T₃ enhances Ang2 in rat aorta in myocardial I/R: comparison with left ventricle

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Abstract

Angiogenesis is important for recovery after tissue damage in myocardial ischemia/reperfusion, and tri-iodothyronine (T₃) has documented effects on angiogenesis. The angiopoietins 1/2 and tyrosine kinase receptor represent an essential system in angiogenesis controlling endothelial cell survival and vascular maturation. Recently, in a 3-day ischemia/reperfusion rat model, the infusion of a low dose of T₃ improved the post-ischemic recovery of cardiac function. Adopting this model, our study aimed to investigate the effects of T₃ on the capillary index and the expression of angiogenic genes as the angiopoietins 1/2 and tyrosine kinase receptor system, in the thoracic aorta and in the left ventricle. In the thoracic aorta, T₃ infusion significantly improved the angiogenic sprouting and angiopoietin 2 expression. Instead, Sham-T₃ group did not show any significant increment of capillary density and angiopoietin 2 expression. In the area at risk (AAR) of the left ventricle, T₃ infusion did not increase capillary density but restored levels of angiopoietin 1, which were reduced in I/R group. Angiopoietin 2 levels were similar to Sham group and unchanged by T₃ administration. In the remote zone, T₃ induced a significant increment of both angiopoietin 1/2. In conclusion, T₃ infusion induced a different response of angiopoietin 1/2 between the ventricle (the AAR and the remote zone) and the thoracic aorta, probably reflecting the different action of angiopoietin 1/2 in cardiomyocytes and endothelial cells. Overall, these data suggest a new aspect of T₃-mediated cardioprotection through angiogenesis.

Key Words

- ▶ thyroid hormones
- ▶ angiogenesis
- ▶ angiopoietin 1/2
- ▶ tyrosine kinase receptor 2
- ▶ myocardial ischemia/reperfusion

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Introduction

In response to stress conditions, such as acute ischemia, heart evokes diverse and complex cellular responses involving both cardiomyocytes and non-muscle cells that sustain processes of structural remodeling of the myocardium (Swynghedauw 1999). In the myocardium, each cardiomyocyte is surrounded by an intricate network of capillaries, and its proximity to the endothelial cells

ensures cardiomyocytes to receive oxygenated blood supply and important protective signals for the I/R organization and survival (Hsieh *et al.* 2006). Insufficient angiogenesis but also compromised endothelial cell survival occurs in the myocardial infarction. Moreover, there is strong evidence of reduced myocardial capillary density in several human heart diseases such as

aortic stenosis, dilated cardiomyopathy and ischemic cardiomyopathy (Karch *et al.* 2005). Thyroid hormones have well-documented effects on angiogenesis, inducing sprouting in heart of adult hypothyroid mice through genomic and non-genomic effects (Chen *et al.* 2012). Non-genomic action of T₃ seems to initiate on plasma membrane integrin $\alpha v\beta 3$ receptor (Mousa *et al.* 2006, Davis *et al.* 2009), promote new blood vessel formation in a model of chick chorioallantoic membrane (CAM) (Davis *et al.* 2004) and tubule formation in human dermal and cardiac microvascular endothelial cells (Mousa *et al.* 2008, Balzan *et al.* 2013). Angiogenesis is important for recovery after tissue damage in cardiac muscle ischemia. Once angiogenic signaling has arisen in the infarcted area, it could also reach a more distant vessel, such as the aorta. In particular, the system of the angiopoietins 1/2 (Ang1/2) and tyrosine kinase receptor 2 (Tie2) is essential in angiogenesis and blood vessel formation. Tie2 is an endothelium-specific tyrosine kinase receptor for the angiopoietin ligands (Ang1/2). Ang1 is a strong Tie2 agonist, produced primarily by perivascular cells, that seems to be involved in blood vessel maturation and stabilization (Thomas & Augustin 2009). Some studies indicate that Ang1 promotes survival of cardiac and skeletal cells (Lee *et al.* 2011). In contrast, Ang2 is produced primarily by the endothelial cells in remodeling vessels and seems to function largely as a Tie2 antagonist to promote tumor angiogenesis and inflammation (Maisonpierre *et al.* 1997, Fiedler *et al.* 2006, Thomas & Augustin 2009). At the same time, both Ang1 and Ang2 could also exert their activity in a Tie2-independent manner through integrins (Fiedler *et al.* 2004, Dallabrida *et al.* 2005, Lee *et al.* 2011, Felcht *et al.* 2012).

Recently, Ang1 and Ang2 were shown to be involved in ventricular ischemia/reperfusion (I/R) injury (Matsunaga *et al.* 2003, Shyu *et al.* 2003, Lee *et al.* 2011). However, up to now, no studies on the effects of physiological dose of T₃ infusion on angiogenic activity and on the expression of Ang1/2–Tie2 system in myocardial I/R are available.

Recently, our group published two studies on experimental model of I/R in the early post-ischemic settings and the T₃ administration started after 24 h from the ischemic event to avoid the risk of the presence of unstable cardiovascular and systemic conditions (Forini *et al.* 2014, Nicolini *et al.* 2015). By this model, that closely mimics human AMI disease, 48-h T₃ administration was able to preserve the global heart function to reduce the infarct size as well as cardiomyocyte apoptosis and to improve the myocyte mitochondrial function (Forini *et al.* 2014). Moreover, T₃ reduced the pro-fibrotic process

leading to adverse cardiac remodeling (CR) (Nicolini *et al.* 2015).

With this model, we investigated whether the administration of near-physiological dose of T₃ in 3-day I/R could influence the angiogenesis on left ventricle (LV) and thoracic aorta of rat heart, studying, in particular, the capillary index and the expression of Ang1/2–Tie2 angiogenic system.

Materials and methods

Animal experimental protocol

All experiments and protocols were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of the Italian Ministry of Health (Endorsement n.240/2011-B). All surgeries were performed under anesthesia (Zoletil 50 mg/kg + xylazine 3 mg/kg ip), and all efforts were made to minimize suffering (Tramadol 10 mg/kg ip, if necessary).

A rat model of left anterior descending (LAD) coronary artery occlusion and reperfusion was used, using adult male Wistar rats 12–15 weeks old and weighing about 300 g, as described previously (Forini 2014). In brief, after 30 min of LAD occlusion, the occluder was removed and reperfusion was allowed 24 h after surgery, I/R rats were treated for 48 h with a constant subcutaneous infusion of 6 μ g/kg/day T₃ (I/R T₃, *n*=8) or saline (I/R, *n*=8) via a miniosmotic pump (Alzet, Model 2ML4, Palo Alto, CA, USA). Four animals for each group were killed, respectively, at 3 and 14 days after I/R surgery. Sham-operated control animals were prepared in a similar manner, except that the LAD was not occluded, and were treated with constant infusion of saline (Sham group, *n*=4). The other three Sham rats were treated for 48 h with a constant subcutaneous infusion of the same dose of T₃. Animals were killed by a lethal KCL injection and hearts explanted. Cardiac tissues were obtained from the LV free wall remote to LAD region (remote zone, RZ) and in the core of the ischemia–reperfusion region (area at risk (AAR)) as described previously (Forini *et al.* 2014). Thoracic aortas were dissected, cleaned from adipose tissues and the descendent branch of aortas was divided in two parts: the samples for gene or protein expression were immediately stored at –80°C, and the fresh sample for angiogenic assay was quickly processed.

Serum thyroid hormone levels

Thyroid hormone levels were measured in blood collected from the femoral vein either before or 3 and 14 days after

LAD occlusion. Free T₃ (FT₃) and free thyroxine (FT₄) levels were measured by a completely automated AIA 600 system (Tosoh Corporation, Tokyo, Japan).

Echocardiography study

To image *in vivo* the presence of regional contraction abnormality and/or to assess the global systolic function, we carried out echocardiography studies at 3 days after I/R. We used a portable ultrasound system (MyLab 25, Esaote SpA, Genova, Italy) equipped with a high-frequency linear transducer (LA52, 12.5 MHz). In animals sedated as described previously, images were obtained from the left parasternal view. Short-axis two-dimensional view of the LV was taken at the level of papillary muscles to obtain M-mode recording. Anterior (ischemia reperfused) and posterior (viable) end-diastolic and end-systolic wall thicknesses, systolic wall thickening and LV internal dimensions were measured following the American Society of Echocardiography guidelines. Parameters were calculated as mean of the measures obtained in three consecutive cardiac cycles. The global LV systolic function was expressed as fractional shortening (FS%).

Capillary labeling

Hearts were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 24h and then stored in 25% w/v sucrose in 0.1M PB overnight at 4°C. They were subsequently embedded in cryo-gel and frozen using liquid nitrogen. Sections of 10µm thickness were cut in a coronal plane using a cryostat (CM3050S; Leica Biosystems, Germany). Sections were permeabilized and incubated with 7µg/mL Fluorescein Griffonia simplicifolia (Bandeiraea) Isolectin B4 (IB4; Vector Laboratories, Burlingame, CA, USA) in 0.1% Triton X-100 in PB for 48h at 4°C. IB4 staining reveals blood vessels by binding to carbohydrate residues on their luminal surface (Alroy *et al.* 1987). Digital images of the LV outer wall of each heart were acquired with a fluorescent microscope (Nikon) equipped with a digital photcamera (Leica dfc 320). Images were normalized to background and analyzed using an image editing software (Adobe Photoshop CS3; Adobe Systems, Mountain View, CA, USA). Subsequently, the same sections were stained with toluidine blue and acquired at low magnification to localize the I/R area.

Ex vivo aortic ring angiogenesis assay

Thoracic aortas were dissected, cleaned from fibro-adipose tissue and blood, and then serially cross-sectioned into

2mm rings (Gelati *et al.* 2008), which were placed in 24-well microplates coated with collagen and cultured in serum-free EBM medium (EBM; Lonza, Basel, Switzerland). Cultures were observed by microscopy (40X Carl Zeiss vision GMBH) and images were captured after 1 week using Axiovision 3.0. The number of capillary networks was visually counted through a blind analysis (seven microscopic fields for sample).

Aortic tissue samples

Aortic tissue extractions (mRNA and protein) were performed directly from frozen thoracic aortic pieces stored at -80°C. Samples were immediately transferred in liquid nitrogen and ground to a fine powder with mortar and pestle and then homogenized in 150µL lysis buffer or 0.7 mL Qiazol (Qiagen) using a Tissue Lyser (Qiagen).

Quantitative real-time PCR analysis of gene expression

Total RNA was extracted with miRNeasy Mini Kit (Qiagen). Real-time PCR analysis were performed in a 384-well CFX384 RT-PCR System (Bio-Rad). Each reaction was carried out in a total volume of 10µL. Reaction mixture included 4µL of template cDNA (1:5 diluted), 0.5µM of each primer and 2X iTaq Universal Sybr Green Supermix (Bio-Rad). Amplification protocol started with 95°C for 30s followed by 39 cycles at 95°C for 5s and 60°C for 15s. To assess product specificity, amplicons were checked by melting curve analysis. Melting curves were generated from 65°C to 95°C with increments of 0.5°C/cycle. Each assay was performed in triplicates, with negative control. The combination of GeNorm and qBase software technology following recent guidelines was used to assess the expression stability of each candidate reference gene and to determine the ideal number of genes required for normalization in order to calculate individual normalization factors for each sample. The average Ct values obtained from each triplicate was converted into a relative quantity and analyzed with CFX384 Manager algorithm. Gene stability is expressed by the M value, which is calculated as the average variation between one of the genes and all the others analyzed. Genes are ranked for their M value, and at each step of the analysis the least stable gene (highest M value) is excluded and M is recalculated. The most stable genes are identified and internal control genes with M value <0.5 were chosen for normalization. Primer details of reference and target genes are summarized in Table 1. A standard curve for each target and housekeeping gene was evaluated to assess amplification efficiency and linearity.

Table 1 Primer sequences.

Gene	Primer sequence (5'→3')	Length bp	GenBank n.
<i>Hmbs</i>	F: TCTAGATGGCTCAGATAGCATGCA R: TGGACCATCTTCTTGCTGAACA	76	NM_03168
<i>Papbn1</i>	F: TATGGTGCAGACAGAGAAGA R: TATGCAAACCCCTTGGGATG	110	116697
<i>Tbp</i>	F: CACCGTGAATCTTGGCTGTAAAC R: CGCAGTTGTTCTGGCTCTC	124	NM_01004198
<i>Ang1</i>	F: CAGTGGCTGCAAAAACCTTGA R: TCCACATCTGTGAGCTTTCG	164	NM_053546,1
<i>Ang2</i>	F: GATCTTGTCTTGGCTCAGC R: ACGGCGTTAGACATGTAGGG	161	NM_134454,1
<i>Hif</i>	F: TCAAGTCAGCAACGTGGAAG R: TATCGAGGCTGTGTCGACTG	198	NM_024359,1
<i>Vegf</i>	F: CTACCTCCACCATGCCAAGT R: TTTCTTGCCTTTCGTTTTT	189	NM_031836
<i>Vegf-r</i>	F: CCAAGCTCAGCACACAAAAA R: CCAACCACTCTGGGAAGTGT	190	NM_U93307,1
<i>Intβ3</i>	F: TGACATCGAGCTGGTGAAG R: GAGTAGCAAGGACAATGAGC	224	NM_153720,1
<i>Tie2</i>	F: CCTAGAGCCAGAGACT R: GCCTTCTGTTTAGGG	263	NM_001105737,1

Western blotting

Aortic tissue lysate proteins were quantified by Bradford method in an Infinite 200PRO microplate reader (TECAN, Milan, Italy). Proteins (30 µg) were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically blotted onto 0.2 mm nitrocellulose membranes. The membrane were blocked for 1 h at room temperature with 1% blocking solution (Roche) in Tween 20/PBS 0.1% vol/vol (PBST) and then incubated overnight at 4°C with primary antibodies against angiopoietin-1 (goat sc-6319, 1:1000; Santa Cruz, CA, USA), angiopoietin 2 (goat polyclonal sc-7017, 1:1000; Santa Cruz, CA, USA), Tie2 (rabbit sc-9026) and Gapdh (mouse; Cell Signaling Technology, 1:2000). After three washes, the appropriate secondary IgG–HRP-linked conjugate (anti-rabbit A0545; Sigma, anti-mouse sc-2005; Santa Cruz, CA, USA) at 1:3000 dilution was applied. Proteins were visualized with the Clarity ECL substrate (Bio-Rad) and acquired images were quantified using Optiquant software.

Cell cultures

Human microvascular endothelial cells (HMEC-1) were cultured in M-199 medium (Lonza) supplemented with 10% (v/v) fetal bovine serum, 1% glutamine, 1% antibiotics, 1% epidermal growth factor (EGF; Sigma Chemical Co, St Louis, MO, USA) and 0.5% hydrocortisone as described previously (Balzan *et al.* 2011). After growing to subconfluence, cells were preincubated in

the presence or absence of Tetrac (0.5 µM) for 15 min in M-199 medium with 1% serum and then exposed to T₃ (10 µM) for 8 h under either normoxia or hypoxia. For the hypoxia study, cells were placed in a Molecular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA). The chamber was filled with 100% N₂ until an oxygen-free environment was created and then placed inside the CO₂ incubator at 37°C for 8 h. After incubation, total RNA was extracted with RNeasy Kit (Qiagen).

Statistical analysis

Differences between the means of two variables were evaluated by Student's *t*-test. For comparison between more than two groups, we used ANOVA, followed by *post hoc* test Bonferroni adjustment for multiple comparisons (StatView 5.0.1). The results are expressed as mean ± s.d. and values of $P \leq 0.05$ were considered statistically significant.

Table 2 FT₃–FT₄ rat serum levels at 3 and 14 days after surgery.

	FT ₃ (pg/mL)	FT ₄ (pg/mL)
Sham	3.4 ± 0.6	13.2 ± 1.6
I/R-3d	2.7 ± 0.3 ^a	12.7 ± 3.5
I/R-14d	3.1 ± 0.2 _{b,c}	10.5 ± 0.5 _{b,c}
I/R T ₃ -3d	6.1 ± 1.0 ^{d,f}	2.9 ± 1.2 ^{e,f}
I/R T ₃ -14d	2.8 ± 0.5	12.1 ± 4.2

^a $P < 0.05$; ^b $P < 0.001$ vs Sham; ^c $P < 0.001$ vs I/R-3d; ^d $P < 0.05$; ^e $P < 0.001$ vs I/R-14d; ^f $P < 0.01$ vs I/RT₃-14d.

Table 3 *In vivo* heart functional parameters by echocardiography at 3 days.

Heart function	Sham	I/R	I/R+T ₃
LVEDd (cm)	0.57 ± 0.02	0.59 ± 0.02	0.55 ± 0.01
LVESd (cm)	0.23 ± 0.01	0.33 ± 0.02 ^{b,c}	0.24 ± 0.01
SAWT %	69 ± 3	50 ± 5 ^{b,c}	65 ± 2
SPWT %	67 ± 4	63 ± 4	66 ± 5
FS%	61 ± 1	45 ± 3 ^{a,c}	57 ± 2
HR	429 ± 13	442 ± 24	496 ± 3

^a*P* < 0.05 and ^b*P* < 0.01 vs sham; ^c*P* < 0.01 vs T₃ treatment.

Values are mean ± S.E.M.; FS%, percent fractional shortening; LVEDd, left ventricular end-diastole diameter; LVESd, left ventricular end-systole diameter; SAWT%, percent systolic anterior wall thickening; SPWT%, percent systolic posterior wall thickening.

Results

T₃ and T₄ blood levels after T₃ infusion

Table 2 presents the circulating values of FT₃ and FT₄, 3 and 14 days after surgery. I/R-3d group shows significantly lower FT₃ levels with respect to Sham (*P* < 0.05) according to the low T₃ state induced by I/R procedure. T₃ infusion significantly increased the FT₃ levels both compared with Sham and I/R-3d (*P* < 0.001), whereas FT₄ levels significantly drops (*P* < 0.001). After 14 days from I/R, the levels of FT₃ and FT₄ decrease and increase, respectively, with respect to T₃ I/R-3d (*P* < 0.01 and *P* < 0.001), coming back to the Sham values.

T₃ infusion improves the post-ischemic recovery of cardiac function

As presented in Table 3, I/R rats exhibited a significant decrease in left ventricular end-systole diameter (LVESd), percent systolic anterior wall thickening (SPWT%) and percent fractional shortening (FS%).

T₃ supplementation restored the normal values. These data suggest that T₃ plays an important role in CR after myocardial infarction.

T₃-induced angiogenic activity after 3 days of I/R in thoracic aorta ring

In 3-day I/R rats, the angiogenic index of the aortic rings did not change when compared with Sham. Instead, I/R rats treated with T₃ showed a significant three-fold increase in sprouting efficiency (*P* < 0.05) when compared with I/R rats or Sham (Fig. 1). Sham-T₃ group did not show a significant increment on capillary density. After 14 days from I/R, the sprouting of T₃ I/R-14d group decreased.

T₃ induces Ang2 expression after 3 days of I/R in thoracic aorta

To explore the action of 48-h T₃ administration after I/R injury on angiogenic gene expression, we evaluated Ang1/2-Tie2 system and other angiogenic genes in the thoracic aorta, respectively, 3 and 14 days after surgery (Fig. 2). When compared with Sham group, the I/R group 3 days after surgery showed a reduction (ranging from 1.7- to 2.6-fold) on the expression of HIF1α, VEGF-R (*P* < 0.0001) and Tie2 (*P* < 0.001), whereas no change was observed for VEGF, Ang1, Ang2 and INTβ3. T₃ administration after ischemia abolished the expression of HIF1α (*P* < 0.0001), VEGF-R (*P* < 0.0001), VEGF (*P* < 0.001) and Tie2 (*P* < 0.001) and reduced INTβ3 of 1.8-fold (*P* < 0.01). T₃ induced a significant (14-fold) increment on the expression of Ang2 (*P* < 0.0001 vs Sham, vs I/R and vs Sham-T₃). After 14 days of surgery, Ang2 expression in I/R-T₃-14d group returned to lower values with respect to I/R T₃-3d (*P* < 0.0001) (Fig. 2).

Figure 1

Representative images of angiogenic sprouting of thoracic aorta ring from Sham, I/R-3d, I/R T₃-3d, Sham-T₃-3d, I/R-14d and I/R T₃-14d groups by an *ex vivo* assay. After surgery, rings were plated on collagen and photographed after 1 week. In the legend, d = days. The histogram represents the mean value ± S.D. of the angiogenesis index, **P* < 0.05 vs Sham; #*P* < 0.05 vs I/R-3d, *n* = 4.

Figure 2

Quantitative analysis of *Ang1*, *Ang2*, *Tie2*, *Hif1α*, *Vegf*, *Vegf-r* and *Intβ3* mRNAs in thoracic aorta of Sham, I/R-3d, I/R T₃-3d, Sham-T₃, I/R-14d and I/R T₃-14d groups, respectively. In the legend, d=days. The histogram represents the mean value ± s.d., **P* < 0.01, ***P* < 0.001, ****P* < 0.0001 with respect to Sham, *n* = 4. #vs I/R-3d; ##vs I/R-14d; ###vs I/R T₃-14d, vs Sham-T₃.

Western blot after T₃ administration and 3 days of I/R

Ang2 protein increased of two-fold in T₃-I/R group after 3 days, compared with Sham and I/R.

Tie2 protein was virtually unaffected by T₃-I/R or I/R group (Fig. 3) and Ang1 protein was undetected (data not shown).

T₃ induces Ang2 expression through integrin αvβ3 in HMEC-1

In HMEC-1 cells under hypoxic condition, T₃ (10 nM) increased the expression of Ang2 of 12-fold and of VEGF, HIF1α and integrin β1 of two- to three-fold (Fig. 4).

Figure 3

Western blot of Ang2 and Tie2 in thoracic aorta tissues of Sham, I/R and I/R T₃, after 3 days. The histogram graph represents a typical example of three experiments of Ang2 analysis normalized to Gapdh.

Ang1 and Tie2 were undetected. The increased expression of Ang2 and VEGF was blocked if the cells were preincubated with Tetrac, meanwhile HIF1α and integrin β1 were only partially blocked.

Figure 4

Determination of *ANG2*, *VEGF*, *HIF1α* and *INTβ1* mRNA in HMEC-1 cells treated with T₃ under normoxic and hypoxic conditions in the presence or absence of Tetrac. Cells were exposed at 10 nM T₃ for 8 h in the absence or presence of hypoxia with or without Tetrac (0.5 μM). Data are expressed as fold of the respective control.

Figure 5

Representative images of capillary densities in the left ventricle of I/R and I/R T₃ hearts stained with IB4. A and B illustrate IB4-labeled capillaries in two different regions of I/R heart tissue (boxed areas in C). D and F illustrate IB4-labeled capillaries in two different regions of I/R T₃ heart tissue (boxed areas in F). C and F are low-power photomicrographs of toluidine blue-stained heart sections. Scale bars: 500 μm in the fluorescence micrographs, 1 mm in the toluidine blue micrographs. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-16-0118>.

T₃ does not induce increment of capillary density in heart

Representative images for capillary density in the infarct area of IR and I/R T₃ rats are shown in Fig. 5. The IB4 staining did not reveal any significant difference between the infarct area of IR and I/R T₃ (mean ± s.d. of gray levels, $n=7$; 3426 ± 1748 vs 3373 ± 1301 ROI/ 500 μm², $P < 0.1$). However, compared with I/R, I/R T₃ samples showed a capillary density arranged in a more homogeneous and organized pattern (compare Fig. 5A with Fig. 5E) and a reduction of infarct area as assessed with toluidine staining (compare Fig. 5C with Fig. 5F).

T₃ differently regulates the expression of Ang1/2 and Tie2 system in cardiac tissue and thoracic aorta

In the LV, AAR showed a significant reduction of Ang1 ($P < 0.05$ vs Sham) and T₃ infusion restored levels of Ang1 in AAR ($P < 0.001$ vs I/R) (Fig. 6). Instead, Ang2 was not changed. In the remote zone (RZ), T₃ induced an increment of two-fold of Ang1 ($P < 0.01$ vs Sham and I/R) and of 2.8-fold of Ang2 ($P < 0.05$ vs Sham and I/R).

Tie2 was increased by T₃ both in RZ and AAR, although no statistical significance was reached.

Discussion

Several studies demonstrated the beneficial effects of thyroid hormones on CR and function in myocardial infarction, acting not only on cardiomyocytes and cardiofibroblasts but also on endothelial cells and microvasculature (Iervasi & Nicolini 2013, Forini *et al.* 2014, De Castro *et al.* 2015, Gerdes *et al.* 2015, Nicolini *et al.* 2015). Angiogenesis is essential for the recovery after tissue damage, particularly after cardiac and skeletal muscle ischemia (Tomanek & Schatteman 2000, Kessler *et al.* 2014). Moreover, thyroid hormones have well-documented effects on angiogenesis (Davis *et al.* 2004, 2009, Moeller & Broecker-Preuss 2011, Savinova *et al.* 2011, Chen *et al.* 2012, Balzan *et al.* 2013).

In this study, we investigated whether I/R could affect the angiogenic activity and the Ang1/2–Tie2 expression of thoracic aorta and LV tissue and we evaluated the possible effects of T₃ infusion after the ischemic event.

Myocardial I/R itself did not affect angiogenic sprouting (Fig. 1). At the same time, I/R induced a significant reduction of genes involved in angiogenesis such as HIF1α, VEGF-R and Tie2 (Fig. 2). After 3-day I/R, T₃ significantly

Figure 6

Quantitative analysis of Ang1, Ang2 and Tie2 mRNAs in area at risk (AAR) and remote zone (RZ) of the left ventricle from Sham, I/R and I/R T₃ group, at 3 days. The histogram represents the mean value ± s.d., * $P < 0.05$, ** $P < 0.01$ with respect to Sham, # $P < 0.05$, ## $P < 0.01$ vs I/R-3d; $n=5$.

increased angiogenesis index and strongly upregulated the expression of Ang2 (Figs 1 and 2). Instead, HIF1 α , VEGF-R, VEGF and Tie2 were completely downregulated (Fig. 2). However, the angiogenic sprouting and Ang2 expression decreased when a group of I/R rats infused with 48-h T₃ were killed after 14 days (Figs 1 and 2). Thus, we can speculate that T₃ stimulation of Ang2 in the early phase of I/R induces premature vessels that, however, regress after T₃ stimulus is gone. After 48 h of T₃ administration, the infarct area of the LV was reduced, when compared with the area of I/R rats (Fig. 5C and F). Moreover, as previously observed (Forini *et al.* 2014, Nicolini *et al.* 2015), T₃ improves cardiac functional recovery (Table 3). However, we did not find a significant difference in capillary density in the two groups, even though a more organized pattern of capillary density was observed after T₃ administration (Fig. 5A and E). However, we cannot exclude an increased capillary density during the following days. In fact, some studies reported that the infarcted territory was highly vascularized by capillaries after 7–14 days of reperfusion, whereas there was a progressive increase in the percentage of pericyte-coated microvessels and arteriolar density up to 4 weeks after reperfusion (Ren *et al.* 2002, Virag *et al.* 2003). Although thyroid hormones promote angiogenic activity, their effects at the microvascular level remain controversial. In fact, an increased capillary density was found in the heart of both hyperthyroid and hypothyroid animals (Rodríguez-Gómez *et al.* 2013). Moreover, in hypothyroidism, Savinova *et al.* (2011) reported unchanged capillary density after T₃ treatment, even if a positive response to angiopoietins was observed.

The Ang1/2–Tie system acts as a vascular-specific ligand/receptor system for endothelial cell survival and vascular maturation. Ang1 acts as an agonist of Tie2 receptor, whereas Ang2 is the antagonist (Maisonpierre *et al.* 1997, Thomas & Augustin 2009). Previously, Shyu *et al.* (2003) have shown that only the expression of Ang2 increased after I/R in the ventricular myocardium, while the expression of Ang1 did not change.

Ang2 is almost exclusively expressed by endothelial cells where it is stored in Weibel–Palade bodies and rapidly released upon stimulation (Fiedler *et al.* 2004). More precisely, Ang2 is present at low levels in resting endothelial cells, whereas it is increased in the endothelial cells of remodeling blood vessels (Maisonpierre *et al.* 1997, Augustin *et al.* 2009, Daly *et al.* 2013). Furthermore, Ang2 expression is upregulated in a wide range of human cancers (Augustin *et al.* 2009, Daly *et al.* 2013) and Tie2 receptor is broadly expressed in the endothelium of

quiescent adult vasculature. In contrast to Ang2, Ang1, which is expressed primarily in smooth muscle cells and other perivascular cells, seems to promote, through its Tie2 receptor activation, blood vessel maturation and stabilization (Davis *et al.* 1996, Thurston *et al.* 2000, Augustin *et al.* 2009). Our results strongly support the idea that T₃ administration in 3-day I/R rats stimulates angiogenesis in the thoracic aorta through the increase in Ang2. In fact, in Sham-operated rats infused with the same dose of T₃, angiogenic activity and Ang2 levels increased in less extent (Figs 1 and 2). Several studies reported an upregulation of Ang2 and Tie2 expression by hypoxia in endothelial cells (Mandriota & Pepper 1998) and also after I/R in the rat ventricular myocardium (Shyu *et al.* 2003, Baoxia 2012). In our experimental conditions, Tie2 and Ang2 in thoracic aorta tissue did not show any change after 3-day I/R. Shyu *et al.* (2003) reported that the variations in the expression of Ang2 and Tie2 have a temporal pattern with a maximal increase at 24–48 h after reperfusion. The most validated model explaining Ang2 role in tumors and in cardiac capillary density modifications proposes that Ang2 inhibits Tie2 signaling facilitating VEGF-dependent angiogenesis (Maisonpierre *et al.* 1997, Daly *et al.* 2013). As in our model Ang2 expression was not associated with VEGF expression, our findings do not suggest that Ang2 and VEGF act in concert in response to T₃ infusion in I/R. Similarly, Ang2 did not correlate with HIF1 α , Tie2 and VEGFR. Instead, our *in vitro* study by HMEC-1 cells in hypoxic condition showed that T₃ directly stimulated VEGF and HIF1 α (Fig. 4). A possible explanation could be that T₃, in I/R condition, plays an indirect inhibitory action on VEGF and HIF1 α through the mediation of some brake factor released from other cells types.

Interesting studies reported that Ang2 and Ang1 could induce angiogenesis independently from Tie2, through their binding to integrins $\alpha\beta 3$, $\alpha\beta 5$ and $\alpha 5\beta 1$ (Dallabrida *et al.* 2005, Lee *et al.* 2011, Park *et al.* 2014). Recently, we reported that the molecular mechanism of the proangiogenic action of thyroid hormones is initiated at plasma membrane level in human microvascular endothelial cells through the interaction with integrin $\alpha\beta 3$. In fact, the exposition of the cells to Tetrac (the inhibitor of T₃ binding to integrin $\alpha\beta 3$) inhibited this effect (Balzan *et al.* 2013). In this study, in HMEC-1 cells under hypoxic condition, we observed a strong upregulation of Ang2 after T₃ administration (Fig. 4). Furthermore, as Tetrac inhibited this activation, we suggested that the mechanism by which T₃ induces Ang2 expression is

through integrin $\alpha\text{v}\beta 3$. At the same time, Ang2 induction by T₃ seems independent from Tie2 mediation, as both in the aorta and in the HMEC-1 model, Tie2 expression was inconsistent (ND=RNA undetected). Moreover, only a two-fold increase in Ang2 protein was observed in T₃ I/R (Fig. 3). The partial discrepancy we found between gene expression and Western blot analysis could be attributed to the rapid secretion of Ang2 from the cells (Fu *et al.* 2006, Thomas & Augustin 2009).

In contrast to Ang2, Ang1 is abundantly expressed in the myocardium (Thomas & Augustin 2009). The important finding of our study is that Ang1 is downregulated in the AAR and T₃ infusion restores Ang1 levels (Fig. 6). Lee *et al.* 2011 have shown that Ang1 coordinated protective effects not only in endothelial cells but also in cardiomyocytes during I/R injury, promoting endothelial integrity through vascular endothelial (VE)-cadherin dephosphorylation and cardiomyocyte survival through the integrin $\beta 1$ /phospho-ERK (pERK)/caspase. These authors proposed that direct, no-Tie2-mediated interactions of Ang1 with cardiac myocytes contributed to this cardioprotection because Ang1 acts locally, associated with cardiac matrix, whereas Ang2 diffuses freely, exerting its influence at a greater distance.

In our paper, T₃ induced a twofold increment of Ang1 also in RZ, suggesting that Ang1 overexpression in the heart could reduce the infarct zone, preserving as much as possible the cardiac function after myocardial infarction. Instead, Ang2 increased 2.8-fold only in the RZ and Tie2 underwent a modest but not significant increment both in AAR and RZ.

These data suggest that T₃ might induce a different response of Ang1/2–Tie system in the heart and in the thoracic aorta that probably reflects the different actions of Ang1/2 in cardiomyocytes, endothelial cell and fibroblasts in healthy and diseased conditions. Notably, some findings put Ang2 in a novel biological context as a potential regulator in the surveillance of quiescent vasculature, rapidly modifying the vascular response upon angiogenic activation (Fiedler *et al.* 2004). Recently, Hu *et al.* (2014) demonstrated the crucial role of liver sinusoidal endothelial cells in the liver regeneration through autocrine-acting Ang2, pointing at the endothelium as a gatekeeper and regulator of tissue homeostasis and regeneration.

In conclusion, we propose that the T₃–Ang1/2 function may act in concert with the effects on the cardiovascular system in order to protect the heart and the peripheral tissues in adverse conditions.

Study limitation

The mechanisms of action and functional significance of this new T₃–Ang2 function remain to be investigated deeper.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Balzan S was involved in the conception and design of the study. Kusmic C was involved in the development of methodology, Balzan S in rat model, Sabatino L in the angiogenic assay and Western blot, Nicolini G in real-time PCR and Amato R and Casini G in immunohistochemistry. Balzan S was involved in the analysis and interpretation of data and writing of the manuscript. Balzan S, Sabatino L and Kusmic C were involved in the revision of the manuscript. Iervasi G was involved in the study supervision.

References

- Alroy J, Goyal V & Skutelsky E 1987 Lectin histochemistry of mammalian endothelium. *Histochemistry* **86** 603–607. (doi:10.1007/BF00489554)
- Augustin HG, Koh GY, Thurston G & Alitalo K 2009 Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nature Reviews. Molecular Cell Biology* **10** 165–177. (doi:10.1038/nrm2639)
- Balzan S, Del Carratore R, Nardulli C, Sabatino L, Lubrano V & Iervasi G 2013 The stimulative effect of T3 and T4 on human myocardial endothelial cell proliferation, migration and angiogenesis. *Journal of Clinical & Experimental Cardiology* **4** 1–7. (doi:10.4172/2155-9880.1000280)
- Balzan S, Del Carratore R, Nicolini G, Befly P, Lubrano V, Forini F & Iervasi G 2011 Proangiogenic effect of TSH in human microvascular endothelial cells through its membrane receptor. *Journal of Clinical Endocrinology and Metabolism* **97** 1763–1770. (doi:10.1210/jc.2011-2146)
- Baoxia C 2012 Angiopoietin-1, Angiopoietin-2 and myocardial ischemia-reperfusion injury. *Journal of Hypertension* **1** e107. (doi:10.4172/2167-1095.1000e107)
- Chen J, Ortmeier SB, Savinova OV, Nareddy VB, Beyer AJ, Wang D & Gerdes AM 2012 Thyroid hormone induces sprouting angiogenesis in adult heart of hypothyroid mice through the PDGF-Akt pathway. *Journal of Cellular and Molecular Medicine* **16** 2726–2735. (doi:10.1111/j.1582-4934.2012.01593.x)
- Dallabrida SM, Ismail N, Oberle JR, Himes BE & Rupnick MA 2005 Angiopoietin-1 promotes cardiac and skeletal myocyte survival through integrins. *Circulation Research* **96** e8–e24. (doi:10.1161/01.res.0000158285.57191.60)
- Daly C, Eichten A & Castanaro C 2013 Angiopoietin-2 functions as a Tie2 agonist in tumor models, where it limits the effects of VEGF inhibition. *Cancer Research* **73** 108–118. (doi:10.1158/0008-5472.CAN-12-2064)

- Davis FB, Mousa SA, O'Connor L, Mohamed S, Lin HY, Cao HJ & Davis PJ 2004 Proangiogenic action of thyroid hormone is fibroblast growth factor-dependent and is initiated at the cell surface. *Circulation Research* **94** 1500–1506. (doi:10.1161/01.RES.0000130784.90237.4a)
- Davis PJ, Davis FB & Mousa SA 2009 Thyroid hormone-induced angiogenesis. *Current Cardiology Reviews* **5** 12–16. (doi:10.2174/157340309787048158)
- Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, *et al.* 1996 Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **871** 1161–1169. (doi:10.1016/s0092-8674(00)81812-7)
- De Castro AL, Tavares AV, Fernandes RO, Campos C, Conzatti A, Siqueira R, Fernandes TR, Schenkel PC, Sartório CL, Llesuy S, *et al.* 2015 T3 and T4 decrease ROS levels and increase endothelial nitric oxide synthase expression in the myocardium of infarcted rats. *Molecular and Cellular Biochemistry* **408** 235–243. (doi:10.1007/s11010-015-2501-4)
- Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, Bartol A, Kienast Y, Vettel C, Loos EK, *et al.* 2012 Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *Journal of Clinical Investigation* **122** 1991–2005. (doi:10.1172/JCI58832)
- Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G & Augustin HG 2004 The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood* **103** 4150–4156. (doi:10.1182/blood-2003-10-3685)
- Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzernath M, Rosseau S, Suttrop N, *et al.* 2006 Angiopoietin-2 sensitizes endothelial cells to TNF- α and has a crucial role in the induction of inflammation. *Nature Medicine* **12** 235–239. (doi:10.1038/nm1351)
- Forini E, Kusmic C, Nicolini G, Mariani L, Zucchi R, Matteucci M, Iervasi G & Pitto L 2014 Triiodothyronine prevents cardiac ischemia/reperfusion mitochondrial impairment and cell loss by regulating mir30a/p53 axis. *Endocrinology* **155** 4581–4590. (doi:10.1210/en.2014-1106)
- Fu P, Wang C & Xiu R 2006 Serum C-reactive protein and soluble angiopoietin receptor Tie-2 in patients with acute myocardial infarction and its detection by optical protein chip. *Clinical Hemorheology and Microcirculation* **34** 169–175.
- Gelati M, Aplin AC, Fogel E, Smith KD & Nicosia R.F 2008 The angiogenic response of the aorta to injury and inflammatory cytokines requires macrophages. *Journal of Immunology* **181** 5711–5719. (doi:10.4049/jimmunol.181.8.5711)
- Gerdes AM 2015 Restoration of thyroid hormone balance: a game changer in the treatment of heart failure? *American Journal of Physiology Heart and Circulatory Physiology* **308** H1–H10. (doi:10.1152/ajpheart.00704.2014)
- Hsieh PC, Davis ME, Lisowski LK & Lee RT 2006 Endothelial-cardiomyocyte interactions in cardiac development and repair. *Annual Review of Physiology* **68** 51–66. (doi:10.1146/annurev.physiol.68.040104.124629)
- Hu J, Srivastava K, Wieland M, Runge A, Mogler C, Besemfelder E, Terhardt D, Vogel MJ, Cao L, Korn C, *et al.* 2014 HG Endothelial cell-derived angiopoietin-2 controls liver regeneration as a spatiotemporal rheostat. *Science* **343** 416–419. (doi:10.1126/science.1244880)
- Iervasi G & Nicolini G 2013 Thyroid hormone and cardiovascular system: from basic concepts to clinical application. *Internal and Emergency Medicine* **8** S71–74. (doi:10.1007/s11739-013-0911-4)
- Karch R, Neumann F, Ullrich R, Neumüller J, Podesser BK, Neumann M & Schreiner W 2005 The spatial pattern of coronary capillaries in patients with dilated, ischemic, or inflammatory cardiomyopathy. *Cardiovascular Pathology* **14** 135–144. (doi:10.1016/j.carpath.2005.03.003)
- Kessler K, Borges LF, Ho-Tin-Noé, Jondeau G, Michel JB & Vranckx R 2014 Angiogenesis and remodelling in human thoracic aortic aneurysms. *Cardiovascular Research* **104** 147–159. (doi:10.1093/cvr/cvu196)
- Lee SW, Won JY, Lee HY, Lee HJ, Youn SW, Lee JY, Cho CH, Cho HJ, Oh S, Chae IH, *et al.* 2011 Angiopoietin-1 protects heart against ischemia/reperfusion injury through VE-cadherin dephosphorylation and myocardial integrin- β 1/ERK/caspase-9 phosphorylation cascade. *Molecular Medicine* **17** 1095–1106. (doi:10.2119/molmed.2011.00106)
- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, *et al.* 1997 Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277** 55–60. (doi:10.1126/science.277.5322.55)
- Mandriota SJ & Pepper MS 1998 Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circulation Research* **83** 852–859. (doi:10.1161/01.RES.83.8.852)
- Matsunaga T, Warltier DC, Tessmer J, Weihrauch D, Simons M & Chilian WM 2003 Expression of VEGF and angiopoietins-1 and-2 during ischemia-induced coronary angiogenesis. *American Journal of Physiology: Heart and Circulatory Physiology* **285** H352–H358. (doi:10.1152/ajpheart.00621.2002)
- Moeller LC & Broecker-Preuss M 2011 Transcriptional regulation by nonclassical action of thyroid hormone. *Thyroid Research* **4** S6. (doi:10.1186/1756-6614-4-s1-s6)
- Mousa SA, O'Connor L, Davis FB & Davis PJ 2006 Proangiogenesis action of the thyroid hormone analog 3,5-diiodothyropropionic acid (DITPA) is initiated at the cell surface and is integrin mediated. *Endocrinology* **147** 1602–1607. (doi:10.1210/en.2005-1390)
- Mousa SA, Bergh JJ, Dier E, Rebbaa A, O'Connor LJ, Yalcin M, Aljada A, Dyskin E, Davis FB, Lin HY, *et al.* 2008 Tetraiodothyroacetic acid, a small molecule integrin ligand, blocks angiogenesis induced by vascular endothelial growth factor and basic fibroblast growth factor. *Angiogenesis* **11** 183–190. (doi:10.1007/s10456-007-9088-7)
- Nicolini G, Forini E, Kusmic C, Pitto L, Mariani L & Iervasi G 2015 Early and short-term triiodothyronine supplementation prevents adverse post-ischemic cardiac remodeling: role of transforming growth factor- β 1 and anti-fibrotic mirNA signaling. *Molecular Medicine* **21** 900–911. (doi:10.2119/molmed.2015.00140)
- Park SW, Yun JH, Kim JH, Kim KW, Cho CH & Kim JH 2014 Angiopoietin 2 induces pericyte apoptosis via α 3 β 1 integrin signaling in diabetic retinopathy. *Diabetes* **63** 3057–3068. (doi:10.2337/db13-1942)
- Ren G, Michael LH, Entman ML & Frangogiannis NG 2002 Morphological characteristics of the microvasculature in healing myocardial infarcts. *Journal of Histochemistry and Cytochemistry* **50** 71–79. (doi:10.1177/002215540205000108)
- Rodríguez-Gómez I, Banegas I, Wangenstein R, Quesada A, Jiménez R, Gómez-Morales M, O'Valle F, Duarte J & Vargas F 2013 Influence of thyroid state on cardiac and renal capillary density and glomerular morphology in rats. *Journal of Endocrinology* **216** 43–51. (doi:10.1530/JOE-12-0208)
- Savinova OV, Liu Y, Aasen GA, Mao K, Weltman NY, Nedich BL, Liang Q & Gerdes AM 2011 Thyroid hormone promotes remodeling of coronary resistance vessels. *PLoS ONE* **6** e25054. (doi:10.1371/journal.pone.0025054)
- Shyu KG, Chang CC, Wang BW, Kuan P & Chang H 2003 Increased expression of angiopoietin-2 and Tie2 receptor in a rat model of myocardial ischaemia/reperfusion. *Clinical Science* **105** 287–294. (doi:10.1042/CS20030025)
- Swynghedauw B 1999 Molecular mechanisms of myocardial remodeling. *Physiological Reviews* **79** 215–262.

- Thomas M & Augustin HG 2009 The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis* **12** 125–137. (doi:10.1007/s10456-009-9147-3)
- Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM & Yancopoulos GD 2000 Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nature Medicine* **6** 460–463. (doi:10.1038/74725)

- Tomanek RJ & Schattelman GC 2000 Angiogenesis: new insights and therapeutic potential. *Anatomical Record* **261** 126–135. (doi:10.1002/1097-0185(20000615)261:3<126::aid-ar7>3.0.co;2-4)
- Virag JI & Murry CE 2003 Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. *American Journal of Pathology* **163** 2433–2440. (doi:10.1016/S0002-9440(10)63598-5)

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