# Effect of hindlimb unweighting on expression of hypoxia-inducible factor-1α, vascular endothelial growth factor, angiopoietin, and their receptors in mouse skeletal muscle

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Short title: Expression of angiogenic factors after hindlimb weighting

**Summary**

Hindlimb unweighting (HU) leads to capillary regression in skeletal muscle. However, the molecular mechanism(s) remains to be elucidated. To gain insight into the regulation of this process, we investigated gene expression of hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), angiopoietin, and their receptors in the atrophied muscle induced by HU. The hindlimbs of mice were unweighted by tail-suspension and then the gastrocnemius muscles were isolated after 10 days. To assess the capillary distribution, the capillary endothelium in frozen transverse sections was identified by staining for alkaline phosphatase. The mRNA levels were analyzed using a real-time reverse transcription-polymerase chain reaction. After 10 days-HU, the number of capillaries around a muscle fiber was significantly decreased by 19.5%, suggesting that capillary regression appears to occur. The expression of HIF-1α was significantly down-regulated after 10 days-HU. The expression of VEGF remained unchanged whereas those of Flt-1, KDR/Flk-1, and neuropilin-1 were significantly down-regulated, suggesting that VEGF signaling through these receptors would be attenuated. The expression of angiopoietin-1, -2, and their receptor, Tie-2 were also significantly down-regulated, suggesting that angiopoietin-1 signaling through Tie-2 would be attenuated. These findings suggest that alterations in expression of VEGF, angiopoietins, and their receptors may be associated with capillary regression after HU.

**Key words**

Angiogenic factor, Capillary regression, Hindlimb unweighting, Muscle atrophy

## Introduction

The microcirculation system is important for supplying oxygen and substrates to cells as well as for removing metabolites produced by cells, so it is essential for the cell to maintain a suitable capillary network under physiological conditions. Skeletal muscle adapts its capillary network to alterations in neuromuscular activity including motor activity and load bearing. Hindlimb unweighting (HU), rodent model is frequently used to simulate and study neuromuscular perturbations occurring in a real microgravity environment during spaceflights (Morey-Holton and Globus 2002). This earth-based model of microgravity is characterized by reduction of motor activity and lack of load bearing (Talmadge 2000). This model is known to affect capillary distribution of antigravity muscle (Desplanches *et al.* 1987, Desplanches *et al.* 1990, Desplanches *et al.* 1991, Kano *et al.* 2000, Dapp *et al.* 2004). For instance, capillary-to-fiber ratio, typically used as a measure of capillary distribution, decreases especially in soleus muscle, suggesting that a reduction in the absolute number of capillaries occurs in antigravity muscle. Although the adaptation of muscular vasculature to microgravity is thought to be physiological response to react to an environmental change, the mechanism(s) are still only partially understood. A possible mechanism has been proposed that apoptotic signaling may play an important role in remodeling of capillary network during HU (Fujino *et al.* 2005). HU spontaneously induces apoptosis of vascular endothelial cell in soleus muscle. The apoptotic process should be mediated, at least in part, by specific angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin. Recently, we investigated the expression of angiogenic factors to gain insight into the regulation of muscle denervation-induced capillary regression (Wagatsuma *et al.* 2005, Wagatsuma and Osawa 2006a). The expression of angiogenic factors was down-regulated in short- and long-term muscle denervation. Muscle denervation is different from HU in that neuromuscular activity is completely inhibited (Talmadge 2000). Therefore, the molecular response to HU may be different from that of muscle denervation. To our knowledge, it remains to be elucidated the expression patterns of angiogenic factors after HU.

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional factor consisting of HIF-1α and HIF-1β subunits (Semenza 1999). HIF-1 acts as a master regulator of numerous hypoxia-inducible genes including VEGF (Levy *et al.* 1995) and Flt-1 (Gerber *et al.* 1997) which are related to angiogenesis. The biological activity of HIF-1 is determined by the expression and activity of the HIF-1α subunit (Jiang *et al.* 1997). Targeted inactivation of HIF-1α in the mice results in abnormal vascular development and embryonic lethality (Iyer *et al.* 1998). In *HIF1α*$^{-/-}$ mice, defects in angiogenesis have been observed in the developing embryonic tissue, suggesting that HIF-1α play a crucial role in angiogenesis. Under normoxia, HIF-1α protein is rapidly degraded by the ubiquitin-proteasome pathway by binding of the von Hipple-Lindau tumor suppressor protein (Salceda and Caro 1997, Maxwell *et al.* 1999). However a previous study has shown expression of HIF-1α protein even in control muscle (Stroka *et al.* 2001), suggesting that HIF-1α protein may be normoxically stabilized in skeletal muscle. Therefore, HIF-1α may be involved in expression of VEGF and Flt-1 in response to HU.

VEGF, the most potent endothelial-specific mitogen, plays a crucial role in vasculogenesis and angiogenesis (Ferrara 1999). VEGF exerts its biological effects through two tyrosine kinase receptors, fms-like tyrosine kinase (Flt-1) and a kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1), expressed predominantly on endothelial cells (Ferrara 2001). These two receptors exhibit markedly different signaling and biological properties (Ferrara 2001). KDR/Flk-1 is considered to be the major mediator of several physiological and pathological effects of VEGF on endothelial cells (Cross *et al.* 2003). KDR/Flk-1 has been implicated in VEGF survival signals in endothelial cells through the phosphatidylinositol 3’-kinase PI3-kinase/Akt-dependent pathway (Gerber *et al.* 1998). This pathway is thought to be important in protection from apoptosis (Yao and Cooper 1995). Neuropilin-1 is also a VEGF receptor that modulates VEGF binding to KDR and may regulate VEGF-induced angiogenesis (Soker *et al.* 1998). Therefore, VEGF signaling, at least in part, mediated by KDR/Flk-1/neuropilin-1 may be attenuated, resulting in capillary regression induced by HU.

Angiopoietins are also angiogenic factors that make essential contributions to the maturation, stabilization, and remodeling of the vasculature (Fam *et al.* 2003). The biological effects of angiopoietin are mediated by another tyrosine kinase receptor, the tyrosine kinase with Ig and EGF homology domain-2 (Tie-2), which, like the VEGF receptors, is expressed primarily on endothelial cells (Schnurch and Risau 1993). The angiopoietins act in concert with VEGF (Asahara *et al.* 1998) and are critically important for angiogenesis. Angiopoietin-1/Tie-2 signaling modulates vessel maturation and maintains vessel integrity through the recruitment of pericyte-endothelial cell whereas angiopoietin-2 blocks angiopoietin-1/Tie-2 signaling, loosening vascular structure, leading vessel regression and apoptosis in the absence of VEGF (Fam *et al.* 2003). Therefore, the hypothesis of the current study is that (1) alterations in expression of HIF-1α may be associated with those of VEGF and Flt-1; (2)

VEGF/KDR/Flk-1/neuropilin-1 and angiopoietin-1/Tie-2 signaling may be attenuated, contributing to capillary regression induced by HU.

To test these hypotheses, we investigated alterations in the expression of these angiogenic factors and their receptors in mouse gastrocnemius muscle after HU. We demonstrated these angiogenic factors would be down-regulated concomitant with capillary regression after HU.

## Materials and Methods

*Animal care and experimental procedure*

Female 7-week-old (age when experiment was started) CD1 mice (Clea Japan, Meguro, Tokyo) were used and were housed in the animal care facility under a 12-h light/12-h dark cycle at room temperature (23 ± 2°C) and 55 ± 5% humidity. The mice were randomly assigned to one of two groups as follows: HU (n=12) or ambulatory control (n=12). For HU, we have modified a rat hindlimb suspension model originally described (Morey-Holton and Wronski 1981) so as to accommodate the use of mice (McCarthy *et al.* 1997). Briefly, each mouse was weighed and anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight). The bandages (Nichiban, Bunkyo, Tokyo) were wrapped around the tail in a helical pattern starting at the base of the tail. After the mouse had recovered from the anesthetic, a swivel hook was placed through the bandage just distal to the tip of the tail. All procedures in the animal experiments were performed in accordance with the guidelines presented in the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, published by the Physiological Society of Japan. This study was also approved by the Animal Committee of the National Institute of Fitness and Sports, Japan.

*Histochemistry*

Gastrocnemius muscle was dissected from mice and frozen in liquid nitrogen-cooled isopentane. Staining of capillaries was performed as described previously (Ziada *et al.* 1984). Frozen transverse sections from the midbelly region of gastrocnemius muscles were fixed for 10 min in acetone at −20°C and air-dried before being stained for alkaline phosphatase, which is present in the capillary endothelium. Morphometric measurements of fiber cross-sectional area (FCSA) and of capillary distribution were performed using light microscopy with CCD camera in randomly selected fields (average of 10 fields/section) of the superficial region of gastrocnemius muscle. The FCSA was measured using ImageJ 1.36b image analysis software (http://www.rsb.info.nih.gov/ij/). To assess the capillary distribution, the number of capillaries around a muscle fiber was directly counted (Wagatsuma 2006b).

*RNA extraction and cDNA synthesis*

The total RNA preparation was performed on the different muscle piece used for the morphological analysis. Superficial regions of the gastrocnemius muscles predominantly composed of fast muscle fibers were carefully isolated. The tissue was then transferred to glass homogenizers on ice, and 1 ml TRI reagent (Molecular Research Center, Cincinnati, OH) was added per 50 mg of tissue. RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm. All samples had an optical density ratio ($OD_{260}/OD_{280}$) of at least 1.9. The DNase-treated total RNA (1 μg) was then converted to cDNA using a First-strand cDNA synthesis system for quantitative RT-PCR (Marligen Biosciences, Ijamsville, MD). The cDNA samples were aliquoted and stored at −80°C.

*Real-time polymerase chain reaction (PCR) analysis*

Real-time PCR was performed using an Opticon™ DNA Engine (MJ Research, Waltham, MA) according to the manufacturer's instructions. Amplification was carried out using SYBR Premix Ex Taq™ (Takara Bio, Otsu, Shiga). All primers used in this study were obtained from Espec Oligo Service (Ibaraki, Tsukuba, Japan). The reactions employed primers for HIF-1α (Simpson *et al.* 2000), VEGF, Flt-1, KDR/Flk-1, angiopoietin-1, -2, Tie-2 (Shih *et al.* 2002), neuropilin-1 (Thijssen *et al.* 2004), and cyclophilin (Shih *et al.* 2002). For each set of primers, PCR thermal cycle conditions were optimized to achieve a single ethidium bromide-stained band following electrophoresis on a 2% agarose gel. Differences in gene expression were calculated relative to the expression of cyclophilin by comparison with a standard curve. To search the appropriate house keeping gene, we investigated several house keeping genes including 18S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase, β-actin, and cyclophilin. We selected cyclophilin as house keeping gene because the expression levels remain unchanged in hindlimb unloading muscle relative to control muscle. Cyclophilin was determined to be appropriate for normalizing the signal by comparing the differences in raw threshold cycle values (the number of amplification cycles at which the signal is detected above the background and is in the exponential phase). A standard curve was constructed from serially diluted cDNA from gastrocnemius muscle. Each sample was normalized by its cyclophilin content. The final results were expressed as a relative fold change compared to control animals.

*Statistical analysis*

Values are means ± standard error (SE). For analysis between control and hindlimb unweighting, Student's *t*-test was used to determine significance. The level of significance was set at $P<0.05$.

**Results**

After 10 days-HU, FCSA significantly decreased by 63.5% (Control: 2789.1 ± 56.5 $\mu m^2$; HU: 1017.9 ± 17.3 $\mu m^2$). Figure 1 shows the effect of HU on capillary distribution of skeletal muscle. Muscle fibers from hindlimb unweighted animals were pathologically atrophied compared to those from control animals. The number of capillaries around a muscle fiber significantly decreased by 19.5% (Control: 2.72 ± 0.05; HU: 2.19 ± 0.03) after 10 days-HU. The expression HIF-1α mRNA transcript significantly decreased by 30% relative to control muscle (Figure 2) after 10 days-HU. The expression of VEGF mRNA transcript remained unchanged whereas those of Flt-1, KDR/Flk-1, and neuropilin-1 mRNA transcripts significantly decreased by 44%, 68%, and 71%, respectively, relative to control muscle (Figure 2). The expression of angiopoietin-1, -2, and their receptor, Tie-2 mRNA transcripts significantly decreased by 64%, 40%, and 76%, respectively, relative to control muscle (Figure 3). Furthermore, we calculated the ratio of angiopoietin-2-to-angiopoietin-1 after 10 days-HU. The angiopoietin-2-to-angiopoietin-1 ratio increased by 2.3-fold in hindlimb unweighted animals compared with control animals.

**Discussion**

This study was designed to gain insight into the possible mechanism(s) of capillary regression induced by HU. The main findings of this study were that (1) the number of capillaries around a muscle fiber was decreased after 10 days; (2) the expression of VEGF receptors, Flt-1, KDR/Flk-1, and neuropilin-1 were down-regulated in the atrophied muscle induced by HU; (3) the expression of angiopoietin-1, -2, and angiopoietin receptor, Tie-2 were also down-regulated in the atrophied muscle. These shifts in gene expression would be related to regression of capillaries after HU.

We observed capillary regression after HU. Capillary endothelial cells are uniquely exposed to the hemodynamic force such as shear stress of blood flow in vivo. HU does not change blood flow in gastrocnemius muscle (McDonald *et al*. 1992, Woodman *et al.* 2001) but decreases erythrocyte concentration in the blood (Overton *et al.* 1989), suggesting that shear stress loaded on endothelial cells may change, resulting in affecting survival of endothelial cells. Indeed, this possibility may be supported by the observations that shear stress suppresses apoptosis in endothelial cells by PI3-kinase/Akt-dependent pathway (Haga *et al*. 2003).

In the current study, we observed that expression of HIF-1α gene paralleled that of Flt-1 gene which is mediated, at least in part, by the binding of hypoxia inducible factor 1 (HIF-1) to an HIF binding site located in the promoters of Flt-1 gene (Gerber *et al.* 1997), suggesting that HIF-1α may play a role in regulation of Flt-1 gene expression in response to HU. However, we observed no significant changes in the expression of VEGF although VEGF is also known as HIF-1 target gene (Levy *et al.* 1995). However, our data does not rule out the possible involvement of HIF-1α in activation of the VEGF gene in skeletal muscle, because the regulation of HIF-1α expression and activity in vivo occurs at multiple levels, including mRNA expression, protein expression, nuclear localization, and transactivation (Semenza 2000). Further studies are needed to elucidate the physiological role of HIF-1α in the expression of VEGF and Flt-1 genes in response to HU.

The expression of VEGF has been down-regulated after short- and long- term muscle denervation (Magnusson *et al.* 2005, Wagatsuma *et al.* 2005, Wagatsuma *et al.* 2006a). We reported that the expression of VEGF was down-regulated immediately after muscle denervation and then remained to be lower than control levels until 30 days (Wagatsuma *et al.* 2006a). Bey and colleagues (2003) report quantitative RT-PCR data on the changes in gene expression in skeletal muscle during short-term HU. They observe the expression of VEGF is immediately down-regulated 12 hours after HU. In the current study, in contrast, we observed no significant changes in VEGF expression levels. We attribute this discrepancy in data to time course of alterations in electromyographic (EMG) activity during HU. The EMG activity of lateral gastrocnemius decreases by half immediately after HU, maintains lower than control levels for 4-5 days, and subsequently tends to recover until 7 days (Alford *et al.* 1987), suggesting that recovery of EMG activity may contribute to VEGF expression observed in the current study. Indeed, VEGF expression may be modulated by electronic stimuli (Hang *et al.* 1995; Skorjanc *et al*. 1998; Brutsaert *et al.* 2002; Tang *et al.* 2004). Additionally, Asmussen and Soukup (1991) suggest that reflex-mediated motoneuronal activity decreases due to decreased muscle spindle feedback during HU, which may affect the expression of VEGF. Therefore, it is likely that alteration in EMG activity during HU may be associated with VEGF expression. In this regard, the response of

VEGF expression to HU is obviously different from that to muscle denervation.

In the current study, we observed the expression of VEGF receptors (Flt-1, KDR/Flk-1, and neuropilin-1) were down-regulated, while that of VEGF remained unchanged in hindlimb unweighted muscle. From the result of the gene expression analysis, our data suggest that VEGF/KDR/Flk-1/neuropilin-1 signaling may be attenuated, resulting in capillary regression after HU. This possibility may be supported by the observation by Tang and colleagues who report that capillary regression is observed in VEGF-inactivated regions of skeletal muscle from VEGF*lox*P transgenic mice in which all three VEGF isoforms were inactivated (Tang *et al.* 2004). They demonstrate capillary regression is accompanied by the appearance of TUNEL-positive apoptotic endothelial cells. Therefore, insufficient VEGF-dependent signal may potentially initiate the apoptotic pathway in endothelial cells and lead to capillary regression.

Although the role of Flt-1 in the adult animal is less clearly defined compared to that of KDR/Flk-1, Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells, suggesting a role for this receptor in the maintenance of endothelial cells (Peters *et al.* 1993). We observed Flt-1 protein was expressed in endothelial cells of normal and regenerating skeletal muscle (Wagatsuma *et al.* 2006c). Therefore, we hypothesize that Flt-1 may contribute to endothelial cell integrity during HU.

We observed the expression of angiopietin-1, angiopietin-2, and Tie-2 were down-regulated in hindlimb unweighted animals, suggesting that angiopietin-1/Tie-2 signaling may be attenuated despite down-regulation of angiopoietin-2. Consequently, we calculated the ratio of angiopoietin-2-to-angiopoietin-1 to compare ambulatory control with HU, because changes in the ratio are thought to determine whether the net effect of the angiopoietins is to stabilize or destabilize the vasculature. Indeed, the ratio of angiopoietin-2-to-angiopoietin-1 is found to increase with angiogenesis induced by exercise training (Lloyd *et al*. 2003) and cutaneous wound hearing (Kampfer *et al*. 2001), due to larger increase in angiopoietin-2 than in angiopoieti-1. Interestingly, we also observed the ratio increased after HU, due to larger decrease in angiopoieti-1 than in angiopoietin-2. This observation is consistent with our previous study using muscle denervation model (Wagatsuma *et al.* 2006a). Although further studies are needed to elucidate the role of angiopoieitn-2 in capillary regression induced by HU, our data suggest that angiopoietin-1/Tie-2 signaling may be attenuated.

In conclusion, we demonstrated that the expression of VEGF receptors, Flt-1, KDR/Flk-1, neuropilin-1, angiopoietin-1, -2, and angiopoietin receptor, Tie-2 were down-regulated in the atrophied muscle where capillary regression occur after HU, suggesting that attenuation of angiopoietin-1/Tie-2 signaling concomitant with decreased VEGF/KDR/Flk-1/neuropilin-1 signaling may result in capillary regression induced by HU. Although the mechanism(s) of reduction of motor activity and lack of load bearing by HU regulates the expression of angiogenic factors remains to be identified, our data may explain, at least in part, capillary regression in the atrophied muscle induced by HU.

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**Legends to figures**

Figure 1. Identification of capillaries by staining for alkaline phosphatase in control (A), and HU (B). Capillaries appear as black dots. Bar = 100 µm.

Figure 2. Effect of HU on expression of HIF-1α, VEGF, Flt-1, KDR/Flk-1, and neuropilin-1 (NRP-1) mRNA transcripts in skeletal muscle. The values are means ± SE (n=6). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control muscle.

Figure 3. Effect of HU on expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and Tie-2 mRNA transcripts in skeletal muscle. The values are means ± SE (n=6). ***$p<0.001$ compared to control muscle.

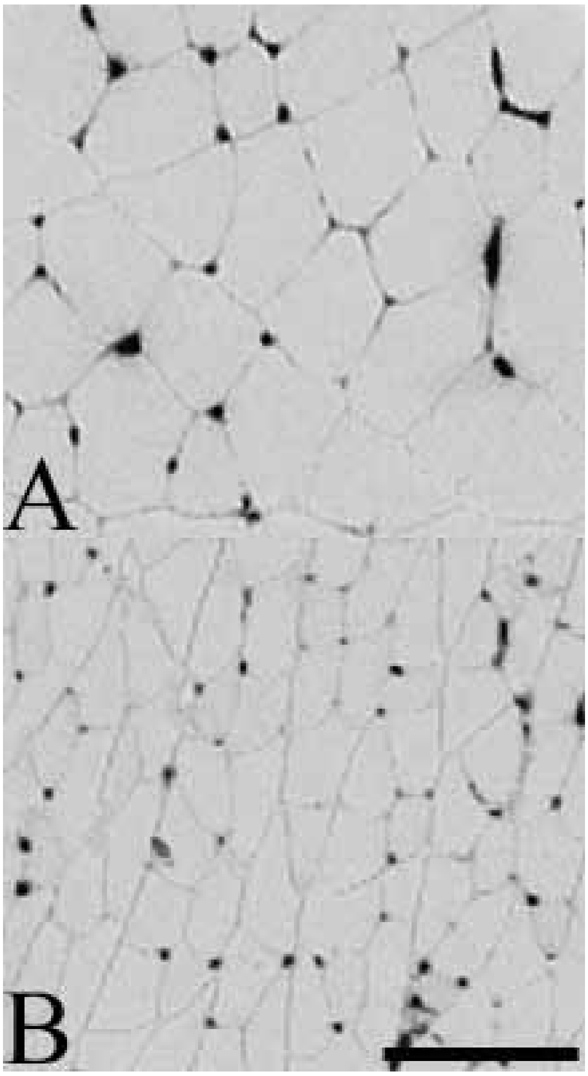
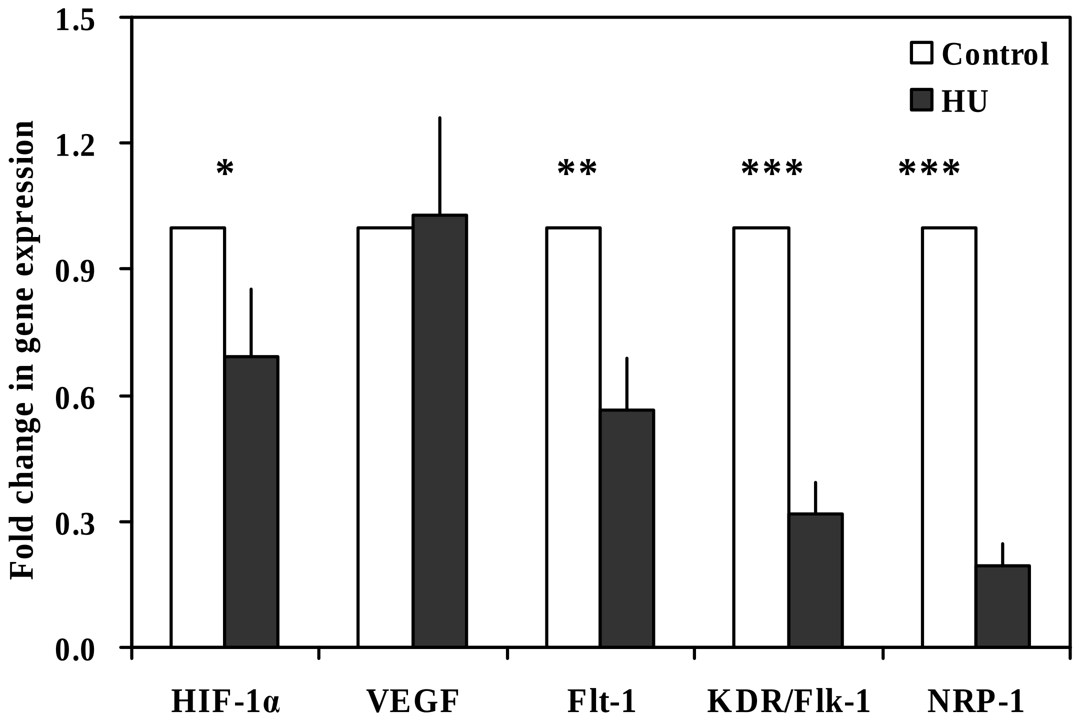



Figure 1.



1.5
1.2
0.9
0.6
0.3
0.0
Fold change in gene expression
Control
HU
*
**
***
***
HIF-1α
VEGF
Flt-1
KDR/Flk-1
NRP-1


Figure 2.

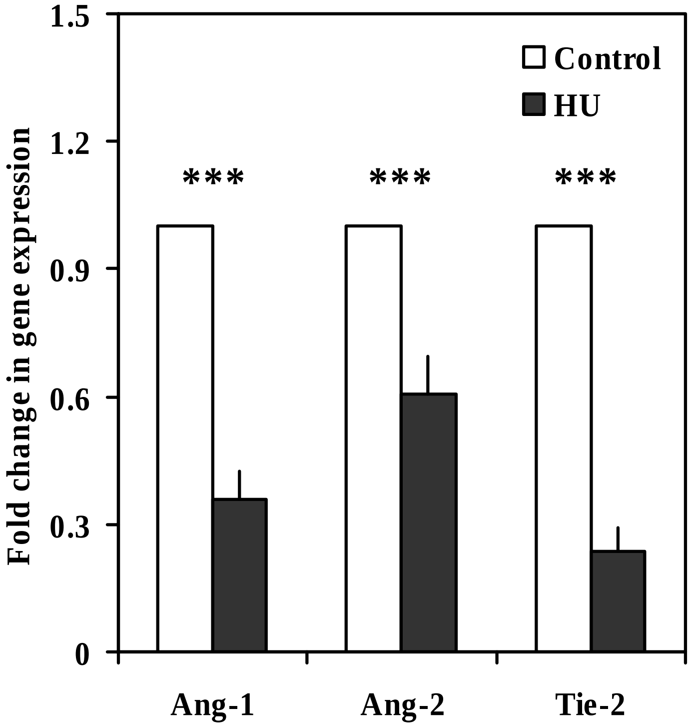

1.5
1.2
0.9
0.6
0.3
0
Fold change in gene expression
Control
HU
***
***
***
Ang-1
Ang-2
Tie-2

Figure 3.