S-glutathionylation of LMW-PTP regulates VEGF-mediated FAK activation and endothelial cell migration

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Summary

Although promising, the ability to regulate angiogenesis through delivery of VEGF remains an unrealized goal. We have shown previously that physiological levels of peroxynitrite (1 μM) are required for a VEGF-mediated angiogenic response, yet the redox-regulated mechanisms that govern the VEGF signal remain unexplored. We assessed the impact of VEGF and peroxynitrite on modifying redox-state, the level of reduced-glutathione (GSH) and S-glutathionylation on regulation of the low molecular weight protein tyrosine phosphatase (LMW-PTP) and focal adhesion kinase (FAK), which are key mediators of VEGF-mediated cell migration. Stimulation of human microvascular endothelial (HME) cells with VEGF (20 ng/ml) or peroxynitrite (1 μM) caused an immediate and reversible negative-shift in the cellular redox-state and thiol oxidation of LMW-PTP, which culminated in cell migration. VEGF causes reversible S-glutathionylation of LMW-PTP, which inhibits its phosphorylation and activity, and causes the transient activation of FAK. Modulating the redox-state using decomposing peroxynitrite (FeTPPS, 2.5 μM) or the GSH-precursor [N-acetylcysteine (NAC), 1 mM] caused a positive-shift of the redox-state and prevented VEGF-mediated S-glutathionylation and oxidative inhibition of LMW-PTP. NAC and FeTPPS prevented the activation of FAK, its association with LMW-PTP and cell migration. Inhibiting LMW-PTP expression markedly enhanced FAK activation and cell migration. Although mild oxidative stress achieved by combining VEGF with 0.1–0.2 mM peroxynitrite augmented cell migration, an acute shift to oxidative stress achieved by combining VEGF with 0.5 mM peroxynitrite induced and sustained FAK activation, and LMW-PTP S-glutathionylation, resulting in LMW-PTP inactivation and inhibited cell migration. In conclusion, our findings demonstrate that a balanced redox-state is required for VEGF to facilitate reversible S-glutathionylation of LMW-PTP, FAK activation and endothelial cell migration. Shifting the redox-state to reductive stress or oxidative stress inhibited the VEGF-mediated angiogenic response.

Key words: Angiogenesis, VEGF, Peroxynitrite, Low molecular weight protein tyrosine phosphatase, S-glutathiolation, Cellular redox-state

Introduction

Angiogenesis plays both beneficial and detrimental roles in cardiovascular diseases. Promoting the angiogenic response has been demonstrated to be beneficial in treating ischemic conditions, such as myocardial ischemia, and delayed wound healing (Xiong et al., 2010; van der Laan et al., 2009). By contrast, abnormally enhanced angiogenic responses are observed during diabetic retinopathy and atherosclerosis (Cao, 2010; Ali and El-Remessy, 2009; Chua and Arbiser, 2009). Although promising, the ability to regulate angiogenesis therapeutically remains an unrealized goal. The role of reactive oxygen species (ROS) as a downstream messenger for vascular endothelial growth factor (VEGF) to promote angiogenesis has been established (reviewed by Colavitti et al., 2002; Colavitti and Finkel, 2005; Ushio-Fukai, 2006; Ushio-Fukai, 2007). In particular, our group demonstrated a crucial role of peroxynitrite, the reaction product of nitric oxide and superoxide anion, in modulating the VEGF signal. Whereas physiologically low levels of peroxynitrite are required to mediate the angiogenic VEGF signal, pathological peroxynitrite levels impair the survival of the VEGF signal and can induce cell death (Gu et al., 2003; El-Remessy et al., 2005; El-Remessy et al., 2007; Abdelsaid et al., 2010). These studies suggest that there is an unidentified redox-regulated mechanism controlling VEGF angiogenic signal and function.

Glutathione (GSH), the most abundant intracellular thiol, not only regulates the redox-state but functions as a signaling molecule to regulate cell proliferation and apoptosis (Rahman and MacNee, 2000; Okamoto et al., 2001). In response to peroxynitrite, protein thiols can undergo post-translational modification by S-nitrosylation, oxidation, formation of disulfides and S-glutathionylation (Wang et al., 2003; Clavreul et al., 2006; Dremina et al., 2007; Sethuraman et al., 2007; Liaudet et al., 2009; Chinta and Andersen, 2011). Under oxidative stress, S-glutathionylation, a reversible protein modification, occurs through the formation of protein-mixed disulfides (protein–SSG) with GSH (Okamoto et al., 2001; Chen et al., 2010) and, hence, S-glutathionylation serves as a mechanism to protect regulatory thiols from irreversible oxidation (Shelton and Mieyal, 2008). De-glutathionylation is catalyzed generally by restoration of the reductive GSH:GSSG ratio and more efficiently by the reducing systems, thioredoxin and glutaredoxin (Dalle-Donne et al., 2009). S-glutathionylation can result in transient activation or inactivation of the protein, and the reversibility of this process dictates its physiological relevance (Shelton and Mieyal, 2008).

Journal of Cell Science

Journal of Cell Science

Low molecular weight protein tyrosine phosphatase (LMW-PTP) regulates rearrangement of the cytoskeleton, endothelial cell growth and differentiation through its ability to bind and dephosphorylate focal adhesion kinase (FAK) and activated growth factor receptors (Huang et al., 1999; Raugei et al., 2002; Shimizu et al., 2005; Kanda et al., 2006). In particular, FAK plays a crucial role in VEGF-induced endothelial cell migration through regulation of focal adhesion assembly and disassembly (Chiarugi et al., 2003; Li and Hua, 2008; Tomar and Schlaepfer, 2009). LMW-PTP shares the CX5R motif with other protein tyrosine phosphatases (PTPs), which render them vulnerable targets for cellular redox changes (Okamoto et al., 2001; Shackelford et al., 2005; Shelton and Mieyal, 2008). Furthermore, a unique property of LMW-PTP is that it has two cysteines that are located in the catalytic pocket; both cysteines must be reduced for enzyme phosphorylation and activity (Chiarugi, 2001; Chiarugi et al., 2001; Xing et al., 2007). Therefore, the activity of LMW-PTP is tightly linked to redox-changes and can be a molecular switch for the regulation of cell migration and angiogenesis (Giannoni et al., 2006). Nevertheless, S-glutathionylation of LMW-PTP in response to VEGF and how this process can regulate FAK migratory signals has not been elucidated.

Here, we attempt to demonstrate the molecular events by which VEGF differentially modulates GSH levels to facilitate S-glutathionylation of LMW-PTP, activation of FAK and cell migration. We also demonstrate the impact of shifting cellular redox-state to oxidative stress or reductive stress on VEGF-mediated S-glutathionylation of LMW-PTP and angiogenic response.

Results

VEGF causes a reversible negative-shift in the redox-state of endothelial cells

We have previously shown that stimulation of endothelial cells with VEGF produces low levels of peroxynitrite (El-Remessy et al., 2007). To examine the effects of VEGF stimulation on cellular redox-state, human microvascular endothelial (HME) cells were stimulated with VEGF (20 ng/ml). The cellular redox-state was assessed by reduced-glutathione (GSH) levels. Stimulation with VEGF caused oxidation of GSH, which resulted in a negative shift in endothelial redox-state as indicated by a 34% and 38% reduction of GSH levels at 1 and 5 minutes, respectively. The cellular redox-state was restored back to its baseline level after 15–30 minutes (Fig. 1A).

VEGF causes reversible thiol oxidation and inactivation of LMW-PTP in endothelial cells

Because LMW-PTP is a redox-regulated phosphatase that plays an important role in the angiogenic process, we investigated the effects of VEGF on the LMW-PTP redox-state. To quantify total free thiols, HME cells were labeled with fluorescein-tagged 5 iodo-acetamide (5-IAM). As shown in Fig. 1B, VEGF caused immediate thiol oxidation of LMW-PTP, indicated by a reduction of free thiol by 40% and 35% at 1 and 5 minutes, respectively. In parallel to the cellular redox-state, LMW-PTP thiol oxidation was transient and free thiols were restored back to normal levels at 15–30 minutes (Fig. 1B). Because oxidation of LMW-PTP can inhibit its activation, we examined the phosphorylation of the LMW-PTP. Indeed, phosphorylation of LMW-PTP was not detected at 1 and 5 minutes, coinciding with its oxidation, whereas it was markedly increased (1.7-fold) after 15 minutes, a time point where free thiols LMW-PTP are reduced (see Fig. 1C).

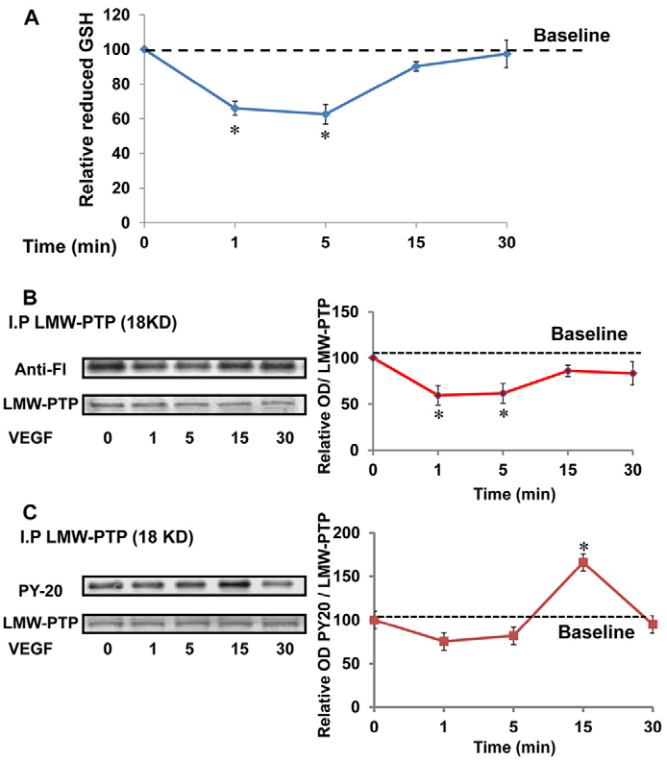

Fig. 1. VEGF causes a reversible negative-shift in the cellular redox state and LMW-PTP redox state in HME cells. (**A**) A graph showing statistical analysis for the relative levels of free GSH detected by DTNB. VEGF (20 ng/ml) caused a transient decrease in GSH levels, which were restored after 15 minutes. (**B**) Thiol oxidation of LMW-PTP detected by immunoprecipitation of 5-IAM labeled lysate with anti-LMW-PTP antibodies and blotted with anti-fluorescein (anti-Fl) antibodies. (**C**) Phosphorylation of LMW-PTP was assessed by immunoprecipitation of LMW-PTP and blotting with anti-PY20 antibodies (n=4–6, *P<0.05 versus zero time).

VEGF causes reversible S-glutathionylation and inhibition of LMW-PTP activity

S-glutathionylation is the final step for protective cysteine modification in response to oxidative stress. VEGF induced an increase in S-glutathionylation of LMW-PTP by 2.6- and 2.4-fold after 5 and 10 minutes, respectively. S-glutathionylation was reversible as the signal was inhibited at 15–30 minutes (Fig. 2A). The specificity of detecting S-glutathionylation was confirmed by complete blocking of the signal when samples were pre-treated with DTT. Assessment of the phosphatase activity of LMW-PTP showed that oxidized-glutathione (GSSG, 10 mM) substantially reduced phosphatase activity to 29.7% of its baseline levels. To further confirm the effect of S-glutathionylation on the enzymatic activity of LMW-PTP, reduced-glutathione (GSH, 10 mM) increased LMW-PTP activity 4-fold (Fig. 2B).

VEGF-induced peroxynitrite stimulates FAK activation and association with LMW-PTP

Because LMW-PTP is the specific phosphatase for FAK, we investigated its tyrosine phosphorylation in response to VEGF. As shown in Fig. 3A, VEGF caused activation of FAK, which peaked at 15 minutes (2.5-fold from the baseline), and which was blocked with the peroxynitrite decomposition catalyst FeTPPS (2.5 μM), suggesting a key role for peroxynitrite (Fig. 3A, lower panel). In

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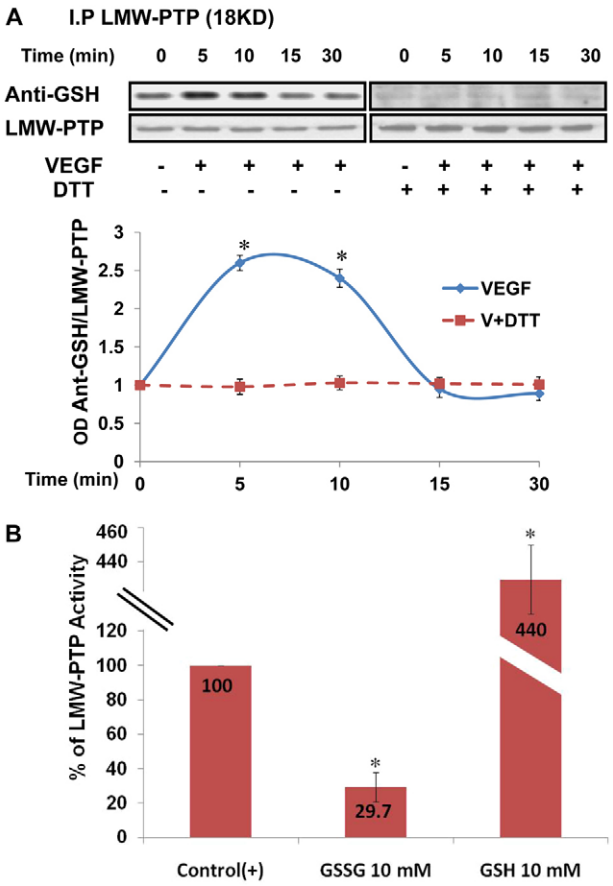

Fig. 2. VEGF causes S-glutathionylation and inhibition of LMW-PTP activity in HME cells. (**A**) Cellular lysate was immunoprecipitated with LMW-PTP, incubated with or without DTT (100 mM) at 70℃ for 10 minutes and then blotted with anti-GSH antibodies. VEGF induced significant and reversible S-glutathiolation of LMW-PTP at 5-10 minutes, which was inhibited after 15 minutes (n=4, *P<0.05 versus zero time). (**B**) The enzyme activity of LMW-PTP was tested against 10 mM of GSSG or GSH and compared with the control. Whereas GSSG caused a significant decrease (70%) in the activity of LMW-PTP, GSH increased the enzyme activity to four times its baseline value (n=4, *P<0.05 versus control).

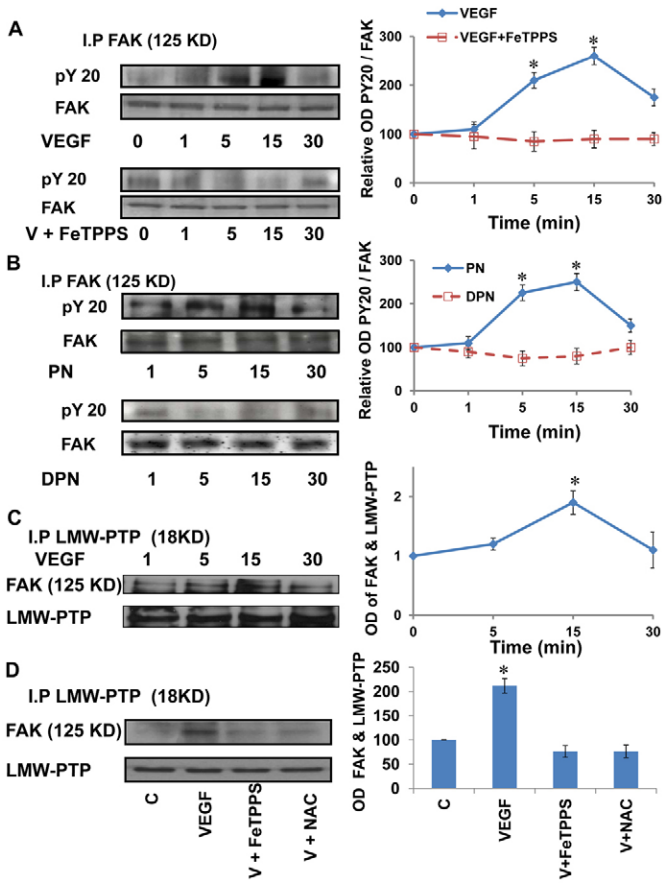

Fig. 3. VEGF-induced peroxynitrite stimulates FAK activation and association between LMW-PTP and FAK in HME cells. (**A**) Total FAK phosphorylation was assessed by immunoprecipitation of FAK and immunoblotting with anti-PY20 antibodies. VEGF (20 ng/ml) caused a time-dependent FAK tyrosine phosphorylation, which that peaked at 15 minutes and was completely blocked with the peroxynitrite decomposition catalyst FeTPPS (2.5 μM). (**B**) Physiological levels of peroxynitrite (PN, 1 μM), but not the decomposed peroxynitrite (DPN), induced the phosphorylation of FAK, which peaked at 15 minutes. (**C**) VEGF stimulates the association between LMW-PTP and FAK (1.9-fold increase in association) at 15 minutes. Cellular lysate was immunoprecipitated with anti-LMW-PTP antibodies and blotted with anti-FAK antibodies. (**D**) VEGF-induced association between LMW-PTP and FAK at 15 minutes was prevented by pre-treatment of HME cells with FeTPPS (2.5 μM) or NAC (1 mM). Cells were treated with VEGF for 15 minutes. (n=4, *P<0.05 versus control).

parallel, peroxynitrite (1 μM) induced FAK activation, which peaked at 15 minutes (2.6-fold), compared with decomposed peroxynitrite (DPN) (Fig. 3B). We then tested the interaction between the LMW-PTP and the FAK in response to VEGF treatment. Fig. 3C shows that VEGF stimulated the association between LMW-PTP and FAK, which peaked at 15 minutes (1.9-fold), a time point at which FAK is fully activated. The association resulted in the blocking of FAK activation (30 minutes). Next, we selected the 15 minute time point to examine the effect of decomposing the peroxynitrite, either by FeTPPS or the general antioxidant NAC, on the association of LMW-PTP with FAK. Fig. 3D showed that treatment with FeTPPS and NAC prevented the association of LMW-PTP with FAK at 15 minutes.

A positive shift in redox-state inhibits S-glutathionylation of LMW-PTP and cell migration

As shown in Fig. 4A, the ability of VEGF to oxidize GSH was inhibited at 1-5 minutes by pre-treatment with FeTPPS or NAC. Moreover, FeTPPS or NAC caused a positive shift in the cellular redox-state at 15-30 minutes with an increase of 1.9-fold. As shown in Fig. 1B, the maximum effect of VEGF on altering the redox-state of LMW-PTP occurred between 1 and 5 minutes. Therefore, we chose the 5 minute time point to examine the effect of the inhibitors. Treatment of HME cells with VEGF caused a 64% increase in LMW-PTP thiol oxidation that was blocked by FeTPPS or NAC (Fig. 4B). In parallel, physiologically low levels of peroxynitrite (1 μM) caused a 65% increase in LMW-PTP thiol oxidation that was blocked by FeTPPS or NAC (Fig. 4C).

Next, we assessed the impact of reductive stress on VEGF-mediated S-glutathionylation of LMW-PTP and cell migration. As shown in Fig. 5A, pre-treatment of HME cells with NAC or FeTPPS inhibited VEGF induced S-glutathiolation of LMW-PTP that peaked at 5 minutes. A wound healing assay revealed an ~1.8-fold increase in cell migration in response to VEGF when

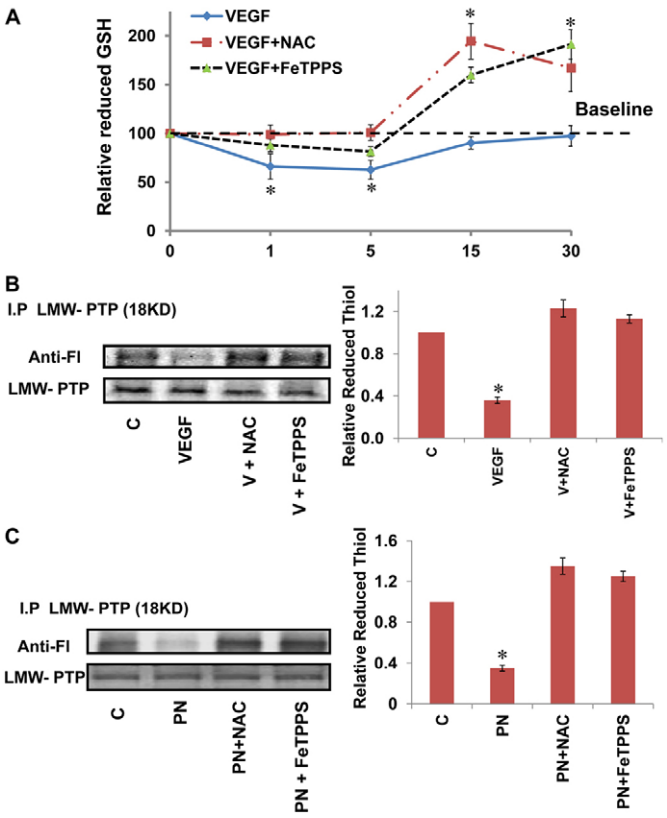

Fig. 4. Reductive stress inhibits VEGF and peroxynitrite-mediated LMW-PTP thiol oxidation. (**A**) A graph showing statistical analysis for the relative levels of free GSH detected by DTNB. VEGF (solid line) induced the reversible oxidation of GSH, which was restored after 15-30 minutes. Treatment with the peroxynitrite decomposition catalyst FeTPPS (2.5 μM, dotted line) or the GSH-precursor NAC (1 mM, dash-dotted line) inhibited the immediate VEGF effect and significantly shifted the redox-state to the positive side within 15-30 minutes (n=4-6, *P<0.05 versus zero time). (**B-C**) FeTPPS and NAC prevented the thiol oxidation of LMW-PTP. Thiol oxidation of LMW-PTP was detected in 5-IAM labeled cell lysate by immunoprecipitation of LMW-PTP and blotting with anti-fluorescein (anti-Fl) antibodies. Cells were pre-treated with NAC and FeTPPS followed by 5 minutes stimulation of VEGF (20 ng/ml) shown in B or peroxynitrite (PN, 1 μM) shown in C (n=4, *P<0.05 versus control).

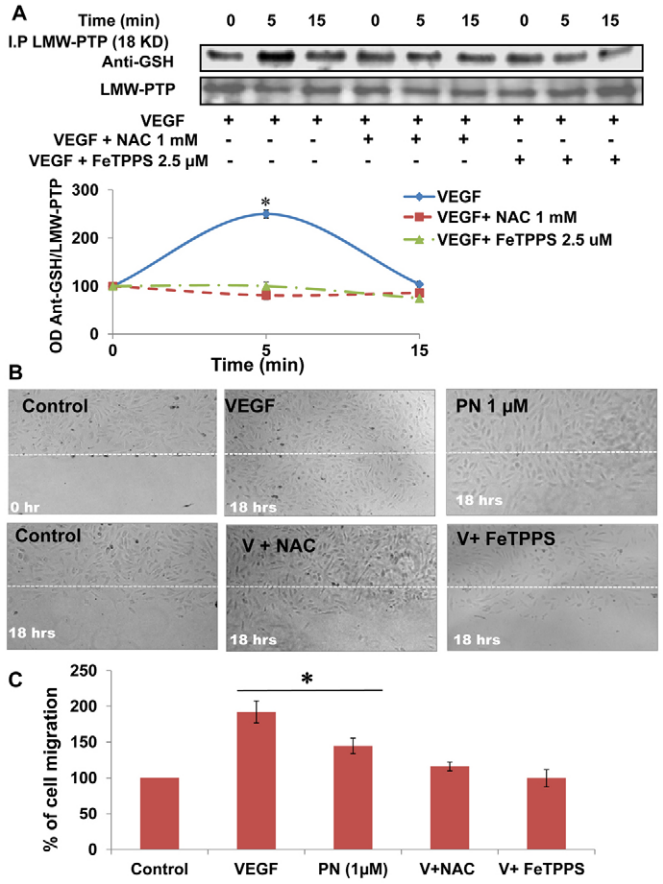

Fig. 5. Reductive stress inhibits VEGF-induced S-glutathionylation of LMW-PTP and HME cell migration. (**A**) Pre-treatment of HME cells with NAC (1 mM) or FeTPPS inhibited VEGF-mediated S-glutathionylation of LMW-PTP, which peaked at 5 minutes. (**B**) Representative micrographs for wounded HME cells after 18 hours of various treatments. (**C**) Statistical analysis showing that peroxynitrite (PN, 1 μM) mimicked VEGF in stimulating cell migration. The effects of VEGF were inhibited by a modulating redox-state by using FeTPPS (2.5 μM) or NAC (1 mM) (n=4, *P<0.05 versus control).

compared with the control. Peroxynitrite (1 μM) mimicked VEGF action and caused an ~1.5-fold increase in cell migration. The treatment of cells with FeTPPS (2.5 μM) or NAC (1 mM) substantially inhibited the VEGF-induced endothelial migration (Fig. 5B–C).

Oxidative stress augments VEGF-mediated effects in inhibiting LMW-PTP activity and stimulating cell migration

To elucidate the impact of oxidative stress milieu on VEGF-mediated angiogenic response, HME cells were stimulated with VEGF (20 ng/ml) in combination with escalating levels of peroxynitrite (0.1 to 0.5 mM). As shown in Fig. 6A, VEGF in combination with 0.1 or 0.2 mM peroxynitrite exerted comparable and reversible effects on the oxidation of GSH compared with VEGF alone. By contrast, combining VEGF with peroxynitrite (0.3-0.5 mM) caused immediate and dramatic oxidation (1-5 minutes), as indicated by the decrease in free GSH levels to 55 and 38% of the baseline value, which remained inhibited at 60-45%, respectively over 30 minutes (Fig. 6A). We then evaluated the action of these combinations of VEGF with peroxynitrite on cell migration. As shown in Fig. 6B, mild oxidative stress (0.1-0.2 mM) enhanced VEGF-mediated cell migration. Increasing levels of peroxynitrite (0.3-0.5 mM) impaired the angiogenic action of VEGF and halted cell migration. To confirm the effect of a high level of peroxynitrite on LMW-PTP enzymatic activity, the phosphatase activity of LMW-PTP was assessed against different concentrations of peroxynitrite. Our results showed a concentration-dependent inhibition of LMW-PTP activity that reached complete inhibition at 0.5 mM peroxynitrite (Fig. 6C). Because LMW-PTP lacks a pharmacological inhibitor, we assessed endogenous phosphatase activity by comparing total enzyme activity in cells that were transduced with scrambled siRNA versus cells transduced with siRNA against LMW-PTP. The analyses show that whereas VEGF alone transiently inactivates LMW-PTP at 5 minutes (a time point for S-glutathionylation), the activity of LMW-PTP is almost restored back to its baseline level by 30 minutes (a time point where

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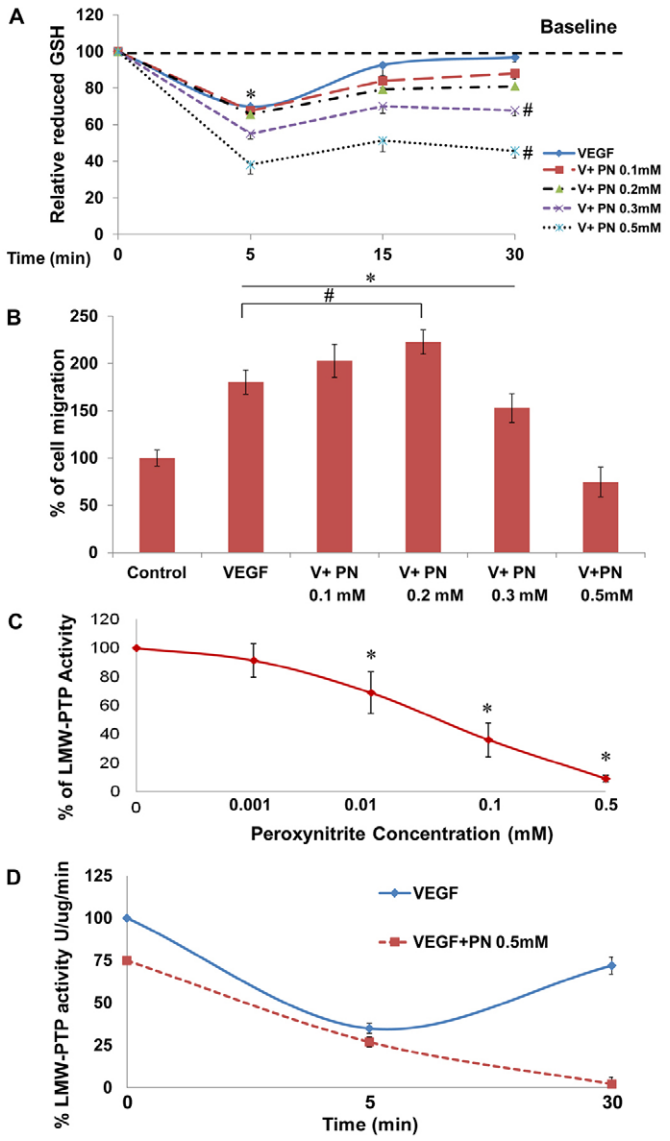

Fig. 6. Oxidative stress augments the VEGF-mediated effects of inhibiting LMW-PTP activity and stimulating cell migration. (**A**) A graph showing the statistical analysis for the relative free GSH levels in response to VEGF (20 ng/ml) with increasing amounts of peroxynitrite (PN, 0.1-0.5 mM). The combination of VEGF and peroxynitrite (PN, 0.1-0.2 mM) produced a comparable pattern to VEGF alone. By contrast, the combination of VEGF with peroxynitrite (PN, 0.3-0.5 mM) shifted the redox-state to the negative side over the 30 minute period. (n=4-6, *P<0.05 versus VEGF). (**B**) A wound healing assay, which shows that whereas the combination of VEGF with mild oxidative stress (PN, 0.1-0.2) enhanced VEGF-mediated cell migration, conditions of high oxidative stress (0.3-0.5 mM) impaired the angiogenic action of VEGF (n=4, *P<0.05 versus zero time, $^{\#}P$<0.05 versus corresponding VEGF points). (**C**) Peroxynitrite causes a concentration-dependent reduction in recombinant LMW-PTP enzyme activity, and maximum inhibition was reached at 0.5 mM of peroxynitrite (n=4–5, *P<0.05 versus control). (**D**) VEGF causes a significant and transient inactivation of endogenous LMW-PTP at 5 minutes, which was restored back to the baseline level by 30 minutes. The combination of VEGF and 0.5 mM peroxynitrite caused a significant and permanent inhibition (5-30 minutes) of LMW-PTP phosphatase activity. (n=3-4, *P<0.05 versus control).

LMW-PTP is phosphorylated instead of S-glutathionylated). The combination of VEGF with 0.5 mM peroxynitrite substantially inhibited the phosphatase activity of LMW-PTP at 5 minutes and continued to decline at 30 minutes.

Acute oxidative stress sustains VEGF-mediated S-glutathionylation of LMW-PTP and activation of FAK in HME cells

To simulate pathological conditions, we evaluated the combination of VEGF with 0.5 mM peroxynitrite on LMW-PTP protein modification. The results showed significant and persistent thiol oxidation of LMW-PTP over a 30 minute time course (Fig. 7A). In parallel, VEGF combined with 0.5 mM peroxynitrite induced significant S-glutathionylation of LMW-PTP, which persisted over 30 minutes (Fig. 7B). This effect was associated with the complete loss of LMW-PTP phosphorylation for 30 minutes (Fig. 7C). Next,

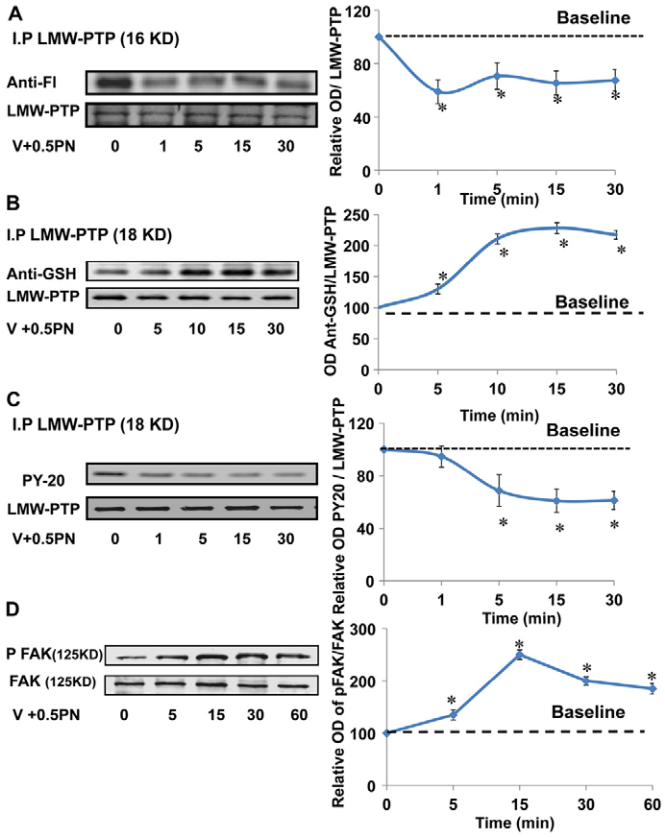

Fig. 7. Acute oxidative stress sustains VEGF-mediated S-glutathionylation of LMW-PTP and FAK activation in HME cells. Thiol oxidation and S-glutathionylation of LMW-PTP were assessed as described in Materials and Methods. (**A**) A combination of VEGF with 0.5 mM peroxynitrite induced an immediate thiol oxidation of LMW-PTP (40%), which persisted over 30 minutes. (**B**) A combination of VEGF with 0.5 mM peroxynitrite induced the S-glutathionylation of LMW-PTP (2-fold), which persisted over 30 minutes. (**C**) A combination of VEGF with 0.5 mM peroxynitrite significantly inhibited phosphorylation of LMW-PTP to 50% of its baseline levels over 30 minutes. (**D**) A combination of VEGF with 0.5 mM peroxynitrite induced activation of FAK-Y397, which persisted over 60 minutes (n=4, *P<0.05 versus zero time).

we examined the activation of FAK^{Y397}, the auto-phosphorylation site of FAK. The combination of VEGF and 0.5 mM peroxynitrite induced the activation of FAK (2.5-fold) at 15 minutes, and this activation was sustained over 30-60 minutes (Fig. 7D).

Silencing LMW-PTP expression stimulates FAK phosphorylation and endothelial cell migration

LMW-PTP expression was silenced in HME cells by using siRNA. As shown in Fig. 8A, the level of LMW-PTP expression

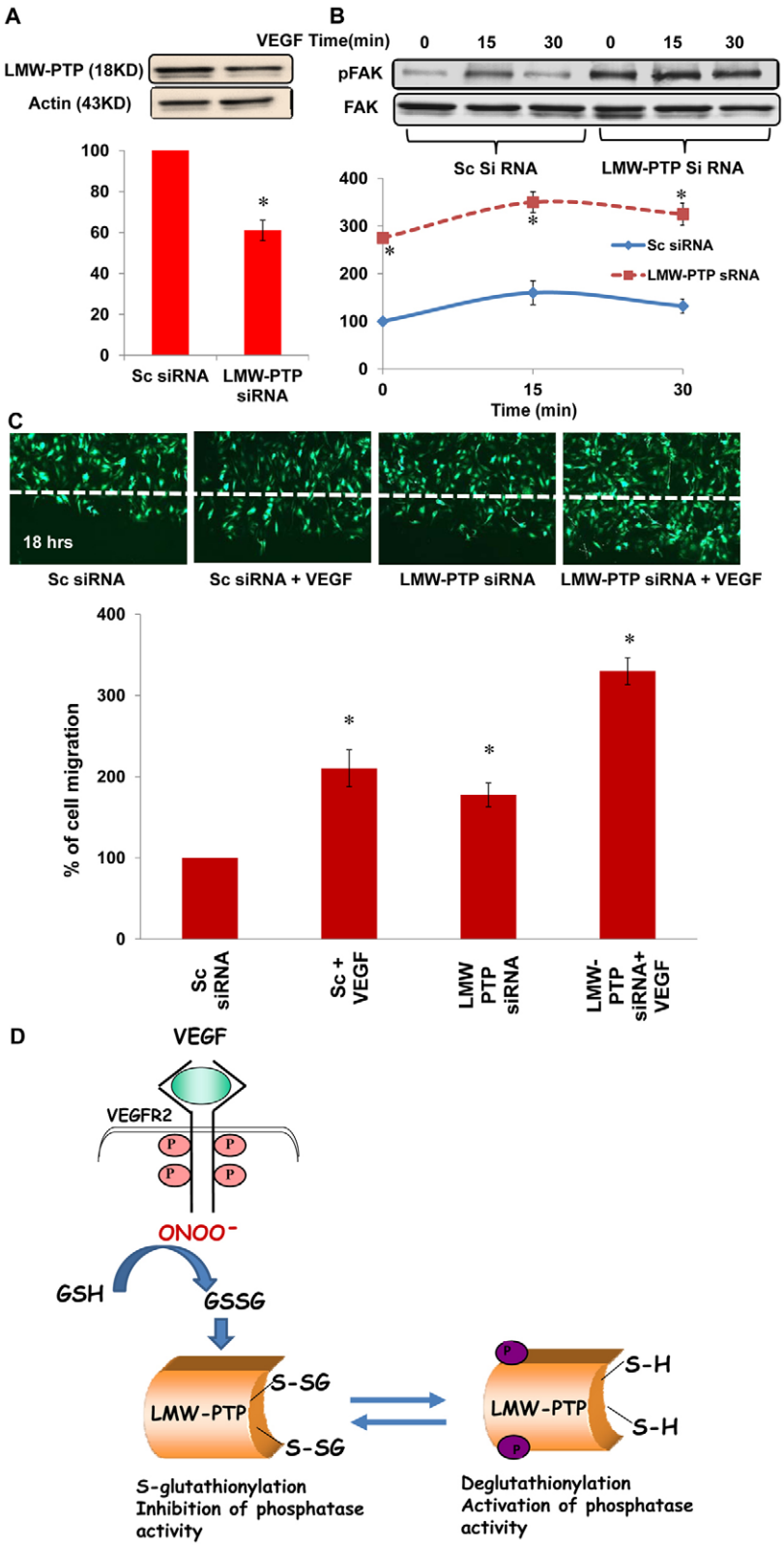

Fig. 8. Silencing LMW-PTP expression stimulates FAK phosphorylation and endothelial cell migration. LMW-PTP expression was silenced in human microvascular endothelial (HME) cells by using siRNA. (**A**) Silencing expression of LMW-PTP caused a 40% reduction in the expression of LMW-PTP in HME cells as shown by western blot analysis. (**B**) Silencing expression of LMW-PTP using siRNA significantly enhanced the activation of FAK at the baseline level (2.7-fold) as well as in response to VEGF (3.5-fold). (**C**) Silencing expression of LMW-PTP using siRNA enhanced endothelial cell migration at the baseline (~2-fold) and in response to VEGF stimulation (3.5-fold) (n=4, *P<0.05 versus zero time). (**D**) A schematic representation of the proposed mechanism for VEGF-mediated S-glutathionylation of LMW-PTP and regulation of the angiogenic response.

Journal of Cell Science

Journal of Cell Science

was reduced by 40%, as indicated by western blot analysis. We then examined the effect of inhibiting the expression of LMW-PTP on FAK activation and cell migration. The results showed that silencing the expression of LMW-PTP significantly enhanced FAK activation at the baseline level (2.7-fold) as well as in response to VEGF (3.5-fold) (Fig. 8B). Moreover, silencing LMW-PTP expression enhanced endothelial cell migration in non-treated cells (~2-fold) and in response to VEGF stimulation (3.5-fold) (Fig. 8C).

Discussion

The major findings of the present study can be summarized as follows: (1) VEGF-induced peroxynitrite transiently reduces the endothelial redox-state to facilitate reversible S-glutathionylation and inhibition of LMW-PTP, and cell migration. (2) Reductive stress prevents S-glutathionylation of LMW-PTP, FAK activation and cell migration. (3) Whereas mild oxidative stress augments the VEGF-mediated cell migration signal, acute oxidative stress sustains VEGF-mediated activation of FAK and S-glutathiolation of LMW-PTP, resulting in the inhibition of LMW-PTP phosphorylation and cell migration.

We, and others, have demonstrated a crucial role for ROS and peroxynitrite in mediating VEGF angiogenic signal (Colavitti et al., 2002; Ushio-Fukai et al., 2002; El-Remessy et al., 2007). The potent inhibitory effect of decomposing peroxynitrite using FeTPPS or the thiol donor and GSH-precursor (NAC), but not a nitration inhibitor, suggested that VEGF modulates GSH levels and induces cysteine modification to regulate the VEGF signal (Ushio-Fukai et al., 2002; El-Remessy et al., 2007). The mechanism of GSH antioxidant activity occurs through the formation of reversible protein-glutathione disulfides, or S-glutathionylation (Shackelford et al., 2005). However, the impact of VEGF on modulating GSH and identifying a redox-sensitive molecular target to regulate angiogenesis remains unexplored. LMW-PTP is a redox-sensitive target that plays a pivotal role in VEGF-mediated cell migration and angiogenesis, both *in vitro* and *in vivo*, as it has been shown to regulate the activation of VEGFR2 (Huang et al., 1999) as well as FAK (Chiarugi et al., 2001). The causal role of LMW-PTP in regulating FAK activation and angiogenic response was demonstrated further by our results showing HME cells in which LMW-PTP expression is silenced have a marked increase in FAK activation and cell migration in non-treated cells as well as in response to VEGF (Fig. 8).

Although previous studies demonstrated the importance of LMW-PTP in regulating VEGF-mediated angiogenesis, the molecular events by which VEGF can regulate the enzyme activation remain poorly understood. In the present study, we assessed the changes of cellular redox-state and in particular of the LMW-PTP in response to VEGF in microvascular endothelial cells. Our results showed that VEGF caused the immediate oxidation of cellular free GSH, which is normally maintained at high levels (Okamoto et al., 2001), and that the oxidation was reversible, as shown by the restoration of reduced GSH within 15-30 minutes. In parallel, VEGF induced the immediate yet reversible thiol oxidation of LMW-PTP (1-5 minutes), which was restored to normal levels after 15 minutes. The activity of LMW-PTP is dually regulated, whereby the two active cysteines (C13 and C18) that are located in the catalytic pocket must be reduced to allow enzyme phosphorylation and activity (Chiarugi, 2001; Chiarugi et al., 2001; Xing et al., 2007). In agreement, VEGF-induced phosphorylation of LMW-PTP was detected only after 15-30 minutes, a time frame where thiols of LMW-PTP are not oxidized. Our results lend further support to a recent report showing that VEGF increased thiol oxidation and formation of cysteine sulfenic acid (Cys-OH) of the scaffold protein (IQGAP1) to promote endothelial migration (Kaplan et al., 2011).

There is growing evidence that active cysteine sites undergo post-translational modification by disulphide bond, S-nitrosylation, oxidation to sulfenic acid (Cys-SOH) or S-glutathionylation. The latter is perceived as the final step for cysteine modification to protect Cys-SOH from further irreversible oxidation to cysteine-sulfinic acid (Cys-SO_2H) and cysteine-sulfonic acid (Cys-SO_3H) (Barrett et al., 1999b). De-glutathionylation of a given protein dictates its physiological relevance (Shelton and Mieyal, 2008) and can be achieved by a reductive GSH:GSSG ratio, and catalyzed specifically and efficiently by the antioxidant systems glutaredoxin and thioredoxin (Dalle-Donne et al., 2009). Here, we tested the hypothesis that VEGF-induced oxidation of GSH can stimulate S-glutathionylation of LMW-PTP and decrease its activity. Indeed, VEGF induced strong S-glutathionylation of LMW-PTP thiols (5-10 minutes), which coincided with inhibited phosphorylation of LMW-PTP (Fig. 1C) and endogenous phosphatase activity at 5 minutes (Fig. 6D). S-glutathionylation declined after 15-30 minutes, a time frame when the redox-state free GSH levels went back to baseline levels, the phosphorylation of LMW-PTP was prominent (Fig. 1) and endogenous phosphatase activity was also restored to baseline levels (Fig. 6D). We then examined the effect of GSH or GSSG on recombinant LMW-PTP phosphatase activity. We used recombinant LMW-PTP for this assay to overcome the technical difficulty to dissect the activity of cellular LMW-PTP from other cellular phosphatases. Oxidized-glutathione GSSG inhibited the LMW-PTP phosphatase activity to one third of its baseline value, whereas the reduced-GSH increased LMW-PTP activity by four times. These results are in agreement with recent reports showing the inhibitory effect of S-glutathionylation and GSSG on VEGF-mediated activation of nitric oxide synthetase in endothelial cells (eNOS) (Langston et al., 2007; Chen et al., 2010).

S-glutathionylation, a protein modification in response to oxidative or nitrative stress can specifically modulate protein function and hence regulate cell signaling (Dalle-Donne et al., 2009). Reversible S-glutathionylation of PTP-1B and LMW-PTP has been demonstrated in response to superoxide anions and platelet-derived growth factor (PDGF), respectively (Barrett et al., 1999a; Kanda et al., 2006). To further illustrate the impact of VEGF-induced S-glutathionylation of LMW-PTP, we examined the activation of FAK and endothelial cell migration. Our results showed that VEGF or physiological levels of peroxynitrite activated FAK, which peaked at 15 minutes. Further to this, FAK activation was detected in endothelial cells treated with H_2O_2 (Vepa et al., 1999). Our results showed that VEGF-mediated association between LMW-PTP and FAK occurred at 15 minutes, a time point where LMW-PTP was phosphorylated (its thiols are not oxidized or S-glutathionylated). Decomposing peroxynitrite using FeTPPS or NAC inhibited VEGF-mediated association between FAK and LMW-PTP. Whereas previous studies support the notion that thiols of LMW-PTP can be oxidized and that its activity is glutathione-dependent (Chiarugi et al., 2001; Kanda et al., 2006; Xing et al., 2007; de Souza

Journal of Cell Science

Malaspina et al., 2009), we believe that our results are the first to demonstrate S-glutathionylation of LMW-PTP as a regulator of VEGF angiogenic signal.

The concept of the 'redox window' whereby achieving a balanced redox-state is essential to allow reparative angiogenesis was recently introduced by Chilian and colleagues (Yun et al., 2009). In support, our results showed that reductive stress or positive shifting of the redox-state by treating the cells with FeTPPS or NAC, inhibited VEGF-mediated thiol oxidation of LMW-PTP, S-glutathiolation of LMW-PTP, its association with FAK and HME cell migration. These results lend further support to previous findings showing that increasing cellular antioxidant defence could impair the VEGF-mediated tube formation *in vitro* (Rocic et al., 2007), choroidal neovascularization (Hara et al., 2010) and tumor angiogenesis (Jo et al., 2011).

Whereas oxidative stress can contribute to pathological neovascularization as in tumor angiogenesis and diabetic retinopathy (Ali and El-Remessy, 2009; Tertil et al., 2010), excessive oxidative stress can exert apoptotic effects (Gu et al., 2003; El-Remessy et al., 2005). To better understand the complex interplay of oxidative stress on the VEGF angiogenic signal, we examined its effect in combination with various levels of exogenous peroxynitrite. Although combining VEGF with modest levels of peroxynitrite (0.1-0.2 mM) gradually shifted the redox-state to the negative side, the redox state was then restored to the baseline level. However, the combination of VEGF with higher levels of peroxynitrite (0.3-0.5 mM) resulted in the redox state remaining permanently negative. In parallel, modest increases of peroxynitrite (0.1-0.2 mM) augmented the VEGF angiogenic effect, whereas excessive levels impaired VEGF angiogenic function. Although generated using an artificial system of oxidative stress, these results demonstrate the impact of local levels of ROS on modifying the outcome of growth-factor signaling. In contrast to VEGF-mediated reversible S-glutathionylation, VEGF with 0.5 mM peroxynitrite induced and sustained S-glutathionylation and thiol oxidation of LMW-PTP. These effects coincided with sustained FAK activation, inhibited LMW-PTP phosphorylation (Fig. 7) and permanently impaired phosphatase activity (Fig. 6D). Moreover, peroxynitrite caused concentration-dependent inhibition of LMW-PTP phosphatase activity, which was completely impaired at 0.5 mM. The implication of irreversible S-glutathionylation on cell fate is not fully understood, although recent findings suggest the accumulation of misfolded or unfolded proteins and activation of apoptotic pathways (Uys et al., 2011).

In summary, we believe that this is the first report to demonstrate the molecular events by which VEGF-induced peroxynitrite modulates intracellular GSH levels and the downstream S-glutathionylation and phosphorylation of LMW-PTP to regulate angiogenic signal under physiological and pathological conditions. These results clearly demonstrate the crucial role of expression and regulation of LMW-PTP, and its function as a molecular switch of FAK activation and endothelial cell migration. Our findings implicate that modulating the redox-state to achieve a balanced milieu is crucial in order to facilitate the angiogenic signal of VEGF and thus achieve the therapeutic goal of VEGF delivery to treat ischemic cardiovascular diseases.

Materials and Methods

Cell culture

Primary cultures of Human microvascular endothelial (HME) cells from retina and cell culture supplies were purchased from Cell Systems Corporations (Kirkland, WA). Experiments were performed using cells between passages (4-6). Cells were switched to serum-free medium 6 hours before stimulation with VEGF 20 ng/ml (R&D, Minneapolis, MN) or peroxynitrite (Calbiochem, Darmstadt, Germany). Peroxynitrite was diluted in NaOH (0.01 M, Sigma, St Louis, MO). The peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTPPS, Calbiochem) was used at concentration of 2.5 μM, whereas the general antioxidant, N-acetyl cysteine (NAC, Sigma) was used at concentration of 1 mM.

Silencing LMW-PTP expression

Transfection of HME cells was performed using Amaxa nucleofector and a kit for primary endothelial cells according to the manufacturer's protocol (Lonza, Germany). Optimization experiments showed that the T005 program and 300 ng of LMW-PTP siRNA (Qiagen, Valencia, CA) gave the maximum transfection efficacy for HME cells. Cells suspended in a nucleofection mixture with the siRNA and pmax-GFP were electroporated and left in complete medium for 48 hours to recover before experiments were performed. Transfection efficiency was between 70 and 80%, as indicated by the number of cells expressing GFP (data not shown) and western blots for LMW-PTP expression (Fig. 8A).

Oxidized and reduced glutathione

Reduced glutathione was measured as described previously (Abdelsaid et al., 2010) using the Northwest Life Science kit (Vancouver, WA). Briefly, reduced-GSH was calculated by subtracting the oxidized-GSSG from the total glutathione. To obtain the total amount of glutathione, cells were lysed in phosphate buffer (100 mM potassium phosphate and 1 mM EDTA) and mixed with an equal amount of 5,5′-dithiobis(2-nitrobenzoic acid) [(DTNB), 10 mM] in the presence of glutathione reductase and NADPH, producing a yellow color that measured at 412 nm. To detect GSSG, samples were treated with 10 mM 2-vinylpyridine (Sigma) in ethanol to sequester all the reduced GSH, and then measured using the same protocol as that used to obtain the total glutathione. The reduced GSH is calculated by subtracting GSSG from the total GSH. The reduced GSH of each time point is then expressed as a relative percentage of the reduced GSH at zero time (100%).

LMW-PTP phosphatase activity

The activity was measured using the CycLex fluorometric kit from MBL (Woburn, MA). Briefly, 3-O-methylfluorescein phosphate was used as a fluorescence substrate for LMW-PTP. For endogenous enzyme activity, cell lysate (5 μl) was used instead of recombinant protein. The specific activity of LMW-PTP was calculated by taking the difference between cells that were transduced with scrambled siRNA and cells that were transduced with LMW-PTP siRNA. The reactions were incubated for 15 minutes at room temperature and the fluorescence intensity was measured using a microtiter plate fluorometer with excitation at 482-502 nm and emission at 510-540 nm. For recombinant protein assays, various concentrations of peroxynitrite (Calbiochem) were prepared by dilution in NaOH (0.01 N, Sigma). Oxidized-glutathione (GSSG, Fisher, Fair Lawn, NJ) and reduced-glutathione (GSH, sigma) were prepared in the assay buffer and used at a concentration of 10 mM. For endogenous activity of LMW-PTP, cells were treated with vehicle, VEGF or VEGF plus 0.5 mM peroxynitrite.

Cell migration assay

The wound-healing assay was performed as described previously (El-Remessy et al., 2007). Briefly, HME cells were grown to confluence and switched to a serum-free medium 6 hours before the experiment. The monolayer was wounded with a single sterile cell scraper of fixed diameter. Images of wounded areas were captured immediately and after 18 hours. Cell migration was calculated by measuring migration distance normalized to the initial distance of wounding using the AxioObserver Zeiss Microscope software (Germany) and was expressed as a percentage of untreated control cells.

Immunoprecipitation and western blot analysis

Isolated retinas and HME cells were harvested after various treatments and lysed in modified RIPA buffer (Millipore, Billerica, MA) for 30 minutes on ice. Insoluble material was removed by centrifugation at 14,000 ***g*** at 4℃ for 30 minutes. 50 μg of total protein were boiled in 6×Laemmli sample buffer, separated on a 10-12% SDS-polyacrylamide gel by electrophoresis, transferred to a nitrocellulose membrane and stained with a specific antibody. Cell lysate (200 μg) was immunoprecipitated with FAK primary antibody (5 μg) and A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. The precipitated proteins were analyzed by SDS-PAGE and blotted with antibodies against PY20 or FAK for equal loading. The primary antibodies were purchased as follows: anti-FAK antibodies (Millipore, Temecula, CA), pFAK (Thermo, Rockford, IL), anti-LMW-PTP antibodies (Exalpha, Maynard, MA), anti-fluorescein antibodies (Invitrogen, Carlsbad, CA), anti-GSH antibodies (Virogen, Boston, MA) and anti-PY20 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected using a horseradish peroxidase-conjugated antibody and enhanced

chemiluminescence (GE Healthcare, NJ). The films were scanned and band intensity was quantified using densitometry software (Alpha Innotech).

Determination of LMW-PTP thiol oxidation and S-glutathionylation

Oxidized thiols of LMW-PTP were determined as described previously (Chiarugi et al., 2003). Cellular or retinal lysates (200 μg) were labeled with fluorescein-tagged 5-iodoacetamide (5-IAM, Sigma) followed by immunoprecipitation with antibodies against LMW-PTP (5 μg) and A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. The beads were washed, analyzed by SDS-PAGE and blotted with anti-fluorescein or anti-LMW-PTP antibodies to check equal loading. S-glutathionylation was detected as described previously (Chen et al., 2007) with minor modifications. Immunoprecipitated LMW-PTP was incubated with or without DTT (100 mM) at 70℃ for 10 minutes, and then immediately loaded onto a 4-20% Tris-glycine polyacrylamide gradient gel (BioRad, Hercules, CA) as described above. The membrane was blotted with antibodies against GSH or LMW-PTP to check equal loading. For LMW-PTP phosphorylation, cell lysates were immunoprecipitated with LMW-PTP antibody as described above and the membrane was blotted with antibodies against PY20 or LMW-PTP to check equal loading.

Data analysis

The results were expressed as mean ± s.e.m. Differences among experimental groups were evaluated by analysis of variance, and the significance of difference between groups was assessed by the post-hoc test (Fisher's PLSD) when indicated. Significance was defined as $P<0.05$.

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Journal of Cell Science