Oxidative stress inhibits caveolin-1 palmitoylation and trafficking in endothelial cells

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During normal and pathological conditions, endothelial cells (ECs) are subjected to locally generated reactive oxygen species, produced by themselves or by other vessel wall cells. In excess these molecules cause oxidative injury to the cell but at moderate levels they might modulate intracellular signalling pathways. We have investigated the effect of oxidative stress on the palmitoylation and trafficking of caveolin-1 in bovine aortic ECs. Exogenous H₂O₂ did not alter the intracellular localization of caveolin-1 in ECs. However, metabolic labelling experiments showed that H₂O₂ inhibited the trafficking of newly synthesized caveolin-1 to membrane raft domains. Several mechanisms potentially responsible for this inhibition were examined. Impairment of caveolin-1 synthesis by H₂O₂ was not responsible for diminished trafficking. Similarly, the inhibition was independent of H₂O₂-induced caveolin-1 phosphorylation as shown by the markedly different concentration dependences. We tested the

effect of H₂O₂ on palmitoylation of caveolin-1 by the incorporation of [³H]palmitic acid. Exposure of ECs to H₂O₂ markedly inhibited the palmitoylation of caveolin-1. Comparable inhibition was observed after treatment of cells with H₂O₂ delivered either as a bolus or by continuous delivery with glucose and glucose oxidase. Kinetic studies showed that H₂O₂ did not alter the rate of caveolin-1 depalmitoylation but instead decreased the 'on-rate' of palmitoylation. Together these results show for the first time the modulation of protein palmitoylation by oxidative stress, and suggest a cellular mechanism by which stress might influence caveolin-1-dependent cell activities such as the concentration of signalling proteins and cholesterol trafficking.

Key words: caveolae, hydrogen peroxide, post-translational modification.

INTRODUCTION

Biochemical and morphological experiments have shown the presence of caveolae, cave-like plasma-membrane subdomains, in many cell types including vascular endothelial cells (ECs) [1,2]. Caveolin-1 is the major protein component of caveolae and is found primarily in two isoforms, caveolin-1 α and caveolin-1 β , that differ in their N-termini and are derived from alternative translation initiation sites [3]. Caveolin-1 is an integral membrane protein with an unusual hairpin-like conformation; the N-terminal and C-terminal regions both face the cytosol and are connected by a membrane-embedded hydrophobic domain containing 33 amino acid residues [4]. Caveolin-1 participates in multiple protein–protein interactions that are critical for its various functions. Interactions of caveolin-1 with adjacent caveolin-1 molecules require residues in both the N-terminal and C-terminal domains and result in the oligomerization of caveolin-1 and the formation of a rigid scaffold that maintains the characteristic cave-like morphology [5–7]. Interactions between caveolin-1 and caveolin-2 in their membrane-embedded domains might also contribute to this structure [8]. In addition to its structural function, caveolin-1 has several important regulatory activities. For example caveolin-1 interacts, via a consensus caveolin-binding motif, with signalling proteins including heterotrimeric G-protein α subunits, protein tyrosine kinases, p21^{H₉-ras} and EC-specific nitric oxide synthase ('eNOS') [9–11]. Interaction with caveolin generally inhibits the signal-transducing activity of the protein [12]. Recent studies suggest that caveolin-1 interacts with chaperones of the immunophilin family to form a soluble complex [13] involved in the transport of cholesterol from the

endoplasmic reticulum to the caveolae and subsequently to non-caveolar plasma membranes [14].

Caveolin-1 is subject to two types of post-translational modification that might be critical for regulating its intracellular activity and localization, namely phosphorylation and palmitoylation. Caveolin-1 was first identified as the major phosphoprotein in Rous-sarcoma-virus-transformed cells [15]. Tyr¹⁴ on caveolin-1 was subsequently shown to be a principal target for Src kinase [16]. The functional consequences of caveolin-1 phosphorylation are not yet known but it might drive intracellular relocation to regions near focal adhesions [17]. Tyrosine phosphorylation of caveolin-1 is stimulated by polypeptide growth factors and by cellular stress, most probably by the activation of p38-mitogen-activated protein kinase [17]. Similarly, treatment of NIH 3T3 cells and ECs with H₂O₂ causes increased Tyr¹⁴ phosphorylation [17–19]. The finding that caveolin-1 is a sensitive target of oxidative stress has been confirmed by functional studies. Smart et al. [20] have shown that the oxidation of caveolar membrane cholesterol causes the translocation of caveolin-1 from the plasma membrane to the Golgi apparatus. In a separate study, treatment of ECs with reactive oxygen species caused a release of caveolin-1 from membranes and also a decrease in the number of caveolae detected by electron microscopy [21]. Oxidative stress might also influence the cellular level of caveolin, as suggested by results showing that oxysterols decrease caveolin-1 mRNA expression in fibroblasts [22].

Caveolin-1, like many caveolae-resident proteins, is susceptible to modification by acylation. Protein palmitoylation is a post-translational modification in which palmitic acid, a 16-carbon

Abbreviation used: ECs, endothelial cells.

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saturated fatty acid, is attached by an *S*-ester linkage to the thiol group of specific cysteine residues. Palmitoylation can influence the membrane interactions, protein–protein interactions, localization, conformation and activity of target proteins [23–25]. Caveolin-1 is triply palmitoylated on cysteine residues at positions 133, 143 and 156 near the C-terminus [26]. Site-directed mutagenesis studies [27] suggest that the palmitoylation of one or more caveolin-1 cysteine residues is required for optimal stability of the oligomeric structures that contribute to caveolar structure [6] and the sequestration of signalling molecules [28]. Caveolin-1 has been implicated in cholesterol transport processes [14]; the palmitoylation of at least two of the three cysteine residues is required for cholesterol binding, for the formation of soluble caveolin–immunophilin complexes and for the transport of cholesterol from the site of synthesis in the endoplasmic reticulum to caveolae [29]. So far there have been no reports on the regulation of caveolin-1 palmitoylation. Here we provide evidence that oxidative stress decreases the trafficking of newly synthesized caveolin-1 to lipid rafts and that this effect is associated with a decreased rate of caveolin-1 palmitoylation.

MATERIALS AND METHODS

Materials

Tran³⁵S-label (1175 Ci/mmol; referred to here as [³⁵S]-methionine) was obtained from ICN (Irvine, CA, U.S.A.) and [9,10-³H]palmitic acid (35 Ci/mmol) was from Dupont New England Nuclear (Boston, MA, U.S.A.). Rabbit polyclonal anti-(human caveolin-1) and mouse monoclonal anti-(phosphocaveolin-1) antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.). Glucose oxidase was from Roche (Indianapolis, IN, U.S.A.). Immunofluorescence reagents were from Vector Laboratories (Burlingame, CA, U.S.A.). Brefeldin A was purchased from Epicentre Technologies (Madison, WI, U.S.A.). Protein A–Sepharose and other assay reagents were from Sigma (St Louis, MO, U.S.A.).

Cell culture

Bovine aortic ECs were maintained in DMEM (Dulbecco's modified Eagle's medium) and F12 medium supplemented with 5% (v/v) fetal bovine serum, 100 i.u./ml penicillin and 100 µg/ml streptomycin, in a humidified air/CO₂ (19:1) atmosphere. Cells were subjected to H₂O₂ either by the direct addition of a dilute H₂O₂ solution, followed by incubation for 15–30 min at 37 °C, or by the addition of glucose oxidase (up to 300 ng/ml for 1 h) to the medium, followed by incubation for 60 min at 37 °C.

Caveolin-1 localization in ECs by immunofluorescence

Subconfluent ECs were grown on glass chamber slides (Falcon; Becton-Dickinson, Franklin Lakes, NJ, U.S.A.). The cells were washed with PBS, fixed for 20 min with 3.7% (v/v) formaldehyde in PBS, then washed three times with PBS (all treatments at room temperature, about 23 °C). The cells were permeabilized for 10 min with 0.1% (v/v) Triton X-100 and washed. To decrease non-specific binding of antibody, ECs were preincubated with 3% (v/v) goat serum (Gibco, Grand Island, NY, U.S.A.) in PBS for 20 min. The cells then were incubated for 60 min with the primary anti-(caveolin-1) antibody (1 µg/ml) in a solution containing 1.5% (v/v) goat serum in PBS. After three washes with PBS and a 5 min incubation with 3% (v/v) non-immune goat serum, the cells were incubated for 45 min with biotinylated goat anti-rabbit antibody (dilution 1:1000; Vector Laboratories, Burlingame, CA, U.S.A.) in 1.5% (v/v) goat

serum. After being washed, cells were incubated for 10 min with Texas Red–avidin (diluted 1:1000 in PBS), washed and placed in mounting medium (Vectashield). No reactivity was observed when the primary antibody was replaced with non-immune rabbit IgG (1 µg/ml) or with buffer, or when both primary and secondary antibodies were replaced by buffer. Autofluorescence was not detectable in ECs under our experimental conditions.

Analysis of caveolin-1 in lipid rafts by sucrose-gradient fractionation

A membrane fraction enriched in lipid rafts was isolated with a discontinuous sucrose gradient [30]. Confluent ECs in two 10 cm-diameter dishes were rinsed twice with PBS and scraped into 2 ml of buffer containing Na₂CO₃ (500 mM), pH 11, aprotinin (20 µg/ml) and leupeptin (5 µg/ml). The cells were homogenized by ten strokes in a Dounce homogenizer followed by sonication for 1 min with a bath sonicator (Laboratory Supplies, Hicksville, NY, U.S.A.). In experiments requiring the immunoprecipitation of caveolin-1, the cells were lysed in MES-buffered saline [25 mM Mes (pH 6.5)/0.15 M NaCl] containing 1% (v/v) Triton X-100, aprotinin and leupeptin [9,20]; the lysate was subjected to Dounce homogenization. For both lysis procedures, the homogenate was adjusted to 42.5% (w/v) sucrose by the addition of 2 ml of 85% sucrose in MES-buffered saline. The mixture was overlaid with 5 ml of 30% (w/v) sucrose solution followed by 3 ml of 5% (w/v) sucrose, both in MES-buffered saline. After centrifugation at 260 000 *g* for 18 h, 1 ml fractions were removed from the top. The caveolin-1-enriched membrane fraction was at the 5–30% sucrose interface in fraction 4.

Determination of caveolin-1 synthesis by metabolic labelling with [³⁵S]methionine

Caveolin-1 synthesis *de novo* by ECs was determined by metabolic labelling. Confluent ECs were incubated with [³⁵S]methionine (60 µCi/ml) in thiol-free medium for 20–30 min. The cells were washed twice with PBS and lysed for 90 min at 4 °C in 0.6 ml of buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 0.5% sodium deoxycholate, 1% (v/v) Nonidet P40, 20 µg/ml aprotinin and 5 µg/ml leupeptin. The lysate was collected by scraping and was cleared by centrifugation at 16 000 *g* for 3 min. SDS (0.1% final concentration) was added to the supernatant and the mixture was incubated first with rabbit polyclonal anti-(caveolin-1) antibody for 90 min, then with Protein A–Sepharose for 60 min. Control cell lysates were immunoprecipitated with rabbit non-immune IgG. Immunoprecipitated complexes were collected by low-speed centrifugation, washed three times with the lysate buffer and then analysed by SDS/PAGE [15% (w/v) polyacrylamide]. The gels were stained with Coomassie Blue dye and radiolabel was detected by autoradiography.

Immunoblot analysis of caveolin-1 and phosphorylated caveolin-1

Proteins from EC lysates or raft-enriched fractions were separated by SDS/PAGE [15% (w/v) polyacrylamide] and transferred to nitrocellulose. Caveolin-1 was detected by immunoblot analysis by using rabbit polyclonal antibody as primary antibody and peroxidase-conjugated secondary antibody. Phosphorylation on caveolin-1 residue Tyr¹⁴ was detected with monoclonal anti-phosphocaveolin-1. Blots were developed by enhanced chemiluminescence with ECL[®] and hyperfilm-ECL[®] (Amersham, Arlington Heights, IL, U.S.A.), and quantified densitometrically with a flatbed scanner and the N. I. H. Image

Figure 1 Effect of H_2O_2 on the subcellular localization of caveolin-1

(A) Caveolin-1 localization in ECs was determined by immunofluorescent labelling. Subconfluent ECs were incubated with medium alone (left panel) or with 200 μM H_2O_2 (right panel) for 20 min and then fixed. The cells were permeabilized and labelled with rabbit polyclonal anti-(caveolin-1) antibody, followed by biotinylated anti-rabbit IgG and Texas Red-avidin. (B) Caveolin-1 in lipid rafts was determined by fractionation on a discontinuous sucrose gradient. ECs were incubated for 20 min with buffer alone (top panel) or with 200 μM H_2O_2 (second panel from top), or for 60 min with glucose and glucose oxidase (100 ng/ml) as a H_2O_2 -generating system (third panel from top) or with glycerol as a vehicle control (bottom panel). The cells were lysed and homogenized in Na_2CO_3 buffer, the homogenate was adjusted to 42.5% (w/v) sucrose and overlaid first with 30% (w/v) and then with 5% (w/v) sucrose solutions. After centrifugation at 260 000 g for 18 h, fractions were collected (and numbered) from the top. An aliquot of each fraction was separated by SDS/PAGE and subjected to immunoblot analysis with rabbit polyclonal anti-(caveolin-1 α) (Cav-1 α) antibody.

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Determination of caveolin-1 palmitoylation in ECs by metabolic labelling with [^3H]palmitate

Caveolin-1 palmitoylation was measured essentially as described [31]. Confluent ECs in 10 cm dishes were washed with serum-free DMEM and then incubated for 2 h in the same medium. ECs were radiolabelled for 15–30 min by incubation with [^3H]palmitic acid (250 $\mu\text{Ci/ml}$) in serum-free medium containing 3.5 mg/ml fatty-acid-free BSA; the cells were then lysed. To examine the palmitoylation of total cellular protein, an aliquot was subjected to SDS/PAGE [12% (w/v) polyacrylamide] under non-reducing conditions. Caveolin-1 was immunoprecipitated from the remainder of the lysate by using rabbit polyclonal anti-(caveolin-1) antibody and subjected to SDS/PAGE as described above. The gels were stained with Coomassie Blue dye and radiolabel was determined by fluorography after a 3–6-week exposure. The identity of immunoprecipitated proteins had been confirmed previously by mass spectrometric analysis [31]. Fragment sequences from the upper band were identical with sequences in

bovine caveolin-1 α . The lower band contained fragment sequences identical with bovine caveolin-1 β and also sequences consistent with the presumptive sequence of bovine caveolin-2 (based on homology with human caveolin-2). The observed palmitoylation of the lower band was most probably due to caveolin-1 β because caveolin-2 does not contain the conserved C-terminal cysteine residues palmitoylated in other caveolin types (and because the palmitoylation of caveolin-2 has not been reported).

RESULTS

Effect of H_2O_2 on subcellular localization of caveolin-1 in ECs

We were interested in the effect of H_2O_2 on the intracellular localization of caveolin-1 because others have shown that H_2O_2 induces caveolin-1 phosphorylation [17–19] and because agents associated with oxidative stress, namely oxysterols and superoxide, alter the expression and membrane association of caveolin-1 [21,22]. We examined the effect of H_2O_2 on the subcellular localization of caveolin-1 in ECs by immunofluorescent labelling. Subconfluent cultures of bovine aortic ECs were incubated with 200 μM H_2O_2 for 20 min. The cells were fixed and

Figure 2 Effect of H₂O₂ on the subcellular localization of newly synthesized caveolin-1

(A) ECs were incubated with [³⁵S]methionine for 20 min in the presence of 100 or 200 μM H₂O₂ or Brefeldin A (BFA, 10 μM). The cells were lysed with Triton X-100 in MES-buffered saline and fractionated on a discontinuous sucrose gradient. An aliquot of the caveolae-enriched fraction at the interface of the 5% and 30% sucrose layers was subjected to immunoprecipitation (IP) with anti-(caveolin-1) antibody (anti-Cav-1) or control antibody (IgG), followed by SDS/PAGE and autoradiography (upper panel). The positions of caveolin-1α (Cav-1α) and caveolin-1β (Cav-1β) are indicated by arrows. The relative amounts of ³⁵S-labelled caveolin-1 in caveolae were quantified by densitometry and are expressed as percentages of the untreated control (lower panel). (B) ECs were treated with H₂O₂ or BFA as above. The relative amount of caveolin-1 in the caveolae-enriched fraction was determined by immunoblot analysis with polyclonal anti-(caveolin-1) antibody. (C) Synthesis of caveolin-1 *de novo* was measured by metabolic labelling. ECs were preincubated for 2 h with cycloheximide (100 μg/ml) or with medium alone, then incubated for 20 min with [³⁵S]methionine in the presence of 200 μM H₂O₂. Caveolin-1 was immunoprecipitated from cell lysates with anti-(caveolin-1) antibody or control IgG; radiolabelled protein was detected by autoradiography.

permeabilized; caveolin-1 was detected with polyclonal rabbit anti-(human caveolin-1) antibody followed by biotinylated anti-rabbit IgG and Texas Red-avidin. The strongest caveolin-1 signal was seen in distinct plasma membrane regions and also in most cellular extensions (Figure 1A, left panel). The relative abundance and distribution of caveolin-1 were not altered visibly in cells treated with a bolus of H₂O₂ (Figure 1A, right panel) or with a H₂O₂-generating system consisting of glucose and glucose oxidase (results not shown). These results are not consistent with a previous study showing caveolin internalization in NIH 3T3 cells after treatment with 1 mM H₂O₂ [32]. However, different cells and a larger amount of H₂O₂ were used in that study. We considered the possibility of a subtle H₂O₂-induced relocalization

of caveolin-1 that was not detectable by whole-cell immunofluorescence, for example a shift from caveolar (or lipid-raft) membranes to non-caveolar plasma membrane domains. To test this possibility we isolated caveolae-enriched membrane fractions on a discontinuous sucrose gradient. As expected, caveolin-1 was found primarily at the boundary between the 5% and 30% sucrose layers (Figure 1B). Neither the addition of H₂O₂ as a bolus nor the addition of a H₂O₂-generating system containing glucose oxidase and glucose caused a significant shift of caveolin-1 from caveolar (fraction 4) to non-caveolar (fractions 9–12) fractions. Taken together, these results suggest that the exposure of ECs to H₂O₂ does not influence the subcellular localization of caveolin-1. However, each of these localization methods detects the entire cellular pool of caveolin-1 and not specific subcellular pools such as newly synthesized caveolin-1. This pool is of special interest because we have shown previously that newly synthesized caveolin-1 is particularly susceptible to palmitoylation [31].

To determine the effect of H₂O₂ on the subcellular localization of newly synthesized caveolin-1, [³⁵S]methionine-labelled cells were lysed in 1% Triton X-100 and fractionated on a discontinuous sucrose gradient. The caveolae-enriched boundary between the 5% and the 30% sucrose fractions was collected and caveolin-1 was immunoprecipitated with polyclonal anti-(caveolin-1) IgG. Treatment of cells with H₂O₂ substantially decreased the amount of both the α and β isoforms of newly synthesized caveolin-1 in caveolae (Figure 2A, upper panel); the inhibition was approx. 40% at 200 μM H₂O₂ (Figure 2A, lower panel). Brefeldin A, which disrupts the secretory pathway [33], was used as a positive control to show decreased trafficking of newly synthesized caveolin-1 to caveolae. Staining of the gel with Coomassie Blue served as a loading control and showed that the amount of caveolin-1 immunoprecipitated from the caveolae-enriched fraction was not altered by treatment with Brefeldin A or H₂O₂ (results not shown). In addition, H₂O₂ was not cytotoxic, as shown by Trypan Blue exclusion after exposure to 500 μM H₂O₂ for 25 min. Immunoblot analysis of the caveolae-enriched fraction confirmed that the total amount of caveolin-1 in this fraction was not decreased by treatment with H₂O₂ (Figure 2B). The absence of the minor (β) isoform of caveolin-1 in immunoblot assays but its presence in metabolic labelling studies might reflect differential sensitivities of the antibody towards the α and β isoforms under the two assay conditions. The observed decrease in newly synthesized caveolin-1 in caveolae was not due to inhibition of *de novo* synthesis of caveolin-1 by H₂O₂. This result was shown in an experiment in which unfractionated lysates from metabolically labelled cells were subjected to immunoprecipitation of caveolin-1. H₂O₂ did not inhibit the incorporation of [³⁵S]methionine into total cellular caveolin-1 (Figure 2C); cycloheximide included as a control effectively inhibited caveolin-1 synthesis. Taken together, these results show that oxidative stress does not affect subcellular localization of pre-existing caveolin-1, which represents most of the caveolin-1 detected in the cell by methods based on mass (i.e. not those based on isotope labelling), but instead it impairs the targeting of newly synthesized caveolin-1 to caveolar domains.

Effect of H₂O₂ on caveolin-1 phosphorylation

H₂O₂ induces the phosphorylation of caveolin-1 on Tyr¹⁴ [18,19]. In other studies, caveolin-1 phosphorylation has been shown to alter trafficking and to cause intracellular relocalization to regions near focal adhesions [17]. We examined whether the impaired trafficking of caveolin-1 to caveolae by treatment with H₂O₂ was associated with caveolin-1 phosphorylation. ECs were incubated

Figure 3 Effect of H₂O₂ on phosphorylation of caveolin-1

ECs were incubated with up to 5 mM H₂O₂ for 20 min or with activated Na₃VO₄ (Van., 200 μM) as a positive control. Phosphorylation on caveolin-1 residue Tyr¹⁴ was assessed by immunoblot analysis with a monoclonal antibody against phosphocaveolin-1 (upper panel). The position of caveolin-1α (Cav-1α) is indicated by arrow. The relative amounts of caveolin-1 phosphorylation were quantified by densitometry and are expressed as fold stimulation compared with untreated control (lower panel).

with up to 5 mM H₂O₂ and lysates were analysed by immunoblotting with an anti-(phosphocaveolin-1) antibody that recognizes phosphorylated Tyr¹⁴. Treatment of cells with H₂O₂ at concentrations between 1 and 5 mM markedly increased the phosphorylation of caveolin-1 on Tyr¹⁴ up to 10-fold compared with the untreated control (Figure 3). However, concentrations up to 500 μM had no effect. Activated Na₃VO₄ was used as a positive control for caveolin-1 phosphorylation on Tyr¹⁴. To verify that the observed phosphorylation was due to caveolin-1, a separate experiment was done in which caveolin-1 from cell lysates was first immunoprecipitated with polyclonal anti-(caveolin-1), then subjected to immunoblotting with anti-phosphocaveolin monoclonal antibody. Essentially identical results were obtained by this method (results not shown). We also considered the possibility that low concentrations of H₂O₂ could stimulate the phosphorylation of caveolin-1 present in specific cellular pools; however, no increase in Tyr¹⁴ phosphorylation was observed in caveolae-enriched fractions isolated by sucrose fractionation (results not shown). The finding that low levels of H₂O₂ inhibit caveolin-1 trafficking to caveolae but do not induce caveolin-1 phosphorylation suggests that the H₂O₂-mediated alteration in trafficking is not mediated by Tyr¹⁴ phosphorylation.

H₂O₂ inhibits palmitoylation of caveolin-1 in endothelial cells

Protein targeting to caveolae often depends on the acylation state of the protein, and particularly palmitoylation [28,34]. To determine whether oxidative stress altered caveolin-1 palmitoylation, ECs were incubated for 20 min with [³H]-palmitate in the presence of H₂O₂. We had previously shown that radiolabelling was due to the covalent modification of caveolin-1 by palmitate, as shown by the release of hydroxylamine and by a TLC analysis of fatty acid released by alkaline hydrolysis [31]. Caveolin-1 was immunoprecipitated from EC lysates with polyclonal anti-(caveolin-1) antibody and [³H]palmitate-labelled protein was detected by fluorography. Treatment with H₂O₂ markedly decreased the incorporation of [³H]palmitate into both caveolin-1α and caveolin-1β; half-maximal inhibition (of the

Figure 4 Inhibition of caveolin-1 palmitoylation by H₂O₂

(A) Bovine aortic ECs were incubated for 20 min with [³H]palmitate in serum-free medium containing 3.5 mg/ml fatty-acid-free BSA and in the presence of up to 500 μM H₂O₂. The cells were lysed and caveolin-1 was immunoprecipitated (IP) with polyclonal anti-(human caveolin-1) antibody (anti-Cav-1) or with purified non-immune rabbit IgG as control. The immunoprecipitate was subjected to SDS/PAGE and fluorography (upper panel). The positions of caveolin-1α (Cav-1α) and caveolin-1β (Cav-1β) are indicated by arrows. The amounts of radiolabelled caveolin-1 were quantified by densitometry (lower panel). (B) An aliquot of the cell lysate was collected before immunoprecipitation and subjected to SDS/PAGE and fluorography as in (A). (C) ECs were treated with H₂O₂ (200 μM) for 20 min. Caveolin-1 was immunoprecipitated from the cell lysates as in (A) and subjected to SDS/PAGE followed by the detection of caveolin-1 by immunoblotting with rabbit polyclonal anti-(caveolin-1). (D) ECs were incubated with [³H]palmitate for 60 min in the presence of up to 300 ng/ml glucose oxidase (GOX) and 4.5 mg/ml glucose in the medium. Caveolin-1 was immunoprecipitated and detected as in (A).

total of both isoforms) was seen at approx. 100 μM H₂O₂ and near-maximal inhibition (approx. 90%) at 500 μM H₂O₂ (Figure 4A). Analysis of the total cell lysate not subjected to immunoprecipitation showed that H₂O₂ did not significantly decrease the labelling of most other detectable proteins, suggesting that the inhibition of [³H]palmitate incorporation was specific with regard to target protein (Figure 4B) and that the inhibition of caveolin-1

Figure 5 Effect of H₂O₂ on caveolin-1 depalmitoylation

ECs were pulse-labelled with [³H]palmitate for 30 min in serum-free medium containing 3.5 mg/ml fatty-acid-free BSA. The cells were washed with medium and chased for up to 120 min in the absence or the presence of H₂O₂ (200 μM) in DMEM containing 5% (v/v) fetal bovine serum and 100 μM cycloheximide. The cells were lysed, caveolin-1 was immunoprecipitated (IP) and [³H]palmitate-modified protein was detected by autoradiography (upper panel). The gel stained with Coomassie Blue is shown as a control (lower panel). The positions of caveolin-1α (Cav-1α) and caveolin-1β (Cav-1β) are indicated by arrows.

palmitoylation was not due to toxicity of the oxidant stress. To exclude the possibility that H₂O₂ decreased the amount or immunoreactivity of the protein under these conditions, caveolin-1 was immunoprecipitated and analysed by immunoassay and found to be unaltered by H₂O₂ treatment (Figure 4C). To mimic the release of H₂O₂ by activated neutrophils *in vivo*, ECs were incubated with [³H]palmitate in the presence of a H₂O₂-generating system. Glucose oxidase at 20 ng/ml uses glucose in the medium (4.5 mg/ml) to generate a flux of approx. 0.18 μmol of H₂O₂ per min [35]. A continuous flux of H₂O₂ decreased the incorporation of [³H]palmitate into caveolin-1 to nearly the same extent as an application of H₂O₂ in bolus form (Figure 4D).

A decreased incorporation of palmitate into caveolin-1 could be due to a combination of a decreased rate of palmitoylation and an increased rate of depalmitoylation. A pulse-chase experiment was performed to determine which of these processes were modified by H₂O₂. ECs were preincubated with [³H]palmitate for 30 min; the exogenous radiolabel was then removed and chased for up to 2 h with medium containing fetal bovine serum to saturate the system with unlabelled fatty acid and cycloheximide to block the *de novo* synthesis of caveolin-1. As reported previously, the turnover of palmitate in caveolin-1 is extremely slow, in fact undetectable even after a chase interval of 24 h [31]. H₂O₂ at 200 μM (Figure 5) or 500 μM (results not shown) did not alter the release of [³H]palmitate from either caveolin-1 isoform, suggesting that the H₂O₂-mediated decrease in the amount of [³H]palmitate incorporated into caveolin-1 was not due to increased depalmitoylation but rather to a decrease in the rate of palmitoylation itself.

DISCUSSION

Agonist-induced alteration of [³H]palmitate incorporation into several proteins, including the β₂-adrenergic receptor [36], G-protein α-subunits [24] and EC-specific nitric oxide synthase [23], has been investigated in detail. In contrast, much less is known about other types of regulation of protein palmitoylation. Inhibition of palmitoylation of several proteins by non-physiological agents has been reported, such as inhibition of p21^{H_a-ras} and p21^{N_{ras}} by cerulenin [37], inhibition of glycoprotein palmitoylation by homologues of tunicamycin [38] and inhibition

of Fyn palmitoylation by 2-bromopalmitate [39]. There is little information on physiological inhibitors of protein palmitoylation. For example, arachidonic and eicosapentaenoic acid inhibit Fyn palmitoylation in COS-1 cells and nitric oxide inhibits palmitoylation of GAP-43 and SNAP-25 in neuronal cells, with functional consequences for neurite growth [40]. In a more recent study, nitric oxide decreased the agonist-stimulated incorporation of [³H]palmitate into G_sα and the β₂-adrenergic receptor, thereby decreasing functional coupling between the receptor and its downstream G-protein target [41]. The mechanism underlying the inhibition of palmitoylation by nitric oxide is not known; regulation of the activity of acylating and deacylating enzymes and modification of cysteine thiols on target proteins have been suggested [40].

The mechanism by which H₂O₂ inhibits caveolin-1 palmitoylation is not yet known. We have considered the possibility that H₂O₂ modifies one or more amino acids in caveolin-1, which decreases the subsequent incorporation of palmitate. Such modifications can be either 'same-site', in which the interference is by competition for a cysteine residue, or 'other-site', in which interference is due to local steric hindrance or to global changes in tertiary structure. In the bradykinin B2 receptor [42], palmitoylation of a cysteine residue and phosphorylation of a nearby tyrosine residue (four residues away) are mutually exclusive. We considered a similar mechanism for caveolin-1 because H₂O₂ stimulates tyrosine phosphorylation and blocks palmitoylation. Although Tyr¹⁴ is far in the sequence from the three palmitoylated cysteine residues near the C-terminus of the protein, the hairpin-like structure proposed for caveolin-1 might bring these residues near to each other. Our results confirm that H₂O₂ induces Tyr¹⁴ phosphorylation of caveolin-1 in ECs; however, it is not likely to inhibit caveolin-1 palmitoylation because the concentration dependences are discrepant. Other protein modifications caused by conditions of oxidative stress include proline hydroxylation [43], oxo-histidine formation [44] and glutathionylation of cysteine [45]. It is tempting to speculate that proline hydroxylation might be involved in view of the proximity of Pro¹³² and Pro¹⁵⁸ to Cys¹³³ and Cys¹⁵⁶ in caveolin-1. However, attempts to use trypsin digestion and analysis by capillary liquid chromatography-electrospray MS to determine the amino acid modification of caveolin-1 have been unsuccessful. In fact, the entire palmitoylated region is undetectable, possibly owing to its hydrophobicity and low volatility (results not shown).

Given that palmitoylation enhances protein trafficking to caveolae [28,46], it is likely that the inhibition in palmitoylation of caveolin-1 mediated by H₂O₂ is responsible for the observed decrease in targeting to membrane rafts. However, it is also possible that the converse mechanism is important, namely that the observed decrease in palmitoylation is a consequence (not the cause) of impaired targeting of caveolin-1 to membrane rafts in the presence of H₂O₂. Indeed, with few exceptions [47], protein palmitoylation occurs primarily at the plasma membrane, where palmitoyl transferase activity is concentrated [48,49]. Newly synthesized caveolin-1 might be incorporated into detergent-resistant membranes in the Golgi apparatus [50]. We have previously shown that inhibition of trafficking of newly synthesized caveolin-1 to the plasma membrane by Brefeldin A or monensin results in a marked decrease in caveolin-1 palmitoylation, indicating that caveolin-1 palmitoylation occurs after the *trans*-Golgi compartment [31]. Thus we cannot exclude the possibility that oxidative stress, by an unknown mechanism, alters the trafficking of nascent caveolin-1, which therefore does not reach its membrane site of palmitate incorporation.

The cellular consequences of the inhibition by oxidative stress of caveolin-1 palmitoylation and trafficking are not known.

Because our results suggest that the inhibition by oxidative stress is restricted to newly made caveolin-1, it is likely that the stress will primarily alter processes requiring the synthesis of caveolin-1 *de novo*. Alternatively, chronic exposure of cells and tissues to oxidative stress might alter the palmitoylation state and cellular localization of most of the protein. Major functions of caveolin-1 require palmitoylation, for example caveolar organization through oligomerization, the regulation of signal transduction, and cholesterol transport [27–29]; however, the specific role of newly synthesized caveolin-1 in these processes has not been investigated. Previous studies on the role of caveolin-1 palmitoylation have used transfection with palmitoylation-defective mutants. The inhibition of caveolin-1 palmitoylation by H_2O_2 could provide an additional tool for investigating these activities and in particular the specific functions of caveolin-1 that require its synthesis *de novo*.

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