UPREGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE BY ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR INVOLVES MAPK AND PKC SIGNALING PATHWAYS

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Nonstandard Abbreviations: P450: cytochrome P450; EET: epoxyeicosatrienoic acids; EDHF: endothelium-derived hyperpolarizing factor; EDRF: endothelium-derived relaxing factor; DHETs: dihydroxyeicosatrienoic acids; NO: nitric oxide; MAPK: mitogen-activated protein kinase; PKC: protein kinase C; eNOS: endothelial nitric oxide synthase; p-eNOS:

phosphorylated eNOS (Thr495); PGI_2: prostacyclin; CYPOR: NADPH-cytochrome P450 oxidoreductase; SDS: sodium dodecyl sulfate; BAEC: bovine aortic endothelial cells; 17-ODYA: 17-octadecynoic acid; L-NMMA: N^G-monomethyl-L-arginine; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PMSF: phenylmethylsulfonyl fluoride; DTT: dithiothreitol; HEPES: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; NADPH: nicotinamide adenine dinucleotide phosphate; GFP, green fluorescent protein.

Abstract

Cytochrome P450-dependent metabolites of arachidonic acid, the epoxyeicosatrienoic acids (EETs), are proposed to be endothelium-derived hyperpolarizing factors (EDHF) that affect vascular tone; however, the effects of EDHF on endothelial-derived nitric oxide biosynthesis remain unknown. We examined the regulation of endothelial nitric oxide synthase (eNOS) by EDHF and investigated the relevant signaling pathways involved. The P450 epoxygenases CYP102 F87V mutant, CYP2C11-CYPOR and CYP2J2 were transfected into cultured bovine aortic endothelial cells and the effects of endogenously formed or exogenously applied EETs on eNOS expression and activity were assessed. Transfection with the P450 epoxygenases led to increased eNOS protein expression, an effect that was attenuated by co-treatment with the P450 inhibitor 17-ODYA. Northern analysis demonstrated that P450 transfection led to increased eNOS mRNA levels consistent with an effect at the pretranslational level. P450 epoxygense transfection resulted in increased eNOS activity as measured by the conversion of L-arginine to L-citrulline. Addition of synthetic EETs (50-200nM) to the culture media also increased eNOS expression and activity. Treatment with MAPK, MAPK kinase, and protein kinase C inhibitors apigenin, PD98059 and H-7, respectively, significantly inhibited the effects of P450 transfection on eNOS expression. Overexpression of P450 epoxygenases or addition of synthetic EETs increased Thr495 phosphorylation of eNOS, an effect that was inhibited by both apigenin and PD98059. Overexpression of P450 epoxygenases in rats resulted in increased aortic eNOS expression providing direct evidence that EDHF can influence vascular eNOS levels *in vivo*. Based on this data, we conclude that EDHF upregulates eNOS via activation of MAPK and PKC signaling pathways.

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Vascular endothelial cells control vascular tone and modulate blood flow to organs by synthesizing and releasing the vasoactive autocoids endothelium-derived relaxing factor (EDRF), which is synonymous with nitric oxide (NO), and prostacyclin (PGI_2) (Furchgott and Zawadzki, 1980; Palmer et al., 1987). Among these, NO probably plays a more important role. In vascular endothelium, NO is produced by a constitutively expressed enzyme known as endothelial nitric oxide synthase (eNOS) which converts L-arginine to L-citrulline (Vallance et al., 1989; Moncada and Higgs, 1993). In addition to endothelium-dependent vasodilatation, NO also has a number of other critical functions in the vascular system including inhibition of platelet aggregation, inhibition of endothelial cell adhesion molecule expression, prevention of vascular smooth muscle cell migration and proliferation, and prevention of intravascular coagulation and thrombosis (Azuma et al., 1986; Freedman et al., 1997; De Caterina et al., 1995; Jeremy et al., 1999; Loscalzo, 2001). Therefore, NO is an important factor in the maintenance of normal vascular homeostasis and the protection of vessels from injuries induced by atherogenic processes such as smooth muscle cell proliferation, platelet aggregation, monocyte adhesion and oxidative modification of low density protein (LDL) (Moncada and Higgs, 1993; Cannon, 1998).

In addition to NO and PGI_2, endothelial cells synthesize and release a third factor called endothelium-derived hyperpolarizing factor (EDHF) which causes hyperpolarization of underlying vascular smooth muscle cells via activation of Ca^{2+}-activated K^+ channels (Cohen and Vanhoutte, 1995; Harder et al., 1995). EDHF-dependent actions may serve as a back-up to NO-dependent vasodilatory mechanisms, but EDHF appears to be a more important regulator of vascular tone under

certain pathologic conditions, and in certain vascular beds such as in the coronary microcirculation (Najibi and Cohen, 1995; Oltman et al., 1998). Epoxyeicosatrienoic acids (EETs), which are cytochrome P450 epoxygenase-derived metabolites of arachidonic acid, display many of the characteristics of EDHF and are thought to be potential candidates for EDHF (Cohen and Vanhoutte, 1995; Harder et al., 1995; Gebremedhin et al., 1998). Indeed, transfection of endothelial cells with an antisense oligonucleotide to a P450 arachidonic acid epoxygenase (CYP2C8/34) results in attenuation of EDHF-mediated vascular responses and treatment with the P450 inducer, β-naphthaflavone, enhances EDHF-mediated vasorelaxation (Fisslthaler et al., 1999).

Both NO and EDHF are important vasodilatory autocoids released by the endothelium, but their mechanisms of action are quite different. Remarkably little is known regarding potential interactions between these two important vascular mediators and the enzymes that are responsible for their biosynthesis. A previous study has demonstrated that NO attenuates the synthesis and/or release of EDHF (Bauersachs et al., 1996); however, the effect of EDHF on eNOS expression and activity in endothelial cells has not been investigated. In the present study, we used cultured bovine aortic endothelial cells (BAECs) to study the effects of EDHF on the eNOS pathway. We found that transfection with several different P450 epoxygenases or addition of physiologic concentrations of synthetic EETs resulted in increased eNOS expression and activity. Moreover, the effects of EDHF on eNOS expression involve activation of both mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) signal transduction pathways.

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Materials and Methods

Materials. All standard cell culture reagents were obtained from GibcoBRL (Life Technologies, Inc., Grand Island, NY) including Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS). PD98059, Apigenin and H-7 were supplied by Calbiochem (Calbiochem-Novabiochem Corp., Darmstadt, Germany). [^{3}H]L-arginine was supplied by New England Nuclear (Boston, MA). Anti-eNOS and anti-phospho-eNOS (Thr495) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ERK1/2 (p44/42 MAPK) and anti-phospho-ERK1/2 or were purchased from New England Biolabs (Beverly, MA). Anti-CYP2C11 and anti-CYP102 antibodies were a generous gift from Dr. Jorge Capdevila (Vanderbilt University, Nashville, TN). Anti-CYP2J2 antibodies were prepared as previously described (Wu et al.,1996). Enhanced chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate) was purchased from Pierce (Rockford, IL). Hybrisol solution was purchased from Intergen (Purchase, NY). PVDF and nylon membranes were purchased from Schleicher and Schuell (Dassel, Germany). The PathDetect™ Signal Transduction Pathway *trans*-Reporting System and the Primer-It II Random Primer Labeling Kit were purchased from Stratagene (Kinsport, TN). [α-^{32}P]dCTP (3000μCi/mmol) was purchased from YaHui Nuclear (Beijing, China). Superfect cell transfection reagent was supplied by Qiagen (Hilden, Germany). The full-length cDNA of human eNOS was a generous gift from Dr. James K. Liao (Harvard University, Boston, MA). The CYP102 F87V mutant cDNA and a cDNA encoding rat CYP2C11 fused to rat NADPH-cytochrome P450 oxide reductase (CYPOR) (Helvig and Capdevila, 2000) were kindly provided by Dr. Jorge Capdevila (Vanderbilt University, Nashville, TN). All other

chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise specified.

Isolation and Culture of Endothelial Cells. Fresh bovine thoracic aortas were obtained at a local slaughterhouse. BAECs were harvested using trypsin (0.25%) and grown to confluence in a growth medium containing DMEM supplemented with 5 mM L-glutamine, 10% FBS and an antibiotic mixture of penicillin (100 units/ml) and streptomycin (100 µg/ml). The identification of cultured cells as endothelial cells was performed by examination for the typical morphology using phase-contrast microscopy and by immunofluorescent staining for factor VIII-related antigen. All passages were performed using 0.05% trypsin and 0.02% EDTA. Only endothelial cells passaged less than four times were used for experiments.

Construction and Preparation of Recombinant Adeno-associated Virus. The recombinant adeno-associated virus (rAAV) vector $pXXUF_1$, packaging plasmid pXX_2, adenovirus helper plasmid pXX_6, and a rAAV plasmid containing the GFP cDNA (GFP-pUF_1) were a generous gift from Dr. Xiao Xiao (University of Pittsburgh, Pittsburgh, PA). The coding regions of CYP102 F87V mutant, CYP2C11-CYPOR and CYP2J2 were subcloned into $pXXUF_1$ downstream from the CMV promoter to produce the constructs CYP102 F87V-pUF_1, CYP2C11-CYPOR-pUF_1 and CYP2J2-pUF_1, respectively. The rAAV was made as previously described (Xiao et al., 1996; Xiao et al., 1998). Briefly, human 293 cells were grown in DMEM supplemented with 10% FBS and antibiotics. One to two hours before transfection, each 15 cm diameter plate of cells (70-80% confluent) was fed with 15 ml of fresh medium. A total of 85 µg of plasmid DNA (molar ratio pUF_1:pXX_2:pXX_6 = 1:1:1) was added to a $CaCl_2$ solution (final concentration, 125 mM)

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and then quickly mixed with BES buffer (final concentration, 25 mM N,N-bis[2-Hydroxyethyl]-2-aminoethane sulfonic acid, 140 mM NaCl, 0.75 mM Na_2HPO4, pH 7.0). The resultant DNA/calcium/BES mixture was incubated at room temperature for 30 min before adding to the cells. After 8-12 hours, the medium containing the DNA/calcium/BES mixture was replaced with fresh medium. After 48-72 hours, the cells were harvested by centrifugation. Cell pellets were resuspended in 1-2 ml of 100 mM NaCl/10 mM Tris-HCl (pH 8.5), subjected to four freeze-thaw cycles and cell debris was removed by centrifugation. For large-scale rAAV preparations, 40 plates, each containing ~5 x 10^6 cells, were used and a single-step gravity-flow column purification method was carried out as described previously (Auricchio et al., 2001). The titer of rAAV was determined by dot blot hybridization. The eluted rAAV was aliquoted and stored at -80^{o}C. The resultant rAAVs were designated rAAV·CYP102 F87V, rAAV·CYP2C11-CYPOR, rAAV·CYP2J2 and rAAV·GFP, respectively.

Overexpression of P450 Epoxygenases in Endothelial Cells. The coding regions of CYP102 F87V mutant, CYP2C11-CYPOR and CYP2J2 were cloned into the Kpn I and Xba I, Cla I, and XbaI and Hind III sites of the mammalian expression vector pCB6 to produce the constructs CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6 and CYP2J2-pCB6, respectively. Bovine rather than human endothelial cells were used for transient transfection because of their higher transfection efficiency (Peng et al., 1995). Cultured endothelial cells were seeded into six-well plates and were grown to ~60% confluence. Cells were then transfected using Superfect Transfection Reagent exactly according to the manufacturer's instructions. After 2-3 hours, the DNA-Superfect mixture was removed and the cells were incubated continuously in the medium containing 10%

FBS for 48 hours. After that, the cells were processed for immunoblotting, northern analysis, eNOS activity assay, EET measurements, and analysis of signal transduction pathways (see below). In some experiments, BAECs were infected with rAAV·CYP102 F87V, rAAV·CYP2C11-CYPOR, rAAV·CYP2J2 or rAAV·GFP (~50 virions/cell), and the cells were processed one week later.

Animals and Treatment. Male Sprague-Dawley rats (200-250 g) were fed standard laboratory chow *ad lib*, given free access to tap water, and maintained on a 12 hour light-dark cycle at constant temperature and humidity. All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the local animal care and use committee. Rats (N=6 per group) were injected intravenously via the tail vein with either vehicle, empty pCB6 vector, CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6 or CYP2J2- pCB6 (5 mg DNA/kg body weight). After 2 weeks, animals were sacrificed by decapitation, and thoracic aortas were harvested, cleaned in PBS from adjacent tissues, snap-frozen in liquid nitrogen, and stored at -80°C until processing.

Nitric Oxide Synthase Activity Assay. NO synthase activity was determined in cell homogenates by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as previously described with minor modifications (Ramasamy et al.,1998). Forty-eight hours after transfection, the cells were washed twice with ice-cold phosphate-buffered saline and harvested by scraping in 200 µl HEPES buffer (20 mM, pH 7.2) containing 0.2 M sucrose, 1 mM EDTA, 1 mM DTT, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM PMSF. In some studies, endothelial cells were pretreated with the NOS inhibitor L-NMMA (1 mM) for 15 min at room temperature. The cells were then homogenized, with a Kontes

MicroUltrasonic Cell Disrupter (Kontes, Vineland, NJ) and the homogenates were used for NOS activity assay. Each sample (200 µl) was added to 50 ul of a medium containing 20 µM L-arginine, 10 µg/ml calmodulin, 3 mM $CaCl_2$, 1 mM NADPH, 10 µCi [^{3}H]L-arginine and was incubated at 37°C for 30 min. The reaction was quenched by addition of 500 µl of ice-cold stop buffer (20 mM HEPES, 2 mM EDTA). The total reaction mixture was loaded onto a column containing 2 ml of Dowex 50WX-8 resin (preequilibrated with NaOH) followed by elution of [^{3}H]L-citrulline with 2 ml of water. Aliquots (100 µl) of the eluents were counted in a liquid scintillation counter (Beckman). The net radioactivity was determined by subtracting the counts per minute observed in the presence of L-NMMA from those observed in the absence of L-NMMA. The protein concentration in the homogenates was measured spectrophotometrically by the method of Bradford. NOS activity was determined as the production of [^{3}H]L-citrulline per minute per mg protein.

Western Blotting. Forty-eight hours after plasmid transfection, or one week after rAAVs infection, or 24 hours after addition of 17-ODYA, PD98059, apigenin or H-7, or 2-12 hours after addition of 14,15-EET, 11,12-EET or 8,9-EET, BAECs were washed twice with ice-cold phosphate-buffered saline and then lysed with an ice-cold buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% NaNS, 0.1% SDS, 1 µg/ml aprotinin, 100 µg/ml PMSF, 1% NP-40 and 0.05% deoxycholic acid. Lysates were centrifuged at 12000g for 2 min and supernatants were used for western blot analysis. Frozen aortas were homogenized individually in the same buffer and centrifuged as above. Protein concentrations of the lysates were determined by the method of Bradford. 10 µg of protein per lane and prestained molecular weight markers (BioRad, Hercules, CA) were separated by SDS/PAGE (8% running, 4% stacking gel). The proteins were

electrophoretically transferred onto PVDF membranes overnight at 4°C. The membranes was incubated at room temperature for 2 hours with a blocking solution containing 5% non-fat dried milk, 100 mM Tris-Cl (pH 7.5) and 0.1%Tween-20 (TBS-T). The membrane was incubated overnight at 4°C with a 1:500 dilution of polyclonal rabbit anti-eNOS and then treated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Lab, West Grove, PA) at room temperature for 2 hours. After incubation with each antibody, the membrane was washed four times with TBS-T at room temperature. Immunodetection was accomplished by using chemiluminescent substrate and then exposure to x-ray film. For some experiments, rabbit anti-phospho-eNOS (1:750 dilution), rabbit anti-phospho-ERK1/2 (1:1000 dilution), rabbit anti-ERK1/2 (1:1000 dilution), rabbit anti-CYP2C11 (1:1000 dilution), rabbit anti-CYP102 (1:1000 dilution) or rabbit anti-CYP2J2 (1:1000 dilution) were used instead of the anti-eNOS primary antibody.

Northern Blotting. Forty-eight hours after transfection, total RNA from endothelial cells was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Equal amounts of total RNA (10 µg/lane) were separated on formaldehyde-1.2 % agarose gels, transferred overnight onto nylon membranes by capillary action, and baked for 2 hours at 80°C prior to prehybridization for 2 hours at 42°C in Hybrisol solution. Radiolabeling of the human eNOS and human GAPDH cDNAs were performed using a Random Primer Labeling Kit and [α-^{32}P]dCTP. The membrane was hybridized with the probes overnight at 42°C in Hybrisol solution. Northern blots were subjected to stringent washing conditions (0.1 X SSC, 0.1% SDS at 42°C) prior to autoradiography with an intensifying screen at –80°C for up to 5 days.

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Measurement of Endogenous EET levels. Forty-eight hours after transfection, endothelial cells were harvested by scraping, rapidly frozen in liquid nitrogen, and maintained at -80^{o}C until analysis. Methods to quantify endogenous EETs in endothelial cells have been previously reported (Node et al., 2001). Briefly, 1.5-2.0 x 10^{7} cells were homogenized in phosphate-buffered saline, extracted under acidic conditions with chloroform/methanol and extracts evaporated in tubes containing [1-^{14}C]EET internal standards (57 μCi/μmol, 30 ng each). Saponification to recover phospholipid-bound EETs was followed by silica column purification, reverse-phase high-pressure liquid chromatography separation of individual EETs, derivatization to corresponding EET-pentafluorobenzyl esters, normal-phase high pressure liquid chromatography purification of derivatized EETs, and gas chromatography/mass spectrometry analysis with selected ion monitoring at *m/z* 319 and *m/z* 321.

Analysis of Signal Transduction Pathways. A PathDetect™ Signal Transduction Pathway *trans*-Reporting System was used according to the manufacturer's instructions to examine whether CYP102 F87V transfection altered MAPK signal transduction pathways. Cultured BAECs were seeded into six-well plates, grown to ~60% confluence and co-transfected using Superfect Transfection Reagent with pFA-ELK$_1$, pFR-Luc and either pCB6-CYP102 F87V or empty pCB6 vector, or positive and negative control plasmids included in the system. If there is activation of MAPK pathway after pCB6-CYP102 F87V transfection, phosphorylation of ELK$_1$ will activate transcription of the luciferase gene from the reporter plasmid (pFR-Luc). Luciferase levels were determined on a luminometer (Lumat LB 9507, Bad Wildbad, Germany) using a Luciferase Assay Kit (Stratagene), and reflect the activation status of the MAPK pathway.

Results

Effect of P450 Transfection on eNOS Expression. Relatively pure (>95%) bovine aortic endothelial cell cultures were confirmed by their morphological features (i.e., cuboidal, cobblestone appearance; contact inhibited) using phase-contrast microscopy and by immunofluorescent staining with antibodies to factor VIII-related antigen (data not shown).

Previous studies have demonstrated that CYP102 F87V and CYP2C11-CYPOR are active arachidonic acid epoxygenases which biosynthesize the EETs (Helvig and Capdevila, 2000; Graham-Lorence et al., 1997; Qu et al.,1998; Capdevila et al.,1992). Transfection of BAECs with CYP102 F87V-pCB6 or CYP2C11-CYPOR-pCB6 resulted in abundant expression of the corresponding recombinant P450 proteins as determined by immunoblotting with specific P450 antibodies (Figure 1A). To examine the effect of overexpression of these P450s on eNOS protein expression *in vitro*, BAECs were transfected with either CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6 or the empty pCB6 vector. Forty-eight hours after transfection, cells were lysed, and eNOS protein levels were determined by immunoblotting using a specific eNOS antibody. As shown in Figure 2A, transfection of BAECs with either CYP102 F87V-pCB6 or CYP2C11-CYPOR-pCB6 resulted in a marked increase in eNOS protein expression compared to transfection with the empty pCB6 vector. Treatment of the cells with the P450 epoxygenase inhibitor 17-ODYA (50 μm and 100 μm) significantly attenuated the upregulatory effect of P450 transfection on eNOS protein expression (Figure 2A). We have previously shown that CYP2J2 is abundant in endothelial cells and likely contributes significantly to the epoxidation of endogenous arachidonic acid pools (Wu et al., 1996;

Node et al., 1999; Node et al., 2001). To examine the effect of overexpression of this endothelial epoxygenase on eNOS expression, we also transfected BAECs with the CYP2J2-pCB6 vector. Transfection of BAECs with CYP2J2-pCB6 resulted in expression of the recombinant CYP2J2 protein (Figure 1A). Expression of CYP2J2 was slightly lower than that of CYP2C11 and CYP102 F87V. As shown in Figure 2B, CYP2J2 transfection also resulted in a significant increase in eNOS protein levels. Densitometric analysis of immunoblots from multiple independent experiments normalized to β-actin expression revealed that CYP102 F87V, CYP2C11-CYPOR and CYP2J2 transfection were associated with a 5.1-, 5.8- and 2.8-fold induction of eNOS protein compared to transfection with pCB6 alone ($p<0.05$) (Figure 2C).

To examine if the effect of P450 transfection on eNOS expression occurred at the pretranslational level, total RNA was extracted from the BAECs and eNOS mRNA levels were assessed by northern analysis using a specific cDNA probe. As shown in Figure 3A, eNOS mRNA levels were significantly increased after CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6 and CYP2J2-pCB6 transfection compared to transfection with the empty pCB6 vector. Densitometric analysis of blots from separate experiments normalized to GAPDH signals to control for loading differences revealed that P450 transfection was associated with a 2- to 3-fold increase in eNOS mRNA levels ($P<0.05$) (Figure 3B). Consistent with the immunoblotting data, CYP2J2 induced eNOS mRNA to a slightly lesser extent than did CYP102 F87V and CYP2C11-CYPOR.

Effect of P450 Transfection on eNOS Activity. To examine if the changes in eNOS mRNA and protein were accompanied by corresponding changes in eNOS activity, we measured the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in P450 transfected

BAECs. Compared to cells transfected with the empty pCB6 vector, cells transfected with either CYP102 F87V-pCB6 or CYP2C11-CYPOR-pCB6 exhibited ~3-fold more eNOS activity ($p<0.05$) (Figure 4).

Effect of Synthetic EETs on eNOS Protein Expression. Cultured endothelial cells were seeded into six-well plates and were grown to ~80% confluence. The cells were treated with physiologically relevant concentrations (50 to 200 nM) of 8,9-EET, 11,12-EET and 14,15-EET or corresponding vehicle (ethanol) for 4 hours. As shown in Figure 5A, addition of exogenous EETs resulted in a significant, dose-dependent increase in eNOS protein levels. Each of the EET regioisomers tested was active in increasing eNOS protein expression. Densitometric analysis of immunoblots from multiple independent experiments normalized to β-actin expression revealed that 8,9-EET, 11,12-EET and 14,15-EET (200 nM) were associated with a significant 2.6-, 2.5- and 3.3-fold increase in eNOS protein expression compared to vehicle ($p<0.05$) (Figure 5B). The induction of eNOS by 8,9-EET and 11,12-EET (200 nm) occurred in a time-dependent manner up to 4 hours (Figure 5C). eNOS expression returned to basal levels 12 hours after EET treatment (data not shown). In agreement with the immunoblotting data, addition of exogenous EETs resulted in increased conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline ($p<0.05$) (Figure 5D). There were no statistically significant differences in the magnitude of conversion by the three EET regioisomers.

Effect of P450 Transfection on Endothelial EET levels. We have previously shown that control BAECs rapidly metabolize exogenous arachidonic acid to EETs (Node et al., 1999) and contain 8.0 ng of total endogenous EET per 10^7 cells (~50 nM total EET) (Node et al., 2001). These levels are similar to those used in the experiments in Figure 5

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which examine effects of exogenous EETs on eNOS expression and activity. We have also shown that CYP2J2-transfected endothelial cells exhibit a 2-fold increase in arachidonic acid epoxygenase activity (Node et al., 1999) and contain significantly increased levels of EETs (Node et al., 2001) compared to control endothelial cells. For example, 14,15-EET levels increased 5% and 11,12-EET levels increased 35% after transfection with CYP2J2. To document that CYP2C11-CYPOR transfected cells also have increased arachidonic acid epoxygenase activity, we measured levels of EETs by gas chromatography/mass spectometry in BAECs under control conditions and after transfection with CYP2C11-CYPOR. Consistent with our previously published data, control endothelial cells contained 8.7 ng of total endogenous EET per 10^7 cells (~55nM). After transfection with CYP2C11, there was a 9% increase in 14,15-EET and a 46% increase in 11,12-EET. This data is consistent with the known regiochemical selectivity of CYP2C11 (Qu et al., 1998) and the presence of an active endothelial epoxide hydrolase with preference for metabolism of 14,15-EET (Zeldin et al., 1993; Fang et al., 2001). The concentration of endogenous EETs in CYP2J2 and CYP2C11 transfected endothelial cells are also within the range used in the experiments in Figure 5. Moreover, these data are consistent with the greater degree of eNOS induction by CYP2C11-CYPOR compared to CYP2J2 (Figure 2B and 2C).

Effect of P450 Epoxygenase Products on eNOS Phosphorylation. Previous studies have demonstrated that eNOS is regulated by phosphorylation which can influence enzyme activity, subcellular trafficking and interaction with other proteins (Garcia-Cardena et al., 1996; Matsubara et al., 1996; Chen et al., 1999; Butt et al., 2000; Fleming et al., 2001a; Michell et al., 2001). For example, phosphorylation of Thr495 has

been shown to deactivate eNOS by hindering binding to calmodulin (Fleming et al., 2001a; Aoyagi et al., 2003). Hence, we conducted experiments using an antibody that reacts with Thr495 phosphorylated eNOS (p-eNOS). Treatment of BAECs with each of the synthetic EETs (100 nM) significantly increased expression of p-eNOS (Figure 6A). Densitometric analysis of immunoblots from multiple independent experiments normalized to β-actin expression revealed that addition of 8,9-EET, 11,12-EET and 14,15-EET was associated with a 1.5-, 2.3- and 2.8-fold increase in p-eNOS expression compared to addition of vehicle ($p<0.05$) (Figure 6B). Moreover, rAAV-mediated overexpression of CYP2C11-CYPOR, CYP2J2 or CYP102 F87V in BAECs (Figure 1B) also significantly increased p-eNOS expression (Figure 7A). Densitometric analysis of immunoblots from multiple independent experiments normalized to β-actin revealed that P450 overexpression was associated with a 1.7- to 1.9-fold increase in p-eNOS expression compared to GFP control ($p<0.05$) (Figure 7B).

Effect of P450 Epoxygenase Overexpression on eNOS and p-eNOS Expression In Vivo. To confirm the physiological relevance of our findings, we conducted *in vivo* experiments in rats to determine the effects of P450 epoxygenase overexpression on vascular eNOS and p-eNOS expression. Rats were injected intravenously with CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6, CYP2J2-pCB6 or the empty pCB6 vector. Two weeks later, animals were sacrificed, aortas were harvested, and immunoblots were performed. Injection of the P450 expression vectors resulted in abundant aortic expression of the corresponding recombinant proteins as determined by immunoblotting with specific P450 antibodies (Figure 1C). Importantly, P450 overexpression was associated with significant increases in aortic eNOS and p-eNOS

levels (Figure 8A). Densitometric analysis of immunoblots from multiple independent experiments normalized to β-actin revealed that P450 overexpression resulted in a significant 1.5- to 2.2-fold increase in aortic eNOS expression and a significant 1.3- to 1.5-fold increase in aortic p-eNOS expression ($p<0.05$) (Figure 8B). These data provide direct evidence that P450 epoxygenases can influence eNOS and p-eNOS expression *in vivo*.

Effect of P450 Transfection on eNOS Expression Involves MAPK and PKC Signal Transduction Pathways. To investigate the involvement of various signaling pathways in the mechanism of P450-induced upregulation of eNOS expression, we pretreated BAECs with the MAPK inhibitor apigenin (25 μm), the MAPK kinase inhibitor PD98059 (20 μm) or the PKC inhibitor H-7 (12 μm), and then evaluated eNOS protein levels by immunoblotting. Pretreatment with either PD98059 or apigenin inhibited both CYP102 F87V- and CYP2C11-CYPOR-induced upregulation of eNOS, thus suggesting involvement of the MAPK signal transduction pathway in this process (Figure 9A and 9B). Pretreatment with H-7 caused a small upregulation of eNOS, but significantly attenuated both CYP102 F87V- and CYP2C11-CYPOR-induced upregulation of eNOS, indicating involvement of the PKC signaling pathway as well (Figure 9C). Importantly, both apigenin and PD98059 significantly limited induction of eNOS by synthetic 14,15-EET (Figure 10A and 10B). Both apigenin and PD98059 also inhibited the increased expression of p-eNOS caused by treatment with 14,15-EET (Figure 10A and 10B). These data provide evidence to support involvement of the MAPK pathway in EET-mediated induction and phosphorylation of eNOS.

The PathDetect™ Signal Transduction Pathway *trans*-Reporting System was used

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to confirm the activation status of MAPK pathway. BAECs were co-transfected with pFA-ELK$_1$, pFR-Luc, and either CYP102 F87V-pCB6 or empty pCB6 vector. As shown in Figure 11A, transfection with CYP102 F87V resulted in a significant increase in luciferase activity compared to transfection with the empty pCB6 vector ($p<0.05$). Since activation of MAP kinases occurs through phosphorylation, we also examined the effect of P450 epoxygenase transfection on the phosphorylation status of MAPK. Forty-eight hours after transfection with either CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6, CYP2J2-pCB6 or empty pCB6 vector, cells were lysed and levels of total or phosphorylated p44/42 MAPK were determined by immunoblotting using antibodies to p44/42 MAPK or phospho-p44/42 MAPK, respectively. As shown in Figure 11B, transfection of BAECs with the three P450 epoxygenases resulted in increased levels of phosphorylated p44/42 MAPK but no significant change in levels of total p44/42 MAPK. Densitometric analysis revealed a 35-45% increase in phosphorylated p44/42 MAPK in P450 transfected cells relative to empty pCB6 vector transfected cells ($P<0.05$) (Figure 11C). Together, these data demonstrate conclusively that P450-induced upregulation of eNOS involves the MAPK signal transduction pathway.

Discussion

In addition to NO and PGI_2, vascular endothelial cells release a third factor termed EDHF which causes hyperpolarization and relaxation of underlying vascular smooth muscle cells (Cohen and Vanhoutte, 1995; Harder et al.,1995). Recent studies from a number of laboratories have confirmed that cytochrome P450 metabolites of arachidonic acid display many of the characteristics of EDHF and are potential candidates for mediating EDHF vascular responses (Cohen and Vanhoutte, 1995; Harder et al., 1995; Gebremedhin et al., 1998; Lu et al., 2001). Cytochrome P450 epoxygenases convert arachidonic acid into four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET) which are converted by epoxide hydrolases to corresponding DHET regioisomers (Capdevila et al., 1992; Fitzpatrick and Murphy, 1988). EETs and/or DHETs have been shown to possess a number of potent biologic activities including effects on peptide hormone secretion, cardiac function, and renal function (Lu et al., 2001; Capdevila et al., 1992; Fitzpatrick and Murphy, 1988; Zeldin, 2001). The EETs have been reported to be potent mitogens which mediate the effects of epidermal growth factor via a mechanism that involves activation of *Src* kinase and initiation of a tyrosine kinase phosphorylation cascade (Chen et al., 1999). Recently, Node et al. have reported that EETs decrease cytokine-induced endothelial cell adhesion molecule expression via inhibition of NF-κB and prevent leukocyte adhesion to the vascular wall (Node et al., 1999). EETs also increase tissue plasminogen activator expression and fibrinolytic activity via activation of Gαs (Node et al., 2001). Hence, EETs possess homeostatic properties in the vasculature in addition to their vasodilatory actions.

EDRF, which is synonymous with NO, is produced from L-arginine by eNOS in the

vascular endothelium. Like EDHF, it plays a pivotal role in the maintenance of normal vascular homeostasis and the regulation of systemic blood pressure (Vallance et al., 1989; Moncada and Higgs, 1993). In addition, it is clear that NO, like the cytochrome P450 epoxygenase products, has a number of other important functions in the vessel wall, including inhibition of platelet aggregation and adhesion molecule expression, prevention of smooth muscle proliferation and modulation of vascular growth, and prevention of coagulation and thrombosis (Moncada and Higgs, 1993; Cannon, 1998). Thus, NO may be considered an antiatherogenic, antiproliferative, and antithrombotic factor. We hypothesized that since both EDHF (EETs and/or DHETs) and EDRF (NO) have similar functions within the vasculature, albeit through different mechanisms, interactions between NO and EDHF in endothelial cells might very well occur. Indeed, a previous study has demonstrated that NO attenuates the synthesis and/or release of EDHF (Bauersachs et al., 1996).

CYP102 (also called $P450_{BM-3}$), isolated from *Bacillus megaterium*, is an enzyme that contains both a cytochrome P450 domain and a reductase domain fused into a single polypeptide chain to form a self-contained catalytic unit (Fulco, 1991). The enzyme catalyzes the high turnover, NADPH-dependent metabolism of arachidonic acid to 18-hydroxyeicosatetraenoic acid (Graham-Lorence et al., 1997). The active site replacement of phenylalanine 87 with valine has been shown to convert CYP102 into a highly regio- and stereoselective arachidonic acid 14(R),15(S)-epoxygenase (99% optical purity) (Graham-Lorence et al., 1997). Similarly, we and others have previously reported that rat CYP2C11 is an active arachidonic acid epoxygenase that forms mixtures of 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET (Helvig and Capdevila, 2000; Qu et al.,

1998; Capdevila et al., 1992). In the present study, these P450s were used as pharmacologic tools to investigate the effects of enhanced endogenous EDHF biosynthesis on eNOS expression and activity in cultured bovine endothelial cells. However, neither CYP102 F87V nor CYP2C11-CYPOR are normally present within the vasculature; hence we also examined the effect of overexpression of the more functionally relevant CYP2J2, an endothelial arachidonate epoxygenase (Node et al., 1999; Node et al., 2001), on eNOS expression and activity. We found that transfection of BAECs with any of these P450s increased the expression of eNOS, both at the mRNA level and the protein level. The effects of CYP102 F87V and CYP2C11-CYPOR were generally more pronounced than that of CYP2J2, consistent with the relative catalytic efficiencies of these P450 enzymes. Moreover, transfection with CYP102 F87V or CYP2C11-CYPOR also increased eNOS activity as measured by the conversion of L-arginine to L-citrulline. Treatment with 17-ODYA, an inhibitor of cytochrome P450s, attenuated the upregulatory effect of P450 transfection on eNOS expression. This supports the hypothesis that the effects of P450 on eNOS expression are not due to a direct interaction between the two heme-containing proteins, but are rather due to an effect of a P450 metabolite on the relevant signaling pathways involved in regulation of eNOS levels. In this regard, we showed that addition of nanomolar concentrations of exogenous EETs to the BAECs also upregulated eNOS expression. Importantly, the biological effects of exogenously added, synthetic EETs occurred at concentrations similar to those which we have previously shown (and confirmed herein) to be present endogenously in BAECs (Node et al., 2001). Based on this data, we conclude that the effects of EETs on eNOS occur at physiologically relevant concentrations.

As discussed above, EETs play an important role in the regulation of vascular homeostasis. A number of studies suggest that EDHF may enhance the relaxing actions of NO but may not be *the primary* mediator of endothelium-dependent relaxation under normal physiological conditions and in most vascular beds (Cohen and Vanhoutte, 1995; Quilley et al., 1997). But, EDHF appears to be a more important regulator in some pathological conditions which are associated with impairment of the nitric oxide biosynthetic pathway such as during hypercholesterolemia (Najibi and Cohen, 1995; Kilpatrick and Cocks, 1994) and in some vascular beds such as the coronary microcirculation (Oltman et al., 1998). Indeed, Bauersachs et al. found that the production of EDHF is dampened by NO under physiological conditions and suggested that when NO synthesis is impaired, EDHF-dependent vasorelaxation may maintain endothelial vasodilator function, at least in part, by alleviating this intrinsic inhibition (Bauersach et al., 1996). Moreover, several studies have shown that the biosynthesis of EETs is increased under certain pathological conditions such as during hypercholesterolemia *in vivo* in rabbits and upon exposure of endothelial cells to oxidized LDL *in vitro* (Pfister et al., 1991; Pritchard et al., 1990). Importantly, a growing list of conditions, including those commonly associated with increased risk of atherosclerosis (e.g. hypertension, hypercholesterolemia and diabetes) are associated with diminished synthesis and/or release of NO (Cannon, 1998). Our finding herein that P450-derived EETs can upregulate eNOS suggest one potential compensatory mechanism for enhancing the production of NO in these conditions.

The EETs have been shown to play critical roles in regulating a variety of intracellular signaling pathways (Node et al., 1999; Node et al., 2001; Chen et al., 1999;

Chen et al., 1998; Rzigalinski et al., 1999). The findings herein that (a) the MAPK pathway is activated in CYP102 F87V transfected BAECs using a *trans*-Reporting System, (b) levels of the phosphorylated MAPK are increased in CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6 and CYP2J2-pCB6 transfected cells, and (c) inhibitors of MAPK, MAPK kinase and PKC can attenuate the upregulation of eNOS induced by P450 transfection or addition of synthetic EETs, suggest that the mechanism of EDHF-mediated upregulation of eNOS involves activation of both MAPK and PKC signal transduction pathways. In this regard, Fleming and co-workers have recently shown that EETs activate the MAPK pathway in human endothelial and vascular smooth muscle cells (Fleming et al., 2001b). Importantly, activation of the MAPK pathway by growth factors can stimulate eNOS expression (Zheng et al., 1999).

eNOS can be phosphorylated at multiple different sites by different kinases; phosphorylation at some of the sites leads to activation of eNOS and phosphorylation at other sites leads to deactivation (Garcia-Cardena et al., 1996; Matsubara et al., 1996; Chen et al., 1999; Butt et al., 2000; Fleming et al., 2001a; Michell et al., 2001). Recent studies have focused on the role of Thr495 phosphorylation in regulating eNOS activity following agonist stimulation (Fleming et al., 2001a; Aoyagi et al., 2003). Thr495 has been shown to be phosphorylated both *in vitro* and *in vivo* in endothelial cells by protein kinase C, AMP-activated protein kinase, and cyclic-nucleotide dependent protein kinases (Fleming et al., 2001a; Butt et al., 2000; Chen et al., 1999; Matsubara et al., 1996). Thr495 phosphorylation deactivates eNOS by hindering the binding of calmodulin (Fleming et al., 2001; Aoyagi et al., 2003). Our data indicate that P450-derived EETs increase Thr495 phosphorylation of eNOS and suggest that this occurs, at least in part,

via the MAPK pathway. Thus, EETs appear to have complex effects on eNOS expression and phosphorylation status. Importantly, the net effect of EETs is to increase eNOS activity. Future studies will examine the effects of P450-derived eicosanoids on phosphorylation of eNOS at other sites.

We present several lines of evidence to support our hypothesis that P450 metabolites of arachidonic acid can influence eNOS expression and activity. First, transfection of endothelial cells with three different P450 arachidonic acid epoxygenases (CYP2C11, CYP102 F87V and CYP2J2) induces eNOS mRNA, protein and activity. Second, the P450 inhibitor 17-ODYA significantly attenuates the effect of P450 epoxygenase transfection on eNOS expression. Third, treatment of endothelial cells with synthetic EETs, at levels that are physiologically relevant and comparable to those present endogenously in endothelial cells, results in the upregulation of eNOS protein and activity. Forth, induction of eNOS by P450 epoxygenases is associated with activation of the MAPK signaling pathway and inhibitors of this signaling pathway attenuate eNOS induction by P450. Fifth, inhibitors of MAPK also inhibit eNOS induction by synthetic EETs. Sixth, P450 epoxygenase transfection or treatment with synthetic EETs increase phosphorylation of eNOS at Thr495. Seventh, overexpression of P450 epoxygenases *in vivo* leads to increased expression of both eNOS and p-eNOS. Taken together, these data provide compelling evidence that important interactions exist between these two functionally relevant biosynthetic pathways.

In conclusion, this study demonstrates that transfection of BAECs with P450 epoxygenases *in vitro* or overexpression of P450 epoxygenases *in vivo* results in increased eNOS expression and increased phosphorylation of eNOS at Thr495. The net

effect in BAECs is enhanced eNOS activity. Treatment with a P450 inhibitor attenuates the upregulatory effect of P450 transfection on eNOS expression suggesting an effect of a P450 metabolite rather than a direct interaction between the two heme-containing proteins. Application of physiological concentrations of synthetic EETs to BAECs also increases eNOS and p-eNOS expression. The upregulation of eNOS by P450 epoxygenase products involves activation of both MAPK and PKC signaling pathways. Together, these data provide new insight into the regulation of endothelial cell function by EDHF and highlight important interactions between two functionally relevant pathways. Given that cardiovascular and related disorders are frequently associated with endothelial dysfunction and diminished NO production, the present study provides a framework for development of novel therapeutics designed to improve endothelial function by modulating cytochrome P450 epoxygenase levels and/or activity.

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Footnotes

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Legends for Figures

Figure 1 - Expression of recombinant P450 proteins *in vitro* and *in vivo*. A, Immunoblots showing expression of recombinant P450 proteins in BAECs transfected with CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6, CYP2J2-pCB6, or the pCB6 empty vector; B, Immunoblots showing expression of recombinant P450 proteins in BAECs infected with rAAV·CYP102 F87V, rAAV·CYP2C11-CYPOR, rAAV·CYP2J2 or rAAV-GFP; C, Immunoblots showing expression of recombinant P450 proteins in aortas from rats injected with CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6, CYP2J2-pCB6, or the pCB6 empty vector. Each lane contains 20 μg of protein. The polyclonal antibodies used are specific to the respective P450 proteins. Molecular weights of the bands are ~125 kDa, 119 kDa and 57 kDa for CYP2C11-CYPOR, CYP102 F87V and CYP2J2, respectively.

Figure 2 - Effect of CYP102 F87V, CYP2C11-CYPOR and CYP2J2 transfection on eNOS protein expression. A, Immunoblot (10 μg protein/lane) showing the upregulatory effect of CYP102 F87V and CYP2C11-CYPOR transfection on eNOS protein expression in BAECs. The blot was stripped and re-probed with an antibody to β-actin. Treatment with the P450 inhibitor 17-ODYA (50 μm and 100 μm) attenuates the upregulatory effect of P450 transfection on eNOS expression. Results are representative of five separate experiments. B, Immunoblot (10 μg protein/lane) showing the upregulatory effect of CYP2J2 transfection on eNOS protein expression in BAECs. The blot was stripped and re-probed with an antibody to β-actin. Results are representative of three independent experiments. C, The blots were scanned and relative eNOS protein levels normalized to β-actin were determined. Values shown are the means ± S.E. of

three to five independent experiments. * p<0.05 vs. transfection with pCB6 empty vector.

Figure 3 - **Effect of CYP102 F87V, CYP2C11-CYPOR and CYP2J2 transfection on eNOS mRNA levels.** A, Northern blots (10 μg total RNA/lane) showing that eNOS mRNA levels are increased in response to transfection of BAECs with CYP102 F87V, CYP2C11-CYPOR and CYP2J2 constructs. RNA loading was determined by hybridization to human GAPDH. Results shown are representative of four separate experiments. B. Autoradiographs were scanned and relative eNOS mRNA levels normalized to GAPDH were determined. Data shown are mean ± S.E. of four separate experiments. * p<0.05 vs. transfection with pCB6 empty vector.

Figure 4 - Effect of CYP102 F87V and CYP2C11-CYPOR transfection on eNOS activity. The eNOS activity was determined by [^{3}H]L-arginine to [^{3}H]L-citrulline conversion as described in Materials and Methods. The experiments were performed three times and the data shown are mean ± S.E. * p<0.05 vs. transfection with pCB6 empty vector.

Figure 5 - Effect of synthetic EETs on eNOS protein levels and activity. A, BAECs were pretreated with various concentrations (0-200 nM) of 8,9-EET, 11,12-EET or 14,15-EET for 4 hours. Cells were then lysed and subjected to protein immunoblotting. The results shown are representative of three independent experiments. Each of the EET regioisomers was active in increasing eNOS protein expression. B, The blots were scanned and relative eNOS protein levels normalized to β-actin were determined. Values shown are the means ± S.E. of three independent experiments. C, The induction of eNOS by 8,9-EET and 11,12-EET (200 nM each) occurred in a time-dependent manner up to 4 hours. D, After treatment with synthetic EETs, eNOS activity was determined by

conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. The experiments were performed three times. * p<0.05 vs. vehicle control.

Figure 6 – **Effect of synthetic EETs on eNOS phosphorylation.** A, Immunoblots (10 μg protein/lane) showing that treatment of the BAECs with 8,9-EET, 11,12-EET or 14,15-EET (100 nM each) increases both eNOS and p-eNOS expression. Results are representative of three separate experiments. B, The blots were scanned and relative eNOS or p-eNOS levels normalized to β-actin expression were determined. Values shown are the means ± S.E. of three independent experiments. ^ p<0.05 vs. vehicle p-eNOS; * p<0.05 vs. vehicle eNOS; ** p<0.01 vs. vehicle eNOS.

Figure 7 – **Effect of P450 epoxygenase transfection on eNOS phosphorylation.** A, Immunoblots (10 μg protein/lane) showing that infection of BAECs with rAAV·CYP102 F87V, rAAV·CYP2C11-CYPOR or rAAV·CYP2J2 increases both eNOS and p-eNOS expression. Results are representative of three separate experiments. B, The blots were scanned and relative eNOS or p-eNOS levels normalized to β-actin were determined. Values shown are the means ± S.E. of three experiments. ^ p<0.05 vs. rAAV·GFP p-eNOS; * p<0.05 vs. rAAV·GFP eNOS; ** p<0.01 vs. rAAV·GFP eNOS.

Figure 8 – **Effect of P450 epoxygenase overexpression on eNOS and p-eNOS expression *in vivo*.** A, Immunoblot (10 μg protein/lane) showing the effect of overexpression of CYP102 F87V, CYP2C11-CYPOR and CYP2J2 on eNOS and p-eNOS expression in vivo in rats. After intravenous injection of CYP102 F87V-pCB6, CYP2C11-CYPOR- pCB6 and CYP2J2-pCB6 into rats, the aortic expression of eNOS and p-eNOS increased. B, Densitometric analysis of immunoblots from six rats per group normalized to β-actin expression. ^ p<0.05 vs. pCB6 empty vector p-eNOS; * p<0.05 vs.

pCB6 empty vector eNOS.

Figure 9 – Roles of MAPK and PKC signaling pathways in induction of eNOS by P450 epoxygenases. A, The MAPK kinase inhibitor PD98059 attenuates the CYP102 F87V- and CYP2C11-CYPOR-induced eNOS upregulation. B, The MAPK inhibitor apigenin attenuates the CYP102 F87V- and CYP2C11-CYPOR-induced eNOS upregulation. C, The PKC inhibitor H-7 attenuates the CYP102 F87V- and CYP2C11-CYPOR-induced eNOS upregulation.

Figure 10 - Roles of MAPK on induction and phosphorylation of eNOS by 14,15-EET. A, The MAPK kinase inhibitor apigenin and the MAPK kinase inhibitor PD98059 both attenuate 14,15-EET-induced expression of eNOS and p-eNOS. Data shown are representative of three separate experiments. B, Densitometric analysis of immunoblots normalized to β-actin expression. Values shown are the means ± S.E. of three independent experiments. ^ $p<0.05$ vs. 14,15-EET alone p-eNOS; * $p<0.05$ vs. 14,15-EET alone eNOS.

Figure 11 – **Activation of the MAPK pathway by P450 epoxygenases.** A, The activation status of MAPK pathway was assessed using the PathDetect™ Signal Transduction Pathway *trans*-Reporting System after transfection of BAECs with CYP102 F87V or the empty pCB6 vector. Experiments were performed three times. * $p<0.05$ vs. pCB6 empty vector. B, BAECs were transfected with CYP102 F87V-pCB6, CYP2C11-CYPOR-pCB6, CYP2J2-pCB6 or empty pCB6 vector. Forty-eight hours after transfection, cells were lysed, and the levels of phosphorylated ERK1/2 were assayed by immunoblotting using a phospho-ERK1/2 antibody (upper panel). The blots were then stripped and reprobed with an ERK1/2 antibody that recognizes both phosphorylated and

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unphosphorylated forms (lower panel). C, The blots were scanned and relative phosphorylated ERK1/2 protein levels were determined. Values shown are the means ± S.E. of three independent experiments. * $p<0.05$ vs. pCB6 empty vector.

Figure 1

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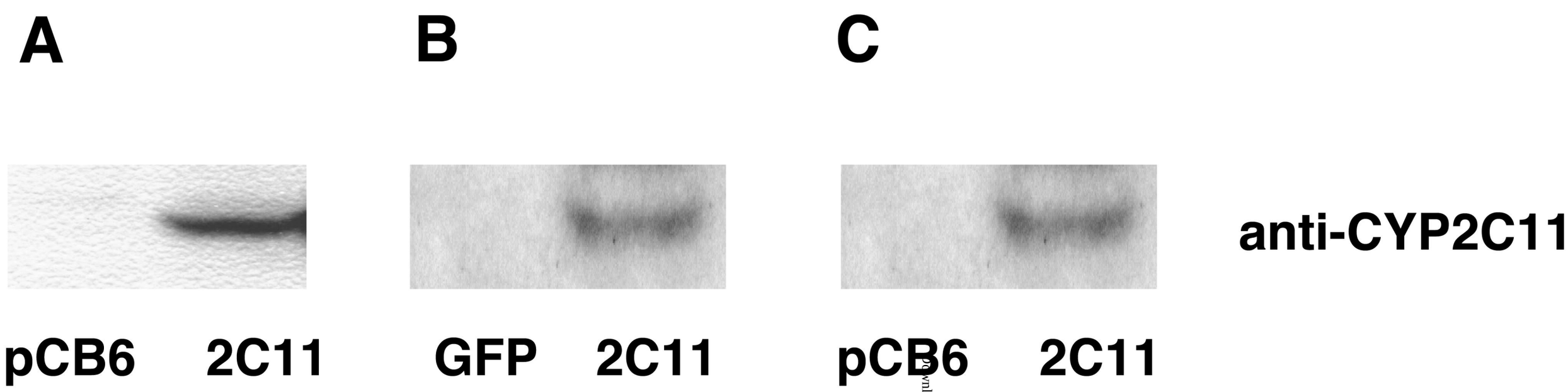

Figure 2

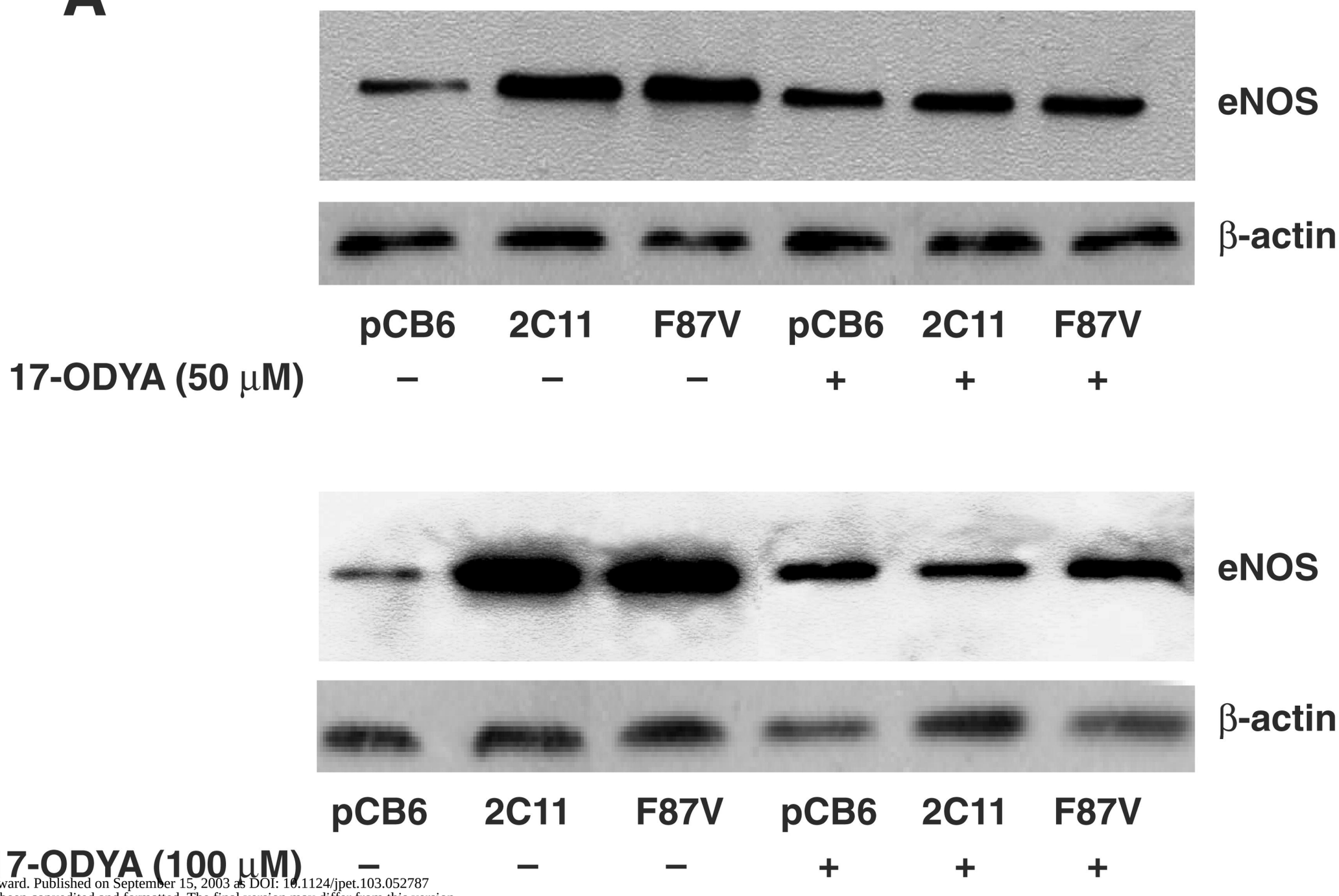

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Figure 3

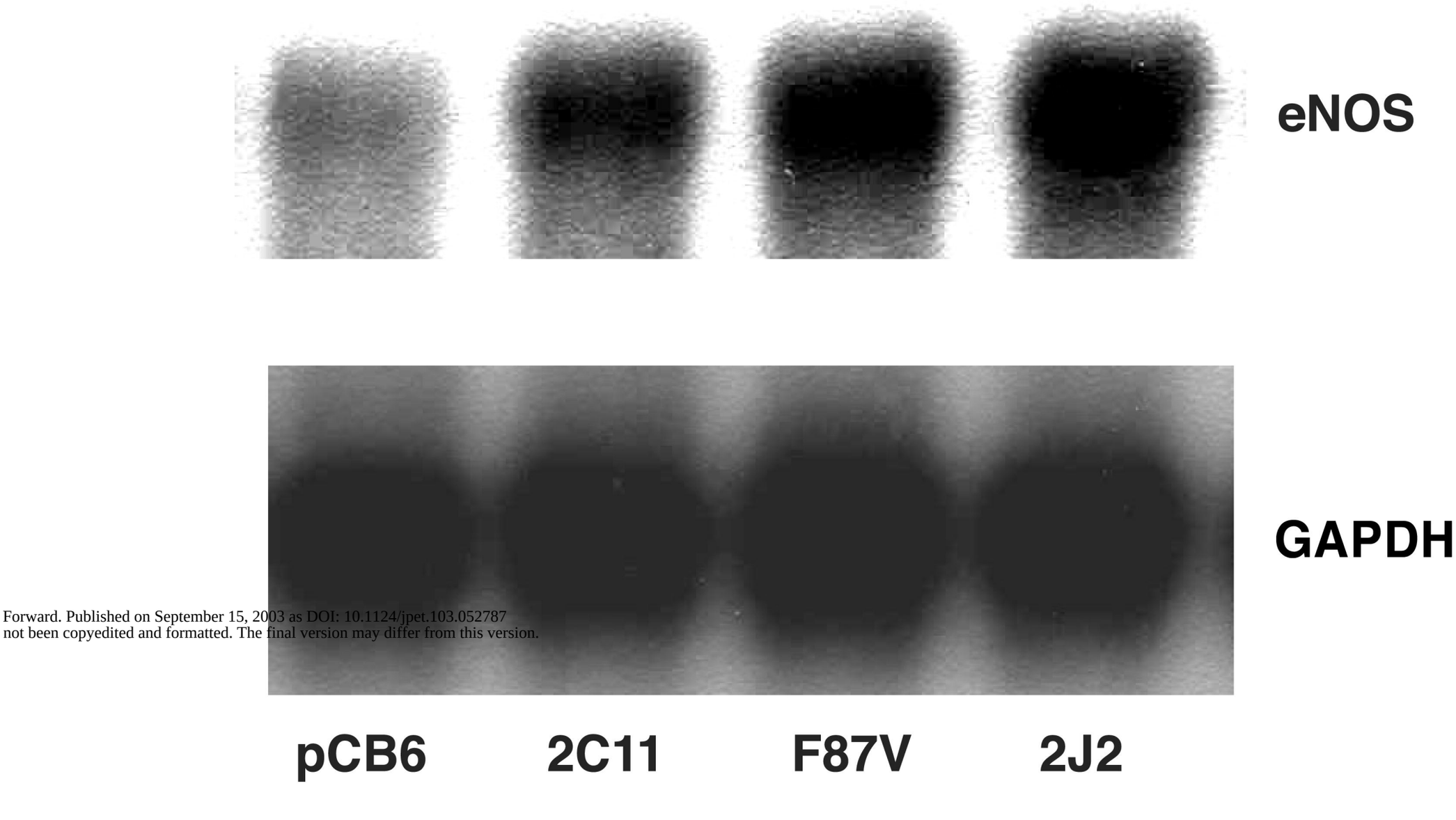

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Figure 4

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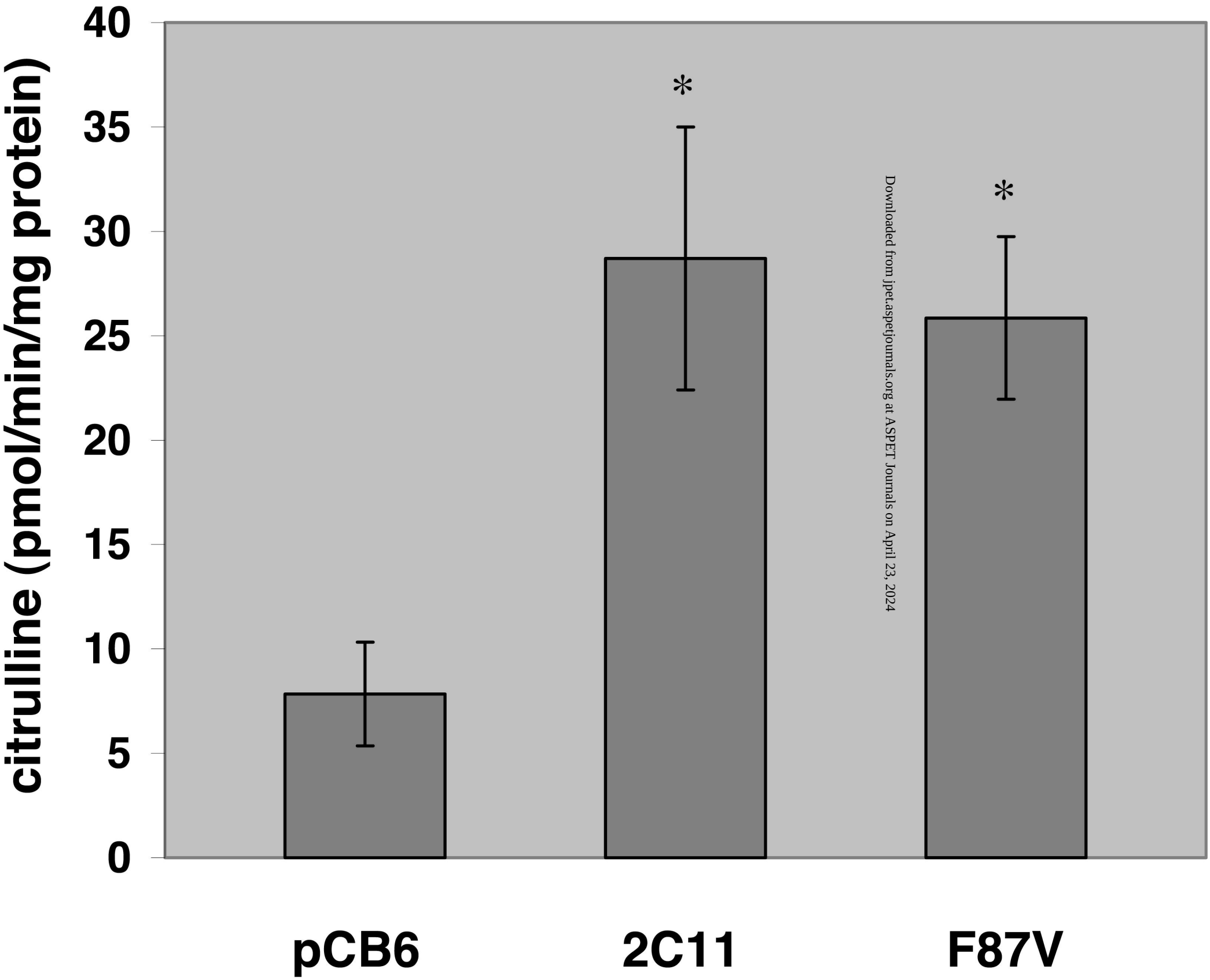

Figure 5

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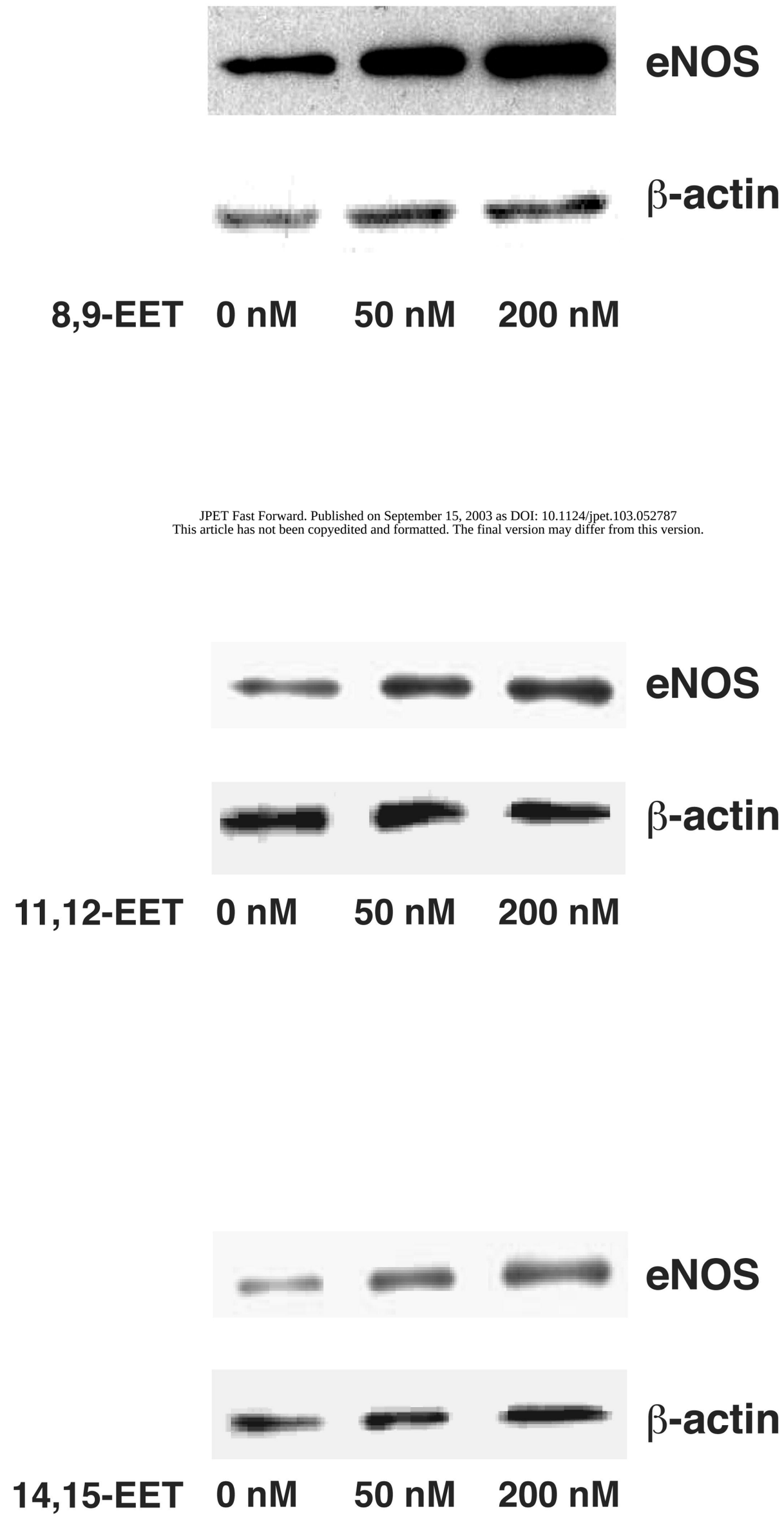

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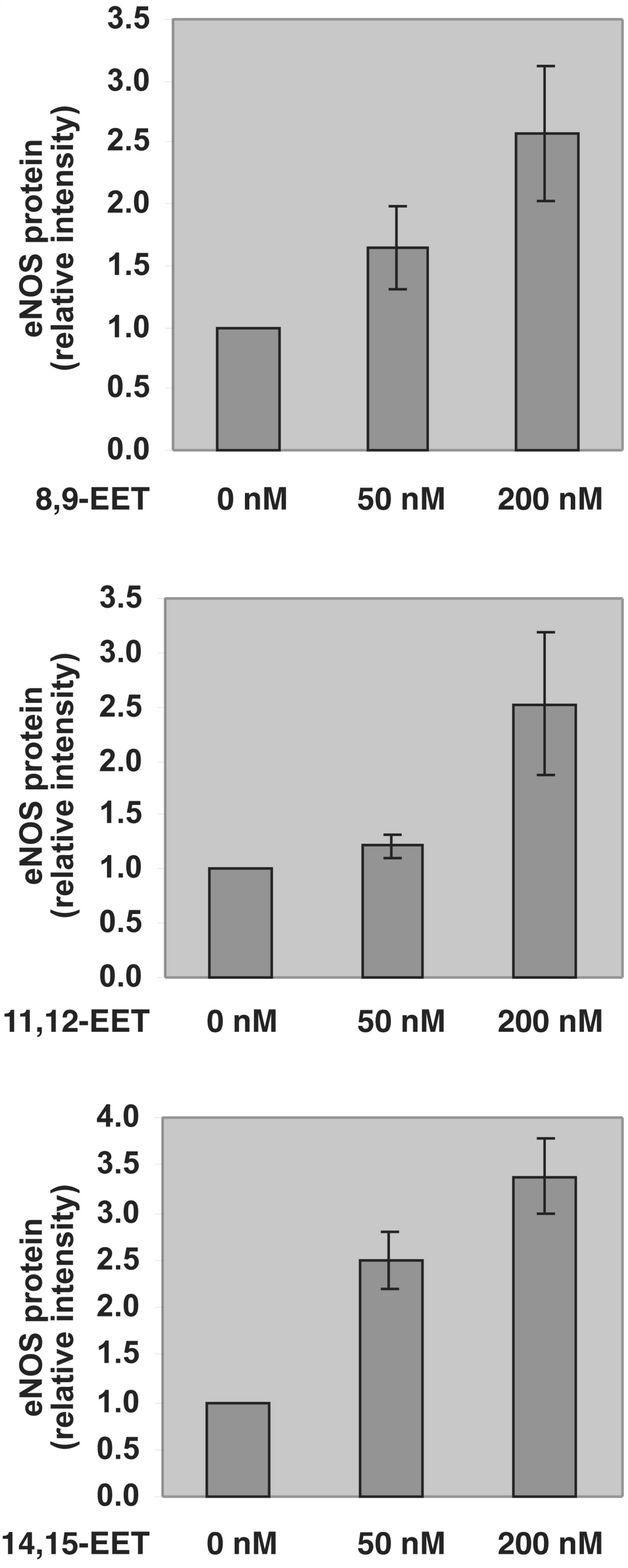
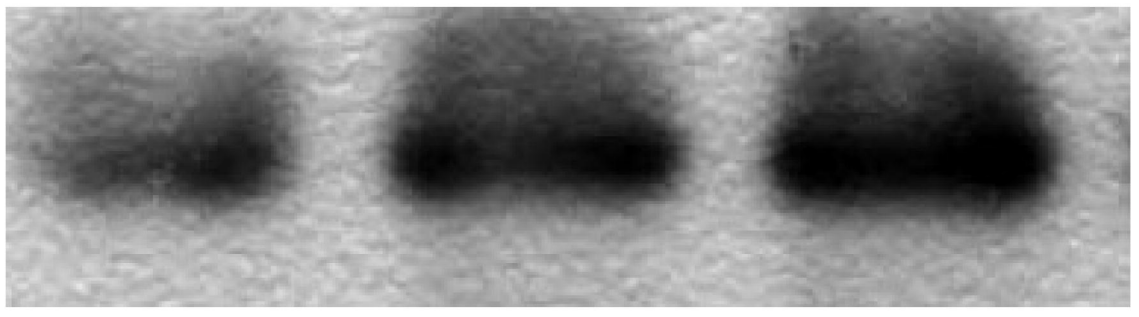
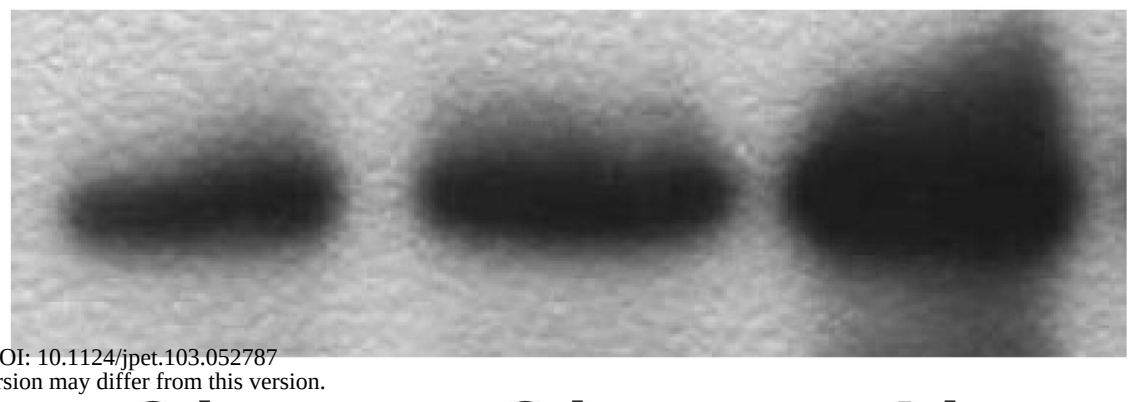


Figure 5

C





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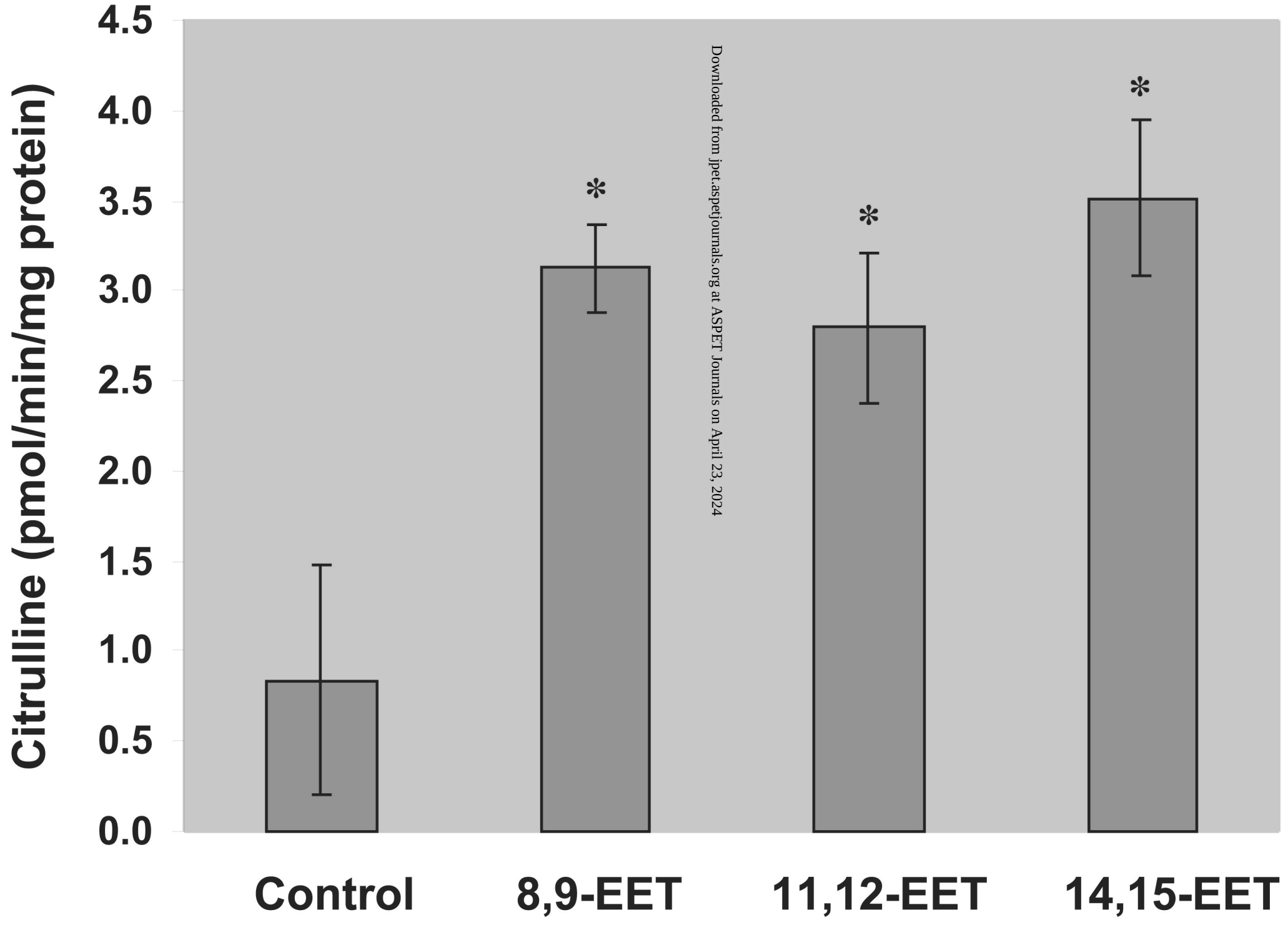

Figure 6

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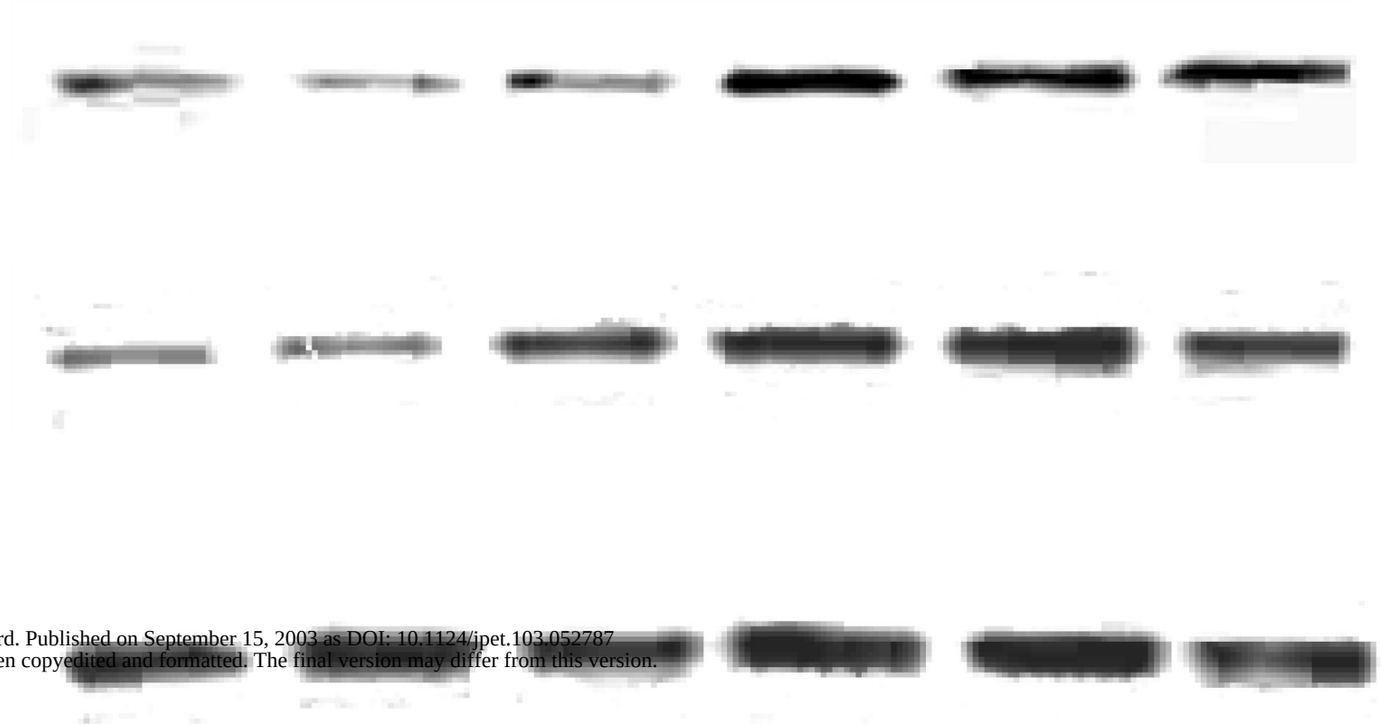

control 17-ODYA vehicle 8,9-EET 11,12-EET 14,15-EET

B

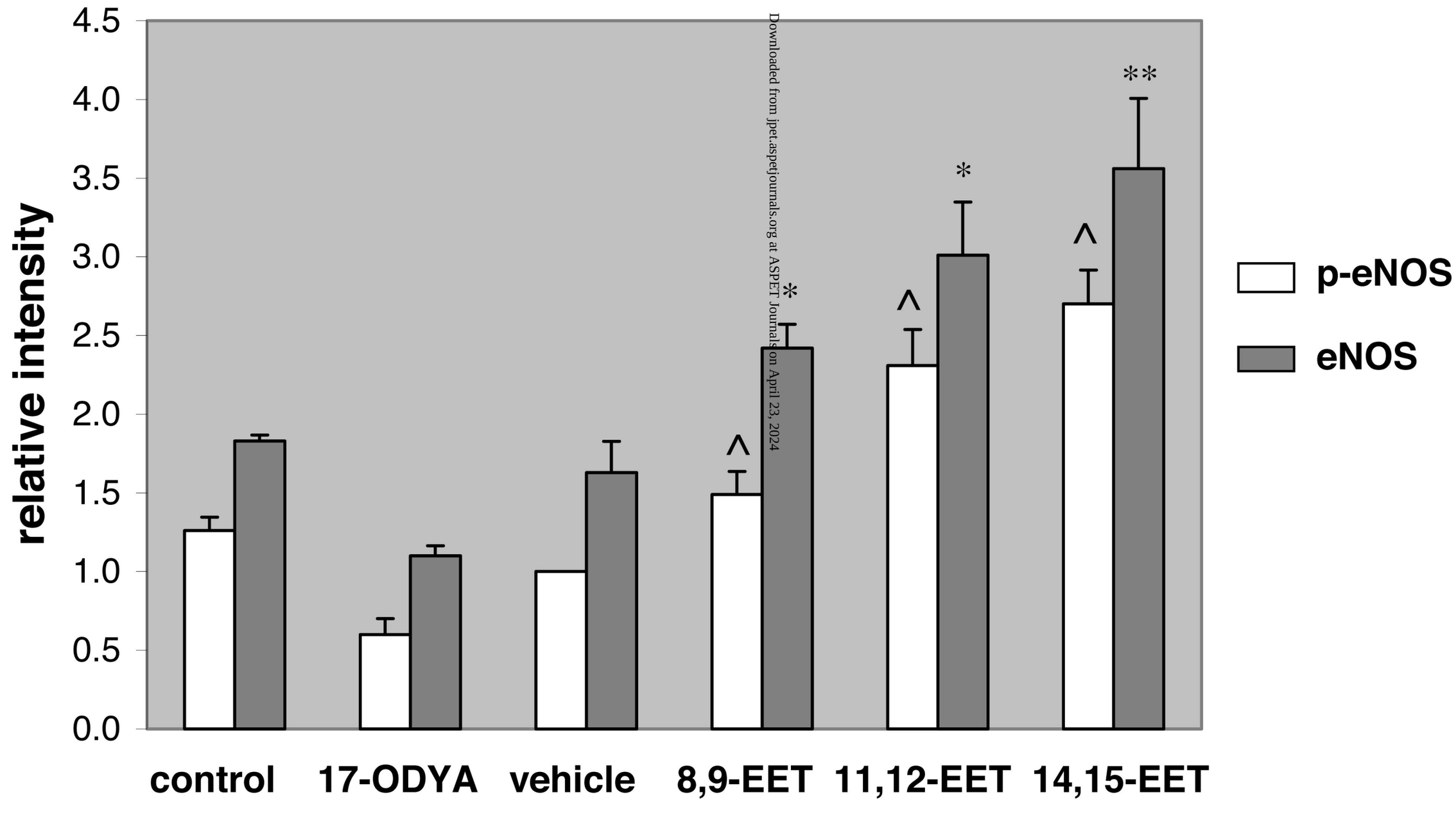

Figure 7

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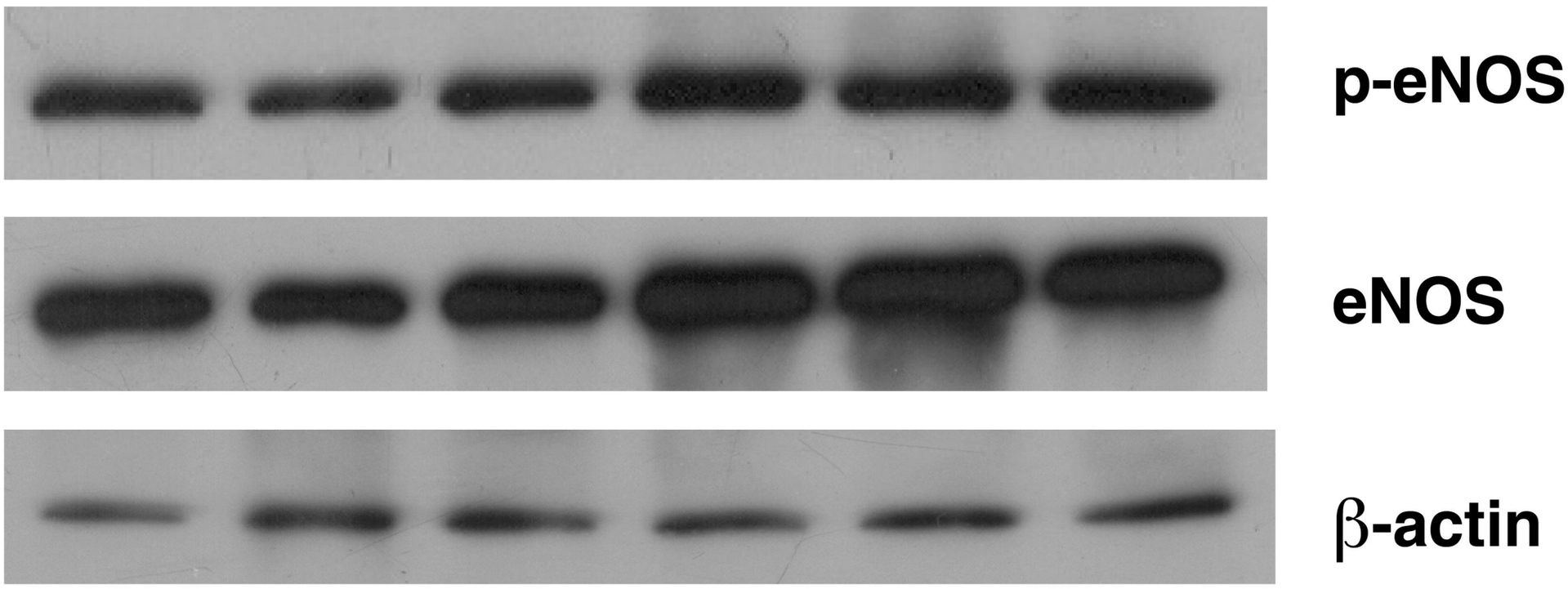

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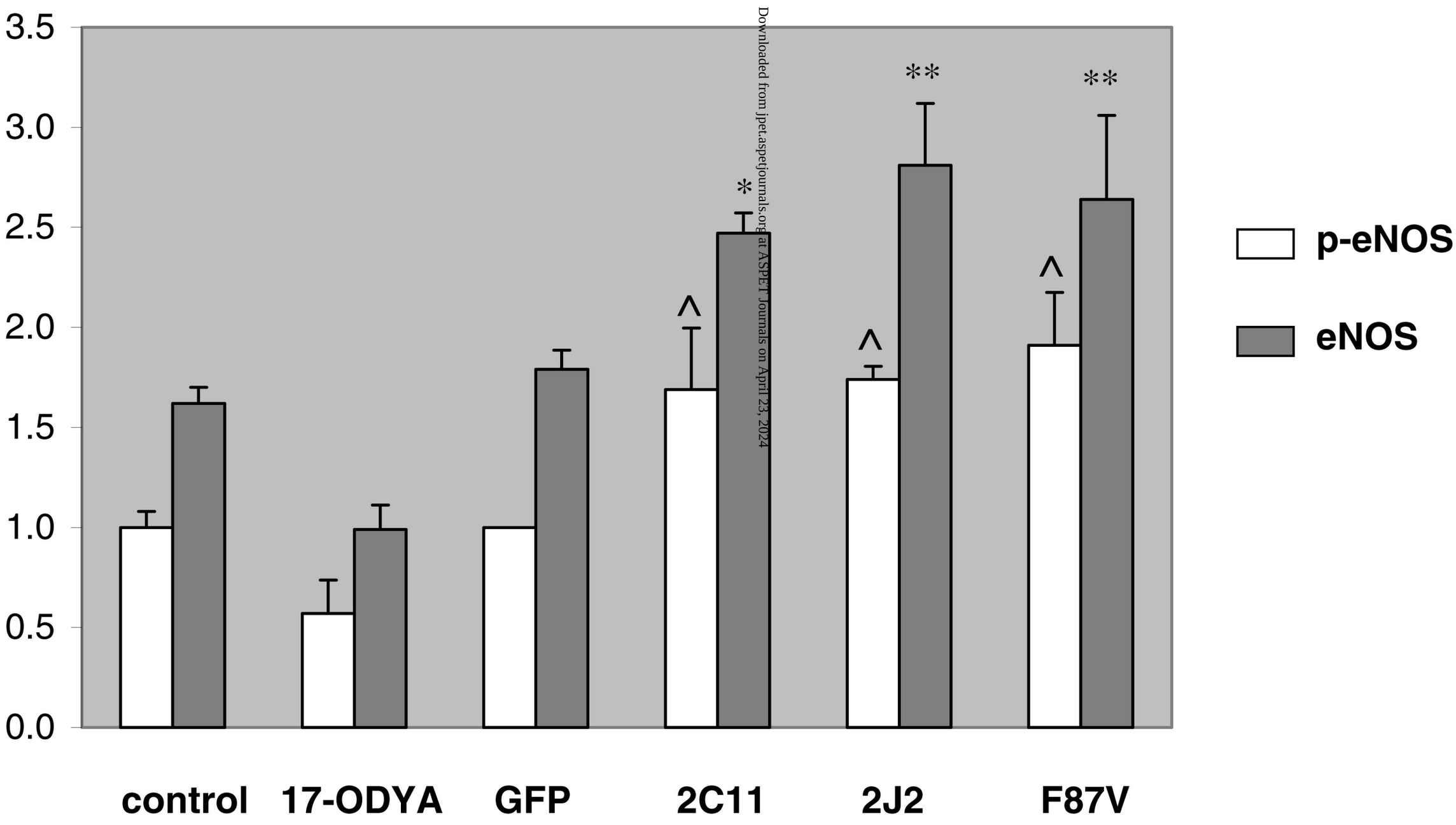

Figure 8

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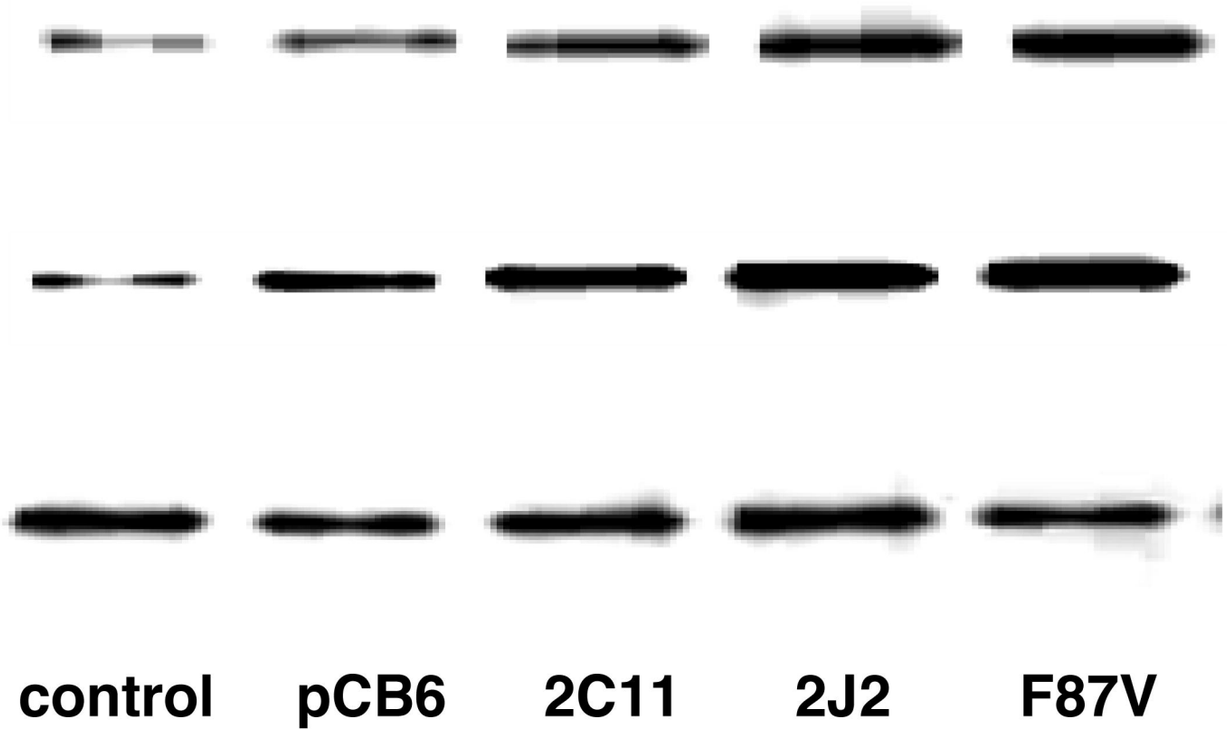

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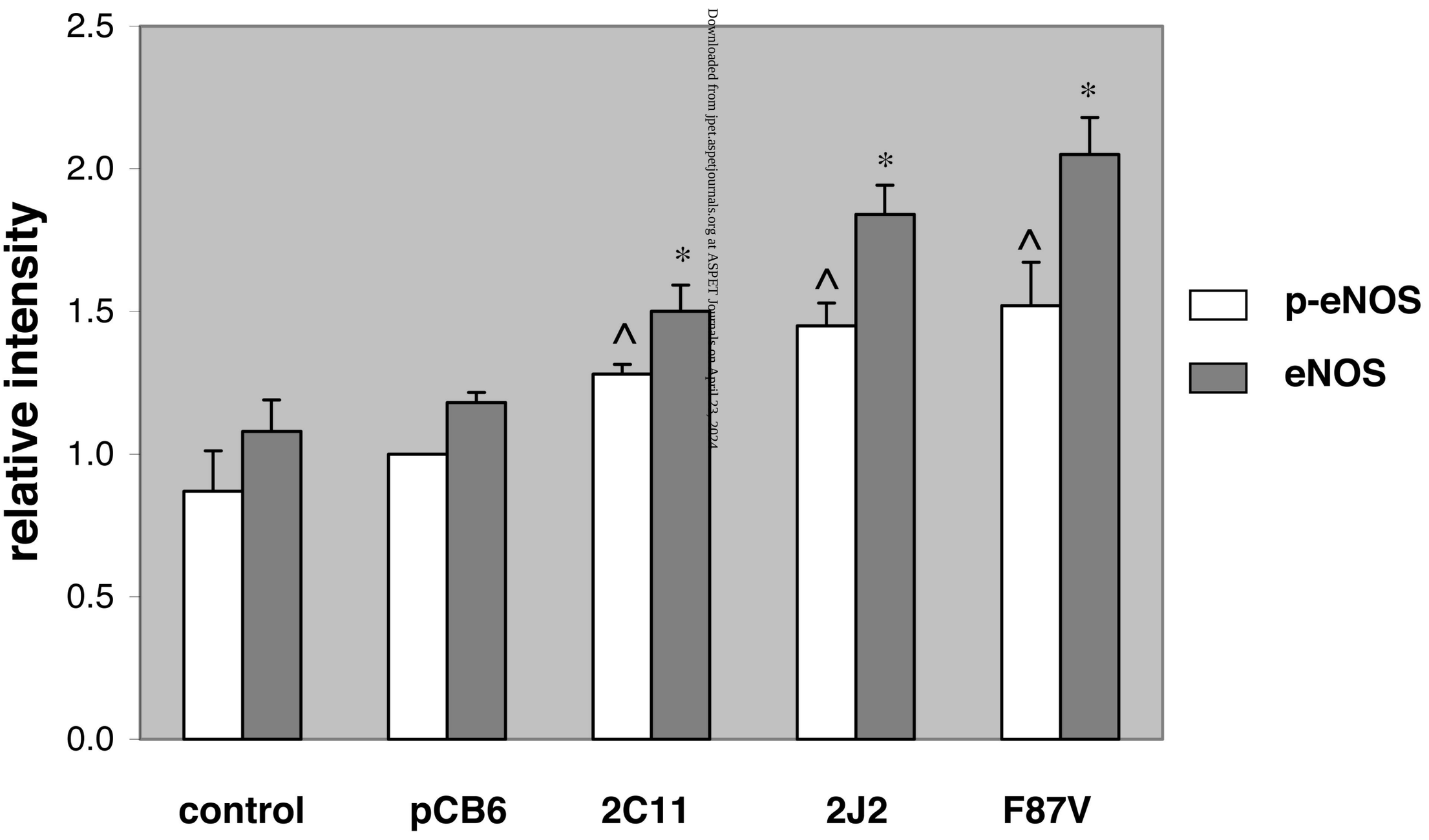

Figure 9

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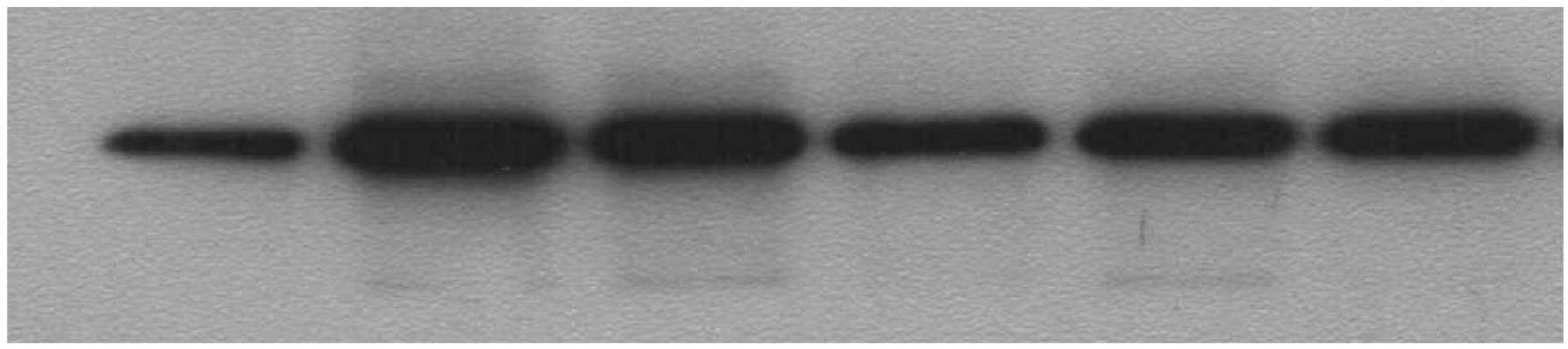
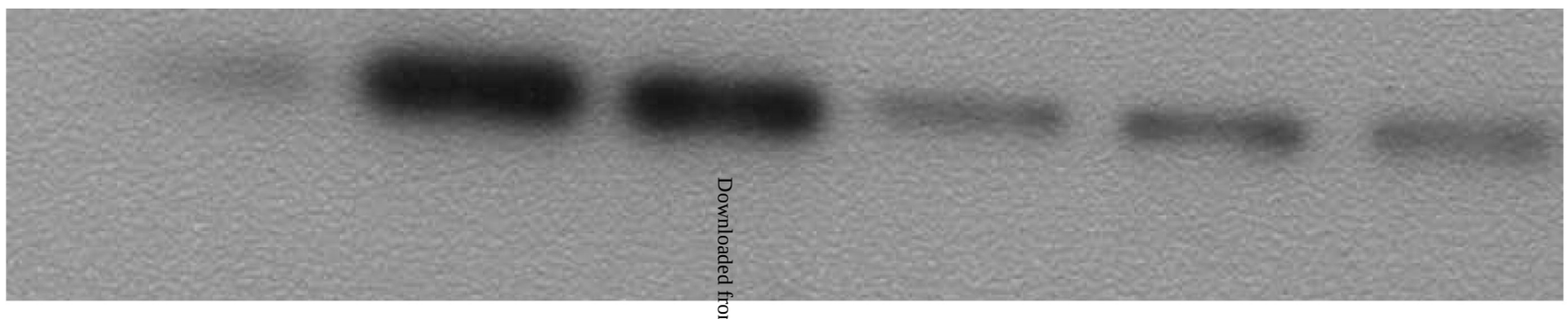


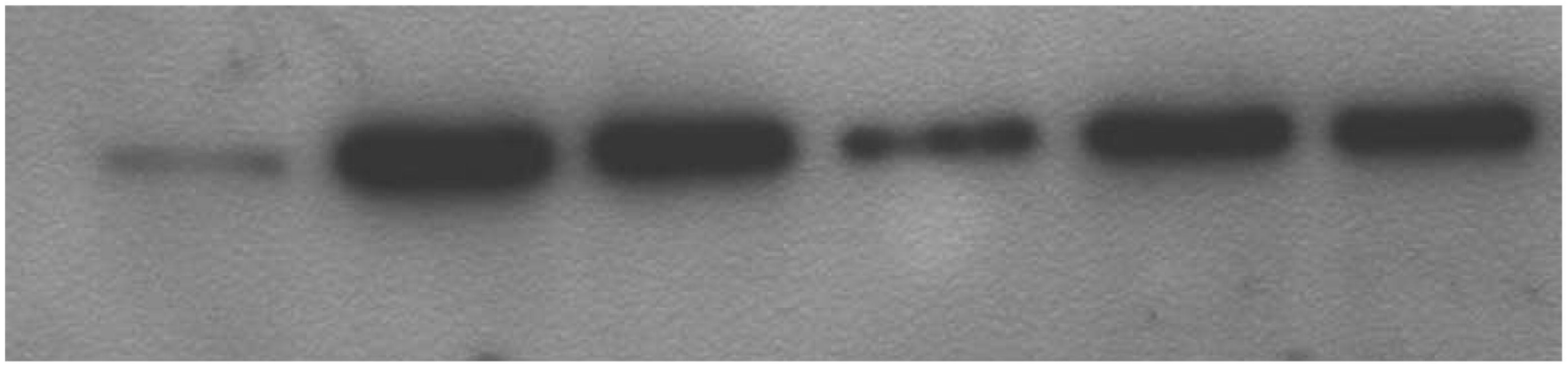
	pCB6	2C11	F87V	pCB6	2C11	F87V
PD98059	–	–	–	+	+	+

B



	pCB6	2C11	F87V	pCB6	2C11	F87V
Apigenin	–	–	–	+	+	+

C



	pCB6	2C11	F87V	pCB6	2C11	F87V
H-7	–	–	–	+	+	+

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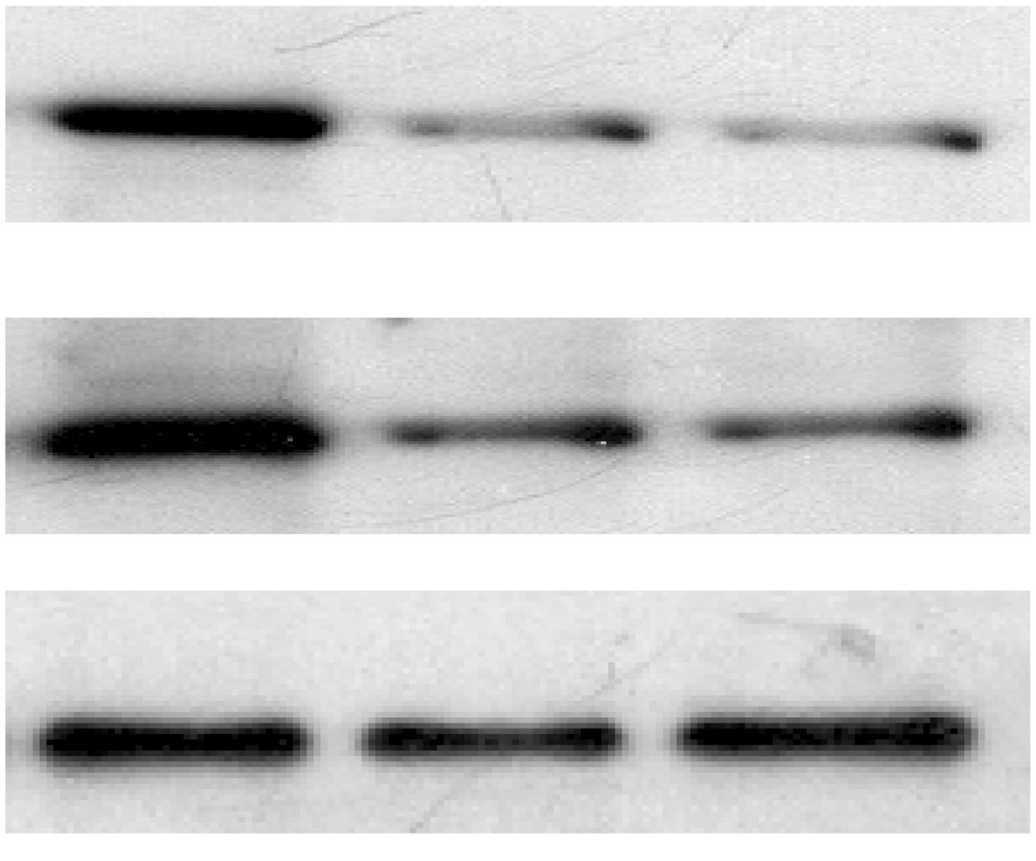
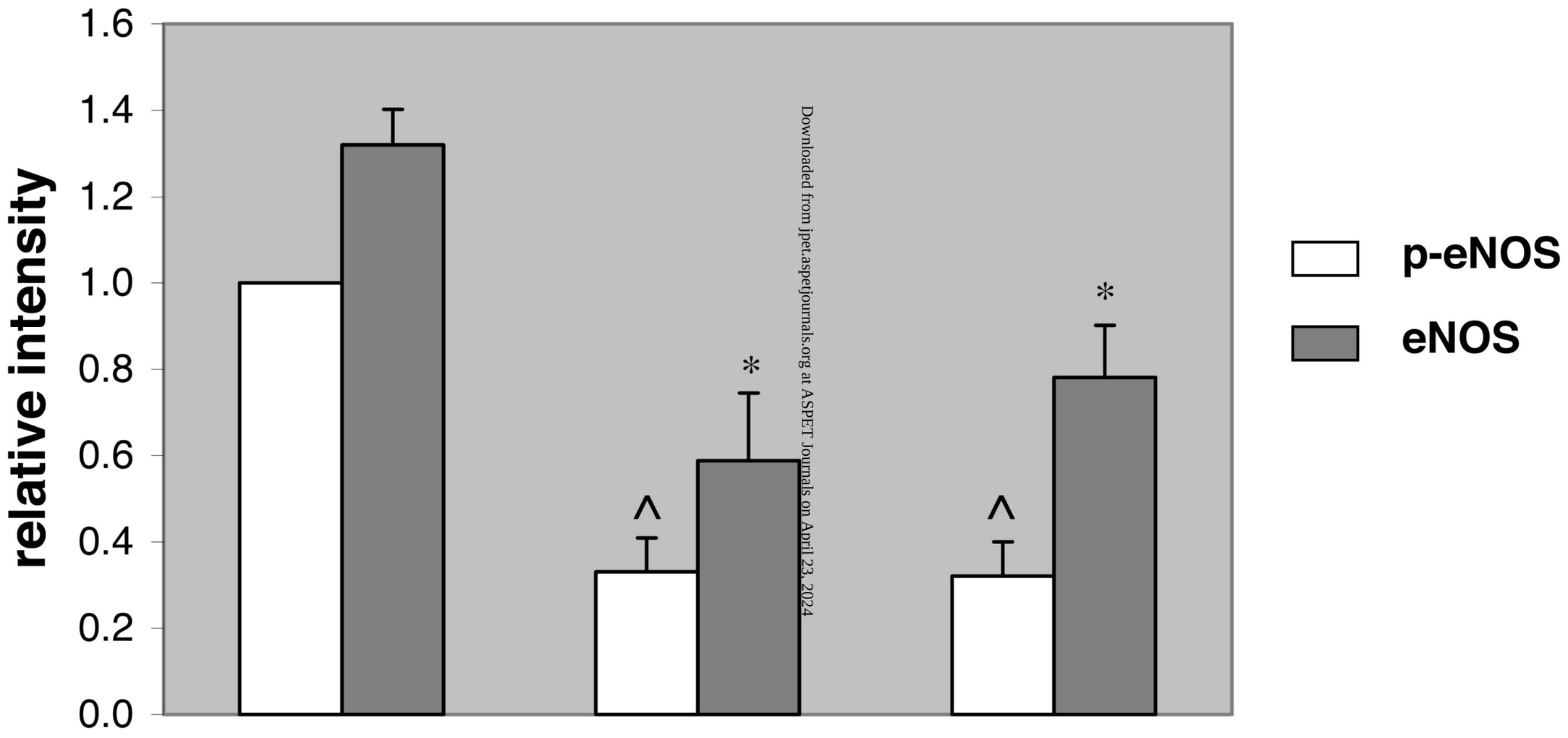
Figure 10

A



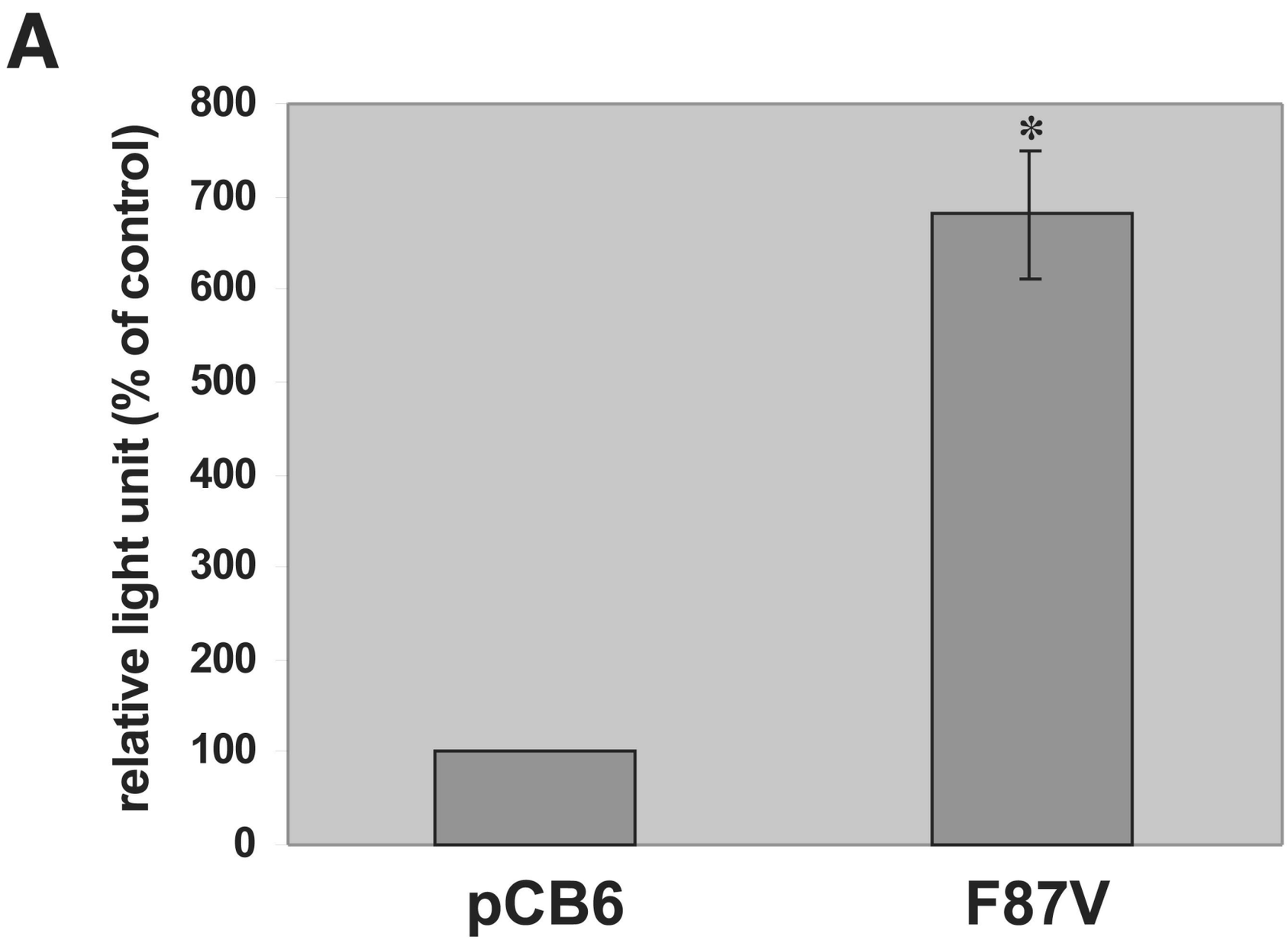
14,15-EET	+	+	+
apigenin	-	+	-
PD98059	-	-	+

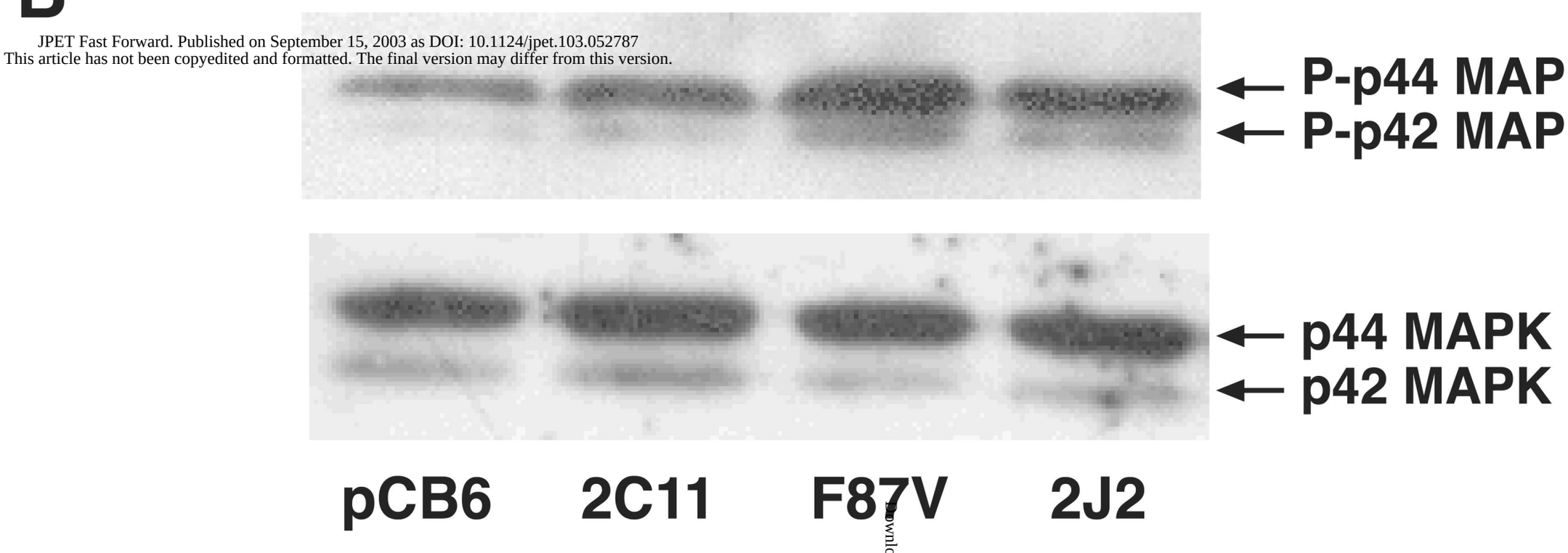
B



14,15-EET	+	+	+
apigenin	-	+	-
PD98059	-	-	+

Figure 11





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