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Interactions of PPAR-alpha and adenosine receptors in hypoxia-induced angiogenesis

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Abstract

Hypoxia and adenosine are known to upregulate angiogenesis; however, the role of peroxisome proliferator-activated receptor alpha (PPARα) in angiogenesis is controversial. Using transgenic Tg(fli-1 :EGFP) zebrafish embryos, interaction of PPARα and adenosine receptors in angiogenesis were evaluated under hypoxic conditions. Epifluorescent microscopy was used to assess angiogenesis by counting the number of intersegmental (ISV) and dorsal longitudinal anastomotic vessels (DLAV) at 28 hours post-fertilization (hpf). Hypoxia (6h) stimulated angiogenesis as the number of ISV and DLAV increased by 18-fold ($p<0.01$) and 100±8 % ($p<0.001$), respectively, at 28 hpf. Under normoxic and hypoxic conditions, WY-14643 (10 μM), a PPARα activator, stimulated angiogenesis at 28 hpf, while MK-886 (0.5 μM), an antagonist of PPARα, attenuated these effects. Compared to normoxic condition, adenosine receptor activation with NECA (10 μM) promoted angiogenesis more effectively under hypoxic conditions. Involvement of A_{2B} receptor was implied in hypoxia-induced angiogenesis as MRS-1706 (10 nM), a selective A_{2B} antagonist attenuated NECA (10 μM)-induced angiogenesis. NECA- or WY-14643-induced angiogenesis was also inhibited by miconazole (0.1 μM), an inhibitor of epoxygenase dependent production of eicosatrienoic acid (EET) epoxide. Thus, we conclude that: activation of PPARα promoted angiogenesis just as activation of A_{2B} receptors through an epoxide dependent mechanism.

Keywords

adenosine receptors; angiogenesis; eicosatrienoic acid; PPARα; zebrafish

1. Introduction

Angiogenesis is a process involving growth of new blood vessels from pre-existing ones. In physiological conditions it occurs during embryonic development, wound healing and in the female reproductive cycle. In pathological conditions, an uncontrolled angiogenesis has been implicated in many diseases including cancer, psoriasis and age-related macular degeneration (Liekens et al., 2001). Currently; many studies are focusing on angiogenesis as a potential target for treatment of many diseases (Maleck, et al., 2005). Inhibition of angiogenesis is a strategy that has been approved for the treatment of diseases associated

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with elevated angiogenesis, such as tumor, cancer, retinopathy, rheumatoid arthritis and psoriasis (Westra et al., 2010). Similarly, promotion of angiogenesis is another strategy to treat diseases associated with reduced angiogenesis, such as ischemic heart disease, cerebral ischemia, peripheral artery disease and wound healing (Sabti, 2007).

Peroxisome proliferator-activated receptor alpha (PPARα), a transcription factor, is a negative regulator of inflammation (Bordiji et al., 2000). However, its role in angiogenesis is still controversial as both pro- and anti-angiogenic effects have been reported. For example, PPARα promotes angiogenesis by suppressing thrombospondin-1 (TSP-1), an inhibitor of angiogenesis (Kaipainen et al., 2007), through prostacyclin-mediated activation of PPARγ angiopoietin-related gene (PGAR); which is a physiological target for both PPARα and PPARγ (Pola et al., 2004) and vascular endothelial growth factor (VEGF)-dependent mechanism (Biscetti et al., 2008). However, PPARα as well as PPARγ elicit anti-angiogenic effects through blockade of VEGFR2 (Meissner et al., 2004). Fenofibrate, an agonist of PPARα, inhibits angiogenesis through PPARα –dependent (Panigrahy et al., 2008) and independent pathways (Araki et al., 2009). Clofibric acid, another PPARα ligand, also caused PPARα-dependent reduction in VEGF expression in human ovarian cancer cell (Yokoyama et al., 2007). WY-14643, a selective PPARα ligand, was reported to inhibit tumor angiogenesis through transcriptional down regulation of cytochrome P450 2C (CYP2C) epoxygenase (Pozzi et al., 2007).

Hypoxia elevates extracellular adenosine by promoting ATP dephosphorylation and by inhibiting equilibrating nucleoside transporter 1 (ENT1) that translocates extracellular adenosine into the cell. The extracellular accumulation of adenosine protects cells from hypoxia-induced injury through four major ways: increased oxygen supply/demand ratio, preconditioning, anti-inflammatory effects and stimulation of angiogenesis. Adenosine exerts these effects through G-protein-coupled receptors: A_1, A_{2A}, A_{2B}, A_3. Endothelial cells predominantly express A_{2A} and A_{2B} receptors. Higher expression and activation of adenosine receptor subtype A_{2B} under hypoxic conditions was reported in many studies (Feoktisov et al., 2002; Feoktisov et al., 2004; Kong et al., 2006L). Unlike adenosine receptor A_{2A} which exerts cell-dependent pro- as well as anti-angiogenic phenotypes (Desai et al., 2005; Olah and Roudabush, 2000), available information indicates that A_{2B} exhibits only pro-angiogenic effect and its stimulation was associated with enhanced expression of VEGF and IL-8 (Ryzhov et al., 2007).

PPARα-dependent up regulation of A_{2A} (Araki et al., 2009) and A_{2B}-mediated down regulation of PPARα is also reported (Peng et al., 2009). However, the interaction of adenosine receptors and PPARα in angiogenesis is not known. Thus, the purpose of this study was to explore the downstream angiogenic mediators of PPARα and adenosine receptors and their possible interaction in angiogenesis.

2. Materials and methods

2.1. Zebrafish

Two to three month old wild type (AB strain) and transgenic friendly leukemia integration 1 a enhanced green fluorescent protein Tg(fli1a:EGFP) zebrafish (*Danio rerio*) from Zebrafish International Resource Center (ZIRC), Eugene , Oregon, (USA).

2.2. Chemicals

WY-14643 (pirinixic acid), tricaine (ethyl 3-aminobenzoate salt), pronase (protease from *Streptomyces griseus*) and miconazole nitrate salt 1-(2,4-Dichloro-β-[(2,4-dichlorobenzyl)oxy]phenethyl)imidazole from Sigma-Aldrich Corp., St Louis, MO (USA).

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NECA (6-Amino-9H–purin-9-yl)-1 -deoxy-N-ethyl-b-D-ribofuranuronamide 5′-N-Ethylcarboxamidoadenosine) was obtained from Fischer Scientific, Pittsburg, PA, (USA). MRS-1706 (*N* -(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*–purin-8-yl) phenoxy]acetamide and MK-886 (3-[3-*tert*-Butylthio-1-(4-chlorobenzyl)-5-isopropyl-1H–indol-2-yl]-2,2-dimethylpropionic acid, sodium salt hydrate) were purchased from Tocris Cooks Inc., St. Louis, MO (USA). Leukotriene B_4 was purchased from Cayman Chemical, Ann Arbor, Michigan (USA).

Stock solutions of WY-14643 (50 mg/ml), MK-886 (25 mg/ml), NECA (50 mg/ml) MRS-1706 (5 mg/ml) and miconazole (10 mg/ml) were prepared in dimethyl sulfoxide (DMSO). All stock solutions were kept at 4°C.

2.3. Maintenance and generation of embryos

Fish were maintained at 28±0.5°C in 14:10h light: dark cycle and fed twice daily with TetraMin tropical flakes. Group mating of 10 pairs of male and female zebrafish was performed around 4:00 PM. Embryos were collected the next morning and examined for viability using a dissecting microscope. 30–50 embryos were incubated in 30 ml of fish water (0.06 g/l of Instant Ocean Salt in distilled water) with or without test compounds at 28±0.5°C. The fish water was replenished every day. For pilot studies, embryos (n=12– 14) 2–4 hour post-fertilization (hpf) were exposed to WY-14643 (1.0, 2.5, 10 and 100 μM), agonist of PPARα, with or without MK-886 (0.5–5.0 μM; IC_{50}=0.5 –1.0 μM) (Tocris Cooks Inc., St. Louis, MO, USA) (Kehrer et al., 2001) an antagonist of PPARα, or NECA (1.0 – 100 μM), a non selective adenosine receptor agonist with or without MRS-1706, a selective antagonist of A_{2B} (10 nM; Ki values for adenosine receptors are 1.39, 157, 112 and 230 nM for A_{2B} , A_1, A_{2a} and A_3 receptors, respectively) (Tocris Cooks Inc., St. Louis, MO, USA) (Desai et al., 2005).

2.4. Epifluorescence microscopy

Transgenic Zebrafish (TG(Fli:EGFP)) expressing green fluorescent protein under the control of the VEGF receptor promoter were used. To monitor the effects of PPARα and adenosine receptor agonists and epoxygenases in hypoxia-induced angiogenesis, based on pilot study, embryos (2–4 hpf) (n=12–14) were exposed to WY-14643 (10 μM; n=14), a PPARα ligand, NECA (10 μM; n=12), a non selective adenosine receptor agonist or miconazole (0.1 μM; n=12–15), an inhibitor of epoxygenase (Dong et al., 2002). For combined administration two groups were made: NECA (10 μM) + miconazole (0.1 μM) (n=12) and WY-14643 (10 μM) + miconazole (0.1 μM) (n=12). All groups were kept under normoxic (20.9 % oxygen) condition for 22–24 h. Embryos (22– 24 hpf) were dechorionated by treating them with a dilute solution of pronase (2 mg/ml in embryo water) (Sigma Aldrich Corp., St Louis, MO, USA) for 2–5 min and then incubated in the hypoxic (5% oxygen) or normoxic chamber at 28 °C for 6 h. Generation of hypoxia (5% oxygen) was accomplished by using an oxygen controller (Coy Laboratory Products, Grass Lake, Michigan, USA).

Embryos were anesthetized with tricaine solution (0.016%) (Sigma Aldrich Corp., St Louis, MO, USA). Blood vessels, namely; intersegmental vessel (ISV) and dorsal longitudinal anastomotic vessel (DLAV) were visualized at 28 hpf using epifluorescence microscopy and images were captured using a Nikon 4X objective with a 30 s Nikon camera exposure. Three parameters were used to assess angiogenesis: (i) total number of ISV (ii) total number of completely formed ISV and (iii) total number of completely formed DLAV. ISVs that reached to the dorsal periphery of the body and DLAVs that formed complete T shaped at the dorsal periphery were considered as completely formed ISV and DLAV, respectively. Angiogenesis was defined as the ratio of the number of completely formed ISV or DLAV to the total number of ISV in the trunk region.

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2.6. Data analysis

Data were expressed as means ± SEM. A two way analysis of variance (ANOVA) followed by Bonferroni's analysis as a post hoc test was performed to compare mean values from different groups. A value of p 0.05 was considered significant.

3. Results

3.1. Effect of hypoxia (6 h) on ISV and DLAV

Compared to normoxia, hypoxia increased angiogenesis in terms of sprout number. The number of primary sprout increased by 119±8% ($p<0.001$) (fig 1a, b & c) and there was an 18-fold ($p<0.001$) or 100±8% ($p<0.001$) increase in number of completely formed ISV (fig 1a, b and d) or DLAV (fig 1a, b and e), respectively, at 28 hpf.

3.2. Effects of PPARα ligand and antagonist on angiogenesis

Lower concentrations of WY-14643; 1.0 and 2.5 μM elicited no effect on angiogenesis under normoxic condition (data not shown). However, WY-14643 (10 μM) increased angiogenesis (primary sprouts) by 41±13% ($p<0.05$) (fig 2a) but there was no change in angiogenesis in ISV or DLAV at 28 hpf. Under normoxic conditions, MK-886 (0.5 μM) alone decreased angiogenesis as manifested by the number of primary sprouts by 84±25% ($p<0.001$) and attenuated WY-14643 (10 μM)-induced increase in primary sprouts by 73±20% ($p<0.001$). MK-886 also inhibited LTB4-induced angiogenic effects on primary sprout by 34±11% ($p<0.05$) (fig 3a), ISV by 68± 20% ($p<0.001$) (fig 3b) and DLAV formation by 70±25% ($p<0.001$) (fig 3c).

Under hypoxic conditions, WY-14643 (10 μM)-stimulated the number of primary sprouts, ISV and DLAV formation by 3±2% ($p>0.05$), 41±9% ($p<0.01$) and 82±15% ($p<0.001$) (fig 2a–c), respectively. Under hypoxic conditions, MK-886 (0.5 μM) alone inhibited angiogenesis in primary sprouts by 55±6% ($p<0.001$), in ISV by 47±17 ($p<0.001$) and in DLAV by 100±8% ($p<0.001$). MK-886 (0.5 μM) also blunted WY-14643-induced ISV and DLAV formation by 65±14% ($p<0.001$) and 87±37% ($p<0.001$), respectively.

3.3. Effects of adenosine receptor agonist and antagonist on angiogenesis

Under normoxic conditions, NECA did not show any angiogenic effect on ISV or DLAV. However, NECA increased primary sprout density 1.9-fold ($p<0.001$) (fig 4a). After exposure to hypoxia (6 h), NECA (10 μM) increased angiogenesis in ISV (41 ±9%; $p<0.05$) (fig 4b) and DLAV (78±17%; $p>0.05$) (fig 4c) at 28 hpf.

In order to evaluate the specific type of adenosine receptor involved in angiogenesis, embryos were pretreated with MRS-1706, a selective antagonist of A_{2B} receptor which selectively inhibited A_{2B}-receptor- induced angiogenesis at 10 nM concentrations (Desai et al., 2005). At 28 hpf, MRS-1706, a selective A_{2B} receptor antagonist, inhibited NECA-induced increase in the number of primary sprouts (fig 4a) by 99+31.8% ($p<0.001$) and 88±20% ($p<0.001$) under normoxic and hypoxic conditions, respectively. MRS-1706 also abolished NECA-induced increase in angiogenesis in ISV at 28 hpf by 100±4% ($p<0.001$) (fig 4b) and in DLAV by 99±8% ($p<0.001$) (fig 4c) under hypoxic but not under normoxic conditions. Compared to NECA (10 μM) alone, MRS-1706 (10 nM) attenuated NECA-induced increase in primary sprouts by 88±20% ($p<0.05$) (fig 4a) and abolished NECA-induced ISV (100±4%; $p<0.001$) (fig 4b).

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3.4. Effect of epoxygenase inhibition on PPARα-mediated angiogenesis

In order to evaluate the involvement of epoxyeicosatrienoic acids (EETs) as a downstream mediator of PPARα-dependent angiogenesis, embryos (2–4 hpf) (n=12) were exposed to WY-14643 (50 μM) with or without miconazole (0.1 μM), an inhibitor of epoxygenase. At 28 hpf, miconazole (0.1 μM) reduced angiogenesis in primary sprout, ISV and DLAV by 5-fold, 2-fold and 6-fold, respectively (fig 5) under hypoxic conditions (fig 5). Compare to miconazole alone, WY-14643 (10 μM) mitigated inhibitory action of miconazole (0.1 μM) in primary sprout by 5-fold ($p<0.05$), in ISV by 4-fold ($p<0.05$) and in DLAV by 6-fold ($p<0.01$) (fig 5).

3.5. Effect of epoxygenase inhibition on adenosine receptor-mediated angiogenesis

The role of epoxyeicosatrienoic acid (EET) in adenosine receptor-mediated angiogenesis was evaluated in embryos (2–4 hpf) (n=12) exposed to miconazole (0.1 μM) or combined miconazole (0.1 μM) + NECA (50 μM). Under hypoxic conditions NECA (50 μM) diminished miconazole (0.1 μM)-mediated antiangiogenic effect. Compared to miconazole (0.1 μM) alone, the combined treatment of miconazole (0.1 μM) with NECA (50 μM) showed about 5-fold ($p<0.05$) angiogenic recovery in spout number and 3-fold ($p<0.05$) angiogenic improvement in ISV and DLAV development, ($p<0.05$ (fig 6) under hypoxic condition.

4 Discussion

Adenosine receptors are the known therapeutic targets in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, wound healing, immune and inflammatory disorders and cancer. Agonists of adenosine receptor have proven effective in the treatment of many cardiovascular disorders including, arrhythmia and cardiac ischemia (Jacobson and Gao, 2006). On the other hand, PPARα, an antiinflammatory transcription factor, is known to play important roles in lipid metabolism and glucose homeostasis and PPARα ligands are currently used for reducing coronary vascular events, decreasing mortality in heart disease (Bishop-Bailey, 2000) and inhibiting inflammation in certain diseases such as atherosclerosis.

Despite the fact that both adenosine receptor and PPARα have been recognized as therapeutic targets for common diseases such as cardiac ischemia, inflammation-related diseases and wound healing, their cross-talk in the normal physiological and pathological conditions is mostly unknown. There is scanty number of studies represented interactions of PPARα and adenosine receptor. The studies of Peng et al., (2009) demonstrated that activation of A_{2B} receptor inhibited expression and activity of PPARα in pathogenesis of fatty liver. Soledade et al., (2007) also reported that A_{2A} adenosine receptor activation stimulated PPARα expression and activity in THP-1 cells and mouse peritoneal macrophages and speculated a possible pathway for A_{2A}-mediated anti-inflammatory mechanism. On other hand, Araki et al. (2009) reported that PPARα up regulated transcription of the A_{2A} adenosine receptor gene in human umbilical vein endothelial cell (HUVEC). While the role of adenosine receptors in angiogenesis is well defined; the role of PPARα in angiogenesis is not definitive. Since both receptors are the therapeutic targets for the cardiovascular diseases, especially cardiac ischemia or peripheral artery disease where recovery depends in part on the ability to make collateral vessels, the interaction of PPARα and adenosine receptor in angiogenesis merits careful evaluation.

The present study evaluated the distinct roles of PPARα and adenosine receptors in angiogenesis and their possible interactions in hypoxia-induced angiogenesis. For this study we used zebrafish embryo that has been used as a preclinical model for assessment of

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angiogenesis (Parng, et al, 2002; Oh, et al., 2008; Lam et al., 2008). Furthermore, this model also showed for the presence of PPARα (Ibabe et al., 2005) and adenosine receptors A_{2A} and A_{2B} (Boehmler et al., 2009) during early stages of embryonic development.

The role of PPARα in angiogenesis is controversial as it acts as an inhibitor as well as an inducer of angiogenic process (Biscetti et al., 2009). Many studies reported an anti-angiogenic effect (Panigrahy et al., 2008; Grabacka et al., 2006) whereas others reported a pro-angiogenic role for PPARα (Kaipainen et al., 2007; Biscetti et al., 2008) Our epifluorescence microcopy data showed that WY-14643, a PPARα ligand, stimulated angiogenesis at 28 hpf. The involvement of PPARα activation in angiogenesis was further confirmed in experiments in which embryos (28 hpf) were exposed to MK-886 with or without WY-14643, at the 4–6 hpf stage. The greater inhibition of basal angiogenesis by MK-886 indicated that MK-886 antagonizes the effect of endogenous PPARα ligands more effectively compared to that of an exogenous activators like WY-14643. This effect likely reflects inhibition of leukotriene B_4 (LTB_4), a known endogenous ligand of PPARα (Narala, et al., 2010) and which synthesis is inhibited by MK-886.

Hypoxia is a known stimulator of angiogenesis. It mainly stimulates angiogenesis by preventing degradation of hypoxia inducible factor-1-α (HIF-1α), a transcription factor, which activates the transcription of many pro-angiogenic genes. However, studies also showed that hypoxia through HIF-1α, reduced PPARα expression and activity in intestinal epithelial cells (Narravula and Colgan, 2001) and cardiac myocytes (Huss et al., 2001; Belanger et al., 2007). Based on the consensus that hypoxia reduces PPARα expression/activity, we expected that PPARα ligands would attenuate hypoxia-induced angiogenesis when compared to normoxia, However, in our experimental setting, administration of PPARα ligands promoted angiogenesis to a similar extent in both normoxic and hypoxic conditions . Although we have not verified yet, however, PPARα-mediated angiogenesis under hypoxic conditions implies that the transcription factors other than HIF-1α such as HIF-2α may be involved to promote PPARα expression (Kelly, 2008), and thereby preserved PPARα-mediated angiogenesis under hypoxic condition.

A protective role for adenosine through its four known receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) in ischemic and hypoxia-induced injury has been documented in many studies (Linden, 2005; Auchampach, 2007). The role of adenosine receptors in angiogenesis has been evaluated in many studies (Auchampach 2007; Clark et al., 2007). Among the four known receptor sub types of adenosine, A_1, A_{2B} and A_3 have been shown to stimulate angiogenesis under physiological as well as pathological conditions (Desai *et al*., 2005; Linden 2005; Auchampach 2007), whereas A_{2A} receptor activation either promotes (Desai et al., 2005; Montesinos et al., 2004) or inhibits angiogenesis (Olah and Roudabush, 2000). Our data demonstrated that adenosine receptor activation stimulated angiogenesis which was greater under hypoxic condition, indicating consistent with a greater generation of adenosine and adenosine receptor expression in hypoxia, (Kong et al., 2006) and greater adenosine receptor-mediated NECA-induced VEGF secretion under hypoxic conditions (Ryzhov et al., 2007)

Since many studies reported that adenosine receptor-mediated angiogenesis contributed up to 50–70% of hypoxia-induced angiogenesis (Adair, 2005) and for the higher expression/activity of A_{2B} receptor under hypoxia-induced angiogenesis Kong *et al*., 2006; Ma et al., 2010) we used MRS-1706, a selective antagonist of A_{2B}, to investigate the involvement of A_{2B} in angiogenesis under hypoxic condition. Although specific involvement of A_{2B} receptor has not been clearly defined, a role for A_{2B} receptor activation was inferred as MRS-1706 abolished NECA-induced angiogenesis under hypoxic condition (fig 3a & b). These results are in agreement with the studies that demonstrated involvement of A_{2B}

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receptor in hypoxia-induced angiogenesis in human microvascular endothelial cells (HMEC-1) (Kong et al., 2006).

EETs are epoxygenase-dependent eicosanoids which have demonstrated a pro-angiogenic effect. (Pozzi et al., 2005; Panigrahy et al., 2010; Michaelis et al., 2008). Indeed, a link between activation of adenosine receptors (A_{2A}) and enhanced generation of CYP2C9-dependent EET in A_{2A}AR-WT mice was reported by Nayeem et al., (2008). In this study, CGS-21680, a selective agonist of A_{2A}R elicited vasorelaxation that was higher in A_{2A}-wild type compared to A_{2A}-knockout mice aortae, and was attenuated by CYP-epoxygenase inhibitor or EET antagonist. Given this background, we examined whether EETs play any role in A_{2B} receptor mediated hypoxia-induced angiogenesis in the zebrafish embryo. Our data demonstrated that, miconazole, an inhibitor of epoxygenase, decreased NECA-induced angiogenesis under normoxic and hypoxic conditions, indicating that EET generation is the downstream mechanism responsible for A_{2B} receptor-mediated hypoxia-induced angiogenesis.

PPARα acts as an inducer (Zhao et al., 2009) as well as inhibitor (Pozzi et al., 2007) of CYP2C expression, our data demonstrating that miconazole, an inhibitor of CYP2C attenuated PPARα-mediated angiogenesis under normoxic as well as hypoxic conditions, indicated that PPARα-activation also stimulates angiogenesis through EET-dependent mechanism.

5. Conclusions

Data from this study demonstrated that: (i) activation of PPARα and adenosine receptors independently promoted angiogenesis in the zebrafish embryo (ii) hypoxia-induced angiogenesis involved in activation of both PPARα and adenosine receptors (iii) both PPARα and A_{2B} receptor promoted angiogenesis through an epoxide-dependent mechanism.

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Abbreviations

CYP2C9	cytochrome P450 2C9
DLAV	dorsal longitudinal anastomotic vessel
EET	eicosatrienoic acid
HIF-1α	hypoxia-inducible factor-1 alpha
hpf	hour post-fertilization
IL-8	interleukin-8
ISV	intersegmental vessel
NECA	5′-N-Ethylcarboxamidoadenosine
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome
PGAR	PPARγ angiopoietin-related gene

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TSP-1	thrombospondin-1
Tg(fli-1:EGFP)	transgenic friendly leukemia integration-1 enhanced green fluorescent protein

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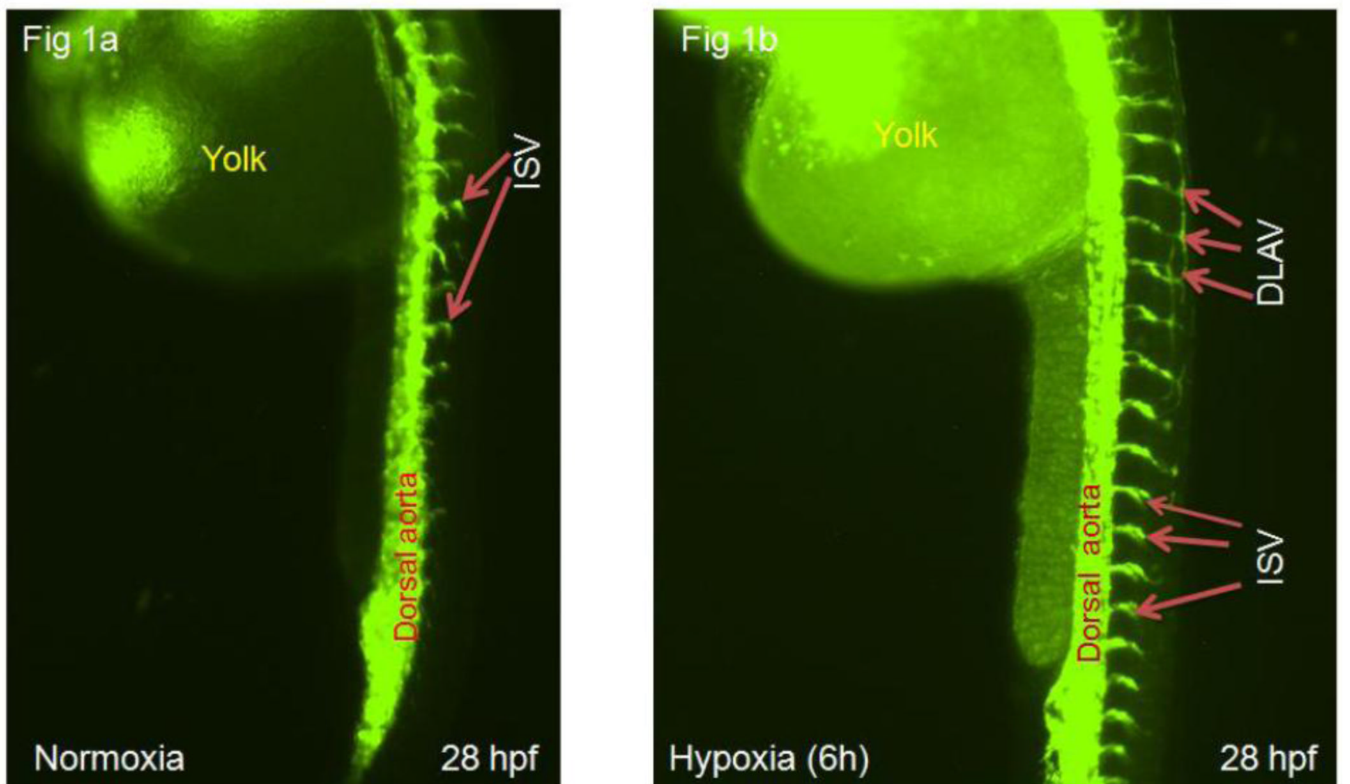

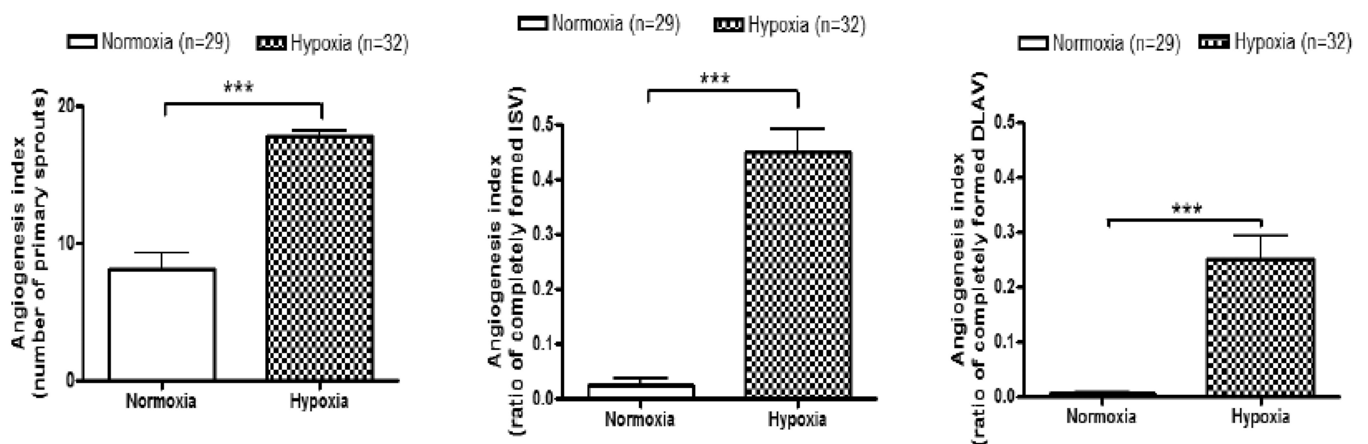

Figures 1.

a–b: Representative epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing ISV and DLAV at 28 hours post-fertilization (hpf) under normoxic (fig 1a) or hypoxic conditions (fig 1b). Intersegmental vessels are represented as ISV and dorsal longitudinal anastomotic vessels as DLAV.

c–e: Quantitative evaluation of angiogenesis index at 28 hpf: primary sprout (figure 1c), ISV (1d) and DLAV (figure 1e). Data are expressed as mean (±SEM) of the total number of primary sprout (c) or the ratio between completely formed ISV (d) or DLAV (e) to the total

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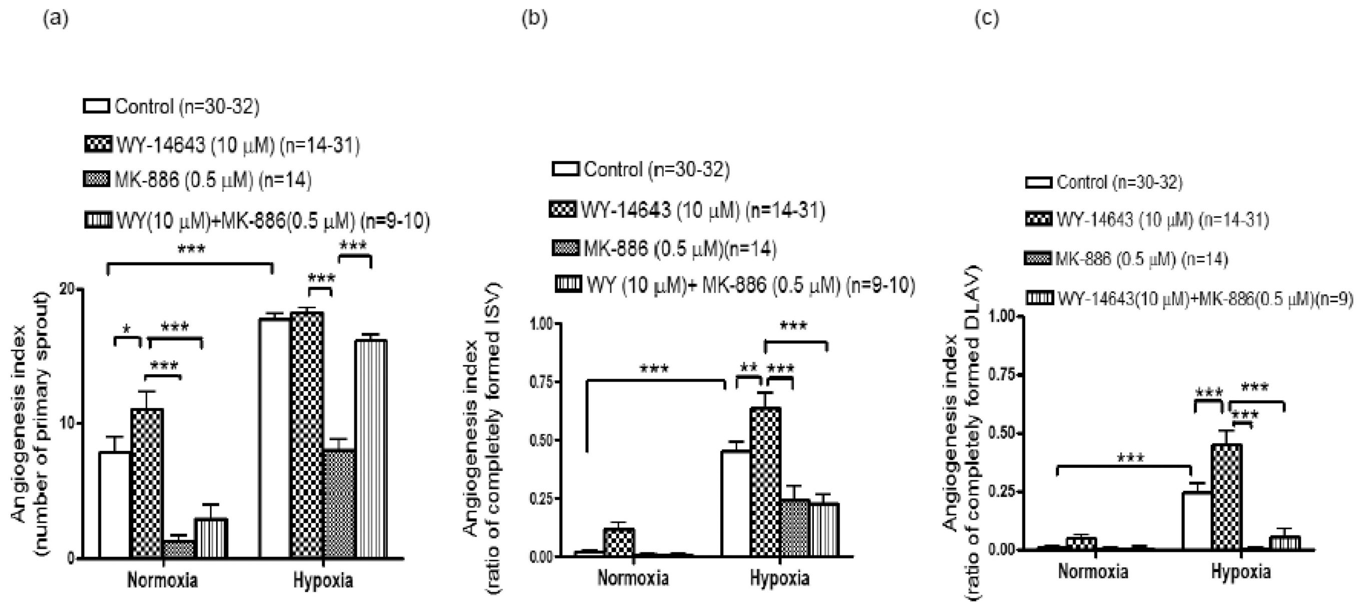
number of ISV. A two way analysis of variance (ANOVA) was used to compare mean of hypoxic group vs. normoxic control. *** $p<0.001$



Figures 2.

a–c: Quantitative evaluation of epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing involvement of PPARα in angiogenesis under normoxic or hypoxic condition. Histograms represent the effects of WY-14643 (10 μM), a selective agonist of PPARα on emergence of primary sprout (ISV) (fig a), ISV development (fig b) and DLAV development (fig c) at 28 hpf in the presence of MK-886 (0.5 μM), an antagonist of PPARα. Data are expressed as mean (±SEM) of total number of primary sprout (fig a) or the ratio between completely formed ISV (fig b) or DLAV (fig c) to the total number of ISV. A two way analysis of variance (ANOVA) was used to compare means of each group. *** p<0.001; **p<0.01; *p<0.05

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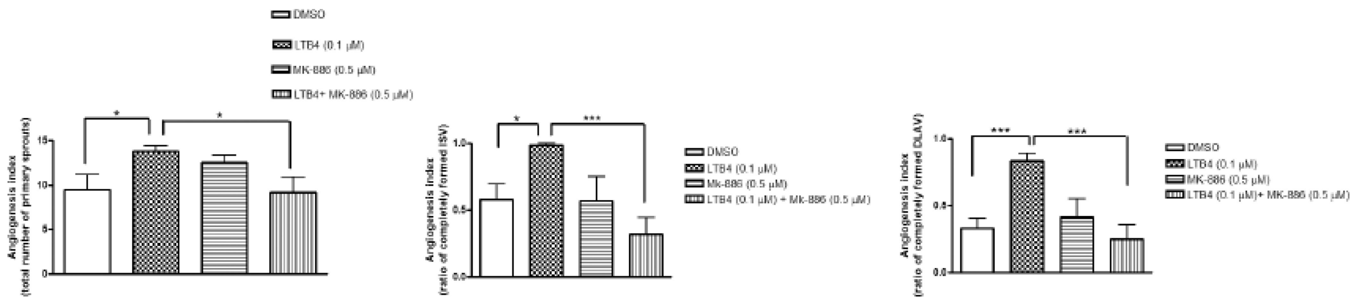

Figures 3.

(a–c); Quantitative evaluation of epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing inhibitory effect of MK-886 on LTB4-induced angiogesis under normoxic condition. Histograms represent the angiogenic effects of LTB4 (0.1 μM), a selective agonist of PPARα on emergence of primary sprouts (fig a), ISV development (fig b) and DLAV development (fig c) at 28 hpf in the presence or absence of MK-886 (0.5 μM). Data are expressed as mean (±SEM) of total number of primary sprout (fig a) or the ratio between completely formed ISV (fig b) or DLAV (fig c) to the total number of ISV. A two way analysis of variance (ANOVA) was used to compare means of each group. *** $p<0.001$; *$p<0.05$

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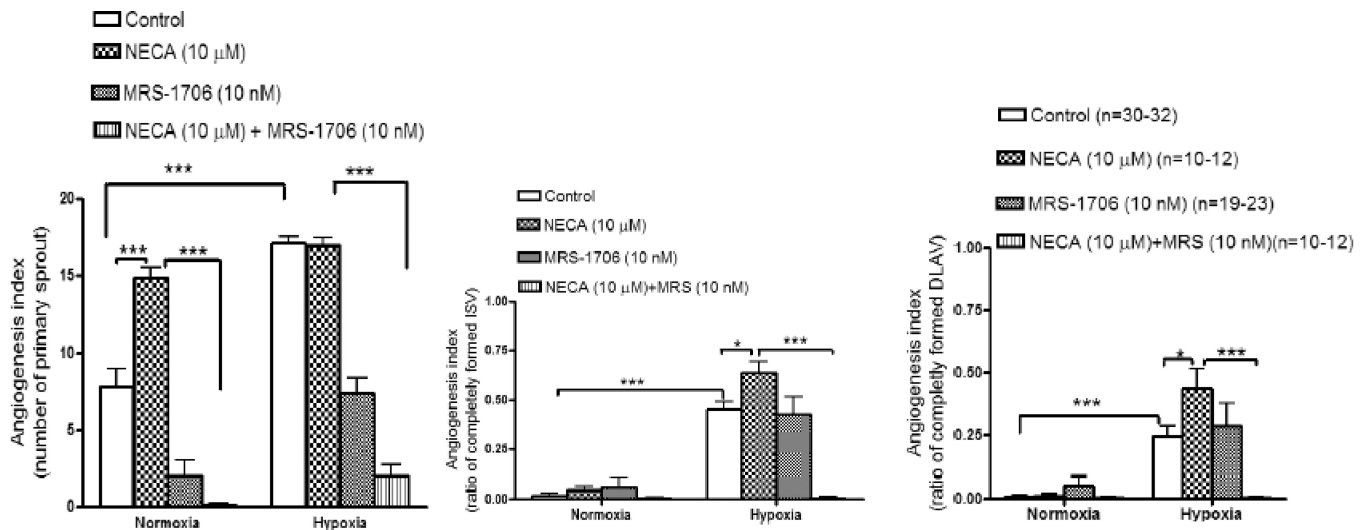
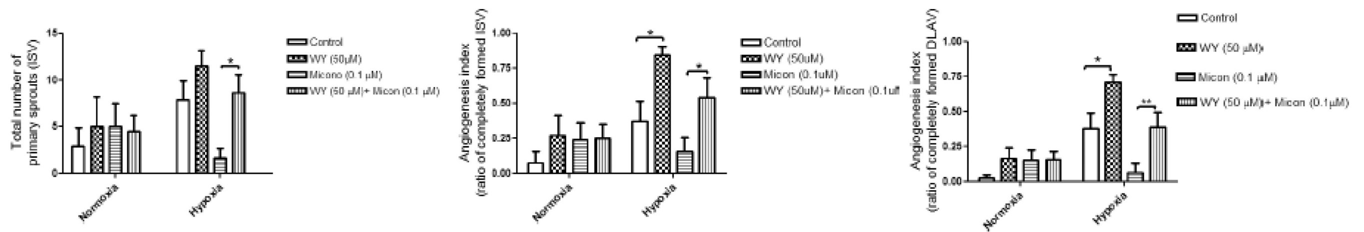


Figures 4.

a–c: cQuantitative evaluation of epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing involvement of adenosine receptor in angiogenesis under normoxic or hypoxic condition. Histograms represent the effect of NECA (10 μM), a non selective agonist of adenosine receptor on emergence of primary sprout (ISV) (fig a), ISV development (fig b) and DLAV development (fig c) at 28 hpf in the presence of MRS-1706 (10 nM), a selective antagonist of A_{2B} adenosine receptor. Data are expressed as mean (±SEM) of total number of primary sprout (fig a) or the ratio between completely formed ISV (fig b) or DLAV (fig c) to the total number of ISV. A two way analysis of variance (ANOVA) was used to compare means of each group. *** $p<0.001$; **$p<0.01$; *$p<0.05$



Figures 5.

(a–c): Quantitative evaluation of epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing the effect of epoxygenase inhibition on WY-14643-induced angiogenesis under normoxic or hypoxic condition. Histograms represent the effect of miconazole (0.1 μM), an inhibitor of epoxygenase, on WY-14643-induced (50 μM) emergence of primary sprout (ISV) (fig a), ISV development (fig b) and DLAV development (fig c) at 28 hours-post fertilization (hpf). Data are expressed as mean (±SEM) of the total number of primary sprout (fig a) or the ratio between completely formed ISV (fig b) or DLAV (fig c) to the total number of ISV. A two way analysis of variance (ANOVA) was used to compare means of each group. ** $p<0.01$; *$p<0.05$

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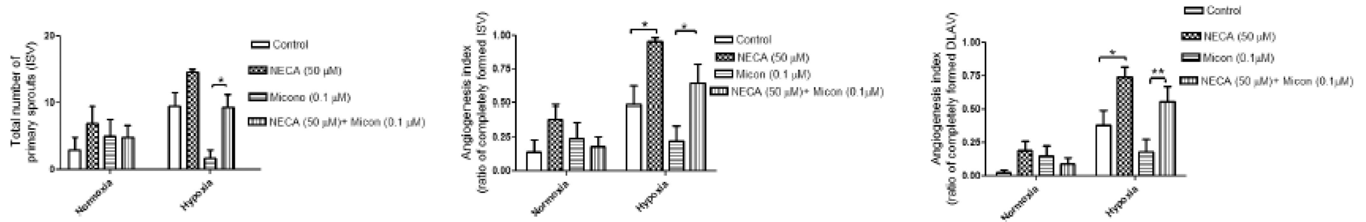

Figures 6.

(a–c): Quantitative evaluation of epi-fluorescent images of Tg(fli:EGFP) zebrafish embryos showing the effect of epoxygenase inhibition on NECA-induced angiogenesis under normoxic or hypoxic condition. Histograms represent the effect of miconazole (0.1μM), an inhibitor of epoxygenase, on NECA-induced (50 μM) emergence of primary sprout (ISV) (fig a), ISV development (fig b) and DLAV development (fig c) at 28 hours-post fertilization (hpf) Data are expressed as mean (±SEM) of the total number of primary sprout (fig a) or the ratio between completely formed ISV (fig b) or DLAV (fig c) to the total number of ISV . A two way analysis of variance (ANOVA) was used to compare means of each group. ** $p<0.01$; *$p<0.05$

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