mu$g/group) were brought up to 1 ml with lysis buffer and precleared by incubation with protein A agarose beads at 4°C for 1 h. After removal of the beads by centrifugation, the supernatants were incubated with 2 $\mu$g of PY99 mAb overnight at 4°C with end-over-end rotation. Protein A agarose beads (50/50 slurry beads, 50 $\mu$L) were added and incubated for 2 h at 4°C. The beads (immunoprecipitates) were then captured by centrifugation (13,000 rpm, 4°C, 5 min), washed, and resuspended in 30 $\mu$l of 2× SDS sample buffer and were heat denatured (95°C, 10 min) and then subjected to immuno-blotting as described below.

**SDS-PAGE and Immunoblotting**

Total cell extracts (20 $\mu$g/lane) boiled in Laemmli buffer or immuno-precipitates were separated on 12% SDS-PAGE with one lane loaded with the same amount of protein marker. The protein was transferred to Immobilon-P membranes electrophoretically (0.3 A, 1.5 h) by using a semidry blotter (Fisher Scientific). Immunoblotting was conducted as described previously [36]. The dilution factor for each primary antibody is listed in the figure leg-ends, whereas the secondary antibodies were diluted at 1:2000 for anti-mouse or 1:3000 for anti-rabbit peroxidase-conjugated IgGs. An anti-biotin secondary antibody (1:2000) was included to reveal the biotinylated protein marker loaded in the first lane of all Western blot analysis. Bound antibodies were visualized using the Chemi-Glow Chemiluminescent sub-
strate (Alpha Innotech Corp., CA), and digital images were captured with the Alpha Innotech ChemiImager Imaging System with a high-resolution charge-coupled device camera and desitometrically analyzed by the Alpha Innotech ChemiImager 4400 software.

Preparation of Triton X-100 Soluble and Insoluble Fractions

Serum-starved oFPAEC (~5 × 10^6 cells/group in 100-mm dishes) were treated with or without M-199-0.1% BSA or with H_2O_2 (0.2 mM) for 20 min and then rinsed with cold PBS twice. Triton X-100-soluble and -insoluble fractions were prepared as described previously [37], with minor modifications. Briefly, the cells were extracted in the dish with 0.5 ml of cold Triton X-100 lysis buffer (25 mM HEPES, pH 7.4, 2 mM MnCl_2, 1 mM phenylmethylsulfonylfluoride, 10 mM Na_3VO_4, 1% proteinase cocktail, and 0.1% Triton X-100) for 10 min on ice. The soluble fractions were transferred to 1.5-ml Eppendorf tubes and centrifuged at 4°C for 5 min at 13,400 rpm. The supernatants were designated as Triton X-100-soluble fractions. The insoluble materials remaining with the cells were washed once with 2 ml of the same buffer without Triton X-100 and then collected in 0.2 ml of the same buffer with a cell scraper and then sonicated. The samples were designated as Triton X-100-insoluble fractions. After protein determination, the samples were mixed with 5X Laemmli buffer and boiled for 10 min for immunoblotting as described above.

Immunofluorescence Microscopy

The oFPAEC were seeded sparsely on gelatin-coated glass coverslips. Following 1-day culture in growth media, the cells were serum starved overnight with M-199-0.1% BSA and then treated with fresh M-199-0.1% BSA plus or minus H_2O_2 (0.2 mM) for 20 or 120 min. Fluorescence immunolabeling was conducted as described previously [38]. Briefly, the cells were rinsed with cold PBS twice and then fixed with 4% paraformaldehyde for 20 min at room temperature. The following procedures were all done at room temperature. After washing with PBS containing 50 mM glycine twice (5 min each), the cells were blocked in PBS containing 1% gelatin, 1% BSA, and 0.075% saponin (a membrane-permeabilizing agent) for 20 min. The cells were then incubated with anti-phospho-CAV1 mAb (2.5 μg/ml) and TRITC-labeled phallolidin (2 μg/ml) in PBS containing 0.5% gelatin, 0.5% BSA, and 0.075% saponin for 45 min. After washing with the same buffer three times, the cells were incubated with Cy2 (green)-labeled anti-mouse IgG (1:250) for 45 min. After three washes (5 min each) with the same buffer and being rinsed with water briefly, the coverslips were mounted with ProLong Gold antifade reagent containing DAPI (Molecular Probes, Eugene, OR) and then analyzed by using the AppliedPrecision softWoRx Explorer software (Issaquah, WA).

Statistical Analysis

Each experiment was repeated at least three times using cells prepared from different ewes. Data are presented as means ± SEM and analyzed by one-way ANOVA using SigmaStat (Jandel Scientific, San Rafael, CA). When a F-test was significant (P < 0.05), treatment responses were compared with their corresponding controls by Fisher multiple comparisons. P < 0.05 was considered significantly different.

RESULTS

H_2O_2 Rapidly Induces Tyrosine Phosphorylation of CAV1 and Many Other Proteins

Ovine fetoplacental artery endothelial cells (oFPAEC) were treated with or without H_2O_2 at 0.2 mM for 20 min. Total protein extracts were profiled for total tyrosine-phosphorylated proteins by Western blot analysis with a specific anti-phosphotyrosine (PY99) antibody. Treatment with 1 mM H_2O_2 provoked remarkable tyrosine phosphorylation of an array of proteins in placental endothelial cells. Notably, three tyrosine-phosphorylated protein bands in the range of molecular weights of 22–28 kDa were observed (Fig. 1a). Next, we immunoprecipitated total tyrosine-phosphorylated proteins in control and H_2O_2-treated cellular protein extracts with the PY99 antibody. By using Western blot analysis with a specific anti-CAV1 antibody, a dramatic increase in tyrosine-phosphorylated CAV1 was detected in the immunoprecipitates of H_2O_2-treated but not control cells (Fig. 1b).

H_2O_2 Rapidly Induces Tyr^{14} Phosphorylation of CAV1 in a Time- and Concentration-Dependent Fashion

Because tyrosine phosphorylation of CAV1 has been recently shown to occur on Tyr^{14} [9] and a phospho-specific antibody recognizing phosphorylated CAV1 on Tyr^{14} is commercially available (Cell Signaling), we next performed detailed time-course and concentration-response experiments to examine the kinetics of CAV1 tyrosine phosphorylation in response to H_2O_2 in oFPAEC by using this specific phospho-CAV1 antibody as a probe. When oFPAEC were treated with 0.5 mM H_2O_2 for up to 120 min, H_2O_2 rapidly stimulated CAV1 phosphorylation on Tyr^{14} in a time-dependent manner. The stimulatory effects of H_2O_2 on CAV1 phosphorylation on Tyr^{14} occurred in as short as 1 min and were remarkably increased at 10 min and reached maximal levels after 30–60 min of treatment, and
FIG. 2. Temporal Tyr\textsuperscript{14} phosphorylation of caveolin 1 (CAV1) by H\textsubscript{2}O\textsubscript{2} in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were treated with 0.5 mM H\textsubscript{2}O\textsubscript{2} for up to 120 min. Total cell extracts were prepared and cellular proteins (20 μg/lane) were subjected to 12% SDS-PAGE fractionation and transferred on Immobilon-P membranes. Phosphorylation of CAV1 on tyrosine\textsuperscript{14} was analyzed by Western blotting with a specific CAV1 polyclonal antibody (pCAV1 pAb, 1:1000) that recognizes Tyr\textsuperscript{14} phosphorylated CAV1, and total CAV1 levels were measured by Western blotting with a specific anti-CAV1 polyclonal antibody (CAV1 pAb, 1:40 000) for monitoring sample loading. Representative blots of Tyr\textsuperscript{14} phosphorylated CAV1 (upper) and total CAV1 (lower, sample loading) are shown in a. Data of means ± SEM (n = 3) of tyrosine phosphorylated CAV1 are presented in b. Means with different letters differ significantly (P < 0.05).

FIG. 3. Concentration-dependent Tyr\textsuperscript{14} phosphorylation of caveolin 1 (CAV1) by H\textsubscript{2}O\textsubscript{2} in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were treated with increasing concentrations (0–20 mM) of H\textsubscript{2}O\textsubscript{2} for 20 min. Total-cell extracts were prepared and cellular proteins (20 μg/lane) were subjected to 12% SDS-PAGE fractionation, and transferred on Immobilon-P membranes. Tyr\textsuperscript{14} phosphorylation of CAV1 was analyzed by Western blotting as described in Figure 2. Representative blots of Tyr\textsuperscript{14} phosphorylated CAV1 (upper) and total CAV1 (lower, sample loading) are shown in a. Data of mean ± SEM (n = 3) of tyrosine phosphorylated CAV1 are presented in b. Means with different letters differ significantly (P < 0.05).

then returned to baseline after 120 min (Fig. 2). When oFPAEC were treated with increasing concentrations (0.002–20 mM) of H\textsubscript{2}O\textsubscript{2} for 20 min, a concentration-dependent CAV1 phosphorylation on Tyr\textsuperscript{14} was also observed. H\textsubscript{2}O\textsubscript{2} at concentrations less than 0.02 mM was unable to induce CAV1 phosphorylation on Tyr\textsuperscript{14}. H\textsubscript{2}O\textsubscript{2} at 0.2 mM again dramatically induced CAV1 phosphorylation on Tyr\textsuperscript{14}. When cells were treated with 2 mM H\textsubscript{2}O\textsubscript{2}, a remarkable increase of CAV1 phosphorylation on Tyr\textsuperscript{14} was observed. In cells treated with 20 mM H\textsubscript{2}O\textsubscript{2} for 20 min, CAV1 phosphorylation was decreased compared with cells treated with 2 mM H\textsubscript{2}O\textsubscript{2} (Fig. 3). In addition, acute (<120 min) treatment with H\textsubscript{2}O\textsubscript{2} did not alter total CAV1 protein levels in oFPAEC (Figs. 2 and 3, lower image panels). Moreover, we noticed that, when cells were treated with more than 2 mM H\textsubscript{2}O\textsubscript{2}, there was a significant increase in cell mortality even in a 20-min treatment (data not shown).

Subcellular Distribution of Tyr\textsuperscript{14} Phosphorylated CAV1 in H\textsubscript{2}O\textsubscript{2}-Treated oFPAEC

Because H\textsubscript{2}O\textsubscript{2} rapidly induced CAV1 phosphorylation in oFPAEC and CAV1 is generally recognized as a plasma-
Antioxidants Inhibit H$_2$O$_2$-Induced Tyr$^{14}$ Phosphorylation of CAV1 in oFPAEC

We next examined the effects of various antioxidants on H$_2$O$_2$-induced CAV1 phosphorylation on Tyr$^{14}$. As illus-
FIG. 5. Effects of antioxidants on Tyr\(^{14}\) phosphorylation of caveolin 1 (CAV1) by H\(_2\)O\(_2\) in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were pretreated with or without increasing concentrations of catalase (\(a\)), superoxide dismutase (SOD, \(b\)), sodium formate (NaFM, \(c\)), and N-acetyl-L-cysteine (NAC, \(d\)) for 60 min, followed by treatment with or without 0.2 mM H\(_2\)O\(_2\) for 20 min. Total-cell extracts were prepared and cellular proteins (20 \(\mu\)g/lane) were analyzed for CAV1 Tyr\(^{14}\) phosphorylation (upper panels) by Western blotting as described in Figure 2. Representative blots of tyr\(^{14}\) phosphorylated CAV1 (upper) and total CAV1 (lower, sample loading) of atypical experiment for each antioxidant surveyed are shown. Data of mean \(\pm\) SEM (n = 4) of tyrosine phosphorylated CAV1 by each antioxidant surveyed are presented in their corresponding panels. Means with different letters differ significantly \((P < 0.05)\).

trated in Figure 5, all the antioxidants tested effectively inhibited \((P < 0.05)\) H\(_2\)O\(_2\)-induced CAV1 phosphorylation on Tyr\(^{14}\) in a dose-dependent manner but with different potency individually. Treatment with as little as 5 U/ml of catalase completely attenuated H\(_2\)O\(_2\)-induced CAV1 phosphorylation (Fig. 5a, upper panel). However, the other three antioxidants tested, i.e., SOD, NaFM, and NAC, were less effective than catalase and they all only partially but still significantly inhibited \((P < 0.05)\) H\(_2\)O\(_2\)-induced CAV1 phosphorylation (Fig. 5, b–d, upper panels). Again, acute (20-min) treatment with H\(_2\)O\(_2\) in the presence or absence of antioxidants did not alter total CAV1 protein levels (Fig. 5, a–d, lower imaging panels). Antioxidants alone did not alter the phosphorylation status of CAV1.

**H\(_2\)O\(_2\)-Induced Tyr\(^{14}\) Phosphorylation of CAV1 in oFPAEC Is Reversible**

Determination of whether H\(_2\)O\(_2\)-induced CAV1 phosphorylation on Tyr\(^{14}\) could be reversed by the removal of H\(_2\)O\(_2\) in the treatment media in a time window in which submaximal response was achieved is shown in Figures 2 and 3a. The oFPAEC were first challenged with 0.2 mM H\(_2\)O\(_2\) for 20 min to induce submaximal CAV1 phosphorylation and then switched to fresh control medium without H\(_2\)O\(_2\) for different time points (0, 1, 5, 10, and 30 min). Total-cell extracts were prepared and Western blot analysis was used to determine CAV1 phosphorylation. Interestingly, H\(_2\)O\(_2\)-induced CAV1 phosphorylation was time dependently lost after H\(_2\)O\(_2\) withdrawal. As illustrated in Figure 6, following the removal of H\(_2\)O\(_2\), tyrosine-phosphorylated CAV1 in H\(_2\)O\(_2\)-treated cells dramatically decreased at 10 min and almost returned to basal levels at 30 min.

**H\(_2\)O\(_2\) Activates Multiple Mitogen-Activated Protein Kinases but None Is Involved in H\(_2\)O\(_2\)-Induced Tyr\(^{14}\) Phosphorylation of CAV1 in oFPAEC**

H\(_2\)O\(_2\) initiates multiple signaling pathways, including all the family members of the MAPKs [40]. MAPKs are serine/threonine kinases and thus the Tyr\(^{14}\) residue in CAV1 protein is unlikely a direct phosphorylation site of active MAPKs. However, of interest, MAPK11 was recently shown to play a role in hyperosmotic shock-induced CAV1 tyrosine phosphorylation, although the mechanism(s) underlying tyrosine phosphorylation of CAV1 through MAPK11 is currently unknown [10]. We asked if H\(_2\)O\(_2\) activates MAPKs, i.e., MAPK1/3, MAPK8/9, and MAPK11 in oFPAEC and, if so, whether either or all of them are involved in H\(_2\)O\(_2\)-induced CAV1 phosphorylation on Tyr\(^{14}\) in oFPAEC. When cells were treated with 0.2 mM H\(_2\)O\(_2\) for 20 min, multiple MAPKs, including MAPK1/3, MAPK8/9, and MAPK11, were phosphorylated (Figs. 7–9). In the presence of increasing concentrations (1–20 \(\mu\)M) of their respective specific inhibitors of MAPK1/3 (PD98059), MAPK8/9 (SP600125), and MAPK11 (SB203580), H\(_2\)O\(_2\)-induced phosphorylation of CAV1 protein was completely inhibited in a dose-depen-
FIG. 6. Tyr14 phosphorylation of caveolin 1 (CAV1) by H2O2 is reversible in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were pretreated with or without 0.2 mM H2O2 for 20 min, washed with fresh media, and then cultured in fresh media for various times for preparing total-cell extracts. Cellular proteins (20 μg/lane) were analyzed for CAV1 phosphorylation by Western blotting as described in Figure 2. Representative blots of Tyr14 phosphorylated CAV1 (upper) and total CAV1 (lower) of atypical experiment are shown in a. Data of mean ± SEM (n = 5) of Tyr14 phosphorylated CAV1 are summarized in b. * P < 0.05 differ significantly from controls.

H2O2-Induced Tyr14 Phosphorylation of CAV1 in oFPAEC Is CSK Dependent

CAV1 was initially identified as a major tyrosine-phosphorylated protein in v-src sarcoma viral oncogene homolog (SRC; also termed v-src)-transformed embryonic fibroblasts [41] and CAV1 is a direct substrate for the tyrosine kinase CSK [9]. We then examined if H2O2-induced tyrosine phosphorylation of CAV1 in oFPAEC is CSK dependent. To this end, we first determined if H2O2 activates CSK in oFPAEC. Total CSK protein was immunoprecipitated from control and cells treated with 0.2 mM H2O2 for 20 min in the absence or presence of increasing concentrations of the specific CSK inhibitor PP2. The immunoprecipitates were subjected to immunoblotting analysis with an anti-phosphotyrosine antibody (PY99) to determine the levels of tyrosine-phosphorylated CSK. As shown in Figure 10, compared with control, treatment with H2O2 for 20 min provoked a significant increase (P < 0.05) in tyrosine-phosphorylated CSK (upper panel, Fig. 10a). Immunoblotting analysis revealed that the levels of immunoprecipitated
FIG. 8. Activation of the MAPK8/9 (JNK1/2) is not involved in Tyr 14 phosphorylation of caveolin 1 (CAV1) by H$_2$O$_2$ in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were pretreated with or without increasing concentrations of the specific MAPK8/9 inhibitor SP600125 for 60 min, followed by treatment with or without 0.2 mM H$_2$O$_2$ for 20 min. Total-cell extracts were prepared and cellular proteins (20 mg/lane) were analyzed for phosphorylation of MAPK8/9 (a, upper panel) and CAV1 (b, upper panel) by Western blotting with an anti-active MAPK8/9 antibody (1:1000) recognizing active MAPK8/9 and an anti-CAV1 antibody (pCAV1 pAb, 1:1000), respectively. Total MAPK8/9 and CAV1 levels were measured by Western blotting with an anti-MAPK8/9 antibody (1:500) or an anti-CAV1 polyclonal antibody (CAV1 pAb, 1:40 000) for monitoring sample loading (lower panels). Representative blots of phospho-MAPK8/9, Tyr14 phosphorylated CAV1, and total MAPK8/9 and CAV1 of atypical experiments are shown. Data of mean ± SEM (n = 3) of phospho-MAPK8/9 and Tyr 14-phosphorylated CAV1 are summarized in their corresponding graphs. Means with different letters differ significantly (P < 0.05).

FIG. 9. Activation of MAPK11 (p38 mapk) is not involved in Tyr 14 phosphorylation of caveolin 1 (CAV1) by H$_2$O$_2$ in fetoplacental artery endothelial cells (oFPAEC). Serum-starved oFPAEC were pretreated with or without increasing concentrations of a specific MAPK11 inhibitor SB203580 for 60 min, followed by treatment with or without 0.2 mM H$_2$O$_2$ for 20 min. Total cell extracts were prepared and cellular proteins (20 mg/lane) were analyzed for phosphorylation of MAPK11 (a, upper panel) and CAV1 (b, upper panel) by Western blotting with a specific anti-active MAPK11-recognizing phosphorylated MAPK11 and an anti-CAV1 antibody (pCAV1 pAb, 1:1000), respectively. Total MAPK11 and CAV1 levels were measured by Western blotting with a specific MAPK11 antibody (1:500) and an anti-CAV1 polyclonal antibody (CAV1 pAb, 1:40 000) for monitoring sample loading (lower panels). Representative blots of phospho-MAPK11, Tyr14 phosphorylated CAV1, and total MAPK11 and CAV1 of atypical experiments are shown. Data of mean ± SEM (n = 3) of phospho-MAPK11 and Tyr 14-phosphorylated CAV1 are summarized in the lower graphs. Means with different letters differ significantly (P < 0.05).

CSK from control and H$_2$O$_2$-treated cells were comparable (lower panel, Fig. 10a). In the presence of increasing concentrations of PP2 (0.2–20 μM), H$_2$O$_2$-induced CSK tyrosine phosphorylation was dose dependently inhibited. We then treated oFPAEC with increasing concentrations (0.2–20 μM) of PP2 for 60 min followed by treatment with or without 0.2 mM H$_2$O$_2$ for 20 min to examine if the blockade of CSK activation inhibits H$_2$O$_2$-induced CAV1 phosphorylation. As illustrated in Figure 10b, H$_2$O$_2$-induced CAV1 phosphorylation was dose dependently inhibited in the presence of increasing concentrations of PP2. In the presence of 20 μM PP2, a concentration effectively inhibited H$_2$O$_2$-induced CSK phosphorylation, as shown in Figure 10a; H$_2$O$_2$-induced CAV1 phosphorylation in oFPAEC.
was completely abolished. PP2 alone did not alter the phosphorylation status of CSK and CAV1.

**DISCUSSION**

We presented herein solid in vitro evidence showing that, upon acute exposure to H$_2$O$_2$-generated oxidative stress, placental artery endothelial cells undergo time-dependent dramatic changes in CAV1 phosphorylation and subcellular redistribution as well as cell shape. Acute treatment with H$_2$O$_2$ activates multiple signaling pathways, including the MAPK family members and the tyrosine kinase CSK in placental endothelial cells. Among these signaling molecules, only CSK is required for CAV1 phosphorylation by H$_2$O$_2$. Notably, H$_2$O$_2$-induced rapid CAV1 phosphorylation can be attenuated by antioxidants and is also reversible rapidly upon the withdrawal of H$_2$O$_2$ in placental endothelial cells. During normal pregnancy, endothelial cells in the uteroplacental and fetoplacental vascular beds and possibly throughout the body must undergo significant physiological adaptations to pregnancy for facilitating the increases in uteroplacental and fetoplacental blood flows obligatory for delivering nutrients and oxygen supplies to support the development of the growing fetus [42, 43]. On the contrary, dysfunctional activation of endothelial cells is a hallmark of pregnancy complications, such as hypertension [23, 26], preeclampsia [27–29], and gestational diabetes [30]. Despite extensive studies having been performed, our knowledge regarding the causes and consequences of endothelial adaptations to normal pregnancy or dysfunctional endothelial activation during complicated pregnancies is still very limited. However, in both cases, endothelial cells are subjected to the dynamic changes in enhanced pregnancy-specific redox status favoring prooxidant production due to increased metabolic activities. Further, although the causes of pregnancy-specific diseases such as preeclampsia are certainly multifactorial and complex and their etiology and pathogenesis are unknown, one emerging hypothesis recently causing significant attention is that placental and maternal factors converge to generate oxidative stress, which in turn causes these disease characteristics of dysfunctional activated endothelial cells [23–27], which provides a reasonable rationale for the antioxidant therapy for preeclampsia [25, 44]. Thus, we believe that these unique features of CAV1 phosphorylation upon oxidative stress exposure implicate an important role of CAV1 phosphorylation in the regulation of placental endothelial cell functions during pregnancy and possibly maternal endothelial adaptations to normal pregnancy and/or dysfunctional activation during complicated pregnancies.

CAV1 is the major structural protein for the Ω-shaped plasma membrane invagination (60–100 nm in diameter), termed as caveolae, first described in endothelial cells ~50 years ago [45, 46]. Initially, caveolae were thought to play an important regulatory role in transcytosis in endothelial cells [47]. However, since the identification of CAV1 a decade ago [2], extensive studies have unraveled that this cell-surface organelle participates in the regulation of numerous cellular functions [8, 48]. In endothelial cells, caveolae occupy ~20% of total volume of endothelial cells [49]. Thus, it is not surprising that CAV1/caveolae are required for the maintenance of normal endothelial cell functions and thus vascular tone. This is best exemplified by the phenotypes of CAV1-null mice, e.g., increased vascular permeability and disorganized lung endothelial cell proliferation, and impaired nitric oxide signaling [50]. CAV1, as an integral membrane protein, has an unusual hairpin-like structure conformation in which its N- and C-terminal regions both face the cytosol and are connected by a membrane-embedded, hydrophobic domain [51]. Most, if not all, of the signaling events in the caveolae are mediated by CAV1 interactions with other proteins or lipids [1, 7–8, 48]. One critical aspect of CAV1 function in the vasculature is that
CAV1 functions as a negative regulator of endothelial nitric oxide synthase [35, 48, 52, 53]. In the uteroplacental and fetoplacental circulations, we believe that CAV1 and caveolae signaling play an important role in the regulation of nitric oxide-mediated vasodilatation and angiogenesis in the uterine and placental vascular beds essential for upregulating uterine and placental blood flows to meet the progressive needs for the growing fetus during pregnancy [35–36].

In living cells, superoxide is the most common reactive oxygen species formed. Once O$_2^\cdot$ is formed, physiological conditions favor the dismutation reaction catalyzed by SOD that rapidly reduces O$_2^\cdot$ to H$_2$O$_2$ [18]. H$_2$O$_2$ is intermediate, relatively stable, and lipid soluble, which is scavenged by catalase and glutathione peroxidase in biological systems [54]. H$_2$O$_2$ can be also converted to OH in the presence of metal-containing molecules [55], and the latter can be scavenged by a pharmacological agent NaFM [56]. In our current study, we have tested the effects of four different antioxidants, i.e., catalase, SOD, NAC, and NaFM on H$_2$O$_2$-induced CAV1 phosphorylation in placental endothelial cells. Our data show that the H$_2$O$_2$ scavenger catalase is the most effective antioxidant in inhibiting H$_2$O$_2$-induced CAV1 phosphorylation. NAC, a precursor of glutathione [57], is much less effective than catalase in inhibiting H$_2$O$_2$-induced CAV1 phosphorylation, although it activates another H$_2$O$_2$ scavenger, glutathione peroxidase [57]. Inhibition of H$_2$O$_2$-induced CAV1 phosphorylation by catalase and NAC is not surprising because they convert H$_2$O$_2$ to water and oxygen. Interestingly, SOD and NaFM both can partially inhibit H$_2$O$_2$-induced CAV1 phosphorylation. These data suggest that the formation of intracellular O$_2^\cdot$- and OH may be involved in H$_2$O$_2$-induced CAV1 phosphorylation because SOD and NaFM are, respectively, scavengers for O$_2^\cdot$ and OH [58].

Numerous data have shown that exposure of H$_2$O$_2$ to a variety of types of cells triggers a large body of signaling pathways, including the nonreceptor tyrosine kinase CSK [59, 60] and all the MAPK family members—MAPK1/3, MAPK8/9, and MAPK11 [40]. In this study, we observed similar stimulatory effects of H$_2$O$_2$ on these signaling pathways in placental artery endothelial cells. When the specific pharmacological inhibitors for each of these signaling pathways was used to examine if one or more of them are involved in H$_2$O$_2$-induced CAV1 tyrosine phosphorylation, we have clearly demonstrated that the blockade of the CSK, but not any of the MAPK pathways, can abolish H$_2$O$_2$-induced CAV1 tyrosine phosphorylation in placental artery endothelial cells. The involvement of CSK in H$_2$O$_2$-induced CAV1 tyrosine phosphorylation is not surprising because CAV1 was initially identified as a phospho-protein in v-src-transformed embryonic fibroblasts [9]. However, none of the MAPK pathways involved in H$_2$O$_2$-induced tyrosine phosphorylation is unexpected. MAPKs are serine/threonine kinases and thus they appear not to play a direct role in the Tyr$^{14}$ phosphorylation of CAV1. However, a recent report has shown that hyperosmotic shock-induced CAV1 tyrosine phosphorylation is through a MAPK11-dependent pathway in NIH3T3 cells [10]. The cause of this discrepancy is currently unknown. Nonetheless, our data suggest that specific signaling mechanism(s) exist for controlling CAV1/caveolae functions in the endothelial cells. At the moment, we do not have any direct data for the functional consequences of MAPK activation by H$_2$O$_2$ in placental endothelial cells, although our recent data suggest that MAPK1/3 activation is important in mediating nitric oxide production and endothelial cell proliferation and differentiation by angiogenic growth factor [33] and angiotensin II [61]. Of interest, MAPK11 has been found recently to play a critical role in placental angiogenesis [62].

Tyrosine phosphorylated by H$_2$O$_2$ CAV1 displays distinct and dynamic subcellular redistribution in a time-dependent manner in endothelial cells. Basal phosphorylated CAV1 was primarily located with the cell protrusions of lamellipodia and filopodia and associated with stress fibers. Following exposure to 0.2 mM H$_2$O$_2$, phosphorylated CAV1 appears internalized with the disorganized actin filaments at 20 min; and at 120 min, the disappearance of phosphorylated CAV1 is associated with further disruption of cellular structures. Simultaneously, dramatic changes in the cell shape also take place with CAV1 phosphorylation following treatment with H$_2$O$_2$. Apparently, oxidative stress exposure disrupts the cell structure in association with disorganized actin filaments and dephosphorylation of CAV1. These subcellular changes in phosphorylated CAV1 might have a role in endothelial cell migration because CAV1 is rapidly phosphorylated in 3T3-L1 fibroblasts plated on fibronectin and this is associated with cell spreading [12] and 2) tyrosine phosphorylated CAV1 was found to be associated with the polarity of migrating cells [17].

Our present study also demonstrates that H$_2$O$_2$-induced rapid CAV1 Tyr$^{14}$ phosphorylation is dose dependent within a concentration range of 0.002–20 mM. As shown in the dose-response study, H$_2$O$_2$ at doses ≤20 μM was unable to induce demonstrable changes in CAV1 tyrosine phosphorylation. At 200 μM, H$_2$O$_2$ can induce a robust increase in CAV1 tyrosine phosphorylation. We also can detect a small increase in CAV1 tyrosine phosphorylation with 50–100 μM H$_2$O$_2$ (data not shown). These data demonstrate that oFPAC cells are more sensitive to H$_2$O$_2$ in stimulation of CAV1 phosphorylation than other cell types, such as fibroblasts [9] and NIH 3T3 cells [10, 11], which requires ≥500 μM H$_2$O$_2$ to induce CAV1 phosphorylation. Because the information regarding the physiological and pathophysiological concentrations of H$_2$O$_2$ in the uteroplacental and fetoplacental circulations is unavailable to date, we cannot conclude from our current data if the dose dependency of H$_2$O$_2$-induced rapid CAV1 Tyr$^{14}$ phosphorylation observed is physiologically and/or pathophysiologically relevant during pregnancy. However, it has been postulated that there might be threshold concentrations for ROS to function as either intracellular intermediates participating in the regulation of normal cellular physiology or to be as toxic byproducts of O$_2$ metabolism [55]. Thus, it is possible that the lower concentrations of H$_2$O$_2$ in the induction of rapid CAV1 Tyr$^{14}$ phosphorylation might be equivalent to the physiological oxidative stress occurring during normal pregnancy, whereas the higher ones might fail in the enhanced pathophysiological oxidative stress occurring during complicated pregnancies. This concept has been supported by other intriguing findings in our current study. For example, rapid CAV1 Tyr$^{14}$ phosphorylation upon exposure to H$_2$O$_2$ at <500 μM is transient and, more interestingly, is reversible rapidly (<10 min) upon the withdrawal of H$_2$O$_2$. Moreover, at a higher concentration (20 mM), H$_2$O$_2$ was less effective than the lower (0.2–2 mM) ones in the stimulation of CAV1 phosphorylation and the former induced cell death (data not shown).

It is commonly recognized that dysfunctional activated endothelium in complicated pregnancies, such as pre-eclampsia and gestational diabetes, is somewhat recoverable upon delivery or removal of the placenta, although whether maternal endothelium is fully recoverable to normo-
mal is under debate [25, 28, 31]. Nonetheless, the reversibility of CAV1 Tyr^{14} phosphorylation by oxidative stress makes CAV1 a potential marker of the dysfunctional activation of endothelial cells associated with the increased ROS-generating products such as lipid peroxides in complicated pregnancies [25, 32, 63]. Thus, it would be interesting and important to test if fetoplacental and uteroplacental endothelial cells from complicated pregnancies are associated with increased CAV1 tyrosine phosphorylation in vivo compared with normal pregnancies. More importantly, revealing a cause-effect relationship between excess ROS generated by the placenta and uteroplacental and fetoplacental endothelial CAV1 tyrosine phosphorylation and its functional consequences in uterine and placental circulations might provide insights for exploring the etiology and pathogenesis of preeclampsia because excess ROS generated from abnormal hypoxia/reoxygenation of the placenta thus impairing placentation is one of most likely causes of preeclampsia [24–26, 28, 29, 44, 64].

REFERENCES


