Skeletal muscle-endothelial cell cross talk through angiotensin II

Leeann M. Bellamy, Adam P. W. Johnston, Michael De Lisio, and Gianni Parise

Departments of 1Kinesiology and 2Medical Physics and Applied Radiation Sciences, McMaster University, Hamilton, Ontario, Canada

Submitted 3 August 2010; accepted in final form 22 September 2010

Bellamy LM, Johnston AP, De Lisio M, Parise G. Skeletal muscle-endothelial cell cross talk through angiotensin II. Am J Physiol Cell Physiol 299: C1402–C1408, 2010. First published September 22, 2010; doi:10.1152/ajpcell.00306.2010.—The role of angiotensin II (ANG II) in postnatal vasculogenesis and angiogenesis during skeletal muscle (SKM) regeneration is unknown. We examined the capacity of ANG II to stimulate capillary formation and growth during cardiotoxin-induced muscle regeneration in ACE inhibitor-treated ANG II type 1a receptor knockout (AT1α−/−) and C57Bl/6 control mice. Analysis of tibialis anterior (TA) cross-sections revealed 17% and 23% reductions in capillarization in AT1α−/− and captopril treated mice, respectively, when compared with controls, 21 days postinjury. Conversely, no differences in capillarization were detected at early time points (7 and 10 days). These results identify ANG II as a regulator of angiogenesis but not vasculogenesis in vivo. In vitro angiogenesis assays of human umbilical vein endothelial cells (HUVECs) further confirmed ANG II as a proangiogenic factor during SKM regeneration in vivo and more importantly demonstrates that ANG II released from SKM can signal endothelial cells and regulate angiogenesis through the induction of endothelial cell migration. ANG II may serve as a proangiogenic regulator during SKM regeneration in vivo and more importantly demonstrates that ANG II released from SKM can signal endothelial cells and regulate angiogenesis through the induction of endothelial cell migration.

captopril; migration; regeneration; vasculogenesis

The development of capillaries during embryogenesis is initiated by the differentiation of endothelial precursor cells (EPC) into mature endothelial cells (EC) in a process known as vasculogenesis (15). This is followed by the proliferation and migration of mature ECs and the sprouting of new capillaries from existing ones in a process referred to as angiogenesis (40). Basal maintenance and remodelling of the vascular network is achieved through angiogenesis; however, following severe injury, neovascularization (or postnatal vasculogenesis) is induced to reconstruct the damaged vascular network due to the low proliferative capacity of differentiated ECs (15). Similarly, in skeletal muscle (SKM), severe myotrauma resulting in muscle regeneration induces the neovascularization of newly formed muscle fibers (15, 33), while expansion of the existing capillary network can be achieved through angiogenesis induced by endurance training (2, 4, 14) or electrical stimulation (1, 45).

Several soluble factors have been identified as regulators of SKM angiogenesis, including vascular endothelial growth factor (VEGF), angioptietin1/2, hypoxia-inducible factor (HIF)-1α, transforming growth factor (TGF)-β1, basic fibroblast growth factor (bFGF), and peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) (8, 9, 25, 28). Interestingly, angiotensin II (ANG II) has been identified as a regulator of angiogenesis in SKM (2) as well as the heart (30), pancreatic tumor (3), and gastric cancer cells (18). ANG II is known primarily for its traditional role as a regulator of blood pressure through modulating vasoreactivity and fluid homeostasis; however, localized renin angiotensin systems have been identified in other tissues including cardiac tissue, digestive organs, the reproductive tract, skin, sensory organs, bone marrow, and adipose tissue (35) and have been implicated in such diverse processes as cell hypertrophy, proliferation, migration, and apoptosis (22, 27, 29, 39). ANG II has the ability to bind two receptor subtypes (AT1 and AT2) with signaling through AT1 mediating hypertrophic and proliferative events while signaling through AT2 generally opposing these functions (7, 17, 32, 42). Importantly, work from our laboratory has identified a locally acting angiotensin signaling system in SKM and muscle satellite cells (20). This local system has been demonstrated to express angiotensinogen, the angiotensin-converting enzyme (ACE), AT1, AT2, and the ability to produce ANG II in the absence of renin expression (20).

Currently, the role of ANG II as either a positive or negative regulator of angiogenesis remains debatable and may be dependent on AT1 or AT2 receptor-mediated signaling. Work from the Greene lab (1, 2) has demonstrated a link between skeletal muscle angiogenesis induced by endurance training and electrical stimulation and AT1 mediated ANG II signaling. Furthermore, ANG II treatment is associated with upregulation of VEGF and its receptor in various cell types (1, 34). Though the mechanism responsible for ANG II-induced capillary growth is currently unknown.

Conversely, negative effects of ANG II on angiogenesis have been reported and appear to be specific to AT2 receptor-mediated signaling. AT2 receptor stimulation has demonstrated a reduction in EC proliferation (42), migration (6), and ischemia-induced angiogenesis (41). These processes appear antagonistic to the functions of ANG II mediated by AT1 receptor signaling, contributing to the controversy that exists regarding the role of ANG II as pro- or anti-angiogenic factor.

Since the role of ANG II in SKM angiogenesis during regeneration remains unknown, the purpose of this study was to 1) elucidate the role of ANG II in regulating neovascularization and angiogenesis in SKM following myotrauma, and 2) investigate cross-talk between SKM and ECs as a potential mechanism for inducing angiogenesis in SKM.

* L. M. Bellamy and A. P. W. Johnston contributed equally to this work.

Address for reprint requests and other correspondence: G. Parise, Depts. of Kinesiology and Applied Radiation Sciences, McMaster Univ., Hamilton, Ontario L8S 4L8, Canada (e-mail: pariseg@mcmaster.ca).
Cervical dislocation. Similarly, day regenerative time points, following euthanization of the mice by injection and continuing throughout the experimental time course. Drinking water (0.5 mg/ml, Sigma) commencing 3 days before CTX injections, with the uninjured TA serving as an internal control. At 7- (early) and 21-day (late) time points post-CTX injection, TA muscles were harvested following euthanization of the mice by cervical dislocation. Similarly, experiment 2 utilized AT1a−/− and age-matched C57Bl/6 mice (n = 4 per group) that received unilateral CTX injections, with the uninjured TA serving as an internal control. At 7- (early) and 21-day (late) time points post-CTX injection, TA muscles were harvested following euthanization of the mice via cervical dislocation to allow for in vivo analysis of vasculogenesis and angiogenesis, respectively. All procedures received approval from the McMaster University Research Ethics Board and were conducted in accordance with guidelines established by the Canadian Council of Animal Care.

Histology. After excision, TA muscles were fixed in neutral buffered formalin, paraffin-embedded, and sectioned for lectin staining for enumeration of capillaries. Briefly, sections were deparaffinized with xylenes and rehydrated with graded ethanol washes. Sections then underwent enzymatic digestion with proteinase K followed by washes of distilled water (0.5 mg/ml, Sigma) commencing 3 days before CTX injection and continuing throughout the experimental time course. Subsequent to bilateral CTX injections of control and captopril-treated mice, the TA muscle was excised at early (10 day) and late (21 day) regenerative time points, following euthanization of the mice by cervical dislocation. Similarly, experiment 2 utilized AT1a−/− and age-matched C57Bl/6 mice (n = 4 per group) that received unilateral CTX injections, with the uninjured TA serving as an internal control. At 7- (early) and 21-day (late) time points post-CTX injection, TA muscles were harvested following euthanization of the mice via cervical dislocation to allow for in vivo analysis of vasculogenesis and angiogenesis, respectively. All procedures received approval from the McMaster University Research Ethics Board and were conducted in accordance with guidelines established by the Canadian Council of Animal Care.

Angiogenesis assay. To assess the effect of ANG II on endothelial cell migration, a transwell assay was performed as previously described (23) with minor modifications. HUVECs were cultured at 10,000 cells per well in a 24-well, 8-μm pore transwell system. The lower transwell compartment was filled with media containing ANG II (10 μM) or nonsupplemented control M-200. After a 12-h incubation, cells were fixed and stained with crystal violet, and cells from the top side of the transwell were removed with a cotton swab, while cells from the bottom surface were solubilized with 2 ml of 1% Triton X-100. The extent of migration was proportional to the absorbance value obtained from an Ultraspec 3000 Pro (GE Healthcare) at 595 nm. Additionally, the migratory effects of ANG II on HUVECs were confirmed using an under agarose assay (16). Three milliliters of a 1.2% agarose solution (dissolved in DMEM) were added to each well in six-well plates; after polymerization and cooling to 4°C, three wells were cut into the agarose gel. HUVECs (10,000) were plated into the center well, whereas ANG II-treated (10 μM) and nonsupplemented DMEM were added to each of the two outer wells. The number of migrating cells toward the ANG II and the nonsupplemented DMEM was counted following a 14-h incubation using the Zeiss Axiovert 200 microscope (Carl Zeiss).

Statistical analysis. Vasculogenesis and angiogenesis were assessed via two-way ANOVAs of capillary to fiber ratio in AT1a−/− mice at 7 and 21 days or in captopril-treated mice at 10 and 21 days versus appropriate controls. Gene expression “fold change” was calculated using the ΔΔCt method (24) and analyzed via t-test between groups. In vitro angiogenesis and migration was assessed by one-way analysis of variance and t-tests, respectively. Significance was considered to be P < 0.05, and data were presented as means ± SE.

RESULTS

Angiotensin II mediates angiogenesis during skeletal muscle regeneration in vivo. A CTX injury model was utilized to induce SKM regeneration encompassing a period of vasculogenesis followed by angiogenesis (15) in wild-type, AT1a−/− and captopril-treated mice. Lectin staining of TA muscle cross-sections revealed that following 7 or 10 days of regeneration, capillary-to-fiber ratio remained unchanged between AT1a−/− mice at 7 and 21 days or in captopril-treated mice at 10 and 21 days versus appropriate controls. These results indicate that the ability to form new capillaries was unaffected by a reduction in ANG II signaling. Consistent with the capillary-to-fiber ratio results, there was no difference in VEGF mRNA expression between control and captopril-treated mice 10 days postinjury (Fig. 1I). Conversely, analysis of capillarization 21 days following injury revealed 17% and 23% decreases in capillary-to-fiber ratio between AT1a−/− (Fig. 1B and D) and captopril-treated (Fig. 1E).
Fig. 1. ANG II mediates angiogenesis during skeletal muscle regeneration in vivo. Representative lectin staining of tibialis anterior (TA) muscle cross-sections in control (A), AT1a−/− (B), and captopril-treated (C) mice 21 days (d21) postinjury. Lectin staining was performed to allow for quantification of capillary-to-fiber ratio (brown indicating capillaries, blue indicating nuclei) at early time points, 7 days (d7) in AT1a−/− group (G) or 10 days (d10) in captopril-treated group (H), and late time points, 21 days for all groups (D/E). Skeletal muscle gene expression analysis of control and captopril mice 10 (F) and 21 days (I) following CTX injection is shown. Arrows indicate positive lectin staining. Note significantly fewer lectin positive capillaries in AT1a−/− mice and following captopril treatment. *Significant difference versus control (P < 0.05).

Angiotensin II is proangiogenic in vitro. Since we demonstrated that ANG II appears necessary for angiogenesis during SKM regeneration in vivo, the effect of ANG II on HUVEC tube formation was assessed. Quantification of total capillary tube length (Fig. 2, A–C) and branch point number (Fig. 2, A, B, and D) per field of view revealed that ANG II treatment
resulted in significant increases of 71% and 124%, respectively, when compared with control conditions. Furthermore, the addition of losartan partially attenuated the proangiogenic effects of ANG II by \( \sim 17.5\% \). These data confirm a role for ANG II as a positive regulator of angiogenesis and suggest that ANG II elicits its effects, at least in part, through AT1-mediated signaling.

**Skeletal muscle cell-derived ANG II signals endothelial cells to regulate angiogenesis.** Given that we have previously demonstrated that muscle satellite cells express angiotensinogen mRNA, which significantly increases upon differentiation (20), the potential for cross talk between SKM and ECs through ANG II was examined. Conditioned medium collected from proliferating and differentiated C2C12s was transferred to HUVECs grown on a basement membrane matrix, and angiogenesis was assessed (see Fig. 3, A–D, for representative in vitro images). No differences in angiogenesis were observed between HUVECs treated with culture media from proliferating muscle cells versus controls (Fig. 3, E and F). However, the addition of conditioned culture media from differentiated myotubes resulted in an 84% increase in total tube length (Fig. 3E) and a 203% increase in the number of branch points formed (Fig. 3F) compared with controls. Importantly, the addition of captopril to differentiated cell culture media to inhibit endogenous ANG II production significantly inhibited the observed proangiogenic response (Fig. 3, C, E, and F). These data provide evidence of a direct signaling interaction, through ANG II, between differentiated muscle stem cells and ECs to stimulate angiogenesis.

**ANG II stimulates endothelial cell migration.** The capacity of ANG II to induce endothelial cell migration as a mechanism for regulating SKM angiogenesis was examined in vitro. Under agarose migration assays revealed a 101% increase \( (P < 0.05) \) in the number of cells that migrated out of the center well toward the ANG II concentration gradient versus controls (Fig. 4A). Additionally, transwell assays were used to further confirm this migratory effect, yielding a 37% increase \( (P < 0.05) \) in EC migration in response to ANG II treatment (Fig. 4B). Therefore, the proangiogenic effect of ANG II can be accounted for, at least in part, by the ability to stimulate EC migration.

**DISCUSSION**

Despite controversy regarding the role of ANG II during angiogenesis, the current investigation further defines ANG II as a necessary pro-angiogenic factor during SKM regeneration. Furthermore, we demonstrate that SKM cells are a source of ANG II production and have the ability to act on endothelial cells inducing angiogenesis.

The regeneration of SKM following injury is an intricate process of multiple events, including the formation of functional myofibers and the revascularization of these fibers. Therefore, the potential for interaction between these two processes is great and regrettably remains poorly understood.

---

Fig. 3. Skeletal muscle cell-derived ANG II interacts with endothelial cells to regulate angiogenesis. Representative phase contrast images of tube formation in HUVECs treated with control (CON, A) and conditioned media collected from differentiated C2C12 muscle cells (DIFF, B), differentiated muscle cells treated with captopril (DIFF + Cap, C), and proliferating muscle cells (PRO, D). The extent of angiogenesis under each condition was quantified based on total tube length (sum of vessels \( >25 \mu \text{m} \)) \( (E) \) and branch point number \( (F) \) per field of view. *Significantly different versus control \( (P < 0.05) \); #Significantly different versus differentiated \( (P < 0.05) \).
An association between muscle satellite cells (SC) and EC has previously been proposed (10) based on the nonrandom proximity between the two cell types in vivo. Importantly, the abundance of SKM capillaries was shown to be proportional to the mean number of SCs associated with each myofiber such that a reduction in capillarization resulted in a depleted SC supply (10). Furthermore, coculture of SCs with ECs resulted in enhancement of SC growth (10). In a similar fashion, we report that treatment of ECs with conditioned media from differentiating myotubes enhanced tube formation, demonstrating a proangiogenic effect of muscle cells on ECs. Importantly, our data demonstrate that ANG II produced by SKM is a focal point, coordinating the cross talk between muscle and ECs.

CTX injection to induce muscle injury allowed us to explore the proposed interaction between muscle and ECs during neovasculogenesis and angiogenesis in vivo (15). Results demonstrate that EPC differentiation and capillary formation was rescued by ANG II infusion (36). Furthermore, they also demonstrate that the typical increase in capillarization associated with endurance exercise is abolished with captopril or losartan treatment in rats (2). Interestingly, these effects were associated with a decrease in VEGF expression and protein content, implying that the angiogenic effects of ANG II are mediated through VEGF (1, 2). However, the source of ANG II production eliciting these effects remained unknown. Recent work from our lab has demonstrated the presence of a local angiotensin signaling system in SKM stem cells and differentiated myotubes (20). Importantly, angiotensinogen expression (the precursor to ANG II) is significantly increased in myotubes following terminal differentiation (20) and therefore may contribute to the bioavailability of ANG II in SKM. After muscle injury, the regeneration process encompasses expansion of the satellite cell pool into precursor myoblasts. These myoblasts subsequently undergo migration, alignment, and differentiation to create functional myofibers. Since angiotensinogen expression is increased at this time, it is attractive to hypothesize that muscle cells can coordinate the growth of a necessary vascular network following the differentiation of myoblasts into myofibers through ANG II release. This is supported by in vitro experiments demonstrating that only conditioned media from differentiated myotubes enhanced capillary growth. Therefore, ANG II mediates the formation of new capillaries from existing ones (angiogenesis), while the process of EPC differentiation during neovasculogenesis appears to be mediated independent of ANG II.

Given the importance of migration during angiogenesis, the ability of ANG II to induce EC migration as a mechanism of regulating angiogenesis was assessed and confirmed through the use of transwell and agarose migration assays. Though ANG II has previously been shown to induce promigratory effects on several cell types (11, 19, 22, 31), the mechanisms underlying these effects are yet to be elucidated. ANG II has been demonstrated to induce matrix metalloproteinase 2 (MMP2) activity in a time- and dose-dependent manner in both HUVECs (19) and cardiomyocytes (11). MMP2 activity plays an integral role in cellular migration, functioning to digest the extracellular matrix (ECM) proteins that anchor cells to their extracellular environment (13). Several additional signaling pathways have been implicated in ANG II-induced migration, including phosphatidylinositol 3 kinase and Src-family tyrosin kinase activation in HUVECs (19), as well as ERK1/2 and p38 mitogen-activated protein kinase signaling in monocytes (22). Recent work from our lab (20) demonstrated the colocalization of lamellipodia and the AT1 receptor on the leading edge of migrating muscle cells, suggesting a chemotactic role for ANG II in cytoskeletal remodeling. Additionally, phosphorylation of cytoskeletal proteins such as Pyk2 and paxillin coincide with ANG II treatment in monocytes (22). Therefore, a promigratory role for ANG II has been established during various stages of cell migration, including signal transduction, ECM digestion, and cytoskeletal reorganization. Further investigation to pin-point the exact mechanism of ANG II-induced EC migration as a mechanism of regulating angiogenesis is warranted.
Our data indicate that ANG II-induced angiogenesis appears to be mediated, at least in part, by signaling through the AT1 receptor. The partial attenuation of capillary formation in vitro with losartan treatment and the reduction in capillary supply observed in the AT1α−/− animals further supports this hypothesis. Furthermore, it has been shown that blockade of AT1 inhibits migration in HUVECs (27). Additionally, stimulation of AT2 in human ECs has been shown to inhibit VEGF-induced EC migration and tube formation in vitro (6). Therefore, these results highlight the antagonistic nature of AT1- and AT2-mediated ANG II signaling.

Clinically, evidence is accumulating suggesting that ACE inhibitors and angiotensin receptor blockers (ARB) may have detrimental secondary effects independent of their roles in vasoregulation. For example, prolonged exposure during fetal development to ACE inhibitors and ARBs, specifically in the second and third trimesters (43), has been implicated in neonatal renal failure, skeletal abnormalities, and central nervous system and cardiovascular malformations (12), which all correlated to a reduction in fetal blood flow (26). The importance of this information is further punctuated by the fact that one in three individuals in the United States are considered clinically hypertensive (44), and ACE inhibitors and ARBs are among the most widely prescribed anti-hypertensive medications (21). Therefore, careful investigation into the secondary effects of these pharmaceuticals on angiogenesis with a human population is needed.

In summary, the results presented in the paper identify SKM as a source of ANG II during regeneration that has the ability to signal ECs and induce angiogenesis as evidenced by the in vitro experiments presented in this paper. Furthermore, evidence suggests that ANG II serves to induce EC migration, which is a critical step during angiogenesis.

ACKNOWLEDGMENTS

We thank Jeff Baker for assistance with cell culture.

GRANTS

This work was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada held by G. Parise. L. M. Bellamy was supported by a NSERC USRA, A. P. W. Johnston was supported by a NSERC Doctoral Post Graduate Scholarship, and M. De Lisio was supported by a CIHR CGS.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


