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Hydrogen Peroxide Regulates Osteopontin Expression through Activation of Transcriptional and Translational Pathways*

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Background: Osteopontin expression is increased in numerous diseases with underlying increases in H_2O_2.
Results: H_2O_2-dependent osteopontin expression is biphasic. Early increases occur through translation via redox-dependent 4EPB-1, whereas late increases require NF-κB- and AP-1-dependent transcription.
Conclusion: H_2O_2-induced osteopontin expression is both translational and transcriptional.
Significance: Understanding how osteopontin is regulated is critical for targeting this inflammatory protein in H_2O_2-dependent pathologies.

Recent *in vivo* studies establish that osteopontin (OPN) expression is hydrogen peroxide (H_2O_2)-dependent. However, the mechanisms by which H_2O_2 increases OPN expression remain poorly defined. OPN protein expression increased in an unusual biphasic pattern in response to H_2O_2. To investigate whether these increases were mediated through transcriptional and/or translational regulation of OPN, smooth muscle cells stimulated with 50 μM H_2O_2 were used as an *in vitro* cell system. Early protein increases at 6 h were not preceded by increased mRNA, whereas later increases (18 h) were, suggesting multiple mechanisms of regulation by H_2O_2. Polyribosomal fractionation assays established that early increases (6 h) in OPN expression were due to increased translation. This increase in translation occurred through phosphorylation of 4E-BP1 at the reactive oxygen species-sensitive Ser-65, which allowed for release and activation of eukaryotic initiation factor eIF4E and subsequent OPN translation. This early increase (6 h) in OPN was blunted in cells expressing a phospho-deficient 4E-BP1 mutant. H_2O_2 stimulation increased rat OPN promoter activity at 8 and 18 h, and promoter truncation studies established that promoter region −2284 to −795 is crucial for H_2O_2-dependent OPN transcription. ChIP studies determined that H_2O_2-dependent transcription is mediated by the reactive oxygen species-sensitive transcription factors NF-κB and AP-1. In conclusion, H_2O_2 stimulates OPN expression in a unique biphasic pattern, where early increases are translational and late increases are transcriptional.

Recent *in vivo* and *in vitro* studies establish that osteopontin (OPN)[2] plays a central role in pathophysiologic conditions, such as atherosclerosis (1–3) and hypertension (4, 5), and physiologic conditions, such as collateral vessel formation (6–8). Reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), have been implicated in the development of vascular disease pathologies, including hypertension, atherosclerosis, and restenosis (4). Physiologically, ROS mediate functions including proliferation, gene expression, migration, differentiation, and cytoskeletal remodeling (9, 10). OPN is an inflammatory cytokine and extracellular matrix protein that is up-regulated in several disease states where H_2O_2 is also increased. H_2O_2 is a downstream effector of many agonists, thus making it a common signaling mediator of both physiologic and pathophysiologic processes, including collateral vessel formation, hypertension, and atherosclerotic plaque formation (1, 6–8, 11). We demonstrated recently that ischemia-induced increases in OPN expression are H_2O_2-dependent in a murine model of hind limb ischemia (8), thus supporting the concept that OPN expression is directly increased in response to H_2O_2. Furthermore, angiotensin II, known to activate NADPH oxidases and increase ROS (12), increases OPN expression *in vitro* (13–15) and *in vivo* (14, 16, 17). Although our recently published data and others demonstrate that H_2O_2 increases OPN expression (8, 15, 18, 19), the mechanisms by which this occurs remain poorly defined. Therefore, further investigation into the signaling events downstream of H_2O_2 and the mechanism by which H_2O_2 influences OPN expression is essential to our understanding of the role of OPN in multiple diseases.

H_2O_2 induces the expression of numerous proteins through the activation of ROS-sensitive transcription factors (20). Moderate amounts of ROS have been shown to increase inflammatory protein expression through the activation of transcription

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[2] The abbreviations used are: OPN, osteopontin; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells; qRT-PCR, quantitative RT-PCR; CHX, cycloheximide; mRNP, messenger ribonucleoprotein.

factors such as NF-κB and activator protein 1 (AP-1) (21–23), among others. Therefore, H_2O_2 may regulate OPN gene expression through modulation of specific transcription factors, such as AP-1 and NF-κB, downstream of redox-sensitive pathways. The ability of H_2O_2 to influence mRNA translation remains controversial (24). Some reports indicate that physiologic levels of H_2O_2 promote increased translation, whereas higher non-physiologic levels of H_2O_2 inhibit translation but still allow for the translation of some target mRNAs (24, 25). One of the critical events required for translation initiation is the dissociation of 4E binding protein 1 (4E-BP1) from eukaryotic initiation factor 4E (eIF4E) (12). Stimulation of cells with various factors, including angiotensin II, leads to the sequential phosphorylation of 4E-BP1 on multiple residues, concluding with the phosphorylation of Ser-65, which results in the release of eIF4E (12) to allow for translation initiation of target mRNAs. Interestingly, eIF4E phosphorylation is increased in response to angiotensin II, oxidized LDL, and H_2O_2 (15, 26, 27). Whether H_2O_2 mediates increases in OPN protein expression through activation of specific translation pathways remains to be investigated.

The goal of this study was to investigate the mechanism(s) by which H_2O_2 confers transcriptional, posttranscriptional, and/or translational regulation of OPN using vascular smooth muscle cells (VSMCs) stimulated with 50 μM H_2O_2 as an *in vitro* cell system. We show, for the first time, that OPN protein expression is increased in an unusual biphasic pattern. Early protein increases at 6 h are not preceded by increased mRNA, whereas later increases are, suggesting multiple mechanisms of regulation of OPN by H_2O_2. We demonstrate that early H_2O_2-dependent increases in OPN protein expression are due to increased translation via phosphorylation of 4E-BP1 at the ROS-sensitive Ser-65, thus allowing for release and activation of eIF4E and subsequent OPN translation. Later H_2O_2-dependent increases in OPN protein expression are mediated through NF-κB and AP-1 binding to the rat OPN promoter, specifically in the −2284 to −795 region, to increase OPN transcription. As a potential therapeutic target, it is critical to understand how OPN expression is regulated in multiple vascular disease pathologies that occur as a result of increases in H_2O_2.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—VSMCs were isolated from rat thoracic aortas by enzymatic digestion as described previously (28). Cells between passages 6 and 14 were cultured in DMEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% calf serum (Invitrogen), 2 mmol/L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. VSMCs were serum-starved for 48 h prior to stimulation with 50 μM H_2O_2 (Sigma) in all experiments, unless indicated otherwise. Ebselen, *N*-acetyl cysteine, and PEG-catalase were purchased from Sigma. For kinase inhibitor studies, VSMCs were serum-starved for 48 h prior and pretreated for 1 h with 30 μM SP600125 (Sigma), a JNK inhibitor, and/or 5 μM panepoxydone (Enzo Life Sciences, Farmingdale, NY), an IκB inhibitor, prior to stimulation with 50 μM H_2O_2. The OPN antibody was from R&D Systems (Minneapolis, MN). β-Actin, total eIF4E, total 4E-BP1, and eIF4E and 4E-BP1 phospho-antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Plasmid Constructs—The plasmid containing the 4E-BP1 rat cDNA ORF was purchased from ORIgene (Rockville, MD) and expresses the Myc-DDK-tagged ORF clone of *Rattus norvegicus* eukaryotic translation initiation factor 4E binding protein (EIF 4E-BP1) in the pCMV6-Entry vector. The pCMV6–4E-BP1 S65A mutant was generated utilizing the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The S65A point mutation was introduced using the mutagenic primers 5′-GGAGTGTCGGAACGCGCCTGTGGCCAA-3′ and 5′-TTGGCCACAGGCGCGTTCCGACACTCC-3′, and mutation was verified by DNA sequencing. The plasmids containing the rat full-length OPN promoter (−2284 rat OPN) in the pGL3-Basic vector that expresses firefly luciferase, as well as −1599 rat OPN and −795 rat OPN, were gifts from Dr. Arihiro Kiyosue (University of Tokyo Hospital, Tokyo, Japan). Luciferase reporter construct −252 rat OPN was generated utilizing the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies), the existing −795 rat OPN plasmid, and the deletion primers 5′-TACCGAGCTCGTTTAGATAGCGCGTGGGTCCCTGATGCTCTTCCAG-3′ and 5′-CTGGAAGAGCATCAGGGACCCACGCGCTATCTAAACGAGCTC-3′. The *Renilla* luciferase in the pGL4.73 vector control plasmid was a gift from Dr. Maziar Zafari (Emory University, Atlanta, GA).

Plasmid Transfection—VSMCs were plated in 10% calf serum at 400,000 cells/9.4 cm^2 (~40% confluency) in 6-well plates 1 day prior to transient transfection with rat OPN promoter, 4E-BP1 S65A, and control plasmid expression vectors by use of enhancer reagent at a ratio of 1 μg of total DNA:8 μl of enhancer and the Effectene transfection reagent at a ratio of 1 μg of total DNA:15 μl of Effectene (Qiagen, Valencia, CA), as reported previously (29). Cells were incubated for 6 h with the DNA-Effectine complexes at 37 °C, and then the medium was changed to serum-free DMEM. Thirty hours post-transfection, cells were stimulated with 50 μM H_2O_2 for indicated times and used for a luciferase reporter assay or Western blot experiments.

Western Blot Analysis—VSMCs were lysed in Hunter's buffer as described previously (30). Briefly, cells were sonicated using a sonic dismembrator at 10 W for ten 1-s pulses. Whole cell lysates were then used for immunoblotting. Band intensity was quantified by densitometry using ImageJ software and expressed/normalized to β-actin.

ELISA—To measure secreted OPN, a rodent OPN ELISA was obtained from Enzo Life Sciences, and the instructions of the manufacturer were strictly followed. Briefly, VSMCs were stimulated with 50 μM H_2O_2 for the indicated times using serum-free DMEM containing protease inhibitor mixture (Sigma). Medium from stimulated cells was collected, clarified by centrifugation at 400 × *g* for 5 min at 4 °C, and diluted 1:16 and 1:32 in assay buffer for use in the ELISA.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction—Total RNA was extracted from VSMCs using the RNeasy kit (Qiagen), unless noted otherwise. OPN mRNA levels were measured by amplification of cDNA using a thermocycler (Applied Biosystems, Foster City, CA), SYBR Green dye, and primers unique for rat OPN (QuantiTect Primers, Qiagen). The copy number was calculated by instrument software from

standard curves of genuine templates. In some cases, the OPN copy number was normalized to 18 S rRNA.

RNA Stability Assay—For RNA stability assays, cells were pretreated for 4 h with 100 μM 5,6-dichloro-1-β-D-ribobenzimidazole (Sigma) to inhibit new transcription prior to no stimulation or stimulation with 50 μM H_2O_2. Total RNA was extracted, and OPN mRNA levels were measured over time by qRT-PCR, as described above.

Cycloheximide Chase Assay—To measure protein half-life, cells were treated with 50 μg/ml of cycloheximide (CHX, Sigma) to inhibit new translation prior to no stimulation or stimulation with 50 μM H_2O_2. Total OPN protein levels were measured over time by Western blot analysis as described above. The curves generated for OPN protein half-life ($t_{1/2}$) were analyzed and compared using a non-linear, second-order polynomial fit for each data set. These curves were used to calculate the $t_{1/2}$ of OPN $\pm$ 50 μM H_2O_2.

Polyribosome Fractionation Assay—VSMCs were serum-starved for 48 h. Cells were treated with 100 μg/ml cycloheximide or 1 mM puromycin for 30 min at 37 °C prior to stimulation with 50 μM H_2O_2 for 0, 1, 2, 4, or 6 h. As described previously (2, 31), cells were harvested in sucrose gradient lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and protease and RNase inhibitors) containing 1% Nonidet P-40, and membranous structures were removed by centrifugation at 20,000 $\times$ *g* for 30 min at 4 °C. The resulting supernatant was loaded on a 15–45% linear sucrose gradient (prepared in 20 mM Tris-HCl (pH 7.5), 100 mM KCl, and 5 mM $MgCl_2$) and spun at 38,000 rpm for 90 min in a SW41 rotor (31, 32). Gradients were separated into 10 fractions. RNA was isolated from each of the fractions by TRIzol (Invitrogen), and OPN mRNA was analyzed by qPCR as described above.

Luciferase Reporter Assay—Detection of luciferase activity in VSMCs was performed using the Dual-Luciferase reporter assay system from Promega, and the instructions of the manufacturer were strictly followed. Briefly, VSMCs were plated in 10% calf serum at 400,000 cells/9.4 cm^2. Twenty-four hours after plating, cells were cotransfected with 2 μg of either rat OPN promoter firefly luciferase construct (in pGL3-Basic), −2284 rat OPN, −1599 rat OPN, −795 rat OPN, −252 rat OPN, or empty vector (control) using Effectine reagent, as described under "Plasmid Transfection." To control for transfection efficiency, cells were cotransfected simultaneously with the *Renilla* luciferase plasmid. Six hours after incubation with the DNA-Effectine complexes, the medium was changed to serum-free DMEM. After 30 h, cells were stimulated with H_2O_2 at the indicated times, harvested, and processed for the luciferase assay. Briefly, for detection of luciferase activity, VSMCs were lysed in 1$\times$ passive lysis buffer, and 20 μl of the cell lysate was used for chemiluminescence signal detection by incubation at room temperature with 100 μl of luciferase substrate. Signals were read in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), and firefly luciferase activity was normalized to *Renilla* luciferase activity.

Chromatin Immunoprecipitation—VSMCs were stimulated for 0, 8, or 18 h with H_2O_2 (50 μM, Sigma). Protein was cross-linked to DNA with 1% formaldehyde for 10 min at 37 °C. The ChIP assay was performed following the Cell Signaling Simple ChIP Enzymatic Chromatin IP protocol (Cell Signaling Technology, catalog no. 9003). Briefly, 4 $\times$ 10^7 cells/treatment group were washed twice with 1$\times$ PBS and harvested in 1$\times$ PBS with PMSF. Cells were centrifuged and suspended in the appropriate buffer with DTT, PMSF, and protease inhibitor mixture. After a 10-min incubation on ice, the cells were pelleted and resuspended in buffer B with DTT. A 1.0-ml sample of this mixture was treated with 2.5 μl of micrococcal nuclease and incubated for 5 min at 37 °C. The mixture was then spun at 13,000 rpm and resuspended in 1 ml of ChIP buffer. DNA was sheared into fragments from 150–900 base pairs by sonication using a Microson Ultrasonic Cell Disruptor XL (Misonix, Inc., Farmingdale, NY) (2 cycles of 4 watts, 10 pulses at 1 s each). 10 μg of DNA-protein complexes was immunoprecipitated overnight at 4 °C using either NF-κB (Cell Signaling Technology (catalog no. 6956), AP-1 (c-FOS, Santa Cruz Biotechnology, catalog no. sc-52X), or IgG control ChIP-grade antibodies. Immunocomplexes were precipitated by incubation for 2 h at 4 °C with ChIP-grade magnetic beads. Incubation with 1$\times$ ChIP elution buffer for 30 min at 65 °C was performed to reverse cross-linked DNA-protein complexes. The samples were digested with proteinase K for 2 h at 65 °C as recommended. After washing, eluting, and DNA purification, the DNA was used for qRT-PCR amplification using the following primers for different regions of the promoter: region −2284 to −1599, 5′-CTGTGCAGGGAAATTCCACAA-3′ and 5′-TCTAGAAATGCTGCCACCGTGT-3′; and region −1599 to −795, 5′-ACACAGGACTTATTACCTTACATCCCC-3′ and 5′-TGCATTCACCAAGAGATAACCCGAA-3′. Primer efficiency was optimized using plasmid DNA standard curves generated using the rat full-length OPN promoter (-2284 rat OPN) in the pGL3-Basic vector and analyzed by linear regression. Each sample was normalized to the individual amount of input DNA.

Statistical Analysis—Results are expressed as mean $\pm$ S.E. from at least three independent experiments. Statistical significance for quantitative results was assessed using analysis of variance followed by post hoc analysis. In some cases, where indicated, Student's *t* test was used to assess significance. The curves generated for mRNA stability assays and the cycloheximide chase assay were analyzed and compared using a non-linear, second-order polynomial fit for each data set, followed by analysis to determine whether the curves were significantly different. ChIP was analyzed using mixed-procedure analysis. For all studies, differences were considered statistically significant at $p < 0.05$.

RESULTS

H_2O_2 Increases OPN Protein Expression in a Biphasic Pattern—We first performed a dose-response experiment (10–200 μM) to establish the dose at which H_2O_2 increases in OPN expression in VSMCs. VSMCs were serum-deprived prior to stimulation with H_2O_2 for 18 h. As shown in Fig. 1*A*, we observed a significant increase in OPN protein expression in response to H_2O_2 that was maximal at 50 μM. To evaluate the time dependence of H_2O_2-induced increases in OPN protein levels, we stimulated VSMCs with 50 μM H_2O_2 over a 24-h time course and measured OPN protein expression by Western blot analysis. OPN protein increased in an unusual biphasic pattern, with an early increase at 6 h and a second increase at 18 h (Fig. 1*B*). A

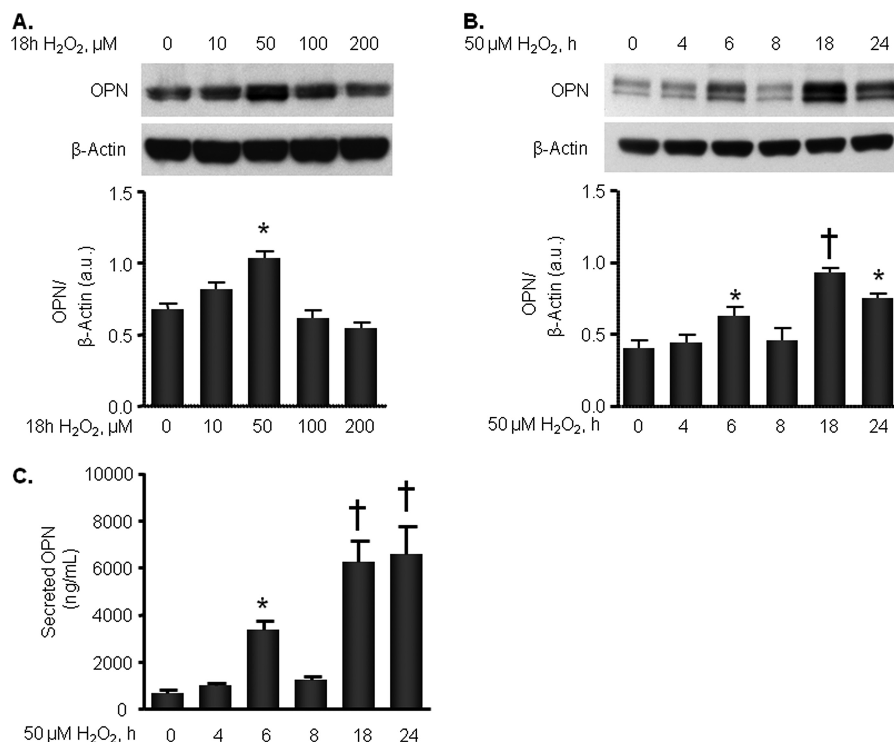

FIGURE 1. **H_2O_2 increases osteopontin expression. Rat aortic smooth muscle cells (VSMCs) were made quiescent in serum-free medium for 48 h prior to stimulation with H_2O_2.** *A*, OPN protein expression (*top row*) was measured by Western blot analysis in a dose-response experiment in which cells were left unstimulated (0 μM, control) or stimulated with 10 μM, 50 μM, 100 μM, and 200 μM of H_2O_2. *Bottom panel*, graph of OPN expression normalized to β-actin and expressed as arbitrary units (*a.u.*). *, $p < 0.05$ *versus* 0 μM control; $n = 4$. *B*, cellular OPN protein expression was measured in response to 50 μM H_2O_2 at 0, 4, 6, 8, 18, and 24 h and increased in a biphasic manner. *Bottom panel*, graph of average data. *, $p < 0.05$ *versus* 0 h; †, $p < 0.001$ *versus* 0 h; $n = 4$. *C*, secreted OPN protein was measured in the media of the same treatment groups as *B* and quantified by ELISA. *, $p < 0.05$ *versus* 0 h; †, $p < 0.0001$ *versus* 0 h; $n = 3$. In *A* and *B*, β-actin was measured to confirm equal loading (*bottom rows*). *Error bars* are mean ± S.E.

similar biphasic pattern was detected in secreted OPN levels, as quantified by ELISA (Fig. 1*C*).

Early H_2O_2-dependent Increases in OPN Are Translational—To determine whether the increases in OPN protein expression are preceded by increases in mRNA levels, serum-deprived VSMCs were stimulated with 50 μM H_2O_2 for up to 18 h. OPN mRNA levels were assessed by qPCR to determine the effect of H_2O_2 on OPN gene expression. H_2O_2 significantly increased OPN mRNA levels at 8 and 18 h (Fig. 2*A*), suggesting that one mechanism by which H_2O_2 increases OPN expression is increased transcription. However, the significant increase in OPN protein expression at 6 h (Fig. 1, *B* and *C*) was not preceded by an increase in mRNA (Fig. 2*A*), thus supporting the idea that multiple mechanisms are responsible for early and late increases in OPN protein in response to H_2O_2. To establish whether the observed H_2O_2-dependent increase in OPN protein expression at 6 h was posttranscriptional or translational, we performed an RNA stability assay. H_2O_2 stimulation did not alter OPN mRNA degradation compared with the control (Fig. 2*B*). To determine whether the H_2O_2-dependent increase in OPN protein expression at 6 h was due to changes in OPN protein stability, we performed a CHX chase assay to measure protein half-life in response to H_2O_2. As shown in Fig. 2, *C* and *D*, H_2O_2 did not alter OPN protein half-life (CHX $t_{1/2} = 49$ min, CHX + H_2O_2 $t_{1/2} = 56$ min, $p = 0.5908$), suggesting that the early increase in OPN protein expression is likely due to altered translation. To further investigate this concept, we performed a polyribosomal fractionation assay to quantify translation of OPN mRNA, where a shift in OPN mRNA from the messenger ribonucleoprotein (mRNP) complexes/monosomal fractions into the polysomal fractions is indicative of increased translation. As shown in the representative tracing (Fig. 2*E*), there was a H_2O_2-dependent shift in OPN mRNA from the mRNP/monosomal fractions into the polysomal fractions that peaks at 2 h before shifting back to the mRNP/monosomal fractions by 6 h (Fig. 2, *E* and *F*). This was the case in three independent experiments and is quantified in Fig. 2*F*. The early significant increase in OPN expression (6 h) in response to H_2O_2 was not sustained throughout the time course (Fig. 2, *E* and *F*), suggesting that H_2O_2-dependent increases in OPN translation did not persist. Taken together, these data indicate that early H_2O_2-dependent increases in OPN protein expression are mediated through increased translation. Therefore, we further investigated the pathway(s) through which this may occur.

H_2O_2-dependent OPN Translation Requires 4E-BP1 Phosphorylation—As discussed above, dissociation of the 4E-BP1-eIF4E complex is a critical event for translation initiation. Angiotensin II has been shown to increase 4E-BP1 phosphorylation at the redox-sensitive Ser-65 (26), resulting in the release of eIF4E. Upon release from 4E-BP1, eIF4E can be phosphorylated, thus allowing eIF4E to initiate translation by binding the 5′ cap of target mRNAs. To determine whether H_2O_2-dependent increases in OPN mRNA translation occur through a 4E-BP1-dependent pathway, we first investigated whether H_2O_2 increases Ser-65 phosphorylation of 4E-BP1. As shown in Fig. 3*A*, 4E-BP1 phosphorylation at Ser-65 increases in

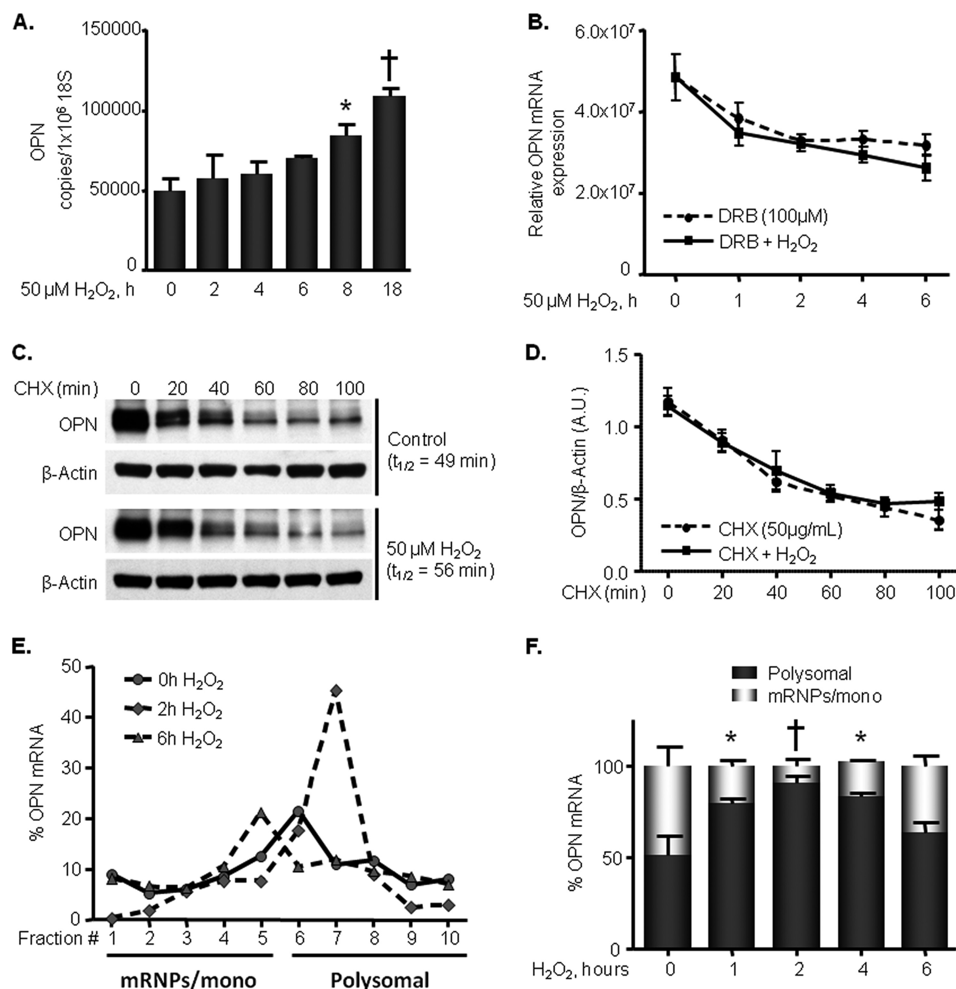

FIGURE 2. **Early H_2O_2-dependent increases in OPN are translational.** *A*, OPN mRNA expression was measured in response to 50 μM H_2O_2 at 0, 2, 4, 6, 8, and 18 h and expressed as copies per 1×10^6 copies of 18 S. *, $p < 0.05$ *versus* 0 h; †, $p < 0.01$ *versus* 0 h; $n = 3$. *B*, VSMCs were serum-deprived for 48 h prior to treatment with 100 μM 5,6-dichloro-1-β-D-ribobenzimidazole (*DRB*) to block new transcription. Stimulation with 50 μM H_2O_2 for 1, 2, 4, and 6 h did not alter the rate of OPN mRNA degradation, as assessed by qRT-PCR. Data shown are from three independent experiments. *C*, VSMCs were treated with 50 μg/ml of CHX to inhibit new translation prior to no stimulation or stimulation with 50 μM H_2O_2. Total OPN protein levels were measured over time by Western blot analysis, normalized to β-actin, and expressed as arbitrary units (*A.U.*). *D*, OPN $t_{1/2}$ was calculated using a non-linear, second-order polynomial fit for each data set. Curves were not statistically different ($p = 0.5908$, $n = 4$). *E*, distribution of rat OPN mRNA on linear sucrose gradients (15–45%) from VSMCs stimulated with 50 μM H_2O_2 for 0, 1, 2, 4, and 6 h. Values shown represent the percentage of the total rat OPN mRNA in each fraction (representative graph). *F*, average data for OPN mRNA distribution in linear sucrose gradients from three independent polysome fractionation time course experiments. *, $p < 0.01$ *versus* 0 h; †, $p < 0.001$ *versus* 0 h; $n = 3$. *Error bars* are mean ± S.E.

response to H_2O_2 as early as 10 min. Furthermore, eIF4E activation, as measured by phosphorylation of eIF4E at Ser-209 (Fig. 3*A*), was also increased in response to H_2O_2, which cannot occur without prior release from 4E-BP1. To determine whether increased phosphorylation of 4E-BP1 at Ser-65 is indeed being mediated by H_2O_2, we pretreated cells with a panel of ROS inhibitors (the antioxidant *N*-acetyl cysteine, the glutathione peroxidase mimetic ebselen, and the H_2O_2 scavenger PEG-catalase) and determined the effects of these antioxidants on Ser-65 phosphorylation. All inhibitors blocked H_2O_2-dependent phosphorylation of Ser-65 (Fig. 3*B*). To establish whether phosphorylation of 4E-BP1 at Ser-65 is required for H_2O_2-dependent increases in OPN translation, we transfected VSMCs with a plasmid expressing an S65A phosphomutant of 4E-BP1 or control vector (Fig. 3*C*). H_2O_2-dependent increases in OPN protein expression at 6 h were blocked significantly in 4E-BP1-S65A-transfected cells. Taken together, these data indicate that H_2O_2 increases phosphorylation of 4E-BP1 at Ser-65, allowing the release and activation of eIF4E and subsequent translation of OPN mRNA.

Late H_2O_2-dependent Increases in OPN Are Transcriptional—The late increases in H_2O_2-dependent OPN protein expression could potentially be due to increased mRNA stability or enhanced promoter activity and transcription because the increase at 18 h is preceded by an increase in OPN mRNA. To establish whether the observed H_2O_2-dependent increase in OPN protein expression at 18 h is transcriptional or posttranscriptional, we performed an RNA stability assay. We found no change in the OPN mRNA $t_{1/2}$ in response to H_2O_2 stimulation (Fig. 4*A*), indicating that late increases in OPN mRNA in response to H_2O_2 stimulation are not due to increased RNA stability. Therefore, using a luciferase reporter assay, we tested whether the mechanism by which H_2O_2 increases OPN mRNA levels is increased promoter activity. VSMCs were transfected with a luciferase reporter construct expressing the full-length rat OPN promoter (pGL3–2284 rat OPN) or empty vector

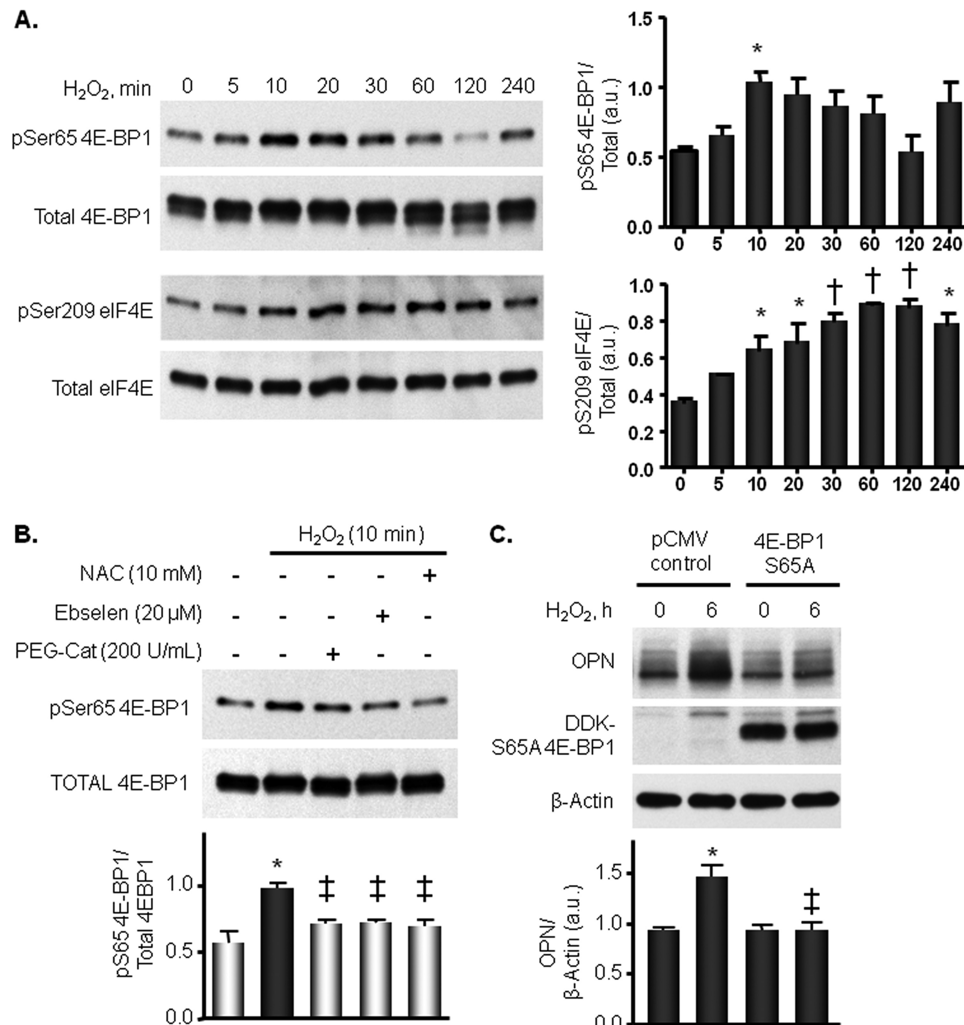

FIGURE 3. **OPN translation requires H_2O_2-dependent phosphorylation of 4E-BP1.** *A*, VSMCs were quiesced for 48 h prior to stimulation with 50 μM H2O2 for 0, 10, 20, 30, 60, 120, and 240 min. Cells were harvested and immunoblotted for protein expression. *Left panel*, *top rows*, phosphorylation of 4E-BP1 at Ser-65, required for release and activation of eIF4E, was increased as early as 10 min (*, $p < 0.05$ *versus* 0 min, $n = 3$, *right panel*, *top graph*). *Left panel*, *bottom rows*, eIF4E phosphorylation at Ser-209 was also increased in response H_2O_2 (*, $p < 0.05$ *versus* 0 min; †, $p < 0.001$ *versus* 0 min, $n = 3$, *right panel, bottom graph*). Phosphorylation was normalized to total protein and expressed as arbitrary units (*a.u.*). *B*, VSMCs were made quiescent for 48 h before pretreatment for 30 min with the ROS inhibitors *N*-acetyl cysteine (*NAC*) (10 mM), ebselen (20 μM), and PEG-catalase (200 units/ml) and subsequently stimulated for 10 min with 50 μM H_2O_2. 4E-BP1 phosphorylation at Ser-65 was measured by Western blot analysis. All inhibitors blocked H_2O_2-dependent phosphorylation of Ser-65 (*, $p < 0.001$ *versus* control; ‡, $p < 0.05$ *versus* 10 min H_2O_2; $n = 4$). *C*, VSMCs were transfected with a pCMV control plasmid or a plasmid containing the S65A phosphomutant of 4E-BP1 (DDK-tagged), made quiescent for 24 h, and stimulated with 50 μM H_2O_2 for 6 h. OPN expression was assessed by Western blot analysis. H_2O_2-dependent early increases in OPN protein expression (*, $p < 0.01$ *versus* 0 h pCMV control; $n = 3$) are blocked in S65A 4E-BP1-transfected cells (‡, $p < 0.01$ *versus* 6 h pCMV + H_2O_2; $n = 3$). *Error bars* are mean ± S.E.

(pGL3). VSMCs were either not stimulated (Fig. 4*B*, *white bars*) or were stimulated with H_2O_2 for up to 18 h (*black bars*). As shown in Fig. 4*B*, rat OPN promoter activity was increased significantly in response to H_2O_2 stimulation at the 8 and 18 h time points. To determine the specific regions of the rat OPN promoter critical for H_2O_2-dependent increases in rat OPN promoter activation, we utilized a series of luciferase reporter constructs with rat OPN promoter truncations (Fig. 4*D*, promoter map) and assessed promoter activity at 18 h after H_2O_2 stimulation. The regions of the rat OPN promoter critical for H_2O_2-dependent OPN promoter activity, according to our data in Fig. 4*C*, are −2284 to −1599 and −1599 to −795. To narrow down which specific transcription factors and binding sites are necessary to increase OPN transcription, we mapped the OPN promoter and found several predicted binding sites for H_2O_2-sensitive transcription factors, including NF-κB and AP-1. As depicted in Fig. 4*D*, the OPN promoter contains one NF-κB and one AP-1 binding site within the −2284 to −1599 region and one NFκB and three AP-1 binding sites within the −1599 to −795 region. Therefore, we determined whether NFκB and AP-1 binding to the OPN promoter increases in response to H_2O_2.

The Role of NF-κB and AP-1 in H_2O_2-dependent OPN Transcription—ROS are known to up-regulate some inflammatory proteins through the activation of NF-κB and AP-1 (21–23). Furthermore, OPN transcription has been shown to be activated by AP-1 and NF-κB (33, 34). Because NF-κB and AP-1 are redox-sensitive transcription factors that also have predicted binding sites within the OPN promoter, we tested whether H_2O_2-dependent OPN promoter activity is mediated through an AP-1- and/or NF-κB-dependent mechanism. Because we lost substantial promoter activity with the −795 truncation, we further examined whether H_2O_2 stimulates increased binding of AP-1

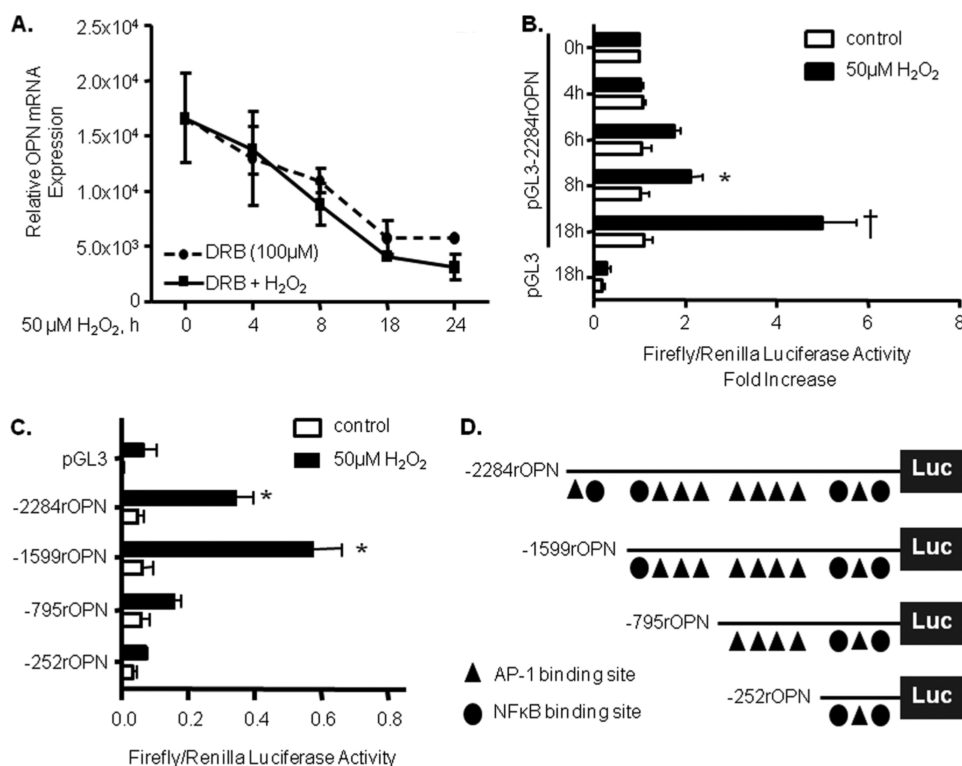

FIGURE 4. **Late H_2O2-dependent increases in OPN are transcriptional.** *A*, VSMCs were serum-deprived for 48 h prior to treatment with 100 μM 5,6-dichloro-1-β-D-ribobenzimidazole (*DRB*) to block transcription. Stimulation with 50 μM H_2O_2 for 4, 8, 18, and 24 h did not alter OPN mRNA degradation, as assessed by qRT-PCR. The data shown are from three independent experiments. *B*, a luciferase reporter assay was carried out using the full-length rat OPN promoter (pGL3–2284 rat OPN) upstream of firefly luciferase. Cells were cotransfected with an empty vector (pGL3) or pGL3–2284 rat OPN firefly and pGL4.73SV40 *Renilla* luciferase. Twenty-four hours post-transfection, cells were not stimulated (*white bars*) or stimulated with 50 μM H_2O_2 for 0, 4, 6, 8, and 18 h (*black bars*). Firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed as fold change compared with 0 h control. 50 μM H_2O_2 significantly increased rat OPN promoter activity at 8 and 18 h (*, $p < 0.05$ *versus* 0 h; †, $p < 0.001$ *versus* 0 h; $n = 4$). *C*, a luciferase reporter assay was carried out using several rat OPN promoter truncations upstream of firefly luciferase (pGL3–2284 rat OPN (full-length), −1599 rat OPN, −795 rat OPN, and −252 rat OPN) and cotransfected with pGL4.73SV40 *Renilla* luciferase. 24 h post-transfection, cells were left unstimulated (*white bars*) or stimulated with 50 μM H_2O_2 for 18 h (*black bars*). Firefly luciferase activity was normalized to *Renilla* luciferase activity and compared with the pGL3 control. 50 μM H_2O_2 significantly increased rat OPN promoter activity in −2284 rat OPN- and −1599 rat OPN-transfected cells (*, $p < 0.001$ *versus* pGL3 control; $n = 3$). *D*, predicted AP-1 (▲) and NF-κB (●) transcription factor binding sites within the rat OPN promoter region for each truncation construct. *Error bars* are mean ± S.E. *Luc*, luciferase.

and/or NF-κB binding sites within the −2284 to −1599 and −1599 to −795 regions of the endogenous promoter using ChIP assays and primers designed to bind specifically within each promoter region (Fig. 5*A*). The ChIP assays confirmed that both AP-1 (Fig. 5*B*) and NF-κB (*C*) binding to the endogenous OPN promoter increases in response to H_2O_2 stimulation. To determine whether JNK and IκB kinase are the upstream kinases responsible for AP-1 and NF-κB activation, respectively, we measured OPN mRNA and protein in response to H_2O_2 in the presence of 30 μM SP600125, a JNK inhibitor, 5 μM panepoxydone, an IκB inhibitor, or both prior to stimulation with 50 μM H_2O_2 for 18 h. H_2O_2-induced OPN mRNA (Fig. 5*D*) and OPN protein expression (*E*) were blocked by JNK (SP600125) and IκB (panepoxydone) inhibitors. However, these effects were not found to be additive. Taken together, we conclude that H_2O_2-dependent activation of JNK and IκB redox-sensitive pathways results in activation of the transcription factors AP-1 and NF-κB, respectively, leading to increased OPN promoter activity and OPN transcription *in vitro*.

DISCUSSION

H_2O_2 is a signaling molecule that influences both physiologic and pathophysiologic processes (1, 6–8, 11), in part through modulation of protein expression. *In vivo* and *in vitro* studies established that OPN expression is increased in physiologic and pathophysiologic conditions that have been linked to increases in H_2O_2, such as atherosclerosis (1–3), hypertension (4, 5), and collateral vessel formation (8, 11). The goal of our study was to define the contribution of transcriptional and/or translational mechanism(s) through which H_2O_2 increases OPN expression. We showed that, in VSMCs, H_2O_2 increased OPN protein expression in a biphasic manner, suggesting multiple potential mechanisms of regulation. We also demonstrated that early H_2O_2-dependent increases in OPN protein expression are mediated through increased translation via phosphorylation of 4E-BP1 at the ROS-sensitive Ser-65, allowing for release and activation of eIF4E and subsequent OPN translation, as depicted in our working model (Fig. 6). Furthermore, late H_2O_2-dependent increases in OPN protein expression are mediated through NF-κB and AP-1 binding to the rat OPN promoter to increase OPN transcription (Fig. 6). Although the physiological/evolutionary benefit to having two mechanisms to up-regulate OPN remains speculative and requires further investigation, we hypothesize that there may be differential functions *in vivo* of acute *versus* chronic increases in inflammation and OPN expression. We propose that rapid increases in the expression of OPN through increased trans-

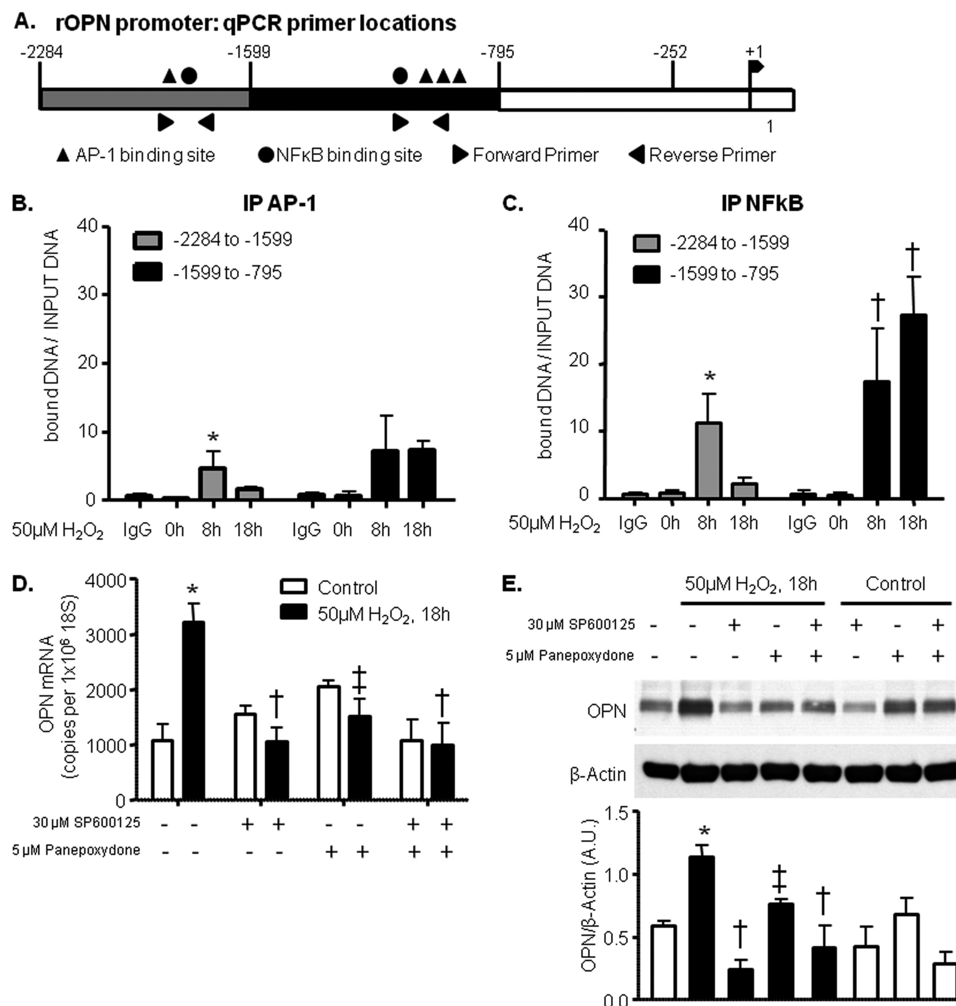

FIGURE 5. **H_2O2-dependent OPN transcription is mediated by NF-κB and AP-1.** *A*, AP-1 (▲) and NF-κB (●) transcription factor binding site localization within the rat OPN promoter from −2284 to −1599 (*gray*) and from −1599 to −795 (*black*). The locations of the ChIP quantitative polymerase chain reaction forward and reverse primers are also depicted (▲). *B* and *C*, VSMCs were made quiescent prior to stimulation for 0, 8, or 18 h with H_2O_2 (50 μM). Protein was cross-linked to DNA, and a ChIP assay was performed on 10 μg of DNA-protein immunocomplexes. Complexes were immunoprecipitated using either IgG control, AP-1 (c-FOS, *B*), or NF-κB (*C*) ChIP-grade antibodies. After the DNA was washed, eluted, and purified, it was used for qPCR. Each sample was normalized to the individual amount of input DNA. $n = 3$. *Error bars* are mean ± S.E. *D* and *E*, VSMCs were made quiescent for 48 h, followed by pretreatment for 1 h with nothing, 30 μM SP600125, a JNK inhibitor, and/or 5 μM panepoxydone, an IκB inhibitor. Cells were subsequently left unstimulated (*white bars*) or were stimulated with 50 μM H_2O_2 (*black bars*) for 18 h. OPN mRNA and protein were harvested and analyzed using qRT-PCR (*D*) and Western blot analysis (*E*), respectively. *D*, OPN mRNA expression is expressed as copies/1 × 10^6 copies of 18 S. *, $p < 0.001$ *versus* control; †, $p < 0.001$ *versus* H_2O_2 alone; ‡, $p < 0.01$ *versus* H_2O_2 alone; $n = 3$. *E*, OPN protein expression was normalized to β-actin and expressed as arbitrary units (*A.U.*). *, $p < 0.01$ *versus* control; †, $p < 0.001$ *versus* H_2O_2 alone; ‡, $p < 0.05$ *versus* H_2O_2 alone; $n = 3$.

lation are required for an immediate, acute response that potentially functions to recruit inflammatory cells to sites of injury, whereas later increases in the expression of OPN through increased transcription are required for chronic responses to injury and inflammation that are more critical for mineralization processes. Understanding the mechanisms by which H_2O_2 influences OPN expression may ultimately be broadly applicable to multiple disease pathologies with underlying increases in H_2O_2 and OPN.

ROS Regulation of OPN Expression—OPN expression has been reported to be increased *in vitro* and *in vivo* downstream of multiple agonists linked to increases in ROS production, including TNFα, angiotensin II, allylamine, aldosterone, and glucose (14–17, 19, 23, 33, 35). Furthermore, our group and others have identified several predicted binding sites within the OPN promoter for ROS-sensitive transcription factors, including NF-κB, AP-1, Ets-1, and cAMP response element-binding protein (Fig. 5*C*) (4, 27). However, because the agonists listed above activate multiple pathways in addition to those that increase ROS production, we chose to stimulate VSMCs directly with H_2O_2 to investigate the H_2O_2-specific effects on OPN expression. H_2O_2 has been linked previously to increased OPN mRNA and protein expression *in vitro* and *in vivo* (8, 18). However, the precise signaling mechanisms downstream of H_2O_2 that mediate increases in OPN expression are unknown. Additionally, the role of ROS-sensitive transcription factors in this process remains controversial. Reports demonstrate conflicting results for the roles of NF-κB and AP-1 in OPN promoter activation, and these conflicting results are likely due to differences in H_2O_2 dose and how rat VSMCs were isolated for experiments. Partridge *et al.* (35) show that NF-κB is a positive mediator of OPN promoter activity in response to stimulation with low-

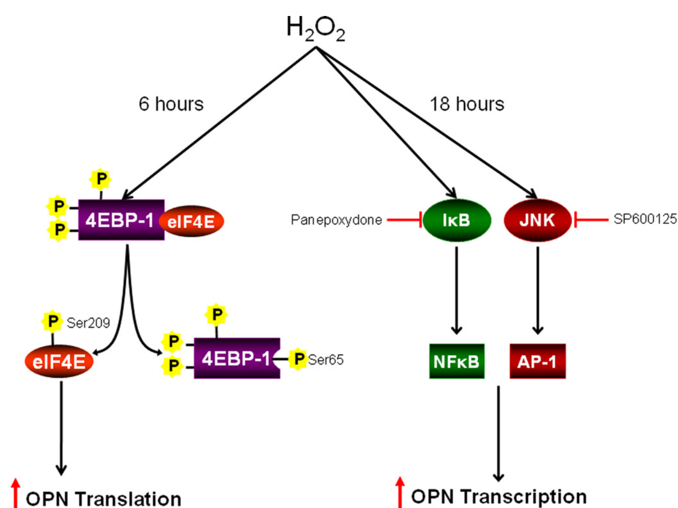

FIGURE 6. **Proposed pathways involved in the regulation of the early (6 h) translational increase in OPN expression and the late (18 h) transcriptional increase in OPN.** H_2O_2 stimulates OPN expression in a unique biphasic pattern where acute increases (6 h) are translational and mediated through the H_2O_2-dependent phosphorylation (*P*) of 4E-BP1 at Ser-65, allowing for release and activation of eIF4E and subsequent translation of OPN. Sustained increases in OPN (18 h) are mediated through activation of the IκB and JNK pathways, resulting in increased NF-κB and AP-1 activation and binding to the OPN promoter to increase OPN transcription.

dose (0.001 μM) H_2O_2. Others have shown that the OPN promoter is negatively regulated by NF-κB, where mutation of one NF-κB binding site returned transcriptional activity to control levels and mutation of one AP-1 site was inert in VSMCs isolated from male Sprague-Dawley rats gavaged with allylamine (70 mg/kg) for 20 days prior to VSMC isolation and report that this is likely due to allylamine-mediated increases in H_2O_2 (35, 36). Furthermore, there is little to no information about the mechanism(s) by which H_2O_2 confers translational regulation of OPN mRNA into protein because most studies focus on transcriptional or posttranslational mechanisms of OPN regulation. Understanding the mechanism(s) by which H_2O_2 regulates OPN expression is relevant to a number of cardiovascular pathologies with increased H_2O_2.

H_2O_2-dependent OPN Translation—Studies of OPN have primarily focused on investigating transcriptional regulation of OPN and posttranslational modifications to OPN, such as glycosylation, phosphorylation, and cleavage. However, little is known regarding OPN regulation at the posttranscriptional or translational levels. Two recent studies indicate OPN expression regulation at the posttranscriptional or translational level. Remus *et al.* (15) recently demonstrated that angiotensin II-induced OPN expression is blunted significantly in the presence of miR181a, which binds to the OPN mRNA 3′ UTR. Furthermore, a recent paper by Shinohara *et al.* (37) described the presence of an intracellular form of OPN generated via alternative translation initiation at a non-AUG site downstream of the canonical AUG sequence and showed that the expression of intracellular OPN does not involve alternative mRNA transcription initiation or splicing. However, the mechanisms regulating OPN mRNA translation into protein remained largely undefined, as does the role of H_2O_2 in this process. Here we report the novel finding that OPN protein increases in response to stimulation with H_2O_2 in a biphasic manner and that early significant H_2O_2-dependent increases in OPN protein expression (6 h) are not due to increased OPN mRNA, alterations in OPN mRNA stability, or changes in OPN protein stability. Indeed, early increases in OPN protein expression are mediated through increased OPN mRNA translation, as indicated by our polyribosome fractionation assay results (Fig. 2, *E* and *F*).

Furthermore, we demonstrate that the increase in OPN translation requires the ROS-dependent phosphorylation of 4E-BP1 at Ser-65, which has been shown to be redox-sensitive in response to angiotensin II (26). Dissociation of the 4E-BP1-eIF4E complex is the critical event for translation initiation, and the release and activation of eIF4E is dependent on phosphorylation of 4E-BP1 at Ser-65 (12). Upon phosphorylation of 4E-BP1 at Ser-65, eIF4E is released and binds to target mRNAs to initiate translation. We demonstrate that, when Ser-65 of 4E-BP1 is mutated to an alanine residue, rendering 4E-BP1 phospho-deficient at this critical ROS-sensitive site, OPN translation/expression is blocked. This early increase in OPN translation is not sustained throughout the 18-h time course (Fig. 2, *E* and *F*), suggesting that H_2O_2-dependent increases in OPN translation decrease after 6 h, correlating with the return to base line in OPN protein levels measured at 8 h and prior to the late increase in OPN protein expression. One could speculate that this is potentially mediated by H_2O_2-dependent changes in OPN mRNA localization or modulation of other translation pathways, such as phosphorylation of eukaryotic initiation factor 2 (eIF2) α subunit, which is a well documented mechanism to down-regulate protein synthesis (19, 35). However, the mechanism responsible for the subsequent decrease following the early increase in OPN translation remains an ongoing area of investigation.

H_2O_2-dependent OPN Transcription—Examination of the potential mechanism(s) responsible for late increases in OPN mRNA and protein expression support the idea that H_2O_2 does not alter OPN mRNA stability. Furthermore, we demonstrated that OPN promoter activity significantly increased in response to H_2O_2 and that this increase in promoter activity was closely followed by a significant increase in OPN mRNA levels. Although we did not observe a biphasic increase in OPN mRNA levels in VSMCs, we do find significant increases in OPN mRNA at 8 and 18 h.

Using promoter truncation studies, we found the nucleotide region −2284 to −795 to be critical to H_2O_2-dependent OPN promoter activity. This region of the OPN promoter contains several predicted binding sites for transcription factors known to be ROS-sensitive, including NF-κB, AP-1, Ets-1, and cAMP response element-binding protein. AP-1 has been linked previously to glucose-mediated transcription of OPN and angiotensin II-mediated transcription of OPN (19, 33). There are conflicting reports about the role of NF-κB as a positive or negative mediator of OPN promoter activation and OPN transcription. Furthermore, which portion of this transcription is H_2O_2-dependent required further investigation. Our results demonstrate, using chromatin immunoprecipitation assays, that both NF-κB and AP-1 binding to the OPN promoter increase in response to H_2O_2 stimulation. The increase in binding of both NF-κB and AP-1 to the −2284 to −1599 and −1599 to −795 regions after H_2O_2 stimulation is an interesting finding because

classical activation of NF-κB and AP-1 is mediated through different pathways in response to other agonists that increase ROS production. This is in line with what was shown by Ahmad *et al.* (34), who demonstrated that AP-1 can positively modulate NF-κB binding downstream of ROS, which may potentially be a mechanism by which both transcription factors participate in OPN promoter regulation. Maps of the OPN promoter (Figs. 4*D* and 5*A*) reveal that the regions of the OPN promoter necessary for H_2O_2-dependent promoter activation contain AP-1 and NF-κB binding sites within close proximity of each other, potentially suggesting transcriptional cooperativity between these two transcription factors in the regulation of OPN transcription. Furthermore, we demonstrate that H_2O_2-dependent activation of JNK and IκB, upstream of AP-1 and NF-κB, respectively, are required for OPN transcription *in vitro* (Fig. 5, *D* and *E*). Inhibition of both JNK and IκB did not have an additive effect on OPN transcription, suggesting that both AP-1 and NF-κB are required and, again, suggesting transcriptional cooperativity. However, further investigation is needed to determine whether this is indeed the case.

These data strongly support a role for H_2O_2 in both the transcriptional and translational regulation of OPN expression. H_2O_2-dependent OPN transcription is mediated by increased binding of both AP-1 and NF-κB, specifically to the −2284 to −795 region of the OPN promoter, and subsequent increase in mRNA and protein levels. H_2O_2-dependent increases in OPN translation occur through phosphorylation of 4E-BP1 at the ROS-sensitive Ser-65, which allows for release and activation of eIF4E and subsequent OPN translation. As a potential therapeutic target, it is critical to understand how OPN expression is regulated in multiple vascular disease pathologies with underlying increases in H_2O_2.

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