The Role of Adrenergic Intervention on Thoracic and Abdominal Perivascular Adipose Tissue Expansion in Rats with and without Heart Failure

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Abstract

Perivascular adipose tissue (PVAT) is a type of fatty tissue that surrounds and interacts with blood vessels, consisting of both brown and white adipose tissue depots (BAT, WAT). BAT is responsible for thermoregulation and energy expenditure while WAT stores energy in the form of triglycerides. Excessive accumulation of WAT has been correlated with obesity – a comorbidity for cardiovascular disease (CVD). Also, WAT adipocytes release inflammatory adipokines which cause inter-arterial inflammation in the form of atherosclerotic depots leading to CVD. The goal of the study was to assess the expansion of WAT and BAT in aortal PVAT isolated from rats with and without heart failure in the presence and absence of β-adrenergic stimulation (isoproterenol). Heart failure was induced via transverse aortic constriction (TAC). PVAT was harvested from SHAM and TAC rats and placed in Matrigel with growth media with and without isoproterenol. After 7 days, WAT from TAC rats grew more compared to SHAM (783±129 vs 519±202 mm$^3$; n=4). Conversely, BAT from SHAM rats expanded more compared to TAC (755±187 vs 523±61 mm$^3$; n=4). In the presence of isoproterenol, angiogenesis decreases in BAT and WAT from both SHAM (from 755±187 to 5±5 and from 519±202 to 370±224 mm$^3$, n=4,2) and TAC (from 523±61 to 29 and from 783±129 to 86 mm$^3$, n=4,1) rats, demonstrating β-adrenergic stimulation blunted the expansion of WAT from TAC rats (89% decrease) more than SHAM rats (29% decrease). These results may form potential therapies for obesity in individuals with heart failure. Further investigation may include additional interventions in order to provide a greater biological understanding of PVAT expansion.

Introduction

Obesity, a disease characterized by a significant gain in unhealthy fat mass, currently affects over 475 million individuals and kills over 2 million worldwide annually (Withers et al.,
Data indicates the prevalence of adiposity has steadily increased in the past few decades, currently impacting over 40% of American adults and 20% of youth in the United States (Ogden, Carroll, Fryar, & Flegal, 2015). The cost of obesity, and obesity related issues, is estimated to be between 147 and 210 billion dollars annually for the U.S. - 20% of the total medical spending in the country (Moore, Amey, & Mpofu, 2018). Since these numbers have been rising, obesity has been deemed a public health emergency with severe financial and economic consequences (Pellegrinelli, Carobbio, & Vidal-Puig, 2016). At its core, obesity is an accumulation of fat mass in the form of adipose tissue (AT), but this accumulation is only the beginning of several medical complications and diseases.

AT growth may occur in two different ways: hyperplasia and hypertrophy. Hyperplasia is the increase in adipocyte number allowing for healthy growth since each cell originates from a progenitor cell (R. Berry, Jeffery, & Rodeheffer, 2014; Fuster, Ouchi, Gokce, & Walsh, 2016). Hypertrophy, on the other hand, is the increase of adipocyte size which leads to large lipid filled, dysfunctional cells (R. Berry et al., 2014; Fuster et al., 2016). A result of hypertrophy is the increased secretion of pro-inflammatory signals, termed adipokines, which impact surrounding adipocytes as well as proximal tissues. The main adipokines linking obesity and CVD are the same inflammatory markers secreted from AT (Padilla, Jenkins, Vieira-Potter, & Laughlin, 2013; Tanaka & Sata, 2018). Specifically, TNF-a, MCP-1, IL-6, and IL-1 are implicated in CVD and have been found in inflamed adipocytes (Padilla et al., 2013). The release of these inflammatory molecules leads to the continuous growth of adipocytes in the region as well as forcing the microenvironment to experience chronic inflammation contributing to CVD (Fuster et al., 2016; Pellegrinelli et al., 2016). This inflammation-induced inflammation cycle is also associated with insulin resistance in the vasculature, skeletal muscles, and liver (Bays, 2011; R.
Berry et al., 2014; Fuster et al., 2016). As a result of these metabolic perturbations, obesity is a co-morbidity for heart disease, type 2 diabetes, atherosclerosis, stroke, and death (Cao, 2010), directly linking CVD, the leading cause of death in America, to AT.

At the cellular and molecular level, AT is comprised of adipocytes, which are droplets of lipids in the form of triglycerides (Arner et al., 2011). While originally thought to only provide structural support and energy storage, the role of AT has expanded to include a wide breadth of functions. AT not only insulates the body, but also has thermoregulatory mechanisms. Even though AT’s main function is a large depot for energy storage, it is able to dispense the energy through lipolysis – a crucial process of lipid breakdown which is impaired in those affected by obesity (Arner et al., 2011). Besides the structural and metabolic functions, AT exhibits paracrine capabilities as well. Specifically, adipocytes secrete a range of adipokines (Catalan, Gomez-Ambrosi, Rodriguez, & Fruhbeck, 2013; Valencak, Osterrieder, & Schulz, 2017), indicating adipocytes interact with their microenvironment and elicit metabolic changes based on the signaling molecules released. This is a key function since AT directly impacts the tissue it borders (Horimatsu, Kim, & Weintraub, 2017; Tanaka & Sata, 2018).

AT is not homogeneous throughout the body and multiple types of AT have been phenotypically sorted and genetically confirmed. Two important types of AT found in humans are brown adipose tissue (BAT) and white adipose tissue (WAT). Depots of both can be found in visceral and subcutaneous locations but they have distinct phenotypic and functional properties (Arner et al., 2011; D. C. Berry, Stenesen, Zeve, & Graff, 2013). As the names suggest, there is a clear color difference between the two since BAT is seen as

![Figure 1: UCP-1. It can act as both a “release valve” and a “leak”](image)
brown and WAT is seen as white. The two different types of AT also have varied stem cell origins (D. C. Berry et al., 2013). BAT’s main function is to generate heat in order to aid in thermoregulation (D. C. Berry et al., 2013). These cells are able to exploit the higher mitochondria content within BAT for this purpose as the protein arrangement in the organelle allows for energy dissipation as a result of uncoupling protein 1 (UCP-1) (D. C. Berry et al., 2017; D. C. Berry et al., 2013; Lowell & Flier, 1997). This system has positive and negative effects, which are largely context dependent, but can be viewed as either a “release valve” or a “leak” within the mitochondria (Fig. 1). The “release valve” is beneficial as it can alleviate the buildup of hydrogen ions within the inner mitochondria membrane, bypassing the ATP synthase complex, and utilizing the electrochemical gradient to produce heat. On the other hand, UCP-1 can act as a leak because it dissipates the mitochondrial membrane potential lowers the production of usable energy in the form of ATP, rendering the mitochondria less efficient in terms of ATP production. WAT is responsible for energy storage in the form of triglycerides and lipids as well as coordinating systemic metabolism (D. C. Berry et al., 2013). The latter function is illustrated by metabolic dysfunctions such as hyperglycemia, hypertension, and diabetes in the presence of an abnormal number of white adipocytes, specifically in those suffering from obesity (D. C. Berry et al., 2013).

Past studies have found another difference between BAT and WAT: the mechanism of remodeling (Hattori, Yamamoto, & Matsuda, 2007; Tonello et al., 1999). It is generally agreed upon that BAT is healthier than WAT though both are required in a limited amount to maintain a healthy homeostasis. The expansion of WAT leads to lypolytic imbalance causing hormone dysfunction leading to a host of other problems including high blood pressure, triglycerides, and a higher risk of developing type 2 diabetes and CVD (Bloor & Symonds, 2014). With a capacity
to impact systemic metabolism and communicate with its microenvironment, WAT, and particularly its expansion, presents a unique opportunity for therapeutic interventions.

As dangerous as excess AT may be, its impact on the CV system depends on its location. Perivascular adipose tissue (PVAT) is identified by its crucial location, surrounding blood vessels. The largest depots of PVAT are located tangent to the aorta without any significant anatomical barrier between the two (Horimatsu et al., 2017). BAT surrounds the lining of the thoracic aorta above the diaphragm, while WAT is found in the abdominal cavity around the distal aorta (Fig. 2). Activated BAT has been shown to reduce plasma triglyceride levels, accelerate uptake of cholesterol-rich remnants, and eliminate atherosclerotic lesions in some rats (Hoeke, Kooijman, Boon, Rensen, & Berbee, 2016). Inflamed WAT becomes a local source of inflammatory cytokines such as TNF-a, MCP-1, IL-6, and IL-18 (Padilla et al., 2013). These adipokines are able to quickly diffuse into the adjacent arterial wall causing inflammation inside the artery in the form of atherosclerotic deposits (Tanaka & Sata, 2018). These atherosclerotic deposits are known to be threatening and indicators of CVD. It has also been noted that an increase in WAT causes circulating lipid profiles to become pro-atherogenic (van Dam, Boon, Berbee, Rensen, & van Harmelen, 2017). Thus, the non-uniform susceptibility to atherosclerosis within the arterial tree is partially attributable to regional phenotypic differences in PVAT. Evidence indicates PVAT can alter vascular function, however the impact of PVAT expansion on its associated regional locations remains elusive, despite the known deleterious effects of excessive AT. Accordingly, PVAT and its capacity for expansion may be a critical intermediate between adiposity and overt CVD.
As mentioned earlier, all AT is healthy in moderation - the issues lie in its expansion. Adipogenesis is the generation and expansion of new and necessary adipocytes (Hausman & Richardson, 2004). All adipocytes interact with the CV system, as AT is the most vascularized tissue (Padilla et al., 2013). Due to this connection, the process of adipogenesis is tightly coupled with angiogenesis. Broadly, angiogenesis is the formation of new capillaries which can occur in two forms: 1) the creation of new capillaries from existing capillaries or 2) the ‘sprouting’ of capillaries from an existing capillary and extending into the surrounding extracellular matrix (Diaz-Flores et al., 2017). The CV system is crucial in this process since it is able to provide the necessary nutrients and growth factors to foster the formation of new blood vessels (Cao, 2010). Though the connection between adipocytes and the CV system is critical, AT is able to take advantage of the relationship in a few ways. Adipocytes can secrete plasminogen activator inhibitor-1 and leptin, key proteins in fatty tissue, which ensure the coordination of adipogenesis and angiogenesis in a synergistic manner (Cao, 2010; Hausman & Richardson, 2004). AT can also induce the release of vascular endothelial growth factor (VEGF) in two different ways. First, the reconstruction of lipids causes the degradation of basal membrane proteins, a type of extracellular proteolysis that is linked to the release of VEGF, causing angiogenesis (Hausman & Richardson, 2004). Second, areas of AT undergoing hyperplasia and hypertrophy experience brief hypoxia, generating a large chemotactic stimuli from these new adipocytes (Gealekman et al., 2014). Hypoxia-induced growth factors, such as VEGF, are released, angiogenesis is initiated, and nutrient delivery to the new AT is established (Gealekman et al., 2014). AT’s ability to expand using the vasculature is a critical part of current research into unhealthy hypertrophy but angiogenic factors delivered by the CV system are not the only route for PVAT expansion.
Previous literature indicates AT retain a large amount of adrenergic innervation, which may be partially responsible for mediating growth of adipocytes (Tonello et al., 1999). α- and β-adrenergic receptors are a class of membrane proteins, ligands for norepinephrine and epinephrine, and an integral part of the sympathetic nervous system. The three β-adrenergic receptors are all activated during a sympathetic response, but the functions elicited are tissue specific. For instance, stimulation of β-1 in the heart is responsible for increasing heart rate, β-2 stimulation in the vasculature is involved in vasodilation, and stimulation of the β-3 receptor has been shown to induce lipolysis (Granneman, Li, Zhu, & Lu, 2005). In particular, the β-3 adrenergic receptor has received the most attention in light of its putative interactions regarding the angiogenic capacity of AT. Notably, stimulating AT with β-3 agonists has been shown to stunt its expansion. In general, treatment with β-3 agonists in rodents has been shown to increase AT-specific energy expenditure and reduce overall obesity (Lowell & Flier, 1997). At the molecular level, stimulation with β-3 agonists stimulates mitochondrial biogenesis by augmenting cAMP production, thus promoting mitotic divisions and eliciting higher respiration rates (Fig. 3) (Granneman et al., 2005; Lafontan & Berlan, 1993; Lowell & Flier, 1997). The overall result is fatty acid combustion and lipolysis and a decrease in AT at the site of stimulation (Fig. 3), however there have been no studies of the effect of β-3 stimulation or inhibition on PVAT (van Dam et al., 2017).

The regional differences of PVAT encourages inquiry into how β-adrenergic agonists and antagonists impact growth. Norepinephrine, a non-

**Figure 3: Diagram of the Lipolysis Pathway due to Beta Agonists**
specific adrenergic receptor agonist, induces thermogenesis in BAT resulting in a high demand for oxygen. More than half of the stored energy in BAT may be used during this process in order to sustain homeostasis (D. C. Berry et al., 2013). This adrenergic-induced process stimulates the release of VEGF to rebuild the lost AT (Fredriksson, Lindquist, Bronnikov, & Nedergaard, 2000). Moreover, a correlation has been shown between the activation of adrenergic receptors and the increase expression of VEGF in BAT (Tonello et al., 1999). Importantly, WAT has a different response to adrenergic receptor agonists. White tissue is seen to remodel in a specific type of process termed browning (Granneman et al., 2005; Harms & Seale, 2013). Browning is the process that transforms WAT into brown-like tissue as seen by the presence of thermogenic genes (Harms & Seale, 2013). This process can occur during cold temperatures which requires a more significant amount of energy for thermoregulation (D. C. Berry et al., 2017). This process changes the AT to adapt to the microenvironment but does not consist of induced lipolysis as is seen in typical BAT. Besides these differences there may be other effects of β-adrenergic agonists and/or antagonists on PVAT.

As the mortality rate increases due to CVD and obesity, it is clear that there is a dire need to better understand the role of AT as it impacts both perivascular and AT. A better understanding of the role of PVAT as well as the impact of β-agonists and antagonists on AT expansion has the potential to translate into viable therapeutic interventions designed to ameliorate and prevent CVD.

**Specific Aims and Hypotheses**

Overall, the goal of this study is to gain a better understanding of PVAT expansion and the regional differences in AT depots along the aorta. In order to capture the putative differences in AT heterogeneity, BAT from the thoracic aorta and WAT lining the abdominal aorta will be
examined (Fig. 2). A rat model to investigate the effect of CVD on PVAT phenotype and growth by incorporating healthy and heart failure rats will be utilized. The impact of β-adrenergic agonists on PVAT expansion will also be tested.

**SPECIFIC AIM #1:** To determine the impact of perivascular adipose tissue regional and phenotypic heterogeneity on PVAT sprouting and growth in rats with and without heart failure.

- **Hypothesis 1.1:** Thoracic PVAT (BAT) will have significantly more expansion compared to abdominal PVAT (WAT) in SHAM rats. VEGF receptor concentrations show BAT relies on VEGF while WAT does not.

- **Hypothesis 1.2:** Thoracic PVAT (BAT) from SHAM rats will have significantly more expansion compared to its respective depot in TAC rats. VEGF receptors are not active in unhealthy rats and will not influence BAT growth to the same degree.

- **Hypothesis 1.3:** Abdominal PVAT (WAT) from SHAM rats will have significantly less expansion compared to its respective depot in TAC rats. WAT in TAC rats will produce more inflammatory adipokines which will cause the PVAT to expand to a greater degree.

**SPECIFIC AIM #2:** To ascertain the role of β-adrenergic agonists and antagonists on PVAT phenotype and expansion in rats with and without heart failure.

- **Hypothesis 2.1:** β-agonists, specifically β-3, in thoracic and abdominal PVAT will decrease PVAT growth. β-adrenergic stimulation will activate lipolysis and reduce PVAT expansion.

- **Hypothesis 2.2:** β-antagonists will cause the β-adrenergic receptors to lose their function resulting in an increase growth of both thoracic and abdominal PVAT. β-inhibitors will make it impossible to activate growth inhibition resulting in uncontrollable expansion.

**Materials and Methods**
**Animal Handling & Transaortic Constriction Surgery:**

For the proposed studies, rats will undergo SHAM and TAC surgery. Rats undergo the surgery at 4 weeks under deep anesthetization. Briefly, a longitudinal cut was made in the sternum and a suture spaced with a 20 gauge needle was tied around their transvers aorta. 22 weeks after SHAM or TAC surgery, the animals were sacrificed, and PVAT was excised for growth/expansion assay. All protocols for animal handling are approved by the Institutional Animal Care and Use Committee (IACUC).

Transverse aortic constriction (TAC) surgery is a common procedure performed to create a CVD model in rats. The operation implants a small ring around the aorta. This constriction ultimately increases blood pressure and leads to cardiac hypertrophy, a type of CVD (deAlmeida, van Oort, & Wehrens, 2010). Provided obesity and its related comorbidities typically manifest simultaneously, this model will provide experimental control to tease apart the impact of normal aging-related obesity and CVD. Thus, we will utilize a surgical intervention, TAC-induced heart failure, to determine its role in PVAT expansion. In order to reduce the number of different variables, we will incorporate SHAM rats that undergo a placebo surgery, as the experimental control.

**PVAT Growth/Expansion Assay:**

Aortas and accompanying PVAT was extracted. Thoracic and abdominal aorta/PVAT was separated, placed in microvascular endothelial growth media (EGM-Mv) until PVAT is further dissected and separated from the aorta. Aortas are stored at -20°C for future assays. Next, PVAT is cut into small slices (< 1 mm³) and embedded into a 96-well plate with Matrigel. After PVAT embedding, the 96-well plate incubates at 37°C with 5% CO₂ for 30 min. Unused PVAT is stored at -20°C for future assays. After incubation, 200 µL of EGM-Mv is added to each well,
and the plate is returned to 37°C with 5% CO₂ for 7 days. Images of PVAT explants were taken on a Zeiss microscope 1, 3, 5, and 7 days after embedding. Images were analyzed by outlining PVAT area on each day (Fiji, ImageJ). Growth area was calculated by subtracting the size of the explant from the area on days 1, 3, 5, and 7. On days 2, 4, and 6 half of the EGM-Mv media is replaced with fresh EGM-Mv. The removed media is stored -20°C for future assays.

**Drug Interventions:**

β-adrenergic agonists isoproterenol was utilized. This drug, with concentrations of 1x10⁻⁸M, was added to EGM-Mv during extraction, preparation, and media changes.

**Statistical Analysis & Power Calculations:**

There are no existing data on which to base power calculations to determine the effect of heart failure, or adrenergic innervation on the regional heterogeneity of PVAT expansion as proposed. Thus, *a priori* power calculations indicate a sample size of 8 is sufficient to detect the effect of all variables on outcome measures estimated with an effect size $f = 0.35$, $1 - \beta = 0.80$, and $\alpha = 0.05$ (analysis via G*Power). Data was analyzed using a $2 \times 2$ (TAC/drug/VEGF × PVAT depot) repeated measures-ANOVA. Fishers LSD test are used for post-hoc comparisons, with $P < 0.05$ as the criterion for statistical significance. All statistical analyses was performed in SPSS.

**Results**

We began our experiments by examining BAT and WAT expansion in SHAM (n=4) and TAC rats (n=4). The results indicate that SHAM BAT grew more than the SHAM WAT. These results are consistent with our hypothesis 1.1 which relied on the impact of VEGF. The results in TAC rats showed that WAT had more growth than BAT (Fig. 4). This was expected provided CVD is linked to the presence of abnormally large WAT deposits (D. C. Berry et al., 2013; Bloor
& Symonds, 2014; Brinkley et al., 2014; Britton et al., 2012; Padilla et al., 2013). A comparison was also made between PVAT in healthy control and TAC rats. The thoracic BAT grew significantly more in the control rats (Fig. 4A) while the abdominal WAT expanded more in the TAC rats (Fig. 4B). We hypothesize that these results are due to the VEGF presence since BAT in obese rats is not able to use VEGF properly (Tonello et al., 1999).

Isoproterenol (ISO), a non-selective β-agonist, was used to induce activity in all β-receptors within the PVAT (Granneman et al., 2005; Lafontan & Berlan, 1993). In healthy rats (n=2), BAT and WAT were impacted differently by the presence of ISO. BAT growth was stunted, while abdominal PVAT was not impacted to the same degree but was still seen to decrease (Fig. 5 A&B). The difference in growth may partially be explained by the distinct effects of β-agonists on the two AT depot: BAT uses UCP-1 to produce energy in the form of heat to compensate for the activation of the β-receptors while WAT does not have UCP-1 and is not as impacted by β-activation (D. C. Berry et al., 2017; D. C. Berry et al., 2013; Granneman et al., 2005; Lafontan & Berlan, 1993; Lowell & Flier, 1997; van Dam et al., 2017). In TAC rats (n=1), BAT and WAT were impacted in the same

![Figure 4. Expansion of Thoracic PVAT and Abdominal PVAT in SHAM and TAC rats. A, Thoracic PVAT growth in SHAM (n=4) and TAC rats (n=4) over seven days. B, Abdominal PVAT growth in SHAM (n=4) and TAC (n=4) rats over seven days.](image)

![Figure 5. Impact of Isoproterenol on Thoracic PVAT and Abdominal PVAT Expansion in SHAM and TAC rats. A, Thoracic PVAT growth in SHAM (n=4), TAC rats (n=4), SHAM with isoproterenol (ISO) (n=2), and TAC rats with ISO (n=1) over seven days. B, Abdominal PVAT growth in SHAM (n=4), TAC rats (n=4), SHAM with ISO (n=2), and TAC rats with ISO (n=1) over seven days.](image)
way by the presence of ISO. Both types of AT had less growth when incubated with ISO (Fig. 5 A&B). These results may be attributed to the CVD and adiposity inherent to TAC rats, potentially causing biological processes to degrade. However, more research is needed to determine the impact of CVD on the function of PVAT β-receptors.

**Discussion**

Our investigations have provided a greater understanding of PVAT as it applies to both CVD and obesity. Completion of these experiments address a critical gap in our biological understanding of β-adrenergic receptors and their role in PVAT expansion, a critical area for therapeutic interventions.

Based on the results from these growth assays, there are a few different paths of inquiry that can be taken. Firstly, it is important to test specific β-agonists and antagonists since Isoproterenol is a non-specific β-agonist. CL-316,243, Fenoterol, and Betaxolol may be used to determine the impact of specific β 1/2/3-agonist on PVAT expansion. Results from these different trials will provide a greater understanding of the role of β-receptors on AT growth and the therapy potential of each drug. Secondly, it is crucial to understand the different biological pathways underlying the process. Determining the relative levels of β-receptors and cAMP at different stages in the process by immunohistochemically analyzing tissue and cell media would quantify the process that is taking place after activation of the different PVAT.

Recent research suggests that there are differences in VEGF receptors on the BAT and WAT which may help explain the results. BAT has a greater amount of VEGF receptors indicating BAT uses this type of growth stimulation while WAT does not (Hattori et al., 2007; Tonello et al., 1999). Provided the potential impact of VEGF presence on our results further investigations are warranted.
Once the most efficacious agonist is determined, the next critical step would be a collaboration with a cardiovascular surgeon to create a human clinical trial. Since rats and humans have different amounts of BAT and WAT, it would be important to test the effects of these drugs ex-vivo on human PVAT before proceeding to a clinical trial. This type of experimentation would be identical to the work that was done in vitro. In clinic, it would be possible to determine the effectiveness of the drug based on obesity progression in the patients. The trial would asses weight loss, heart health, and other typical obesity factors before and after administration of the drug. Based on the current results, a therapy may be more successful for patients with obesity and cardiovascular disease but clinical work is critical before conclusions can be made.
References


