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Post-transcriptional regulation of placenta growth factor mRNA by hydrogen peroxide

Jennifer H. Shaw and **Pamela G. Lloyd**
Department of Physiological Sciences, Oklahoma State University

Abstract

In tissues containing pre-existing collateral vessels, occlusion of an upstream supply artery results in diversion of blood flow through these vessels, protecting the distal tissue from ischemia. The sudden rise in blood flow through collateral vessels exerts shear stress upon the vessel wall, thereby providing the initial stimulus for arteriogenesis. Arteriogenesis, the structural expansion of collateral circulation, involves smooth muscle cell (SMC) proliferation which leads to increased vessel diameter and wall thickness. Since shear is sensed at the level of endothelial cells (EC), communication from EC to the underlying SMC must occur as part of this process. We previously reported that endothelial cells (EC) exposed to shear stress release hydrogen peroxide (H_2O_2), and that H_2O_2 can signal vascular SMC to increase gene and protein expression of placenta growth factor (PLGF), a known mediator of arteriogenesis. The purpose of the current study was to further elucidate the mechanism whereby PLGF is regulated by H_2O_2. We found that a single, physiological dose of H_2O_2 increases PLGF mRNA half-life, but has no effect on PLGF promoter activity, in human coronary artery SMC (CASMC). We further demonstrated that the H_2O_2–induced increase in PLGF mRNA levels partially relies on p38 MAPK, JNK and ERK1/2 pathways. Finally, we showed that chronic exposure to pathological levels of H_2O_2 further increases PLGF mRNA levels, but does not result in a corresponding increase in PLGF secreted protein. These data suggest that PLGF regulation has an important translational component. To our knowledge, this is the first study to characterize post-transcriptional regulation of PLGF mRNA by H_2O_2 in vascular SMC. These findings provide new insights into the regulation of this important growth factor and increase our understanding of PLGF-driven arteriogenesis.

Keywords

Reactive oxygen species; arteriogenesis; angiogenesis; PLGF; p38; JNK; ERK1/2

Introduction

A critical determinant for survival following an acute coronary event caused by atherosclerotic vascular occlusion is the ability to develop coronary collateral arteries (1).

Address correspondence to: Pamela G. Lloyd, Ph.D., Dept. of Physiological Sciences, 264 McElroy Hall, Oklahoma State University, Stillwater, OK 74078, Phone: (405) 744-9019, FAX: (405) 744-8263, pamela.lloyd@okstate.edu.

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None.

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This coronary collateralization process (termed arteriogenesis) functions as a compensatory process which allows continued blood flow to tissues distal to an arterial occlusion. Arteriogenesis is distinct from angiogenesis, or capillary proliferation, in both its initial stimulus and functional response. Angiogenesis is primarily driven by ischemia, leading to increased vascular endothelial growth factor-A (VEGF-A) production followed by endothelial cell proliferation and migration. In contrast, arteriogenesis involves the structural enlargement of pre-existing collateral vessels that form intra-arterial connections. These vessels have low flow rates in the absence of an upstream occlusion, due to the lack of a pressure gradient across the vessel length. However, they experience a substantial increase in blood flow following an upstream occlusion due to the decrease in pressure in the occluded vessel. This hemodynamic change exerts increased shear stress on the collateral vessel wall. Increased shear stress is generally considered to be the key initiator of the arteriogenic process, and activates signaling processes that lead to eventual increases in collateral artery diameter and wall thickness. Since shear stress is sensed by the endothelial layer, but migration and proliferation of vascular smooth muscle are key components of arteriogenesis, it is evident that shear stress activates endothelium-dependent signaling to vascular smooth muscle cells (2, 3). Arteriogenesis also depends upon the recruitment and activation of monocytes, which penetrate the vessel wall and differentiate into macrophages, creating an inflammatory environment that is thought to be essential for collateral growth (4–7).

Placenta growth factor (PLGF), a member of the VEGF family, is an important mediator of arteriogenesis (8–10). Although PLGF was first identified in placental tissue (11), we and others have shown that it is also expressed in adult vasculature (12) by both endothelial cells (EC) and smooth muscle cells (SMC)(10). The ability of PLGF to induce arteriogenesis requires recruitment of monocytes to the vessel wall(4–6). Since PLGF is chemotactic for monocytes and the vascular wall is largely composed of SMC, we hypothesized that SMC PLGF expression may play an important role in arteriogenesis. We further hypothesized that regulation of PLGF expression in SMC would be endothelium-dependent, since the shear signal is sensed by endothelium as discussed above. In agreement with this hypothesis, we previously reported that physiological levels of hydrogen peroxide (H_2O_2) are released from EC in response to shear stress, and that H_2O_2 can act as a paracrine mediator to increase PLGF gene and protein expression in vascular SMC (13). H_2O_2 has received increasing attention as an important physiological signaling molecule in vascular biology. Our previous findings are consistent with the important role of H_2O_2 in coronary collateral growth (7).

Clinical trials of the use of growth factors to stimulate arteriogenesis have reported little or no beneficial effect of growth factor treatment (1). Better understanding of the complex and interrelated signaling pathways that govern arteriogenesis may allow development of more efficacious pro-arteriogenic therapies for patients with ischemic cardiovascular disease. Despite the clearly important role of PLGF in adaptive arteriogenesis, little is known about its regulation. In the current study, we sought to further characterize the mechanism by which H_2O_2 increases PLGF expression in human coronary artery smooth muscle cells (CASMC). We report that treatment of human CASMC with a single dose of a physiological level of H_2O_2 extends the PLGF mRNA half-life. This post-transcriptional effect is partially dependent on the p38 MAPK, JNK and ERK1/2 pathways. Furthermore, we found that sustained exposure to pathological levels of H_2O_2 further increases PLGF mRNA levels, in comparison to a single dose of H_2O_2. Despite the large increase in PLGF mRNA, PLGF secreted protein levels were not increased by sustained exposure to high levels of H_2O_2. This finding suggests that pathological levels of H_2O_2 may affect translation of PLGF . Overall, these results reveal novel features of the PLGF regulatory mechanism and suggest important avenues for further research.

Materials and Methods

Primary cells

Human coronary artery smooth muscle cells (CASMC) were purchased from Lonza (Walkersville, MD). Cells were grown in a humidified incubator containing 5% CO_2 in Smooth Muscle Cell Basal Medium (SmBM, Lonza) plus Smooth Muscle Growth Medium (SmGM-2 SingleQuots, Lonza) until 80% confluent. Culture medium was changed every other day. CASMC used for experiments were between passages 4 and 8. Donors include a 12 year old male and a 56 year old female. Human coronary artery endothelial cells (CAEC) were purchased from Lonza. Cells were grown in a humidified incubator containing 5% CO_2 in Endothelial Cell Basal Medium (EMB, Lonza) plus Endothelial Cell Growth Medium (SingleQuots, Lonza). Culture medium was changed every other day. CAEC used for experiments were at passage 5. Donor cells were from a 21 year old male.

H_2O_2 treatment of human CASMC

CASMC were grown in a CO_2 incubator in 6-well plates containing SmBM plus SmGM-2 SingleQuots (Lonza, Walkersville, MD) until 80% confluent. Cells were then serum-starved (DMEM, 1% FBS) for 48 h followed by the addition of a single dose of H_2O_2 (50 μM) directly to the medium. For chronic exposure to pathological levels of H_2O_2, cells were treated with glucose oxidase (5 U/mL, Sigma, St. Louis, MO) which continually catalyzes the oxidation of β-D-glucose to form D-glucono-1,5-lactone and hydrogen peroxide (β-D-glucose + O_2 + $H_2O \rightarrow$ D-glucono-δ-lactone + H_2O_2). Following 8 h of treatment, medium was collected for ELISA and cells were lysed in TriZol (Invitrogen, Carlsbad, CA) for RNA isolation.

Plasmids

To assess PLGF promoter activity, the PLGF promoter sequence was inserted into the firefly luciferase pGL3 basic plasmid (Promega) at the at the Sst1 restriction site (14). This plasmid (PLGF-luc) was the generous gift of the late Dr. Brian Murphy (SRI International, Menlo Park, CA). The sequence of the PLGF promoter was confirmed by the OSU DNA sequencing facility. The pRL *Renilla* luciferase (pRL) plasmid (Promega) was used as the transfection efficiency control. A 50:1 ratio of firefly luciferase plasmid to *Renilla* luciferase plasmid was used, as recommended by the manufacturer.

Transfections

Human coronary artery smooth muscle cells (passage 5 – 8; 1×10^6 cells) were co-transfected with 2 μg PLGF-luc and 40 ng pRL using the Amaxa Nucleofector System, program A-033 (Lonza, Walkersville, MD). After transfection, cells were seeded in 6 well plates and left undisturbed for ~20 h. Next, 2 mL of fresh SmBM plus SmGM-2 was added to each well and co-transfected (PLGF-luc + pRL) cells were treated with H_2O_2 (50 μM) for 8 h. Medium was removed from the plates and 500 μL Passive Lysis Buffer (Promega) was added per well. Cells were scraped from the plate and immediately frozen at −20°C to ensure complete lysis. Frozen lysates were thawed and assayed for PLGF promoter activity using the Dual Luciferase assay system (Promega). Luminescence was measured using a Synergy HT multimode plate reader (BioTek).

Human coronary artery endothelial cells (passage 5; 5×10^5 cells) were co-transfected with 2 μg of PLGF-luc and 40 ng pRL using the Amaxa Nucleofector System, program S-005 (Lonza) to assess the basal PLGF promoter activity in endothelial cells, which constitutively produce relatively high levels of PLGF (data not shown) and thus serve as our positive control for the PLGF promoter activity assay. After transfection by electroporation, cells were seeded in 6 well plates and left undisturbed for ~20 h. Next, 1 mL of fresh EBM plus

EGM-2 was added to each well. 24 h later, medium was removed from the plates and 500 μL Passive Lysis Buffer (Promega) was added per well. Cells were scraped from the plate and immediately frozen at −20°C to ensure complete lysis. Frozen lysates were thawed and assayed for PLGF promoter activity (as reported by luciferase protein expression) using the Dual Luciferase assay system (Promega). Luminescence was measured using a Synergy HT multimode plate reader (BioTek, Winooski, VT).

PLGF mRNA half-life assay following H_2O_2 treatment

CASMC were grown in 6 well plates until 80% confluent, serum starved for 48 h, and treated with a single dose of H_2O_2 (50 μM). Untreated and H_2O_2-treated CASMC were then exposed to the transcription inhibitor actinomycin D (10 μg/mL, Sigma, St. Louis, MO) for 0, 2, 4, 8 or 16 h. RNA was harvested using TriZol (Invitrogen) at each time point for measuring PLGF mRNA levels by real time PCR.

Inhibition of kinase pathways following H_2O_2 treatment

CASMC were grown until 80% confluent, serum-starved for 48 h, and treated with a single dose of H_2O_2 (50 μM). H_2O_2-treated CASMC were exposed to either a p38 MAPK inhibitor (SB202190, 10 μM, Tocris, Ellisville, MO), a JNK inhibitor (SU3327, 10 μM, Tocris) or an ERK1/2 inhibitor (FR180204, 10 μM, Tocris) for 8 h. RNA was then isolated using TriZol (Invitrogen) for real time PCR.

Anisomycin Treatment

CASMC were grown until 80% confluent, serum starved for 48 h, and treated with a single dose of H_2O_2 (50 μM). H_2O_2-treated CASMC were exposed to anisomycin (300 ng/mL, Sigma) or with anisomycin (300 ng/mL) alone for 8 h. RNA was then isolated using TriZol (Invitrogen) for real time PCR.

Analysis of gene expression

At the conclusion of the experimental treatments, culture medium was removed and total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. RNA was assayed for quality and quantity in a Take 3 plate in a Synergy HT multimode plate reader (BioTek, Winooski, VT). RNA was then treated to remove genomic DNA (Turbo DNAFree, Ambion, Austin, TX) and converted to cDNA (qScript cDNA SuperMix, Quanta BioSciences, Gaithersburg, MD) according to manufacturers' protocols. Real time PCR was performed on an ABI 7500 Fast instrument for quantification of gene expression. Primers for human PLGF and β-actin were designed using Primer Express and custom-synthesized (Invitrogen) in accordance with our previous work (13). PCR product formation was detected using SYBR Green chemistry (PerfeCTa SYBR Green FastMix, Low ROX, Quanta BioSciences). ΔΔCt values were used to determine the RQ value (Applied Biosystems 7500 software) for PLGF gene expression relative to the expression of the internal housekeeping gene β-actin (results expressed as PLGF/β-actin mRNA, fold of control). Our lab has previously determined that β-actin gene expression is not significantly affected by H_2O_2 treatment (data not shown).

ELISA

CASMC were grown in 6 well plates until 80% confluent, serum starved for 48 h and treated with a single dose of H_2O_2 (50 μM) or glucose oxidase (5 U/mL, Sigma) for 8 h. To protect secreted protein from degradation during sample processing, protease inhibitor cocktail (Sigma) was added (1:250) to the medium during collection on ice following the 8 h treatment. Total protein was quantified using the BCA Protein Assay (Pierce, Rockford, IL).

Secreted PLGF protein levels were measured using the Human PLGF ELISA System (R&D Systems, Minneapolis, MN).

Statistical analyses

The effects of H_2O_2, glucose oxidase, and kinase inhibitors on PLGF mRNA level following H_2O_2 treatment were detected with one-way ANOVA followed by the Holm-Sidak test. Significance of anisomycin treatment and co-transfections was determined using one-way analysis of variance (ANOVA) on ranks followed by Dunn's post-hoc test. The effect of actinomycin D on PLGF mRNA half life and the effect of glucose oxidase on H_2O_2 levels in medium over time were assessed by two-way ANOVA followed by the Holm-Sidak test. Differences were considered significant at $p<0.05$. Statistical analyses were performed with SigmaStat software.

Results

A single dose of hydrogen peroxide has no significant effect on PLGF promoter activity

We previously reported that PLGF mRNA levels are increased in human CASMC at 8 h following a single dose of exogenous H_2O_2 (13). In other cell types (human embryonic kidney cells, choriocarcinoma cells, placental villi), pro-inflammatory stimuli have been shown to increase PLGF promoter activity via metal responsive transcription factor (MTF-1), NF-κB, and CREB (14–16). It has also been well established that H_2O_2 activates transcription factors such as NF-κB and CREB (17, 18). In addition, the PLGF promoter region contained in the plasmid (PLGF-luc) used in this study was previously characterized and was reported to contain binding sites for MTF-1 and NF-κB (14). Therefore, we hypothesized that H_2O_2 treatment would increase PLGF mRNA levels in vascular smooth muscle cells by activation of PLGF gene transcription.

Currently, there are no positive controls known that drive PLGF promoter activity in smooth muscle cells. However, our lab has found that endothelial cells constituitively express PLGF mRNA and protein at levels higher than those found in smooth muscle cells (data not shown). Therefore, in order to demonstrate that the PLGF-luc plasmid can be used to detect changes in PLGF promoter activity, we transfected human coronary artery endothelial cells (CAEC) with PLGF-luc for comparison with CASMC. Co-transfected (PLGF-luc + pRL) CAEC had a higher basal level of PLGF promoter activity than CASMC co-transfected with PLGF-luc + pRL (Fig 1). Next, we assessed the effect of a single, physiological dose of H_2O_2 (50 μM) on PLGF promoter activity in CASMC. There was no significant difference in PLGF promoter activity between H_2O_2-treated CASMC and untreated CASMC at 8 h post treatment (Fig. 1). We conclude that a single physiological dose of H_2O_2 does not activate PLGF gene transcription in human CASMC.

Hydrogen peroxide increases the half-life of PLGF mRNA in human CASMC

As shown in Fig. 1, there was no increase in PLGF promoter activity 8 h after treatment with a single dose of 50 μM H_2O_2. Therefore, we hypothesized that H_2O_2 acts to increase PLGF mRNA levels by extending the half-life of PLGF mRNA transcripts. To test this hypothesis, we treated human CASMC with either actinomycin D (10 μg/mL) alone to inhibit transcription or actinomycin D (10 μg/mL) plus a single dose of H_2O_2 (50 μM), then measured PLGF mRNA levels at 0, 2, 4, 8 and 16 h. We found that PLGF mRNA transcript half-life was significantly ($p<0.01$, $n=3$) increased by H_2O_2 at 2, 4, and 8 h post treatment (Fig. 2). There was no significant difference in PLGF mRNA levels between H_2O_2-treated and untreated groups at 0 or 16 h (Fig. 2).

The level of H_2O_2 in the medium of CASMC following the addition of a single dose of 50 μM H_2O_2 had returned to baseline by 30 minutes post-treatment (data not shown). Therefore, brief exposure of CASMC to a single physiological dose of H_2O_2 appeared to exhibit a transient ability to stabilize PLGF mRNA that persisted for up to 8 h post-treatment and was no longer evident 16 h post treatment.

Anisomycin treatment increases the level of PLGF mRNA

RNA stability often requires continuous translation of RNA stabilizing proteins that bind target transcripts and prevent degradation (19). Therefore, we hypothesized that ongoing protein translation might contribute to the increase in the PLGF mRNA levels following treatment with H_2O_2. To test this hypothesis, we treated cells with either anisomycin or cycloheximide, two inhibitors of protein synthesis. PLGF mRNA was significantly increased in CASMC treated with either H_2O_2 (50 μM) plus anisomycin (10 μg/mL) or anisomycin alone, compared to untreated control CASMC (Fig. 3). In contrast, neither cycloheximide alone nor H_2O_2 (50 μM) plus cycloheximide significantly affected PLGF mRNA levels, compared to the corresponding non-cycloheximide treated groups (data not shown).

Although anisomycin is an inhibitor of protein translation, it is also widely accepted as a potent activator of MAP kinases (20). Since the protein synthesis inhibitor cycloheximide did not upregulate PLGF expression, we conclude that the increased PLGF mRNA levels seen following anisomycin treatment occurred secondary to activation of MAPK pathways. Combination of anisomycin and H_2O_2 (50 μM) did not further increase PLGF mRNA levels over either treatment alone, suggesting that anisomycin and H_2O_2 (50 μM) act via similar pathways to affect PLGF expression.

Kinase pathways contribute to the peroxide-induced increase in PLGF mRNA levels

Since anisomycin treatment caused an increase in PLGF mRNA levels, we further characterized the role of kinase signaling in this mechanism. Oxidative stressors such as H_2O_2 are known to activate multiple kinase pathways which can affect transcription, RNA stability and translation (21). Thus, we hypothesized that kinases which are known to be activated by oxidative stress (such as p38 MAPK, JNK and/or ERK1/2), would contribute to the increase in PLGF mRNA following treatment with H_2O_2. Human CASMC were treated with a single dose of H_2O_2 (50 μM) in the presence of a p38 MAPK inhibitor (10 μM), a JNK inhibitor (10 μM) or an ERK1/2 inhibitor (10 μM). At 8 h post treatment, we found that all three kinase inhibitors significantly ($p<0.05$, $n=3$) inhibited (by ~40%) the peroxide-induced increase in PLGF mRNA levels (Fig. 4). We conclude that all three of these kinase pathways are partially responsible for the increase in PLGF mRNA levels following exposure to a single dose of a physiological level of H_2O_2.

Chronic exposure to highly elevated levels of peroxide further increases PLGF mRNA levels

Exogenous treatment with a single dose of H_2O_2 (50 μM) reflects a mild burst of physiological oxidative stress, such as a short bout of moderate exercise. In the context of cardiovascular pathologies, however, H_2O_2 levels may be chronically elevated in diseased vasculature. We hypothesized that sustained exposure of CASMC to high levels of H_2O_2 would further increase PLGF mRNA levels. Human CASMC were treated with the glucose oxidase enzyme (5 U/mL) to provide a continuous source of H_2O_2 (by driving the enzymatic reaction β-D-glucose + O_2 + H_2O → D-glucono-δ-lactone + H_2O_2). Levels of H_2O_2 were highly elevated at 2 h (~750 μM) and gradually returned to control levels by 24 h as substrates for the enzymatic reaction were depleted (Fig 5). Whereas a single exogenous low dose of H_2O_2 caused a ~4–6 fold increase in PLGF mRNA levels, sustained exposure to

highly elevated levels of H_2O_2 for 8 h resulted in a ~20-fold increase ($p<0.001$, n=3) in PLGF mRNA (Fig. 6). We conclude that chronic exposure to H_2O_2 further increases PLGF mRNA levels.

Chronic exposure to highly elevated levels of peroxide does not result in a corresponding increase in secreted PLGF protein

We next sought to determine whether the large increase in PLGF mRNA following glucose oxidase treatment resulted in a corresponding increase in PLGF protein. Previously, our lab reported a modest but significant increase in PLGF protein levels in human CASMC following treatment with a single physiological dose of H_2O_2 (50 μM) (13). Therefore, we hypothesized that chronic elevation of H_2O_2 would further increase PLGF protein levels. Surprisingly, we found that CASMC exposed to sustained high levels of H_2O_2 showed no significant change in secreted PLGF protein compared to untreated cells (Fig. 7), despite the substantial increase in PLGF mRNA (Fig. 6). These data raise the possibility that translation of PLGF mRNA may be inhibited in a highly oxidative environment.

Discussion

PLGF is a VEGF-family protein that specifically mediates arteriogenesis rather than capillary proliferation (5, 9, 22). As such, modulation of PLGF expression may be useful in the setting of ischemic cardiovascular disease, where enlarged collateral arteries could provide an alternate route for blood flow to compensate for atherosclerotic obstruction of upstream arteries. Alternatively, inhibition of PLGF expression could potentially have an anti-tumor effect by preventing tumor blood vessel growth. However, despite the potential therapeutic utility of modulating PLGF expression, little is known about the signaling mechanisms that regulate its expression in adult vasculature.

We previously reported that conditioned medium from endothelial cells was able to induce PLGF gene expression in smooth muscle cells, and that this effect appeared to be mediated by H_2O_2 (13). Furthermore, exogenous H_2O_2 induced a dose-dependent increase in PLGF mRNA expression in both established and primary vascular SMC, along with a mild but significant increase in PLGF protein. This effect was specific for PLGF, as mRNA for VEGF-A was only transiently upregulated by exogenous H_2O_2, and VEGF-A protein did not increase following H_2O_2 treatment. The goal of the present study was to establish further details of this novel signaling pathway.

The results presented here demonstrate that a single dose of exogenous H_2O_2 acts to increase PLGF mRNA levels in SMC by increasing the half-life of PLGF mRNA, rather than by activating PLGF gene transcription. We also treated cells with anisomycin, which caused an increase in PLGF mRNA. In addition to its action as an inhibitor of protein synthesis, anisomycin is known to activate stress-related MAP kinases (20). Since cycloheximide did not upregulate PLGF, these results suggested that kinase signaling could be involved in the H_2O_2–induced increase in PLGF mRNA. Thus, we tested whether protein kinase inhibition could block the increase in PLGF mRNA following H_2O_2 treatment. Consistent with previous reports demonstrating that H_2O_2 upregulates p38 MAPK, Erk1/2, and JNK signaling (21), the effect of H_2O_2 on PLGF mRNA levels was found to be partially dependent on all three kinase pathways. Although H_2O_2 is more stable than superoxide (O_2^-) and is thus considered to be a more likely mediator of signaling in the vascular wall than O_2^-, it nonetheless has a relatively short half life. Analysis of H_2O_2 levels in media of cells treated with a single dose of 50 μM H_2O_2 demonstrated that H_2O_2 had returned to basal levels within 30 min of treatment (data not shown). To determine whether exposure to chronically elevated H_2O_2 produced similar effects to transient exposure, we utilized an enzymatic system to generate H_2O_2 in culture medium of SMC (glucose oxidase). Glucose

oxidase treatment produced a marked increase in H_2O_2 levels (to ~750 μM after 2 h). This increase was associated with greatly elevated PLGF mRNA levels (~20 fold of untreated control at 8 h post-glucose oxidase). Surprisingly, however, we did not observe a corresponding increase in PLGF protein expression.

There are several possible explanations for the failure of increased PLGF mRNA to lead to a corresponding increase in PLGF protein. First, it is possible that the age of the primary human cells used in the glucose oxidase experiment influenced the results. In our previous paper (in which upregulation of PLGF mRNA led to a small, but significant increase in PLGF protein), we used primary human CASMC which were obtained from a young (12 yo) donor. When assessing PLGF expression in response to glucose oxidase, the source of the primary human CASMC was an older (56 yo) donor. Although we cannot draw any definitive conclusions about possible age effects on PLGF translation from this limited comparison, it is possible that aged cells fail to upregulate PLGF protein expression in response to increased PLGF mRNA levels. This hypothesis is consistent with the known reduction in arteriogenic capacity of aged animals (23–25), and with the observation that aged endothelium releases pathologically elevated levels of H_2O_2 (26). The question of whether age affects PLGF expression at the translational level therefore is an important area for further research.

Alternatively, there may be additional age-independent mechanisms controlling the translation of PLGF mRNA that are active in our model system. For instance, PLGF mRNA could potentially be protected from translation by microRNA (miRNA) binding to the transcript. It has recently been reported that H_2O_2 treatment (at concentrations similar to those used in our current study) upregulates numerous miRNA species in vascular SMC (27). It is unknown whether any of these miRNA regulate PLGF translation. If PLGF translation is regulated by miRNA, then H_2O_2 treatment may be able to simultaneously increase PLGF mRNA levels (by stabilizing the transcript), and inhibit its translation into protein (by upregulating inhibitory miRNA). The role of miRNA in regulation of PLGF translation is an interesting question for further investigation.

Although the details of mechanisms regulating PLGF translation remain to be revealed, our present findings are consistent with reports in the literature showing that the dependence of arteriogenesis on oxidative signaling is finely balanced; adaptive collateral artery enlargement is prevented by both pathologically high levels of ROS and by insufficient levels of ROS (7). Thus, it is possible that mild oxidative stress results in increased PLGF protein expression and a resulting enhancement of arteriogenesis, whereas a greater degree of oxidative stress prevents production of PLGF protein and hinders arteriogenesis.

Conclusions

In summary, these studies expand on our previously published results to demonstrate that H_2O_2 treatment increases PLGF gene expression by increasing the half-life of PLGF mRNA, rather than by increasing the rate of PLGF mRNA transcription and that the H_2O_2-induced increase in PLGF mRNA is partially dependent upon kinase signaling. Finally, we found that a high level of oxidative stress induces even greater elevation of PLGF mRNA than does mild oxidative stress, without a corresponding increase in PLGF protein expression. These results suggest that regulation of PLGF expression has an important translational component. These data further increase our understanding of the regulation of this key arteriogenic protein, and generate important questions for further research.

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Highlights

- A single, low dose of H_2O_2 increases the half-life of PLGF mRNA in human CASMC
- The H_2O_2 induced increase in PLGF mRNA levels depend, in part, on kinase signaling
- Chronic exposure to H_2O_2 induces an even greater elevation of PLGF mRNA
- Elevated PLGF mRNA due to chronic exposure does not result in increased PLGF protein

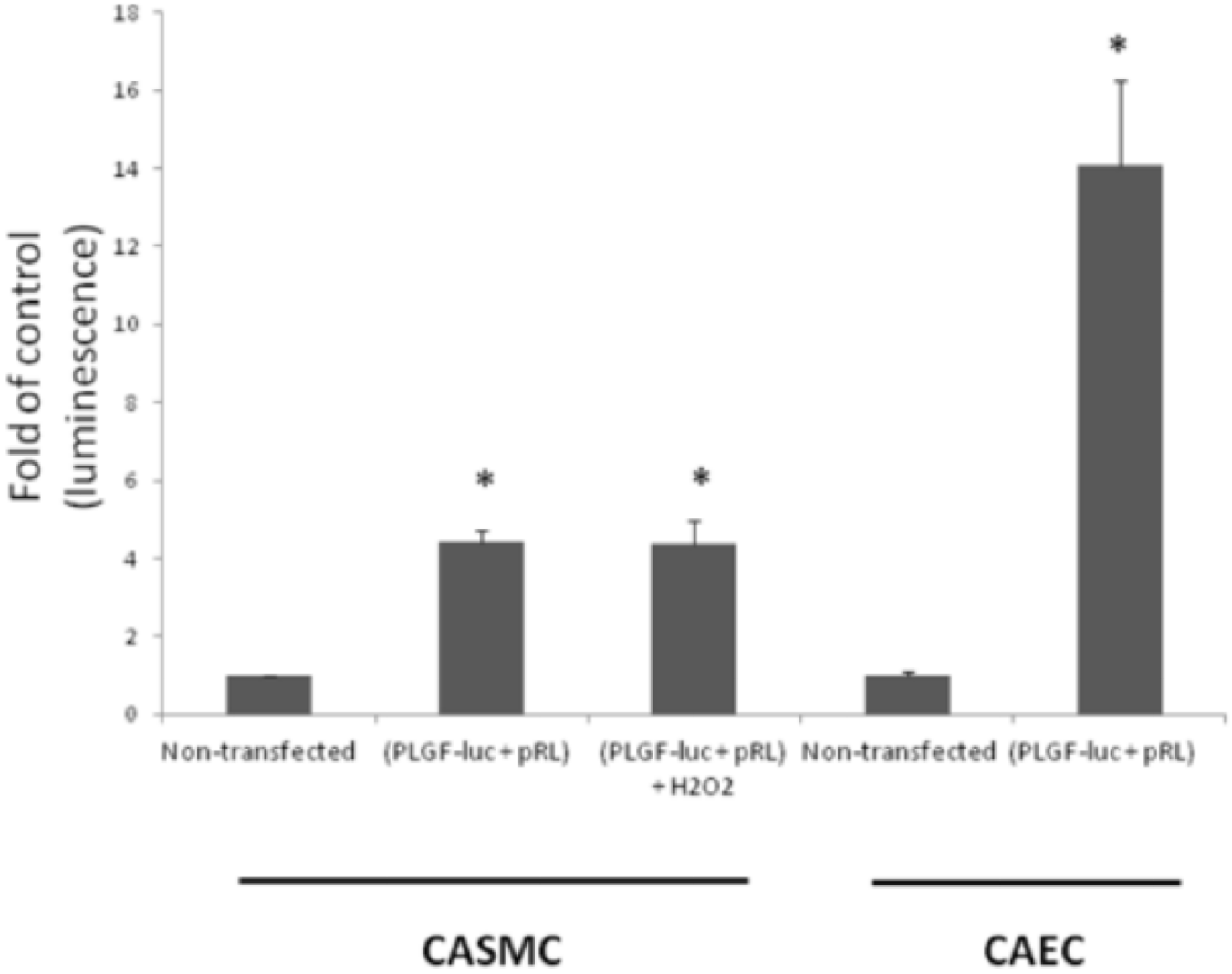

Figure 1. Hydrogen peroxide has no significant effect on PLGF promoter activity

Human CASMC were co-transfected with 2μg plasmid containing the PLGF promoter linked to the firefly luciferase reporter gene (PLGF-luc) and 40 ng *Renilla* luciferase control plasmid (pRL). Human CAEC (n=3) were also co-transfected with PLGF-luc and served as a positive control for PLGF promoter activity. PLGF promoter activity was not statistically different in co-transfected CASMC treated with H_2O_2 compared to the basal level of promoter activity observed in untreated co-transfected CASMC (n=6). (*$p<0.05$, non-transfected cells vs. respective transfected cell type).

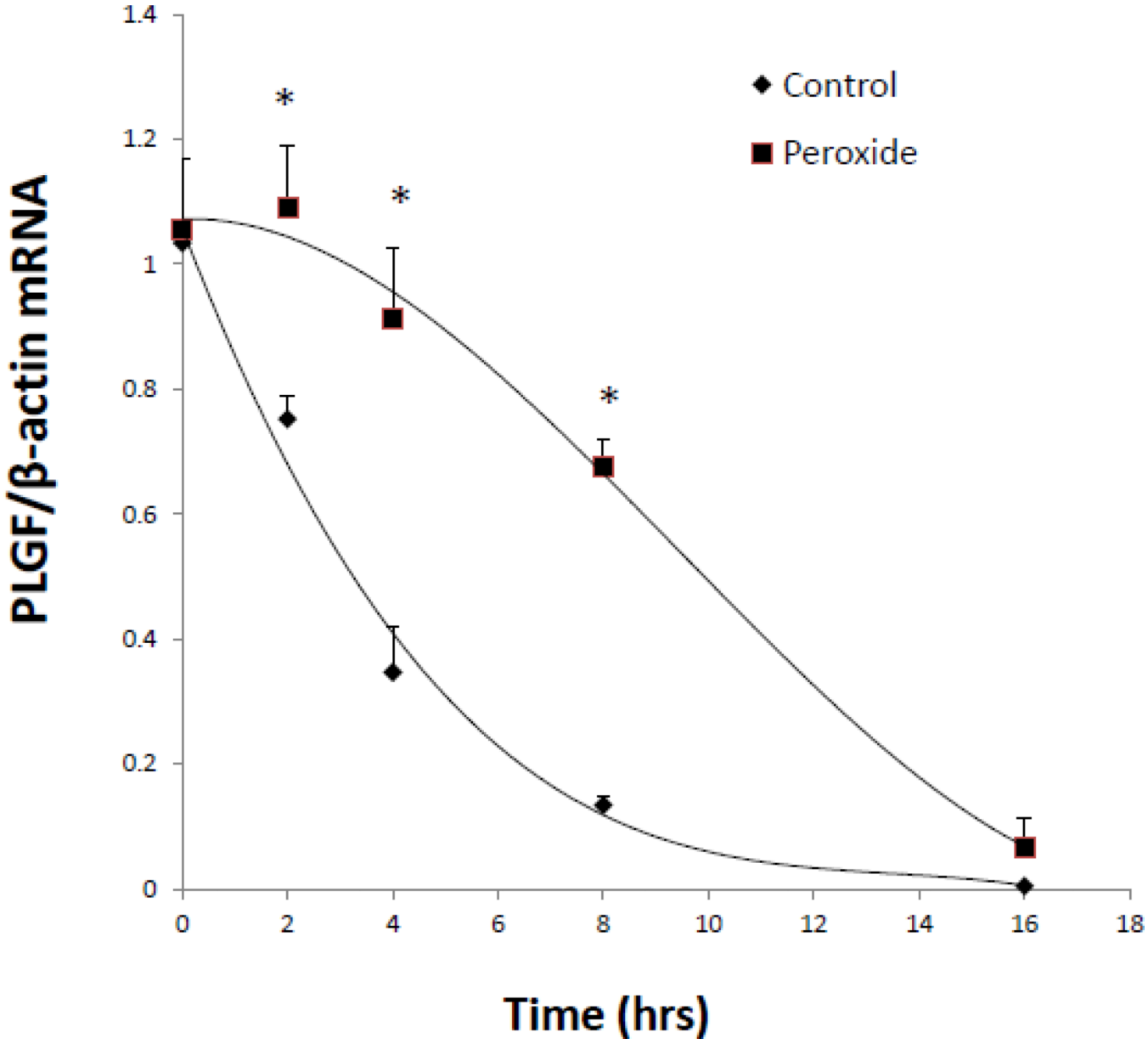

Figure 2. Hydrogen peroxide increases the half-life of PLGF mRNA

Human CASMC were treated with either actinomycin D (10 μg/mL) alone or with actinomycin D (10 μg/mL) plus a single dose of H_2O_2 (50 μM) and mRNA was collected at 0, 2, 4, 8 and 16 h later. PLGF mRNA transcript half-life was significantly (*$p<0.01$, n=3) increased at 2, 4, and 8 h post treatment with H_2O_2. There was no significant difference in PLGF mRNA levels at 0 and 16 h (n=3).

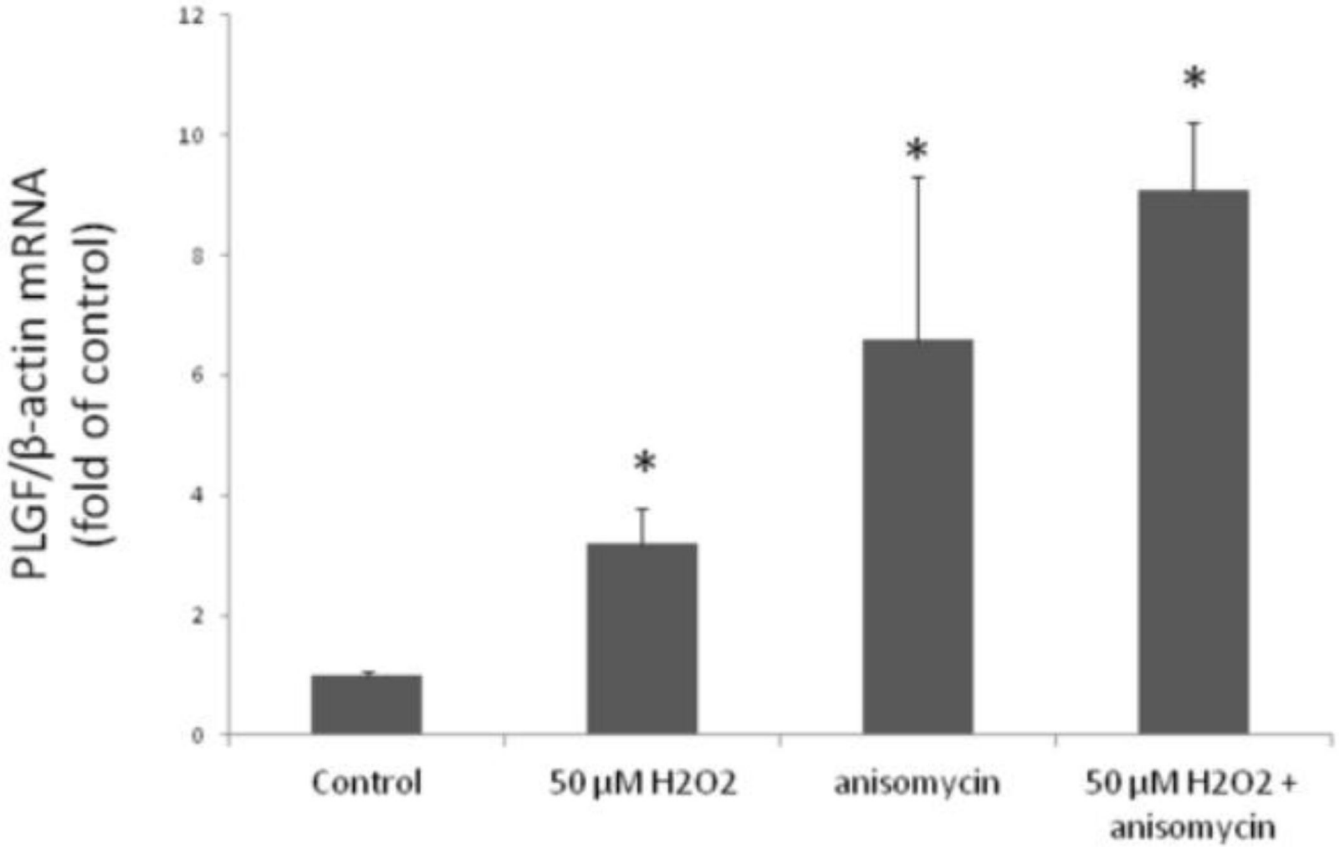

Figure 3. Anisomycin increases PLGF mRNA

Human CASMC treated with a single dose of H_2O_2 (50 µM), H_2O_2 plus anisomycin (10 µg/mL), and anisomycin alone showed a significant (*$p<0.05$, $n=3$) increase in PLGF mRNA, compared to control. There was no statistical difference in PLGF mRNA levels following treatment with H_2O_2 plus anisomycin vs. anisomycin alone.

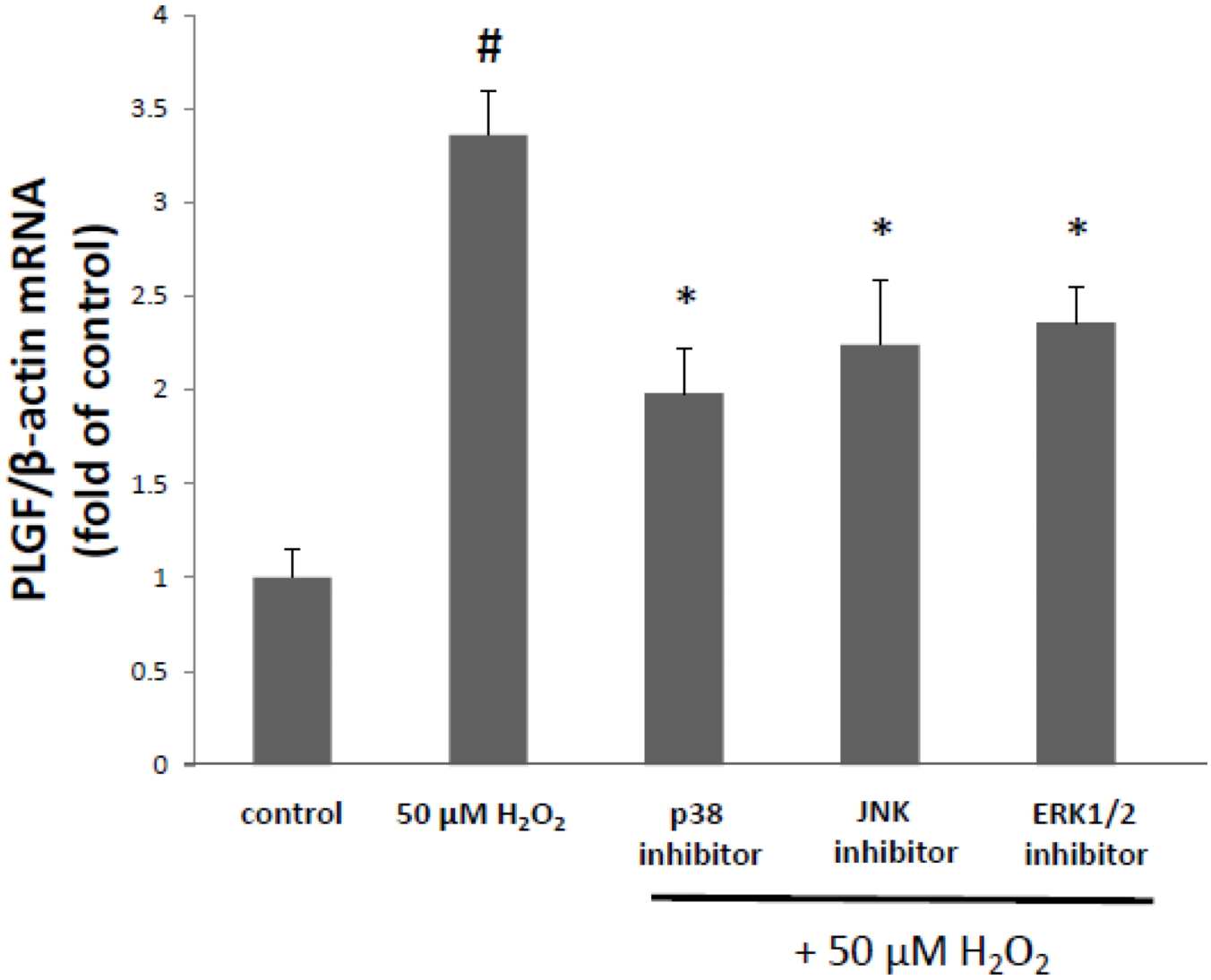

Figure 4. Kinase pathways are involved in the peroxide-induced increase in PLGF mRNA

Human CASMC were treated with a single dose of H_2O_2 (50 μM) in the presence of a p38 MAPK inhibitor (10 μM), JNK inhibitor (10 μM) or ERK1/2 inhibitor (10 μM) for 8 h. All three kinase inhibitors significantly reduced the H_2O_2-induced increase in PLGF mRNA, compared to treatment with H_2O_2 alone (*$p<0.05$, n=3; #$p<0.05$, H_2O_2 treated vs. untreated).

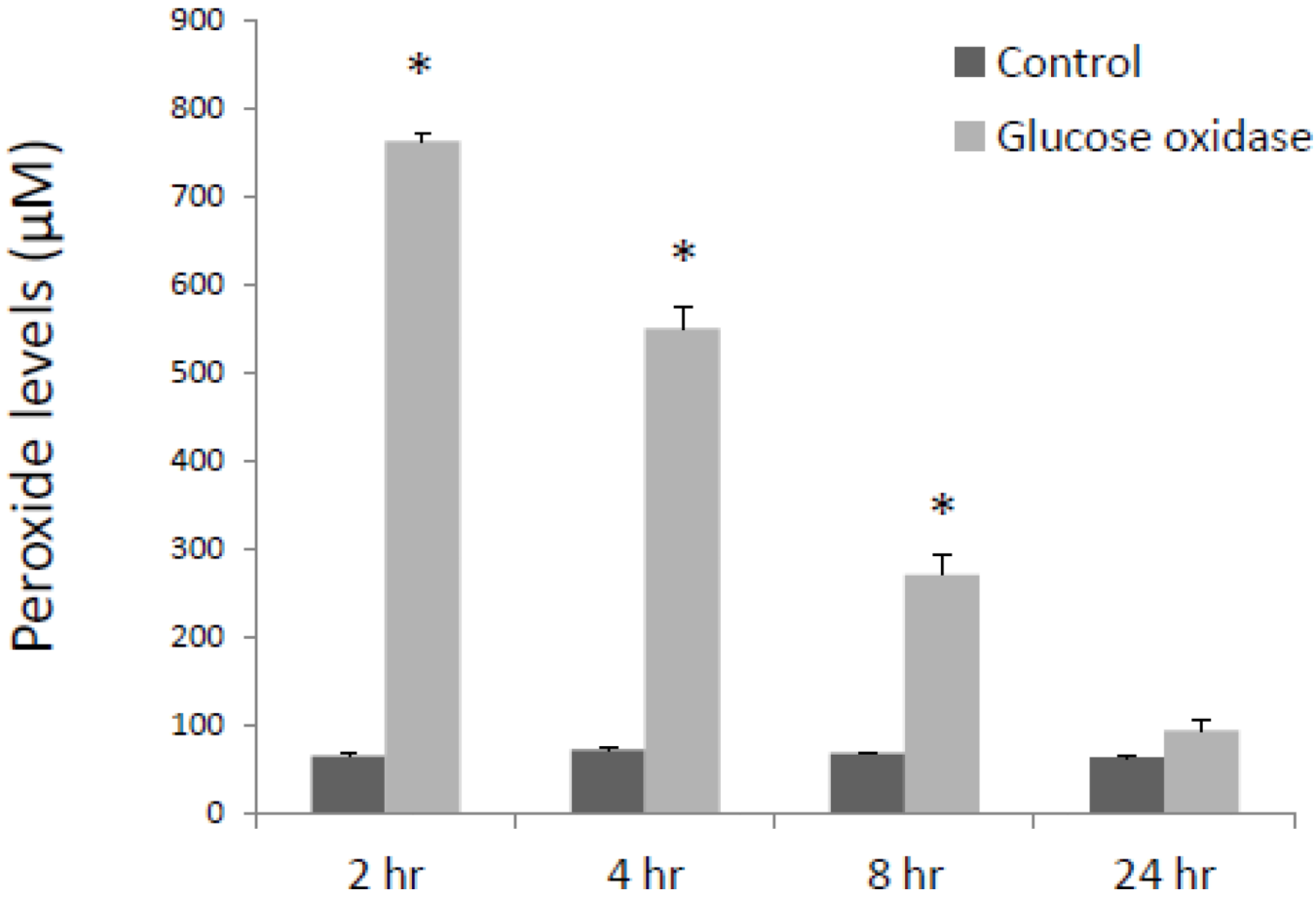

Figure 5. Time course of hydrogen peroxide concentration generated by glucose oxidase

Medium was collected from wells containing human CASMC treated with glucose oxidase (5U/mL) for 2, 4, 8 and 24 h and assayed for peroxide. A large increase in hydrogen peroxide (~750 µM) was observed at 2 h and gradually declined, reaching ~250 µM by 8 h and ~60 µM by 24 h (*$p<0.05$, n=3, control vs. glucose oxidase).

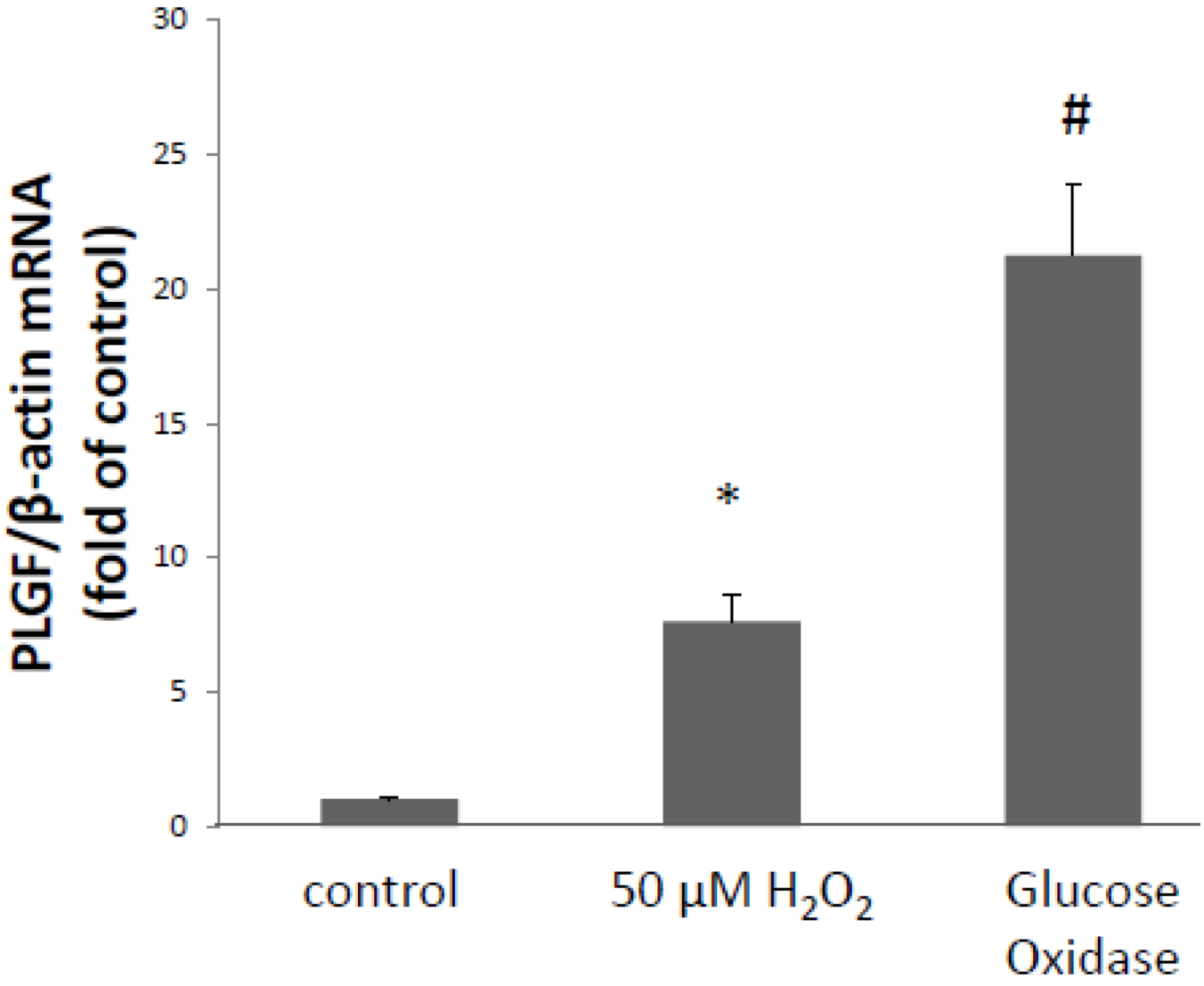

Figure 6. Exposure to continuously elevated levels of peroxide further increases PLGF mRNA

Human CASMC treated with glucose oxidase enzyme (5 U/mL) for 8 h exhibited a ~20-fold increase (#$p<0.001$, n=3) in PLGF mRNA compared to untreated cells, while a single dose of H_2O_2 (50 μM) produced a ~6 fold increase in PLGF mRNA (*$p<0.05$, n=3).

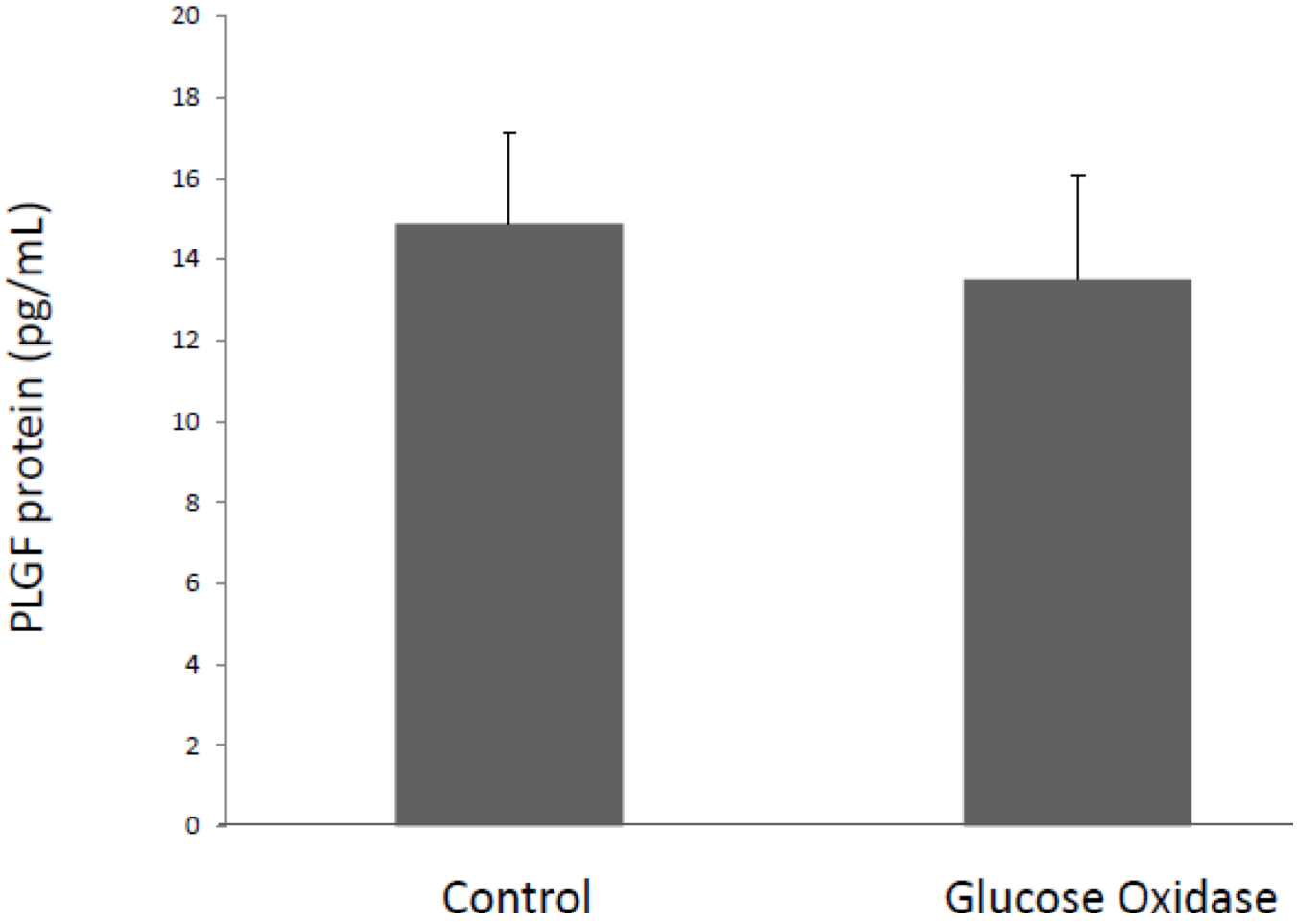

Figure 7. Chronic exposure to highly elevated levels of peroxide does not increase PLGF protein level in CASMC medium

Human CASMC exposed to a sustained high dose of H_2O_2 via treatment with glucose oxidase (5U/mL) showed no significant change in secreted PLGF protein, compared to untreated cells (n=3).