Pre-Diabetes Augments Neuropeptide Y$_{1-}$ and $\alpha_{1-}$ Receptor Control of Basal Hindlimb Vascular Tone in Young ZDF Rats

Nicole M. Novielli$^1$, Baraa K. Al-Khazraji$^1$, Philip J. Medeiros$^1$, Daniel Goldman$^{1,2}$, Dwayne N. Jackson$^{1,2*}$

1 Department of Medical Biophysics, The University of Western Ontario, London, Ontario, Canada, 2 Biomedical Engineering Program, The University of Western Ontario, London, Ontario, Canada

Abstract

**Background:** Peripheral vascular disease in pre-diabetes may involve altered sympathetically-mediated vascular control. Thus, we investigated if pre-diabetes modifies baseline sympathetic Y$_{1-}$receptor (Y$_{1R}$) and $\alpha_{1-}$receptor ($\alpha_{1R}$) control of hindlimb blood flow (Q$_{fem}$) and vascular conductance (VC).

**Methods:** Q$_{fem}$ and VC were measured in pre-diabetic ZDF rats (PD) and lean controls (CTRL) under infusion of BIBP3226 (Y$_{1R}$ antagonist), prazosin ($\alpha_{1R}$ antagonist) and BIBP3226+prazosin. Neuropeptide Y (NPY) concentration and Y$_{1R}$ and $\alpha_{1R}$ expression were determined from hindlimb skeletal muscle samples.

**Results:** Baseline Q$_{fem}$ and VC were similar between groups. Independent infusions of BIBP3226 and prazosin led to increases in Q$_{fem}$ and VC in CTRL and PD, where responses were greater in PD ($p<0.05$). The percent change in VC following both drugs was also greater in PD compared to CTRL ($p<0.05$). As well, Q$_{fem}$ and VC responses to combined blockade (BIBP3226+prazosin) were greater in PD compared to CTRL ($p<0.05$). Interestingly, an absence of synergistic effects was observed within groups, as the sum of the VC responses to independent drug infusions was similar to responses following combined blockade. Finally, white and red vastus skeletal muscle NPY concentration, Y$_{1R}$ expression and $\alpha_{1R}$ expression were greater in PD compared to CTRL.

**Conclusions:** For the first time, we report heightened baseline Y$_{1R}$ and $\alpha_{1R}$ sympathetic control of Q$_{fem}$ and VC in pre-diabetic ZDF rats. In support, our data suggest that augmented sympathetic ligand and receptor expression in pre-diabetes may contribute to vascular dysregulation.

Citation: Novielli NM, Al-Khazraji BK, Medeiros PJ, Goldman D, Jackson DN (2012) Pre-Diabetes Augments Neuropeptide Y$_{1-}$ and $\alpha_{1-}$Receptor Control of Basal Hindlimb Vascular Tone in Young ZDF Rats. PLoS ONE 7(10): e46659. doi:10.1371/journal.pone.0046659

Editor: Michael Bader, Max-Delbruck Center for Molecular Medicine (MDC), Germany

Received July 5, 2012; Accepted September 5, 2012; Published October 5, 2012

Copyright: © 2012 Novielli et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding source: National Institutes of Health, R33 HL089094 and Natural Sciences and Engineering Research Council, R4218A03. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dwayne.jackson@schulich.uwo.ca

Introduction

In the peripheral vasculature, sympathetic neurons regulate arteriolar tone through the release of norepinephrine (NE) and neuropeptide Y (NPY). NE has been considered the primary neurotransmitter in maintenance of baseline arteriolar tone [1] through its interaction with alpha-adrenergic receptors ($\alpha_{R}$) located on vascular smooth muscle cells, causing vasoconstriction. NPY is co-stored and co-released with NE and acts on neuropeptide Y$_{1}$ receptors (Y$_{1R}$), to cause potent and prolonged vasoconstriction [2,3,4]. Interestingly, post-synaptic co-activation of Y$_{1R}$ and $\alpha_{1R}$ by NPY and NE leads to synergistic vasoconstrictive effects [4]. Although recent evidence has shown that NPY contributes modestly to baseline vascular tone in skeletal muscle of male rats [5], its effects are suggested to predominate under conditions of elevated sympathetic nerve activity [6,7,8].

A large proportion of the body’s resistance vasculature lies within skeletal muscle, which is highly regulated by sympathetic nerve activity (SNA) to maintain blood pressure and blood flow distribution under healthy conditions. However, in type 2 diabetes, sympathetic regulation of vascular tone can become augmented, leading to alterations in normal blood flow control. Type 2 diabetes is commonly associated with vascular disease, however recent findings indicate that cardiovascular complications may be initiated in the pre-diabetic state, before the diagnosis of type 2 diabetes [9,10]. Pre-diabetes is characterized by the concomitant presence of hyperinsulinemia, impaired glucose tolerance and insulin resistance and occurs prior to overt pancreatic $\beta$-cell failure. Of note, hyperinsulinemia stimulates SNA and may play a role in autonomic and vascular dysfunction associated with the disease [11]. In humans, hyperinsulinemia is associated with elevated SNA and correlates with the degree of insulin resistance [12,13,14]. Moreover, systemic infusion of insulin in rats has been shown to preferentially increase lumbar SNA [15,16,17,18]. Studies using animal models of pre-diabetes and the metabolic syndrome have reported augmented $\alpha$-adrenergic vascular responsiveness to adrenergic agonists in isolated vascular preparations [19,20]. As noted above, NPY-mediated vascular modu-
mediated vascular control in pre-diabetes are lacking. Furthermore, there have been no studies investigating NPY and α-adrenergic co-modulation of vascular control in pre-diabetes.

The overall aim of the present study was to investigate if pre-diabetes modifies sympathetic Y1R and α1R control of basal skeletal muscle blood flow (Qfem) and vascular conductance (VC). Thus, we tested the independent and dependent (synergistic) functional contributions of endogenous Y1R and α1R activation on Qfem and VC in vivo and hypothesized that pre-diabetes augments Y1R and α1R vascular modulation. Concurrently, we hypothesized that skeletal muscle NPY concentration and Y1R expression would be upregulated in pre-diabetic rats.

Materials and Methods

All animal procedures were approved by the Council on Animal Care at The University of Western Ontario (protocol number: 2008-066). All invasive procedures were performed under α-chloralose and urethane anesthetic, and all efforts were made to minimize animal suffering.

Animals

Nine seven-week-old male ZDF rats (PD) and 8 age-matched lean controls (CTRL) (Charles River Laboratories, Saint-Constant, Quebec, Canada) were used in this study. The inbred ZDF rat is affected by a homozygous mutation of the leptin receptor (fa/fa), therefore leptin is unable to suppress appetite [21]. When fed a high fat diet (i.e., Purina 5008 rat chow), these animals become obese, hyperinsulinemic, insulin resistant and hyperglycemic by 7 weeks of age [20,21], characteristic of the pre-diabetic condition in humans [22,23]. This phenotype is absent in the ZDF Lean rats heterozygous for the leptin receptor mutation (fa/+), and thus served as the control group in this study. Animals were housed in animal care facilities in a temperature (24°C) and light (12-hour cycle)-controlled room, fed Purina 5008 rat chow (Ralston Purina, St. Louis, MO, USA) and allowed to eat and drink water ad libitum. Prior to surgery, animals were anesthetized with an intraperitoneal injection of α-chloralose (80 mg/kg) and urethane (500 mg/kg). This anesthetic was ideal for this study as it leaves autonomic, cardiovascular and respiratory function intact [24]. Internal body temperature was monitored via a rectal temperature probe and maintained at 37°C with the use of a thermally controlled water-perfused heating pad.

Surgery

A mid-neck incision was made and a tracheal cannula was introduced to facilitate spontaneous respiration. End-tidal CO2 and O2 measures were made from expired air between pharmacological perturbations throughout the experiment using a breath-by-breath gas analyzer (ADInstruments, Colorado Springs, CO, USA). The left common carotid artery was cannulated (PE-50 tubing) and the cannula was advanced to the bifurcation of the ascending aorta. This cannula was used for localized drug delivery to the left hindlimb. Following cannulation, gauze was removed and care was taken to reposition the gut. Incisions were closed with sterile wound clips (9 mm stainless steel wound clips). A blood sample was then taken from the carotid cannula in order to evaluate blood glucose levels, lactate levels, and pH using an iSTAT portable clinical analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Using microscopic assistance, the left femoral artery was carefully isolated from surrounding nerves and vessels. Qfem was measured beat-by-beat using a Transonic flow probe (0.7 PSB) and flowmeter (model TS420 Perivascular Flowmeter Module; Transonic Systems, Ithica, NY, USA). The flow probe was placed around the left femoral artery ~3 mm from the femoral triangle and innocuous water-soluble ultrasound gel was applied over the opened area of the left hindlimb to keep tissue hydrated and to maintain adequate flow signal.

Experimental protocol

Once surgery was completed, animals recovered for 1 hour. Prior to drug treatments, vehicle (160 µl of 0.9% saline) was delivered, followed by a 15-minute recovery period. Baseline data were recorded for 5 minutes followed by five separate drug infusions [5,25,26,27]. Using a repeated measures design, drug infusions were delivered at a rate of 16 µl/sec in the following order: 1) 250 µl of 0.2 µg/kg acetylcholine chloride (ACh, Sigma-Aldrich, St. Louis, MO, USA), 2) 160 µl of 100 µg/kg BIBP3226, a specific Y1R antagonist (TOCRIS, Ellisville, MO, USA), 3) 160 µl of 20 µg/kg prazosin, a specific α1R antagonist (Sigma-Aldrich, St. Louis, MO, USA), 4) combined 100 µg/kg BIBP3226+20 µg/kg prazosin, and 5) 160 µl of 5 µg/kg sodium nitroprusside (SNP, i.e., sodium nitroprussiate dihydurate, Sigma-Aldrich, St. Louis, MO, USA). Since the hemodynamic effects of prazosin are long lasting, BIBP3226 (Y1R antagonist) was administered first in all experiments. When hemodynamic variables returned to baseline (30–40 minutes), prazosin (α1R antagonist) was infused. Once responses to prazosin peaked and stabilized (~5 minutes), combined blockade (Y1R+α1R antagonist) was achieved by a subsequent infusion of BIBP3226 (100 µg/kg). In a previous study (using a similar protocol), we addressed the effects of randomized versus fixed delivery of BIBP3226 and prazosin and reported no effect of randomization [27].

Insulin immunoassay

Insulin levels were determined from plasma samples using an ELISA and by following manufacturer’s instruction (ALPCO Immunoassays, Salem, NH, USA). All samples and standards (10 µl) were distributed in duplicate in the provided 96-well immunoplate. Seventy-five microliters of horseradish peroxidase (HRP)-labeled monoclonal anti-insulin antibody was added to each well and incubated at room temperature for 2 hours. The immunoplate was then washed 6 times with assay wash buffer. Following washing, 100 µl of tetramethylbenzidine (TMB) peroxidase substrate solution was added to each well and incubated for 15 minutes at room temperature. The reaction was then terminated with 100 µl of stop solution, and the optical absorbance of each well was read at 450 nm (Bio-Rad iMark Microplate Reader, Bio-Rad, Hercules, CA, USA).
TMB peroxidase substrate solution was added to all wells. After a 300 minute incubation at room temperature with 100 μl of streptavidin-HRP (10 ng/ml or 2–3 pg per well (manufacturer’s data). White and red vastus skeletal muscle mass was removed from the hindlimb and flash frozen in liquid nitrogen. Approximately 100 mg of tissue was cut from the whole muscle and homogenized in 2 mL of radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPA, 1% Sodium deoxycholate, 0.1% SDS, 100 mM EDTA) containing protease inhibitor cocktail (104 mM AEBSF, 80 mM aprotinin, 2.1 mM leupeptin, 3.6 mM betatatin, 1.5 mM pepstatin A, 1.4 mM ME-64, Sigma-Aldrich, St. Louis, MO, USA). Samples were then centrifuged at 4°C for 25 minutes at 14000 rpm and supernatant was collected and then stored at −80°C until ready for use. Protein concentration was determined using the Bradford protein assay [32]. Fifty micrograms of protein from each sample was loaded on a 4% to 12% gradient gel and separated by SDS-PAGE. After electrophoresis, proteins were transferred at a constant voltage to polyvinylidene fluoride membranes. Membranes were blocked in 5% milk in tris-buffereed saline+Twee20 (0.5%) (TTBS) at 4°C for 5 hours. Membranes were washed in TTBS and incubated overnight at 4°C in one of two primary antibodies in 5% milk in TTBS, specific to rat, human or mouse: 1) Y1R (rabbit polyclonal to NPY1R, Cat no. ab73897, Abcam, Cambridge, MA, USA), and 2) αR (rabbit polyclonal to alpha 1 adrenergic receptor, Cat no. ab3462, Abcam, Cambridge, MA, USA). After incubation, membranes were washed in TTBS then incubated in secondary antibody conjugated to HRP (goat antirabbit IgG, Cat no. A0545, Sigma Aldrich, St. Louis, MO, USA) in 5% milk in TTBS for 1 hour at room temperature. Membranes were washed and bands were detected using Immun-Star WesternC® Chemiluminescent kit (Bio-Rad, Hercules, CA, USA) and imaged with a ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Membranes were immediately washed, stripped, and blocked in 5% bovine serum albumin for 1 hour at room temperature. Membranes were washed and incubated in primary antibody specific to β-actin (loading control, anti-beta actin, rabbit polyclonal, Cat no. ab16039, Abcam, Cambridge, MA, USA) for 1 hour at room temperature. Membranes were then washed, incubated in secondary antibody and imaged (as above). Densitometric band analysis was performed with Quantity One.

Table 1. Physical and physiological characteristics of CTRL and PD rats.

|                | CTRL            | PD               |
|----------------|-----------------|------------------|
| Weight (g)     | 196±4           | 253±5*           |
| Blood glucose (mmol/L) | 9.3±0.6       | 14.1±0.9*       |
| Insulin (nmol/L) | 0.1±0.03       | 5.6±0.7*        |
| Blood lactate (mmol/L) | 1±0.1         | 2±0.1*          |
| Expired CO2 (mmHg) | 35±0.5         | 39±0.5*         |
| Expired O2 (%)  | 17±0.1          | 17±0.1          |
| Respiratory rate (breaths/min) | 68±2          | 82±2*          |
| Blood pH       | 7.4±0.01        | 7.4±0.01        |

Values are mean ± SE. CTRL, control, n=7–8; PD, pre-diabetic, n=7–9.
*p<0.001 vs. CTRL.

Table 2. Blood pressure and heart rate responses associated with each condition.

|                | BIBP3226 | Prazosin | BIBP3226+Prazosin |
|----------------|----------|----------|------------------|
|                | CTRL     | PD       | CTRL             | PD          |
| Mean arterial pressure (mmHg) | Baseline | 95±2     | 102±6            | 88±4        | 102±5 |
| Drug           | 89±4     | 94±8     | 72±5*            | 76±6*       | 71±3* | 71±6* |
| Heart rate (beats/min) | Baseline | 375±7   | 414±7†          | 371±6      | 409±8† | 371±6 | 409±8† |
| Drug           | 379±6   | 430±11†  | 368±7           | 413±10†    | 368±6 | 406±7† |

Values are mean ± SE. CTRL, control, n=8; PD, pre-diabetic, n=9.
*p<0.05 vs. Baseline.
†p<0.025 vs. Baseline.
doi:10.1371/journal.pone.0046659.t002
Table 3. Baseline values of hindlimb blood flow and vascular conductance before pharmacological treatments.

|                      | BIBP3226 | Prazosin | BIBP3226+Prazosin |
|----------------------|----------|----------|-------------------|
|                      | CTRL     | PD       | CTRL              | PD                      |
| Hindlimb blood flow (μl/min) | 385±69   | 364±42   | 364±61            | 358±34                  |
| Vascular conductance (μl/min/mmHg) | 4.0±0.6 | 3.7±0.5 | 4.1±0.6           | 3.6±0.4                 |

Values are mean ± SE. CTRL, control, n = 8; PD, pre-diabetic, n = 9.

doi:10.1371/journal.pone.0046659.t003

Table 4. Hindlimb blood flow and vascular conductance at baseline and following acetylcholine and sodium nitroprusside interventions.

|                      | Acetylcholine | Sodium Nitroprusside |
|----------------------|---------------|----------------------|
|                      | CTRL          | PD                   | CTRL                | PD                   |
| Hindlimb blood flow (μl/min) | 380±50        | 395±30               | 385±66              | 377±47               |
| Drug                 | 708±66*       | 760±93*              | 503±72*             | 582±53*              |
| Vascular conductance (μl/min/mmHg) | 4.2±0.6   | 3.7±0.5              | 4.2±0.7             | 3.6±0.6              |
| Drug                 | 10±1*         | 10±1*                | 12±2*               | 11±2*                |

Values are mean ± SE. CTRL, control, n = 6–8; PD, pre-diabetic, n = 6–8.

*p<0.05 vs. Baseline.

doi:10.1371/journal.pone.0046659.t004
Figure 1. Representative mean vascular conductance (0.1 second averaging of 1 kHz beat-by-beat tracing) over 50 seconds for BIBP3226, prazosin and BIBP3226+prazosin treatments in CTRL (top) and PD (bottom).
doi:10.1371/journal.pone.0046659.g001

Figure 2. Sympathetic receptor blockade elicits greater vascular responses in PD. Panel A: Change in hindlimb blood flow (Qfem) and Panel B: vascular conductance (VC) from baseline following Y1R and \( \alpha_1 \)R blockade. With Y1R blockade, the increase in \( Q_{fem} \) and VC was greater in PD (n = 9) versus CTRL (n = 8) \((p<0.05)\). \( \alpha_1 \)R blockade elicited an increase in \( Q_{fem} \) as well as an increase in VC that was greater in PD compared to CTRL \((p<0.05)\). * Indicates different from CTRL \((p<0.05)\).
doi:10.1371/journal.pone.0046659.g002
Effect of simultaneous Y1R and α1R blockade (BIBP3226+ prazosin). Following combined Y1R and α1R antagonism, MAP decreased 17±6 and 31±6 mmHg, for CTRL and PD respectively (p<0.05, Table 2), whereas HR remained unchanged. Qcem and VC increased from baseline in CTRL (ΔQcem = 191±39 μl/min; ΔVC = 3.8±0.7 μl/min/mmHg) and PD (ΔQcem = 279±44 μl/min; ΔVC = 3.8±0.6 μl/min/mmHg) (p<0.05), however the increase in Qcem and VC following combined Y1R and α1R blockade was greater in PD compared to CTRL (p<0.05, Figure 2). Percent change in VC was greater in PD (170±20%) versus CTRL (109±24%) (p<0.05, Figure 3).

To determine the potential synergistic interaction between endogenous Y1R and α1R activation, the sum of the VC responses from the BIBP3226 and prazosin conditions was compared to the VC responses elicited by combined Y1R and α1R blockade within each group. Compared to the sum of the independent effects of BIBP3226 and prazosin infusion, combined blockade resulted in a similar increase in VC within groups (Figure 4).

Tissue NPY concentration and Y1R and α1R expression

Tissue NPY concentration. NPY concentration was 155±32% and 68±32% greater in white and red vastus respectively in PD compared to CTRL (p<0.05, Figure 5).

Tissue Y1R and α1R protein expression. Compared to CTRL, Y1R protein expression was 43±15% and 30±9% greater in PD white and red vastus muscle respectively (p<0.05, Figure 6). α1R expression was 94±43% greater in PD compared to CTRL in red vastus muscle (p<0.05), however expression in white vastus muscle was similar between groups (Figure 7).

Discussion

As hypothesized, we observed heightened sympathetic influences on baseline vascular control in pre-diabetes, as blockade of sympathetic receptors elicited greater Qcem and VC responses in PD compared to CTRL. This is the first study to report that pre-diabetes promotes an overall increase in Y1R and α1R vascular control under baseline conditions. Accordingly, increases in skeletal muscle NPY concentration and Y1R expression were observed in PD. However, in contrast to our hypothesis, we did not unmask Y1R and α1R synergistic effects on VC with combined receptor blockade.

In the current study we demonstrated that modifications in sympathetic vascular control occur before the manifestation of endothelial and/or vascular smooth muscle dysfunction generally observed in overt type 2 diabetes. Our current data are supported by past studies (using the same model of pre-diabetes) showing no differences in responses to ACh or SNP in PD versus CTRL [20,33]. These data indicate that endothelium dependent and independent responses to such pharmacological stimuli (ACh and SNP) are intact in PD, supporting the hypothesis that vascular dysregulation in early pre-diabetes is mainly due to modifications in sympathetic control.

Past work from our group suggests that sympathetic vascular control involves interactions between Y1R and α1R [27]. Until
presently, there was a lack of research investigating the role of NPY in pre-diabetic vascular dysfunction. In fact, past investigations addressing augmented sympathetic vascular control in pre-diabetes have relied predominantly on the functional responses to infusion/application of α-adrenergic agonists in vivo, or responses of isolated vascular preparations treated with these agents [20,34]. Although essential for determining the existence of receptors and their independent function(s) within physiological systems, the infusion of agonists does not address autogenous ligand–receptor interactions. In the current investigation highly selective Y1R and α1R antagonists (BIBP3226 and prazosin respectively) were delivered alone and in combination to address endogenous independent and synergistic Y1R/α1R control under baseline conditions. Although responses to Y1R, α1R, and combined blockade were markedly augmented in PD, we did not unmask endogenous Y1R and α1R synergism in either CTRL or PD (Figure 3). This was surprising, as we have previously reported endogenous synergy between Y1R and α1R in adult male Sprague Dawley rats [27]. Thus, it seems that such receptor interactions are not present in the young ZDF rat or they were not robust enough to resolve in the current study.

Despite similar baseline Q_{fe}m and VC among groups, we observed that both Y1R and α1R sympathetic antagonist treatments resulted in greater vascular responses in PD. Under conditions of heightened sympathetic influence, it seems unexpected that similarities in baseline Q_{fe}m and VC would exist. However, our observations are supported by other work where isolated vessels from pre-diabetic rats (with similar baseline tone) demonstrated greater responses to sympathetic agonists compared to controls [20]. Thus, in the current study, it appears that compensatory dilatory mechanisms served to maintain normal blood flow under baseline conditions in PD. The presence of high blood lactate (a potent vasodilator [35]) in PD likely contributed to buffering the effects of augmented sympathetic vascular modulation. In support of our data, others have shown that insulin resistance [36] and type 2 diabetes [37] are associated with heightened lactate levels.

Our observations of augmented baseline Y1R and α1R activation in PD are complemented by our findings that PD had greater NPY concentration and Y1R and α1R expression in hindlimb skeletal muscle. Neuropeptide Y is produced in sympathetic neuronal cell soma and packaged into secretory large dense-cored vesicles and undergoes axonal transport (the rate of which is SNA level dependent) to the axon terminal where it is released and eventually degraded by enzymes in the synaptic cleft [38]. This is in contrast to NE, which is produced in sympathetic nerve terminal, released, and eventually taken back up into the nerve terminal [39]. Based on the unique origin and fate of NPY, it can be reasonably inferred that increased skeletal muscle NPY concentration measured in PD was a result of one or a combination of the following: i) augmented sympathetic neuronal density; ii) increased production and axonal transport of NPY; and/or iii) increased NPY release into skeletal muscle interstitium. This line of reasoning falls in line with work by others who reported sympathetic nerve hyperactivity in insulin resistant and type 2 diabetic subjects, as well as heightened plasma NPY levels in type 2 diabetic patients [40,41]. Beyond this, in vivo studies investigating NPY levels and Y1R/α1R expression in pre-diabetes are limited, however increased Y1R mRNA expression has been reported in cardiac tissue of diabetic rats [42] and it was shown that rat vascular smooth muscle cells treated with high levels of insulin resulted in upregulation of α1R [43].

**Limitations**

We used hindlimb muscle homogenate in order to quantify the receptors located along downstream resistance arterioles, as these vessels are responsible for modulating flow at the level of the femoral artery. Previous work indicates that peripheral Y1Rs are predominantly associated with vasculature [44]. In contrast, α1Rs have been identified on skeletal muscle fibers in rats, however the density of those located in muscle fibers is negligible compared to α1R expression on resistance arterioles [45]. Based on past reports and the internal consistency between our functional and cellular data, we are confident that our reported differences in ligand concentration and receptor expression reasonably reflect what is occurring at the level of the vasculature.

---

**Figure 6. Y1R expression is augmented in PD.** Western blot analysis of Y1R expression (~43 kDa) in hindlimb muscle homogenate of CTRL (n = 6 per muscle group) and PD (n = 6 per muscle group). PD had greater overall expression of Y1R in both white and red vastus muscles. * Indicates different from CTRL (p < 0.05). doi:10.1371/journal.pone.0046659.g006

**Figure 7. α1R expression is augmented in PD.** Western blot analysis of α1R expression (~42 kDa) in hindlimb muscle homogenate of CTRL (n = 6 per muscle group) and PD (n = 6 per muscle group). PD had greater α1R expression in red vastus muscle, compared to CTRL. * Indicates different from CTRL (p < 0.05). doi:10.1371/journal.pone.0046659.g007
We measured skeletal muscle tissue NPY concentration instead of plasma NPY levels for several reasons. Indeed, repeated blood sampling poses the risk of evolving hypotension and increases in sympathetic nerve activity. As well, plasma NPY levels represent a mixed sample originating from several sources throughout the body. In contrast, the skeletal muscle samples used in this study were promptly harvested from anesthetized animals (with minimal hemodynamic stress) under the same conditions that functional data were acquired. Thus, we feel that our reported NPY levels are an accurate representation of the local skeletal muscle environment under baseline conditions.

Due to limitations in detection, NE levels were not measured in the current study. However, this investigation and previous from our group [26,27] used a sensitive enzyme immunoassay optimized to detect NPY in skeletal muscle homogenates. NPY is co-released and co-stored with NE [4] and plasma NPY release correlates with NE release [46], especially under conditions of elevated sympathetic nerve activity; thus, it is reasonable to postulate that our measures of increased skeletal muscle NPY concentration in PD reflect a concomitant increase in skeletal muscle NE.

In conclusion, we provide the first report that Y1R and Y2R vascular regulation is augmented in the hindlimb of pre-diabetic ZDF rats. Our findings are supported by increased skeletal muscle NPY concentration and Y1R/Y2R expression in PD versus CTRL. Future studies are required to ascertain the long-term cardiovascular consequences of our findings and their functional significance in contracting skeletal muscle.

Acknowledgments

We would like to thank Elizabeth Bowles of Dr. Randy Sprague’s laboratory (Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, Saint Louis, MO, USA) for the insulin ELISA, as well as Stephanie Milkovich for technical assistance and Dr. Christopher Ellis for his valuable advice (Department of Medical Biophysics, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada).

Author Contributions

Conceived and designed the experiments: DNJ. Performed the experiments: MNM. Analyzed the data: MNM DNJ. Contributed reagents/materials/analysis tools: BKA PJM. Wrote the paper: MNM DNJ. Revised manuscript critically for important intellectual content: DG BKA PJM. Participated in performing Western blot experiments for the study: BKA PJM.

References

1. Zukowska-Grojec Z (1995) Neuropeptide Y: A novel sympathetic stress hormone and more. Ann N Y Acad Sci 771: 219–233.
2. Malmstrom RE (1997) Neuropeptide Y Y1 receptor mechanisms in sympathetic vascular control. Acta Physiol Scand Suppl 636: 1–55.
3. Ekelund U, Erlling D (1997) In vivo receptor characterization of neuropeptide Y-induced effects in consecutive vascular sections of cat skeletal muscle. Br J Pharmacol 120: 387–392.
4. Zukowska-Grojec Z, Walheest C (1993) Origin and actions of neuropeptide Y in the cardiovascular system. The Biology of Neuropeptide Y and Related Peptide, ed Colmers WF & Walheest. Totowa: Humana Press. pp.315–328.
5. Jackson DN, Noble EG, Shoemaker JK (2004) Y1- and alpha-receptor control of basal hindlimb vascular tone. Am J Physiol Regul Integr Comp Physiol 287: R226–R233.
6. Bartfai T, Iervolf K, Fusone G, Sforzo P (1998) Regulation of the release of coexisting neurotransmitters. Annu Rev Pharmacol Toxicol 28: 205–310.
7. De Camilli P, Jahn R (1998) Pathways to regulated exocytosis in neurons. Annu Rev Physiol 52: 623–645.
8. Lundberg JM, Franco-Cereceda A, Lou YP, Modin A, Pernow J (1994) An accurate representation of the local skeletal muscle environment during sympathetic nerve activity: a potential role for neuropeptide Y. J Physiol 478: 89–105.
9. Faeh D, William J, Yerly P, Paccaud F, Bover P (2007) Diabetes and prediabetes are associated with cardiovascular risk factors and carotid/femoral intima-media thickness independently of markers of insulin resistance and adiposity. Cardiovasc Diabetol 6: 32.
10. Haffner SM, Steen MP, Hazuda HP, Mitchell BD, Patterson JK (1990) Cardiovascular risk factors in confirmed prediabetic individuals. Does the clock have one without the other. Diabetes Care 31: 1433–1438.
11. Kim SH, Reaven GM (2008) Insulin resistance and hyperinsulinemia: you can't have one without the other. Diabetes Care 31: 547–552.
12. Soma LR (1983) Anesthetic and analgesic considerations in the experimental animal. Ann N Y Acad Sci 406: 32–47.
13. Jackson DN, Milne JK, Noble EG, Shoemaker JK (2005) Neuropeptide Y biosubavailability is suppressed in the hindlimb of female Sprague-Dawley rats. J Physiol 567: 573–581.
14. Jackson DN, Ellis CG, Shoemaker JK (2010) Estrogen modulates the contribution of neuropeptide Y to baseline hindlimb blood flow control in female Sprague-Dawley rats. Am J Physiol Regul Integr Comp Physiol.
15. Jackson DN, Milne JK, Noble EG, Shoemaker JK (2005) Gender-modulated endogenous baseline neuropeptide Y Y1-receptor activation in the hindlimb of male Sprague-Dawley rats. J Physiol 562: 283–294.
16. Terjung RJ, Engbertson BM (1980) Blood flow to different rat skeletal muscle fiber type sections during isometric contractions in situ. Med Sci Sports Exerc 20: S124–S130.
17. Armstrong RB, Laughlin MH (1984) Exercise blood flow patterns within and among rat muscles after training. Am J Physiol 246: H59–68.
18. Armstrong RB, Laughlin MH (1985) Rat muscle blood flows as a function of time during prolonged slow treadmill exercise. Am J Physiol 244: H114–H21.
19. Armstrong RB, Phelps RO (1984) Muscle fiber type composition of the rat hindlimb. Am J Anat 171: 259–272.
20. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 2: 248–254.
21. Ellis CG, Goldman D, Hanson M, Stephenson AH, Milkovich S, et al. (2010) Defects in oxygen supply to skeletal muscle of prediabetic ZDF rats. Am J Physiol Heart Circ Physiol 298: H1661–H1670.
22. Frischer JC (2004) Enhanced arteriolar alpha-adrenoceptor constriction impairs dilator responses and skeletal muscle perfusion in obese Zucker rats. J Appl Physiol 97: 764–772.
23. Chen YL, Wolin MS, Messina EJ (1996) Evidence for cGMP mediation of skeletal muscle arteriolar dilation to lactate. J Appl Physiol 81: 349–354.
24. Lovejoy J, Newby FD, Gebhart SS, DiGirolamo M (1992) Insulin resistance in obesity is associated with elevated basal lactate levels and diminished lactate appearance following intravenous glucose and insulin. Metabolism 41: 22–27.
37. Crawford SO, Hoogeveen RC, Brancati FL, Astor BC, Ballantyne CM, et al. (2010) Association of blood lactate with type 2 diabetes: the Atherosclerosis Risk in Communities Carotid MRI Study. Int J Epidemiol.

38. Lundberg JM (1996) Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 48: 113–178.

39. Eisenhofer G, Goldstein DS, Kopin IJ (1989) Plasma dihydroxyphenylglycol for estimation of noradrenaline neuronal re-uptake in the sympathetic nervous system in vivo. Clin Sci (Lond) 76: 171–192.

40. Huggett RJ, Scott EM, Gilbey SG, Stoker JB, Mackintosh AF, et al. (2003) Impact of type 2 diabetes mellitus on sympathetic neural mechanisms in hypertension. Circulation 108: 3097–3101.

41. Matyal R, Mahmood F, Robich M, Glazer H, Khabbaz K, et al. (2011) Chronic type II diabetes mellitus leads to changes in neuropeptide Y receptor expression and distribution in human myocardial tissue. Eur J Pharmacol 665: 19–28.

42. Chottova Dvorakova M, Wiegand S, Pesta M, Slavikova J, Grau V, et al. (2008) Expression of neuropeptide Y and its receptors Y1 and Y2 in the rat heart and its supplying autonomic and spinal sensory ganglia in experimentally induced diabetes. Neuroscience 151: 1016–1028.

43. Hu ZW, Shi XY, Hoffman BB (1996) Insulin and insulin-like growth factor I differentially induce alpha1-adrenergic receptor subtype expression in rat vascular smooth muscle cells. J Clin Invest 98: 1826–1834.

44. Franco-Cereceda A, Liska J (1998) Neuropeptide Y Y1 receptors in vascular pharmacology. Eur J Pharmacol 349: 1–14.

45. Martin WH, 3rd, Tolley TK, Saffitz JE (1990) Autoradiographic delineation of skeletal muscle alpha 1-adrenergic receptor distribution. Am J Physiol 259: H1402–1408.

46. Zukowska-Grojec Z, Konarska M, McCarty R (1988) Differential plasma catecholamine and neuropeptide Y responses to acute stress in rats. Life Sci 42: 1615–1624.