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Kinetics of neuropeptide Y, catecholamines, and physiological responses during moderate and heavy intensity exercises

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ABSTRACT

Neuropeptide Y 1–36 (NPY1–36) is a vasoconstrictor peptide co-secreted with norepinephrine (NE) by nerve endings during sympathetic activation. NPY1–36 potentiates NE action post-synaptically through the stimulation of the Y1 receptor, whereas its metabolite NPY3–36 resulting from DPP4 action activates Y2 presynaptic receptors, inhibiting NE and acetylcholine secretion. The secretions of NPY1–36 and NPY3–36 in response to sympathetic nervous system activation have not been studied due to the lack of analytical techniques available to distinguish them. We determined in healthy volunteers NPY1–36, NPY3–36 and catecholamine kinetics and how these neurotransmitters modulate the physiological stress response during and after moderate- and heavy-intensity exercises.

Six healthy males participated in this randomized, double-blind, saxagliptin vs placebo crossover study. The volunteers performed an orthostatic test, a 30-min exercise at moderate intensity and a 15-min exercise at heavy intensity each followed by 50 min of recovery in two separate sessions with saxagliptin or placebo. Oxygen consumption (VO_2), ventilation and heart rate were continuously recorded. NE, epinephrine, NPY1–36 and NPY3–36 were quantified by tandem mass spectrometry.

We found that exercise triggers NPY1–36 and NE secretion in an intensity-dependent manner and that NE returns faster to the baseline concentration than NPY1–36 after exercise. NPY3–36 rises during recovery parallel to the decline of NPY1–36. Saxagliptin reverses the NPY1–36/NPY3–36 ratio but does not affect hemodynamics, nor NPY1–36 and catecholamine concentrations.

We found that NPY1–36 half-life is considerably shorter than previously established with immunoassays. NPY1–36 and NE secretions are finely regulated to prevent an excessive physiological Y1 stimulating response to submaximal exercise.

1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide involved in the central and peripheral control of blood pressure and is considered a biomarker of stress, together with norepinephrine (NE) (Pedrazzini et al., 2003). Centrally, NPY is associated with stress resilience and is under clinical investigation for treating post-traumatic stress disorders as well as neurodegenerative diseases (Reichmann and Holzer, 2016). Furthermore, NPY is found at all levels of the gut–brain axis, operating as neural and endocrine messenger (Holzer et al., 2012). Peripherally, NPY is co-stored with NE in peripheral sympathetic nerve endings (Ekblad et al., 1984). The adrenal medulla is another source of NPY, from which it is co-secreted with NE and epinephrine (E), contributing to adrenosympathetic stimulation (Allen et al., 1983).

Previous studies in humans (Lind et al., 1994) and in rats (Joksimovic et al., 2017; Joksimovic et al., 2019) have shown that exercise intensity and NPY are intimately linked. In humans, NPY and NE plasma concentrations rise simultaneously during exercise, and the more intense the exercise, the greater the increase in the NPY concentration (Lind et al., 1994). After the end of exercise, the NPY concentration remains at a high level for more than 15 min, whereas NE returns to basal values within 10 min (Lacroix et al., 1997).

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Variations in plasma NPY concentrations over time correlate with post-exercise nasal vasoconstriction better than NE, indicating that endogenous NPY could be involved in the prolonged post-exercise nasal vasoconstriction and act as a non-adrenergic, non-cholinergic modulator of nasal airway reactivity (Lacroix et al., 1997). These results confirm pharmacological studies demonstrating that NPY is a co-transmitter for catecholamines (CATs) released during physical stress to maintain cardiovascular homeostasis (Pedrazzini et al., 2003).

Unfortunately, the radioimmunoassay (RIA) and the sandwich enzyme-linked immunosorbent assay (ELISA) used by the authors were unable to distinguish NPY fragments and cross-reacted with PYY, another member of the NPY family (Grouzmann et al., 1992; Lind et al., 1994).

NPY stimulates Y1, Y2, Y4, and Y5 (Michel et al., 1998). Stimulation of Y1 receptors causes an increase in blood pressure and post-synaptically potentiates the action of other vasoactive substances such as NE (Wahlestedt et al., 1985). Y2 receptors are mainly located pre-synaptically and, upon stimulation, inhibit neurotransmitter release, including NE and acetylcholine (Westfall et al., 1987).

Once secreted, NPY undergoes proteolysis. In vitro experiments have shown that NPY1–36 is cleaved into three main fragments with the following order of efficacy: NPY3–36> > NPY3–35 > NPY2–36 (Abid et al., 2009). NPY3–36 is produced by dipeptidyl peptidase 4 (DPP4) through the cleavage of the N-terminal Tyr-Pro dipeptide (Mentlein et al., 1993; Wagner et al., 2016). This fragment loses its affinity for the Y1 receptor and becomes a Y2/Y5 receptor agonist (Medeiros Dos Santos and Turner, 1996). Receptor binding assays have revealed that NPY3–35 is unable to bind to Y1, Y2, and Y5 receptors; thus, NPY3–35 may represent the major metabolic clearance product of the Y2/Y5 receptor agonist, NPY3–36.

CATs secretion depends on multiple criteria, such as posture, type, duration, and intensity of exercise. Exercise intensity may be the most important parameter (Wolpern et al., 2015). This can be divided into several domains, including moderate and heavy intensities (Burnley and Jones, 2007; Poole and Jones, 2012).

In the moderate intensity domain, oxygen consumption (VO_2) and blood lactate reach a steady state. Demarcating the boundary between moderate and heavy intensities, the first ventilatory threshold (VT_1) is a physiologically crucial point. VT_1 corresponds to the intensity at which the VE/VO_2 ratio (VE: minute ventilation) begins to increase without a concomitant increase in VE/VCO_2 (VCO_2: carbon dioxide production) or partial pressure of end tidal CO_2 ($PetCO_2$), therefore compromising the steady state.

The heavy intensity domain spans from VT_1 to the secondary ventilatory threshold (VT_2) and is characterized by a delayed heart rate (HR) and oxygen consumption (VO_2) responses (VO_2 slow component that stabilizes later). VT_2 (or respiratory compensation point) represents the onset of hypocapnic hyperventilation, which can be determined by increases in VE/VO_2 and VE/VCO_2 in conjunction with a decrease in $PetCO_2$ caused by metabolic acidosis, resulting from an insufficient buffering capacity.

Given that the previous studies on exercise and NPY did not identify the various fragments, it is still unknown as to whether NPY1–36 is co-secreted with CATs during exercise and contributes to the activation of the sympathetic system. It is also unknown as to whether NPY3–36 is involved in either prolonging the cardiovascular activation by inhibiting acetylcholine release to the heart or activating the vagal pathway upon effort cessation by slowing down the pre-synaptic secretion of CATs through Y2 receptor stimulation.

Therefore, to clarify the modulatory role of NPY during exercise and recovery, a randomized, double blind, crossover study in eight healthy volunteers receiving a DPP4 inhibitor or a placebo was performed. NPY1–36 and NPY3–36 (NPYs), NE and E concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) during and after moderate and heavy intensity exercise.

The main objective of the present study was to establish the kinetics of secretion and the clearance of NPYs and CATs and assess their relationship with VO_2, VE, and HR kinetics during and after exercise. Whether the suppression of NPY3–36 formation by a DPP4 inhibitor may alter the vagal reactivation observed during the post-exercise recovery period was also evaluated.

2. Material and methods

2.1. Volunteer recruitment

An a priori analysis of the sample size based on the variations in NPY concentrations of a pilot study done with one volunteer indicated that six volunteers would enable a statistical power of 0.95, an alpha risk of 0.05, and an effect size of 1, in the case of repeated analysis of variance (ANOVA) measurements. The true statistical power is greater than 0.99. Calculations were performed with G*Power v3.1.9.2. The published literature on the NPY distribution indicates an inter-individual variability of less than 25% (Ahlborg et al., 1992; Pernow et al., 1987; Schuerch et al., 1998). The intra-individual pharmacokinetic variability of similar magnitude in a 6-volunteer crossover study is expected to show a significant variation in metabolic ratios of NPY1–36/NPY3–36 as well as in absolute NPY concentrations of 30%, with the smallest difference being considered clinically significant (the use of 6 volunteers is also appropriate to balance the design for potential confounding factors, such as a period effect). We recruited eight volunteers in case two volunteers withdrew from the study.

Eight healthy, non-smoking male volunteers, aged 18 to 30, who practiced at least 3 h of training per week and took no medication, were recruited at the University of Lausanne, Switzerland. This study was approved by the ethical committee of the Etat de Vaud (CER-VD study 2018–00569). All volunteers provided informed written consent before starting the study. The volunteers were asked to arrive at the investigational room at 7 am at each session and received a light standardized breakfast.

2.2. Experimental design

This study was a randomized, double-blind, placebo-controlled, crossover study with eight volunteers. During, the initial session (session 0), a maximal cycling incremental test to exhaustion with gas exchanges was performed to determine the power output, HR, and VO_2 at VT_1 and VT_2.

Participants were than randomized to receive orally either 5 mg of saxagliptin (Onglyza, AstraZeneca) corresponding to the recommended dose for diabetics or a placebo (mannitol) per os on the preceding night and in the early morning before sessions 1 and 2. A washout period of two weeks was established between these two sessions to ensure that the medication was eliminated. Each volunteer was his own control to limit inter-individual variability. The 16 saxagliptin and the 16 placebo capsules were prepared by the hospital pharmacy. The randomization of the study was carried out beforehand by the pharmacist, who held the capsule allocation codes in a sealed envelope that was only opened at the end of the study.

During the sessions 1 and 2, the volunteers had to pedal on a cycle ergometer for 30 min at moderate intensity (80% VT_1) and then 15 min at heavy intensity (120% VT_1). Each exercise bout was followed by a 50-min recovery period.

During all sessions, the volunteers wore a chest belt (Polar H7, Finland) and a facial mask (Hans Rudolph, Inc., USA) linked to a gas analyzer to measure their HR (beat-to-beat interval for heart rate variability (HRV) computations), VO_2, carbon dioxide production VCO_2, and their breath-by-breath ventilation VE. Moreover, all sessions were performed on the same cycle ergometer (eErgoselect 200, Ergoline, Germany) with breath-by-breath measurement of the gas exchanges via the same gas analyzer (Jaeger Oxycon Pro, Acertys Healthcare Ltd., Belgium) by the same investigator (TN) to minimize the methodological

variability.

2.3. Protocol

2.3.1. Session 0 - maximal incremental test to exhaustion

During session 0, a maximal incremental test on a cycle ergometer was carried out to determine the power output, HR, and VO_2 at VT_1 and VT_2. After 3 min seated on the ergometer, the volunteers cycled at 60 W for 3 min, and then increments of 1 W every 2 s were applied until the volunteers could no longer sustain an enforced cycling frequency of 70 rpm despite the vocal encouragements of the investigator. During the entire test, VO_2 and VCO_2 were recorded on a breath-by-breath basis through a facemask connected to the gas analyzer.

At the end of this test, two investigators determined independently VT_1 and VT_2 using the method of ventilatory equivalents (Beaver et al., 1986; Wasserman et al., 1973). These two thresholds made possible the precise determination (typical error $< 3\%$) of individualized zones of exercise intensity during sessions 1 and 2. Each threshold was determined as the average between the two investigators. In case the two investigator's assessments for a given threshold differed by more than 3%, a third investigator was required to determine the threshold. The average of the two most concordant investigators was then used.

2.3.2. Sessions 1 and 2 - randomized control and experimental sessions

The protocol of both sessions 1 and 2 was identical except that, in randomized order, volunteers received either saxagliptin or a placebo. The study consisted mainly of completing the exercises at two defined intensities interspersed with resting phases. The insertion of a peripheral venous catheter allowed the collection of 37 samples of 2.6 mL of blood each in Li-Hep Monovette tubes containing a mixture of proteases inhibitors to prevent ex vivo peptide degradation (Vocat et al., 2020) to measure the concentrations of NPYs and CATs regularly during the exercise bouts and the recovery periods. An additional tube of blood was collected without proteases inhibitors before performing the exercises for DPP4 activity determination. Heart rate variability (HRV) and gas exchange measurements were continuously monitored throughout the entire protocol, the latter through a facial mask that could be briefly removed for hydration and consumption of a slice of bread during pre-exercise periods.

Both sessions were divided as follows: (1) an orthostatic test (7 min in the supine position followed by 6 min in the standing position); (2) a 30-min moderate intensity (i.e., 80% VT_1) exercise preceded by a 5-min period seated on the ergocycle and followed by a 50-min period in the supine position; (3) a 15-min heavy intensity (i.e., 120% VT_1) exercise preceded by a 5-min period seated and followed by a 50-min period in the supine position. Blood samples were taken at precise moments throughout the session to measure NPYs and CATs concentrations in plasma. The whole duration of a study day was 168 min.

2.4. Assays

The quantification of NPYs was performed by LC-MS/MS using a validated assay (Vocat et al., 2020). CATs were measured using an LC-MS/MS method derived from a method reported previously (Bergmann et al., 2017). LC-MS/MS data were processed using the add-on TargetLynx V. 4.1, SCN 905 (Waters). DPP4 activity was determined in triplicate by a fluorogenic 7-amino-4-methyl-coumarin (AMC) assay as follows: 35 µL pre-warmed H-Gly-Pro-AMC 100 µM in HEPES 200 mM with BSA 0.2 mg/mL pH 7.5 was incubated in 35 µL of pre-warmed plasma at 37 °C. The release of AMC was measured during 2.3 min at 37 °C under agitation using a Hidex Sense microplate reader (Turku, Finland) with $\lambda_{ex} = 390$ nm and $\lambda_{em} = 460$ nm (Malik et al., 1996).

2.5. Data processing

2.5.1. Kinetic parameters

The kinetics of the parameters were determined as follows: areas under the curve (AUC) were determined by the logarithmic trapezoidal method, half-life ($t_{1/2}$) values were calculated from the log-linear slope term of the curves, and maximal concentration (C_{max}) and time to reach C_{max} (t_{max}) were purely descriptive. Three AUCs values were calculated: for the 5 and 15 min after the start of the exercise ($AUC_{0\text{-}5min}$ and $AUC_{0\text{-}15min}$), and for the first 45 min of recovery ($AUC_{0\text{-}45min\ recovery}$). Calculation of AUC over two different time durations was required during exercise because the two NPYs strongly differed in terms of their $t_{1/2}$ and t_{max}. $AUC_{0\text{-}5min}$ was used for comparisons involving NPY1–36, and $AUC_{0\text{-}15min}$ for NPY3–36. However, all values were calculated for the sake of clarity. Kinetic parameters were calculated using Microsoft Excel.

The breath-by-breath data of VE, VO_2, VCO_2 and beat-to-beat HR were 1-min averaged for analysis of their change during exercise and during the post-exercise recovery period.

2.5.2. Heart rate variability

The RR-interval is defined as the time between two QRS peaks and was recorded using a Polar RS800CX set on the RR-interval recording mode. The data were extracted via an infrared interface to the Polar ProTrainer software (POLAR, Finland) and converted into a .txt document. All the HRV analysis followed the recommendations of the Task Force of The European Society of Cardiology (Malik et al., 1996). The RR intervals were first analyzed to remove ectopic beats from the recordings. Ectopic beats were then compensated by means of interpolation to calculate normal-to-normal (NN) intervals. From the NN intervals, mean HR and root mean square of the successive differences (RMSSD) were extracted. All computations were performed separately for the resting, exercise, and recovery periods. HR and RMSSD were averaged each 30 s (Buchheit et al., 2007). All analyses were performed using MATLAB® (R2019a, MathWorks, Natick, MA, USA). The RMSSD was calculated for each of the 30-s segments of recovery as an index of the parasympathetic reactivation post-exercise (Billman, 2002; Buchheit et al., 2008; Buchheit et al., 2007; Goldberger et al., 2006).

2.5.3. Cross-covariance

Cross-covariance is a measure of similarity between two signals. In the present study, it was assumed that there was a transition from a steady state (resting before exercise) to either a steady state or a drift depending on the exercise intensity. Breath-by-breath gas exchange signals, beat-to-beat RR signals, and blood samples were resampled at 4 Hz using cubic spline interpolation. Cross-covariance was then calculated between signals to report which was changing first after the onset of exercise (Orfanidis, 1996).

2.5.4. Statistical analysis

Individual comparisons of NPYs and CATs concentrations were carried out using Student's *t*-test. Comparisons of multiple groups were carried out by two-way ANOVA with repeated measures using Prism 9.1.0 (GraphPad Software, San Diego, CA, USA). The kinetic parameters were analyzed using Student's t-test.

The HRV (HR, RMSSD) and gas exchange (VE, VO_2, and VCO_2) data presented in the figures were analyzed using a two-factor repeated measures ANOVA, with one between factor (placebo vs. saxagliptin) and one within factor (time) for the exercise and the recovery periods, separately. For each ANOVA, if a significant interaction was identified, Tukey's post hoc test was used.

The NPY-NE correlation was studied using Prism, and the best fit was determined using an extra sum-of-squares F-test.

3. Results

Two of the eight healthy volunteers aged 18 to 30 were withdrawn from the study. One volunteer had forgotten to take the tablet the evening before the study, and the other was unable to perform heavy intensity exercise due to poor recovery from the flu between the two sessions. Our calculation showed that six volunteers was a sufficient number to obtain a sufficient statistical power (see section 2.1).

DPP4 activities were determined in the six remaining volunteers at sessions 1 and 2 to assess the efficacy of DPP4 blockade by saxagliptin. The DPP4 activities expressed as unit (U) per L of plasma (1 U is 1 μmol of substrate cleaved by min) demonstrated the full blockade of enzyme activity (0.2 ± 0.3 vs. 12.7 ± 1.6 U/L, $p < 0.0001$) and that the washout period of two weeks was sufficient to restore the initial DPP4 concentration.

3.1. Pre-exercise condition

NPYs concentrations measured in plasma collected in the supine position were at 0.13 ± 0.06 and 0.60 ± 0.20 pM for NPY1–36 and NPY3–36, respectively (Fig. 1 and Table 1), with an NPY1–36/NPY3–36 ratio of 0.22 ± 0.08, indicating that NPY mainly circulates in its truncated form during rest (data already published previously (Vocat et al., 2020)). Treatment with saxagliptin significantly increased NPY1–36 (0.30 ± 0.10 pM, $p = 0.005$), significantly lowered NPY3–36 (0.26 ± 0.12 pM, $p = 0.003$), and reversed the ratio to 1.30 ± 0.42 ($p = 0.0006$). CATs concentrations were not modified by treatment with saxagliptin (0.73 ± 0.46 vs. 0.64 ± 0.27 nM, $p = 0.33$ for NE and 0.19 ± 0.16 vs. 0.14 ± 0.05 nM, $p = 0.27$ for E) (Fig. 1 and Table 1), suggesting that basal secretion of NE and E in the resting condition is not under the control of the NPY1–36 concentration.

3.2. Orthostatic test

Concentrations of NPY1–36 (0.24 ± 0.10 pM or + 82%, $p = 0.03$), NE (1.44 ± 0.40 pM or + 126%, $p = 0.001$), and E (0.38 ± 0.21 pM or + 165%, $p = 0.02$) rose simultaneously after standing. In contrast, the NPY3–36 concentration was unchanged (0.66 ± 0.16 pM or + 9%, $p = 0.31$), indicating that NPY3–36 production was delayed by at least six min after the secretion of NPY1–36 (Fig. 1). In volunteers treated with saxagliptin, the NPY1–36 concentration was also significantly higher after standing (0.45 ± 0.16 vs. 0.24 ± 0.10 pM, $p = 0.01$). The NPY1–36/NPY3–36 ratio increased similarly during postural change with the placebo (0.36 ± 0.09 vs. 0.22 ± 0.08, p = 0.01) and saxagliptin (2.17 ± 0.83 vs. 1.30 ± 0.42, p = 0.03). The orthostatic increases in NE and E were similar in the saxagliptin and placebo groups (NE: 1.40 ± 0.62 vs 1.44 ± 0.40 pM, $p = 0.44$ and E: 0.50 ± 0.53 vs. 0.38 ± 0.21 pM, p = 0.31, respectively). The ANOVA revealed significant effects of treatment for both NPYs, but not for CATs, and significant effects of postural changes for both NPYs and NE, but not for E. The ANOVA also showed no interaction between both factors (see Table 1 for statistics). HR and BP were not significantly modified by DPP4 inhibition during the orthostatic test.

3.3. Period seated on the ergocycle before the exercise

Both sessions 1 and 2 were preceded by a 5-min period of sitting on the ergocycle during which NPY1–36 concentrations fluctuated little (mean 0.33 ± 0.13 pM, 2.5th to 97.5th percentiles at 0.16 and 0.61 pM) and remained within the previously reported reference intervals (Vocat

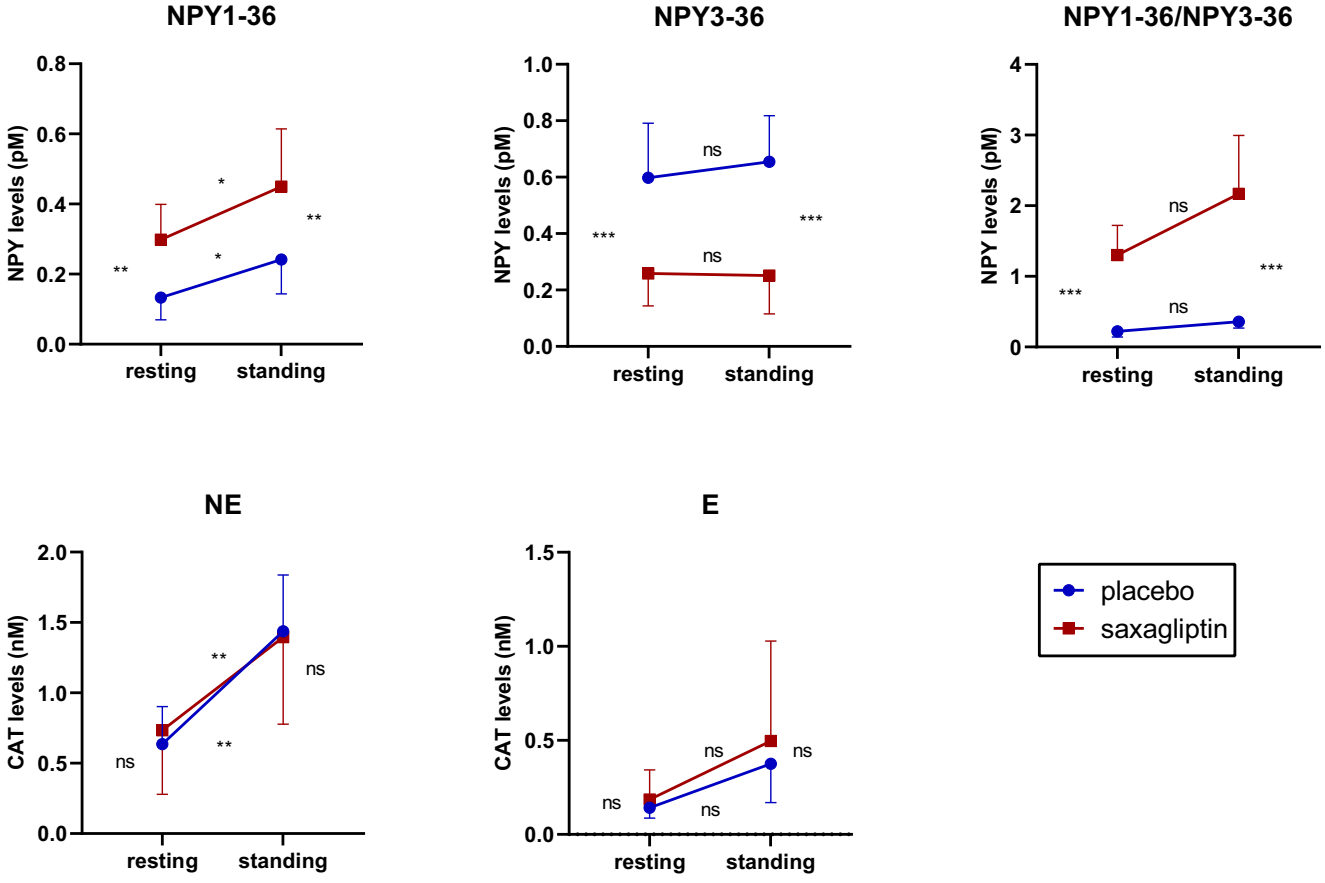

Fig. 1. NPYs and CATs concentrations measured in the supine and standing positions with placebo (blue circles) or saxagliptin (red squares) treatment. Error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
ANOVA models with repeated measures of NPYs and CATs concentrations in resting and standing positions for the placebo and saxagliptin treatments, during the orthostatic test. NPYs concentrations are expressed in pM, and CATs are in nM.

Marker		Concentrations (pM or nM)		Treatment		Position		Interaction	
		Placebo	Saxagliptin	F-value	*p*-value	F-value	p-value	F-value	p-value
NPY1–36	Resting	0.13 ± 0.06	0.30 ± 0.10	37.8	0.002	7.43	0.04	0.635	0.46
	Standing	0.24 ± 0.10	0.45 ± 0.16						
NPY3–36	Resting	0.60 ± 0.19	0.26 ± 0.11	47.2	0.001	5.52	0.07	1.57	0.27
	Standing	0.66 ± 0.16	0.25 ± 0.14						
NE	Resting	0.64 ± 0.27	0.74 ± 0.46	0.07	0.80	74.3	0.0003	2.31	0.19
	Standing	1.44 ± 0.40	1.40 ± 0.62						
E	Resting	0.14 ± 0.06	0.19 ± 0.16	0.77	0.42	4.89	0.08	0.360	0.57
	Standing	0.38 ± 0.21	0.50 ± 0.53						

et al., 2020). Saxagliptin significantly increased basal NPY1–36 concentrations (mean 0.40 ± 0.13 pM, $p = 0.028$) and significantly lowered NPY3–36 concentrations before exercise (0.31 ± 0.18 vs. 0.98 ± 0.59 pM, $p < 0.0001$). The sum of NPY1–36 + NPY3–36 concentrations was systematically lower in the treated group than in the placebo group (0.72 ± 0.28 vs. 1.31 ± 0.65 pM, $p < 0.0001$). The NE and E concentrations were not influenced by DPP4 inhibition ($p = 0.11$ and 0.53, respectively).

3.4. Moderate intensity exercise

Fig. 2 shows the concentrations of the biomarkers as well as the kinetics of VO_2, HR, and RMSSD measured during moderate intensity exercise and the subsequent recovery period in the placebo and the saxagliptin conditions. Table 2 reports the kinetic parameters of the biomarkers. NPY1–36, NPY3–36, VO_2, and HR increased during exercise (Fig. 2A). As expected, NE and E also increased with the demand in oxygen required by exercise, and RMSSD was blunted (Fig. 2B). At the end of the exercise period, RMSSD abruptly rose and, as a mirror, NPY1–36 declined to reach the pre-exercise concentration at 40 min recovery when NPY 3–36 concentrations peaked.

Saxagliptin treatment (Fig. 2C and D) increased the NPY1–36 concentration during exercise compared with the placebo ($AUC_{0\text{-}5min}$ 2.27 vs. 1.66 pM × min, $p = 0.035$) and strongly decreased the NPY3–36 concentration ($AUC_{0\text{-}15min}$ 4.01 vs. 13.0 pM × min, $p < 0.001$), but had no effect on VO_2, HR, NE, and E ($p > 0.05$). During recovery after moderate intensity exercise, RMSSD did not differ between the saxagliptin and placebo conditions, although the AUCs of NPY1–36 were significantly higher ($AUC_{0\text{-}45min\ recovery}$ 23.1 vs. 12.6 pM × min, $p = 0.015$) and the AUCs of NPY3–36 were robustly decreased ($AUC_{0\text{-}45min\ recovery}$ 17.6 vs. 60.5 pM × min, $p < 0.001$) (Fig. 2C and D).

3.5. Heavy intensity exercise

Fig. 3 shows the concentrations of the biomarkers as well as the kinetics of VO_2, HR, and RMSSD measured during heavy intensity exercise and the subsequent recovery period.

The volunteers receiving saxagliptin and the placebo had a similar concentration of NPY1–36 at the end of the exercise without affecting the NE, E, VO_2, and HR trajectories, which were similar in the placebo (Fig. 3A+B) and saxagliptin treated groups (Fig. 3C+D). Interestingly, we observed a delay of 233 s between the onset of the increase in NPY1–36 and in VO_2. The concentrations of NPY3–36 clearly rose with heavy intensity exercise, and were strongly decreased by DPP4 inhibition ($AUC_{0\text{-}15min}$ 5.82 vs. 24.0 pM × min, $p = 0.002$), without affecting the RMSSD. Overall, saxagliptin treatment very clearly decreased DPP4 activity, as evidenced by the decrease in the ratios of the C_{max} of NPY3–36 to NPY1–36 (0.256 vs. 1.31, $p = 0.0001$ for the saxagliptin and placebo groups, respectively). After exercise, the AUCs of NPY1–36 did not differ in the saxagliptin and the placebo groups (42.0 vs. 32.9 pM × min, $p = 0.24$). The half-life of elimination of NPY1–36 at the end of exercise was similar for the two conditions ($t_{1/2}$ 4.78 vs. 4.66 min, $p = 0.42$). Overall, neither the amplitudes nor the kinetics of HR, VO_2, and RMSSD were significantly altered by DPP4 inhibition during exercise and recovery (Fig. 3).

3.6. Comparison of the moderate vs. heavy intensity exercise

We observed that NPY1–36 secretion is dependent on the exercise intensity ($AUC_{0\text{-}5min}$ 1.66 vs. 2.51 pM × min during the first five min of moderate vs. heavy intensity, $p < 0.019$) to reach C_{max} at 1.06 and 4.30 pM ($p = 0.033$), 10.5- and 33.7-fold higher ($p = 0.033$) than the NPY1–36 concentrations measured in the supine position for exercises at moderate and heavy intensity, respectively (Fig. 4, black bars). NPY1–36 concentrations were also higher in volunteers receiving saxagliptin than when receiving a placebo during moderate intensity exercise ($AUC_{0\text{-}5min}$ 2.27 vs. 1.66 pM × min, $p = 0.035$) but not during heavy intensity exercise ($AUC_{0\text{-}5min}$ 3.12 vs. 2.51 pM × min, $p = 0.18$). The same observation was made for the resting period following exercise.

NPY3–36 concentrations were also correlated to the exercise intensity ($AUC_{0\text{-}15min}$ 13.0 vs. 24.0 pM × min, $p = 0.018$ and C_{max} 2.25 vs 5.07 pM, $p = 0.031$ for the moderate and heavy intensity exercises, respectively) and appeared with a delay with respect to NPY1–36 at moderate (t_{max} 38.7 vs. 27.2 min, $p = 0.019$) but not at heavy intensity exercise (21.5 vs. 17.5 min, $p = 0.10$). NPY3–36 concentrations were strongly reduced during the two exercises in the saxagliptin-treated group ($AUC_{0\text{-}15min}$ 4.01 vs. 13.0 pM × min, $p < 0.001$ at moderate intensity exercise and 5.82 vs. 24.0 pM × min, $p < 0.001$ at heavy intensity exercise; C_{max}: 0.677 vs. 2.25 pM, $p < 0.001$ at moderate intensity exercise and 1.07 vs. 5.07 pM, $p = 0.009$ at heavy intensity exercise). After exercise, the AUCs of NPY3–36 differed considerably between the conditions ($AUC_{0\text{-}45min\ recovery}$ 17.6 vs. 60.5 pM × min, $p < 0.001$ at moderate intensity exercise and 30.9 vs. 133 pM × min, $p = 0.010$ at heavy intensity exercise). The half-life of elimination of NPY3–36 after the end of the heavy intensity exercise was similar for the saxagliptin- and placebo-treated groups ($t_{1/2}$ 25.5 vs. 26.9 min, $p = 0.36$). Interestingly, the sum of the C_{max} of NPY1–36 and NPY3–36 was lower in the saxagliptin group compared with the placebo group, without reaching significance (−29%, $p = 0.05$ and − 43%, $p = 0.09$ for moderate and heavy intensity exercises, respectively).

NE was significantly higher during heavy intensity exercise than during moderate intensity exercise (C_{max} 7.89 vs. 3.82 nM, $p = 0.012$). Conversely, the C_{max} of E was unchanged by the exercise intensity ($p = 0.17$). The C_{max} of NE and E was not affected by saxagliptin for both exercise intensities ($p > 0.05$ for all). Interestingly, the relative increases in the NPY1–36 and NE concentrations were dependent on the exercise intensity, as shown by the ratios of C_{max} vs. the concentrations measured in the supine position (Fig. 4). This ratio was not significantly different for NPY1–36 and NE during the orthostatic test (2.1 and 2.4, respectively, $p = 0.22$), but was higher for NPY1–36 than for NE during the moderate (10.5 and 6.31, respectively, $p = 0.015$) and the heavy

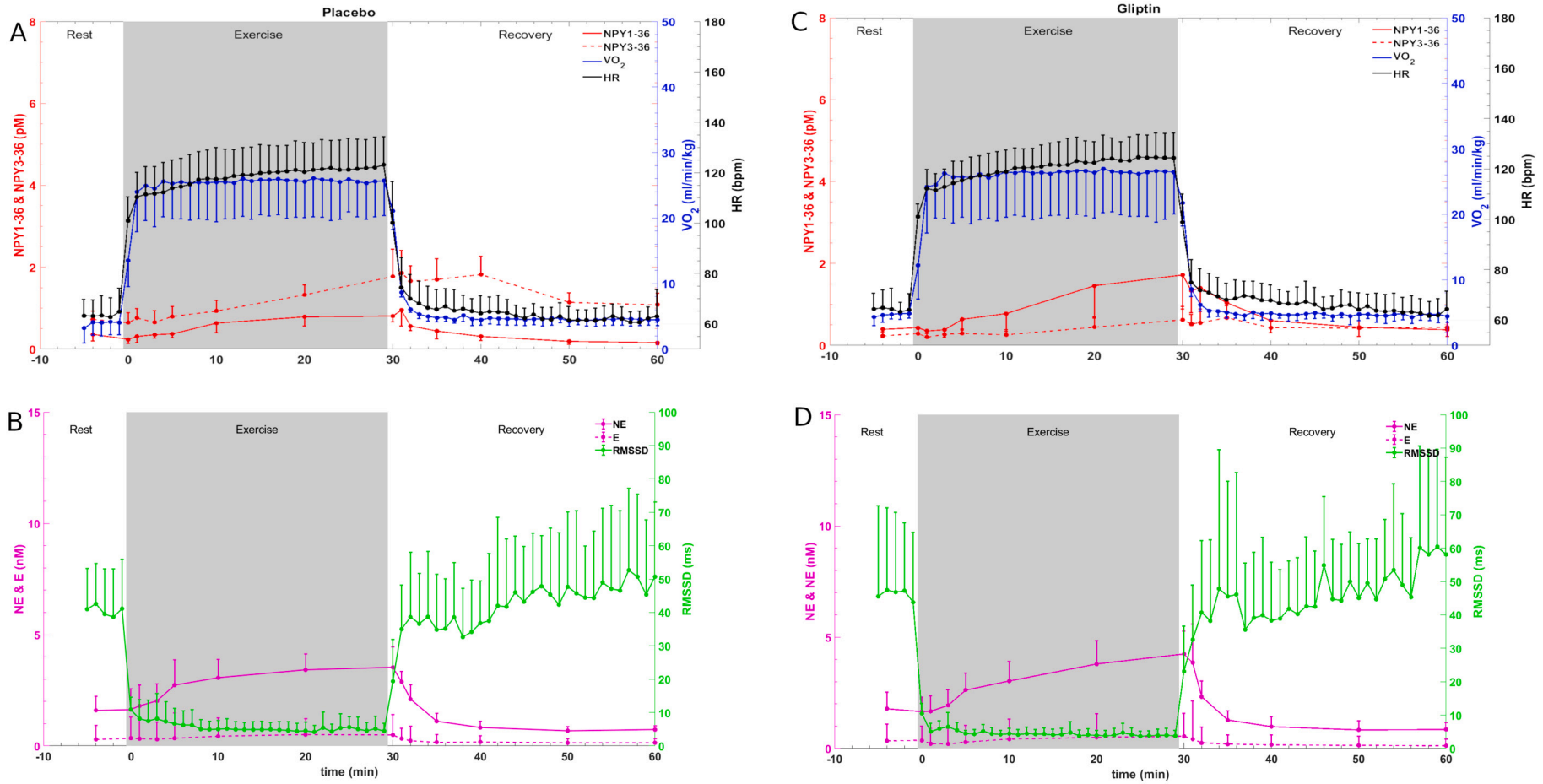

Fig. 2. Kinetics of VO_2 (blue), HR (black), NPY1–36 (red), NPY3–36 (dotted red), NE (pink), E (dotted pink), and RMSSD (green) measured during moderate intensity exercise and the subsequent recovery period in the placebo (panels A and B) and saxagliptin (panels C and D) groups. The amplitudes and kinetics of HR, VO_2, and RMSSD were not significantly altered by DPP4 inhibition during exercise and recovery. Error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Kinetic parameters for NPY1–36, NPY3–36, NE, and E during moderate- and heavy-intensity exercises. Values are mean ± SD.

		NPY1–36			NPY3–36			NE			E		
		moderate	heavy	p-value	moderate	heavy	p-value	moderate	heavy	p-value	moderate	heavy	p-value
$AUC_{0\text{-}5min}$ (−)	placebo	1.66 ± 0.48	2.51 ± 0.70	0.019	3.53 ± 0.98	4.52 ± 0.83	0.045	10.5 ± 4.1	13.0 ± 3.5	0.13	1.56 ± 0.71	1.34 ± 0.56	0.28
	saxagliptin	2.27 ± 0.55	3.12 ± 1.26	0.11	1.31 ± 0.54	1.30 ± 0.80	0.49	10.2 ± 4.1	12.1 ± 2.7	0.19	1.21 ± 0.65	0.927 ± 0.408	0.20
	p-value	0.035	0.18	–	0.001	<0.001	–	0.45	0.31	–	0.19	0.10	–
$AUC_{0\text{-}15min}$ (−)	placebo	7.07 ± 1.75	23.9 ± 15.8	0.024	13.0 ± 3.0	24.0 ± 9.6	0.018	40.3 ± 12.5	71.8 ± 24.9	0.013	5.61 ± 1.58	7.44 ± 3.97	0.17
	saxagliptin	10.2 ± 4.0	24.5 ± 9.4	0.011	4.01 ± 1.73	5.82 ± 2.76	0.12	39.5 ± 12.9	72.1 ± 21.9	0.012	4.83 ± 2.53	7.02 ± 3.61	0.15
	p-value	0.062	0.47	–	<0.001	0.002	–	0.46	0.49	–	0.27	0.43	–
$AUC_{45min\ recovery}$ (−)	placebo	12.6 ± 4.1	32.9 ± 23.5	0.044	60.5 ± 9.5	133 ± 76	0.033	42.7 ± 11.8	61.1 ± 31.9	0.12	7.37 ± 2.92	8.06 ± 1.91	0.32
	saxagliptin	23.1 ± 8.5	42.0 ± 17.8	0.025	17.6 ± 8.3	30.9 ± 16.0	0.055	49.3 ± 17.8	60.6 ± 18.2	0.15	6.95 ± 4.07	8.06 ± 3.44	0.31
	p-value	0.015	0.24	–	<0.001	0.010	–	0.23	0.49	–	0.42	0.50	–
$t_{1/2}$ (min)	placebo	(a)	4.66 ± 0.70	–	(a)	26.9 ± 6.2	–	(a)	2.94 ± 1.75	–	(a)	2.82 ± 0.95	–
	saxagliptin	(a)	4.78 ± 1.07	–	(a)	25.5 ± 6.2	–	(a)	2.78 ± 0.52	–	(a)	2.59 ± 0.80	–
	p-value	(a)	0.42	–	(a)	0.36	–	(a)	0.42	–	(a)	0.35	–
C_{max} (pM or nM)	placebo	1.06 ± 0.34	4.30 ± 3.38	0.033	2.25 ± 0.57	5.07 ± 2.87	0.031	3.82 ± 0.93	7.89 ± 3.17	0.012	0.614 ± 0.185	0.809 ± 0.417	0.17
	saxagliptin	1.67 ± 0.69	4.25 ± 1.97	0.011	0.677 ± 0.292	1.07 ± 0.51	0.069	4.21 ± 1.13	8.17 ± 2.62	0.006	0.574 ± 0.352	0.788 ± 0.401	0.17
	p-value	0.045	0.49	–	<0.001	0.009	–	0.26	0.44	–	0.41	0.47	–
t_{max} (min)	placebo	27.2 ± 9.4	17.5 ± 2.3	0.027	38.7 ± 6.6	21.5 ± 6.6	0.001	21.9 ± 9.9	16.7 ± 0.8	0.13	18.0 ± 13.9	14.9 ± 3.1	0.30
	saxagliptin	29.8 ± 4.7	16.6 ± 1.3	<0.001	32.5 ± 2.4	21.0 ± 7.5	0.006	29.1 ± 4.1	14.6 ± 2.4	<0.001	20.2 ± 11.0	13.8 ± 2.7	0.11
	p-value	0.28	0.21	–	0.037	0.45	–	0.074	0.043	–	0.38	0.28	–
fold change (b) (−)	Placebo	10.5 ± 7.7	33.7 ± 19.1	0.015	4.27 ± 2.50	8.49 ± 3.93	0.028	6.31 ± 0.92	13.0 ± 4.6	0.010	4.61 ± 1.31	5.93 ± 2.21	0.12
	saxagliptin	14.8 ± 6.5	34.7 ± 14.9	0.010	1.11 ± 0.23	1.73 ± 0.51	0.016	6.97 ± 1.43	13.6 ± 3.5	0.002	3.80 ± 1.13	5.71 ± 1.88	0.032
	p-value	0.16	0.46	–	0.013	0.004	–	0.18	0.41	–	0.14	0.43	–

(a) Half-life ($t_{1/2}$) values could not be calculated for the moderate-intensity exercise because of the low concentration change.
(b) Fold change = ratio of C_{max} vs. concentration measured in supine position.

intensity exercise (33.7 and 13.0, respectively, $p = 0.008$), as shown in Fig. 4.

An exponential correlation between NPY1–36 and NE concentrations was observed during heavy intensity exercise, when the NE concentration threshold of 5 nM was reached (Fig. 5). An F-test confirmed that the exponential model explained the data significantly better than the single linear one ($F = 83.8$, $p < 0.0001$). NE returned faster to a baseline concentration than NPY1–36 during the post-exercise recovery period ($t_{1/2}$ 2.94 vs. 4.66 min, respectively, $p = 0.03$ for heavy intensity exercise). No similar observation was made for E.

3.7. Hemodynamic changes with increasing efforts

Table 3 reports the physiological values for the two exercises in the placebo and the saxagliptin conditions. All physiological values in the resting phase, during exercise, and during recovery were unmodified by saxagliptin (all $p > 0.05$).

Figs. 2 and 3 display the kinetics of the main physiological responses to exercise and post-exercise recovery for moderate and heavy intensity, respectively. Note that for moderate intensity exercise, the steady state was achieved rapidly; for heavy intensity exercise, the steady state was delayed, and we observed a slight drift in HR and VO_2 (i.e., the VO_2 slow component). Of greater importance is that neither the kinetics of HR, VO_2, or VE, nor parasympathetic reactivation, were different between the two conditions, since no significant interaction was found for any of these variables, i.e., their changes during exercise or during recovery were similar between the placebo and the saxagliptin conditions for any given exercise intensity.

NPY1–36 and NE concentrations increased with a delay of 21 and 28 s when compared to the increase in VO_2 and HR, respectively, at moderate intensity (see Fig. 2) and with a delay of 250 and 233 s at heavy intensity (see Fig. 3), indicating that the kinetics of physiological responses (e.g., VO_2 and HR) precede neurohumoral activation and are dependent on the exercise intensity. The observed discrepancy between the above-reported delays and the kinetics were not significantly different between the placebo and saxagliptin groups.

4. Discussion

In this study, we report for the first time the kinetics of NPY1–36, NPY3–36, and CATs during exercise at two exercise intensities in healthy volunteers. More specifically, we found that the vasoconstrictor NPY1–36 was secreted simultaneously with CATs during physical effort and that NPY3–36 production was delayed and peaked when the exercise session ended and NPY1–36 returned to baseline. This is an important, question since the dogma of the potentiating effect of NPY on catecholamine-induced vasoconstriction relies on a longer half-life of NPY than catecholamine. Therefore, previous studies using unspecific RIA or ELISA were in fact simultaneously measuring NPY1–36 and NPY3–36, leading to an overestimation of the persistence of NPY1–36 in target tissues after exercise stops (Lacroix et al., 1997; Lind et al., 1994; Pernow et al., 1986; Ulman et al., 1997). We observed that NE, but not E, was higher during heavy intensity exercise than during moderate intensity exercise, indicating a greater contribution of the sympathetic nerves than the adrenal medulla, as postulated in other studies (Lundberg et al., 1985). We observed during heavy exercise that NPY1–36

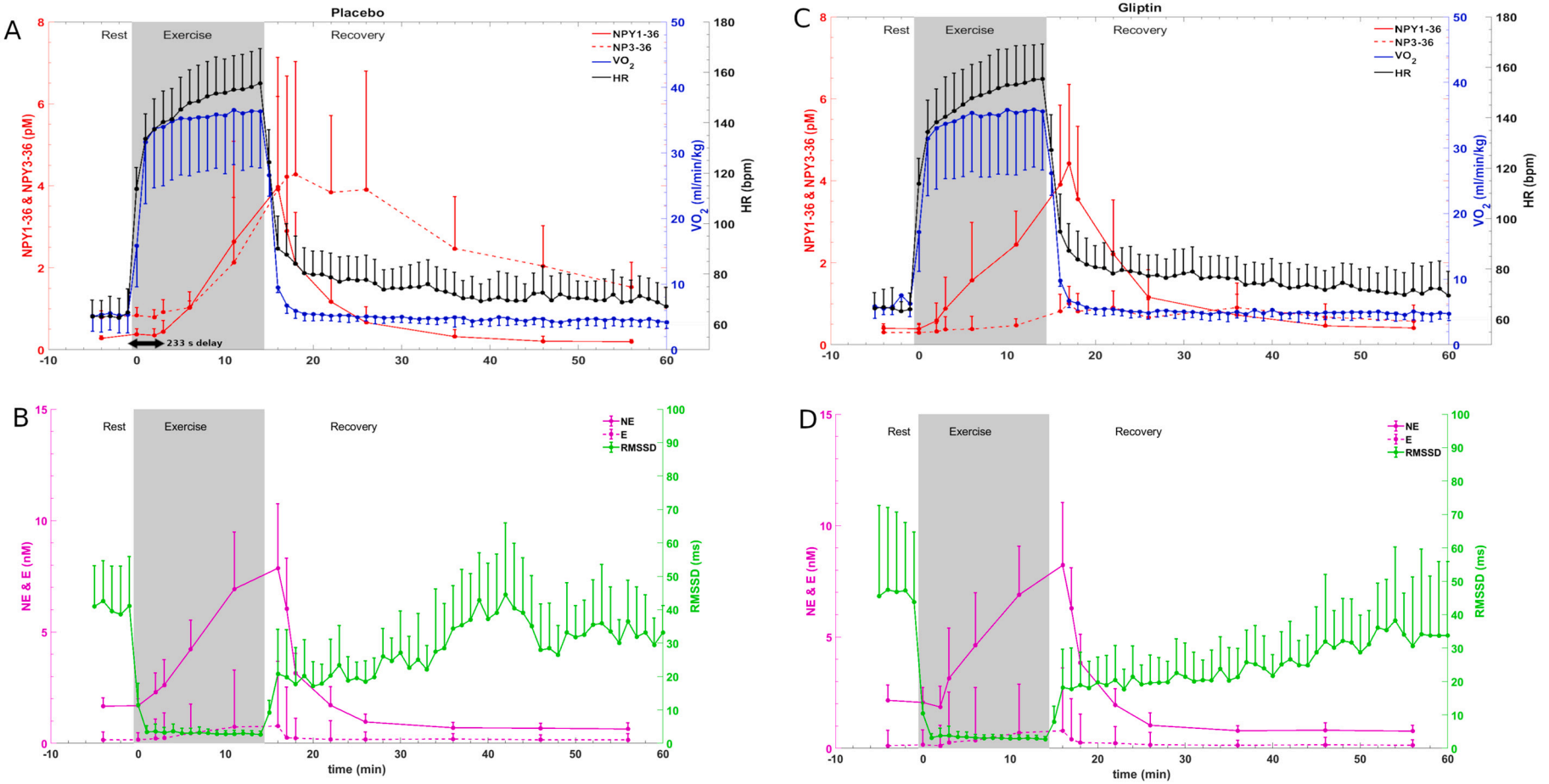

Fig. 3. Kinetics of VO_2 (blue), HR (black), NPY1–36 (red), NPY3–36 (dotted red), NE (pink), E (dotted pink), and RMSSD (green) measured during heavy intensity exercise and the subsequent recovery period in the placebo (panels A and B) and saxagliptin (panels C and D) groups. A delay of 233 s between the onset of the increase in NPY1–36 and in VO_2 is shown as an example. The amplitudes and kinetics of HR, VO_2, and RMSSD were not significantly altered by DPP4 inhibition during exercise and recovery. Error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

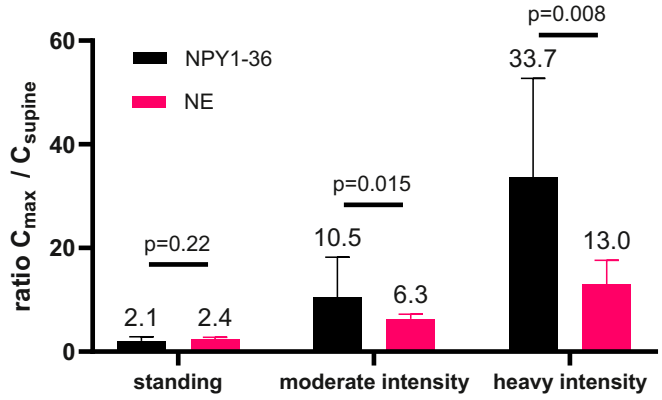

Fig. 4. Ratio of the C_{max} of NPY1–36 (black bars) and NE (pink bars) measured during an orthostatic test and two exercises at moderate and heavy intensity over concentrations measured at pre-exercise in the supine position in the placebo group. A similar or slightly more pronounced effect was observed in the saxagliptin group (data not shown). Error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

onset was delayed compared with VO_2, suggesting that the native peptide may originate from pools distinct from secreted NE vesicles. We also found that, during the heavy intensity exercise, the relative increase of NPY1–36 was higher than that of NE, suggesting that an additional pool of peptide vesicles is available when adrenergic receptors need potentiating Y1 receptor stimulation independently of CATs storage. The threshold of NE secretion required for an exponential NPY release was about 5 nM, which is in agreement with studies indicating the need for exercise at a vigorous intensity (average ~70% VO_2 maximum) to induce a significant release of immunoreactive NPY in healthy men and women. These results also show that immunoreactive NPY is released during maximum load exercise testing at the anaerobic/lactate threshold (Pernow et al., 1986; Ramson et al., 2012; Scioli-Salter et al., 2016).

An in vivo study in healthy volunteers demonstrated that an infusion of NPY to reach a steady state concentration 22-fold higher than endogenous concentrations potentiates the α_1-adrenergic constriction induced by an infusion of phenylephrine in the forearm (Schuerch et al., 1998). However, this threshold may be questioned since it was measured by a sandwich ELISA that is unable to distinguish NPY isoforms and because a ten-fold lower NPY infusion rate, which resulted in a four-fold increase in circulating NPY concentrations, did not increase the blood pressure response to phenylephrine. Nevertheless, these data suggest that circulating NPY may participate in the local or regional control of vascular tone, since NPY1–36 concentrations were 8- and 32-fold higher compared to the basal concentration during moderate and heavy exercises and since saxagliptin may potentiate this effect. However, our study was not designed to evaluate the interaction between vascular resistance and the NPY1–36 concentration, so this question remains open (Schuerch et al., 1998).

The greater increase in the C_{max} of NPY1–36 than of NE during exercise (Fig. 4) can be explained either by the different metabolism of these compounds, or by the additional release of NPY1–36. Assuming that the metabolism (half-life) of NPY1–36 and of NE is not concentration-dependent, this greater increase in C_{max}/C_{supine} for higher intensity exercise suggests that the adrenal medulla is also a non-neural source of NPY released upon exercise, together with NE and E, and may contribute to supporting cardiovascular needs during exercise (Cavadas et al., 2002).

In agreement with the studies of Pernow et al., we confirmed that NE concentrations return to baseline faster than NPY concentrations after exercise, suggesting that the peptide is an important co-transmitter to maintain sympathetic activity for a significant duration (Pernow et al.,

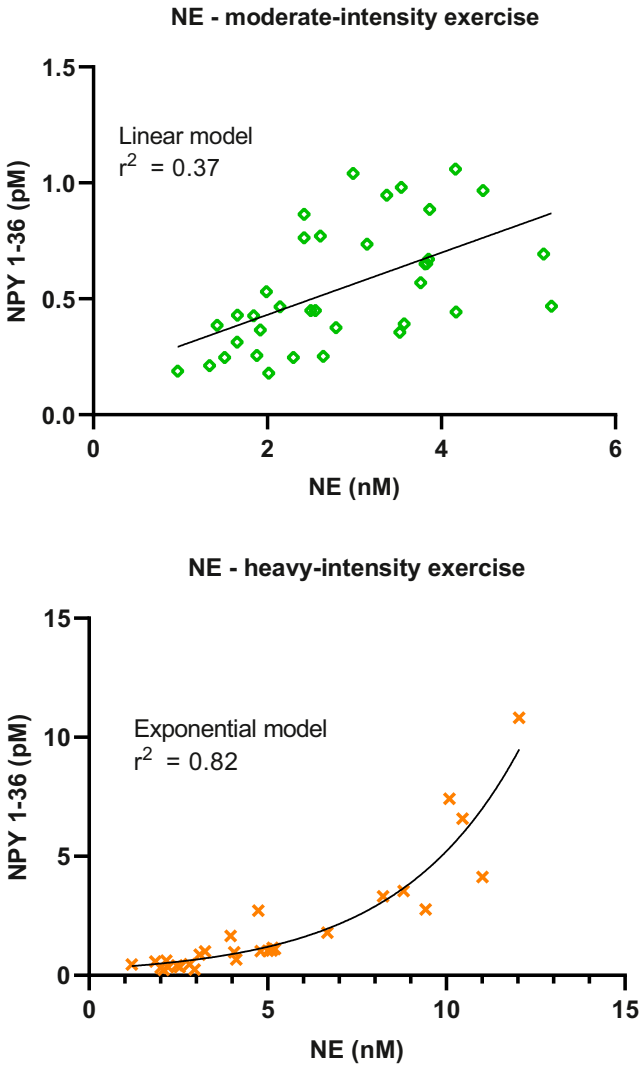

Fig. 5. NPY1–36 vs. NE concentrations measured in the plasma samples of the six participants collected during exercise at moderate (upper panel) and heavy intensity (lower panel) in the placebo group. The best fit for the moderate intensity exercise is a linear model. The best fit for the heavy intensity exercise is an exponential function with a 1/y weighting, as confirmed by an F-test ($F = 83.8$, $p < 0.0001$, compared with the linear model).

1986). However, our findings suggest a more modest contribution of NPY at the postjunctional response to vascular smooth muscles, since the half-life of NPY1–36 was only 160% that of NE (about 4.7 min vs. 2.9 min), and the immunoreactive NPY measured in the Pernow study reflected the increase in the non-vasoconstrictive NPY3–36 with a long half-life ($t_{1/2}$ 26.9 min in our study). The effect of Y2 receptor stimulation on classical transmitter secretion is complex, since NPY is also produced by the heart (Gu et al., 1983) in intracardiac ganglia and parasympathetic neurons where the Y2 receptor is preferentially expressed (McDermott and Bell, 2007).

As expected, there was parasympathetic withdrawal during exercise, and this was reversed during the recovery period (Arai et al., 1989). This is indicated by the post-exercise increase in RMSSD, considered a non-invasive index of parasympathetic activation (Billman, 2002; Buchheit et al., 2008; Buchheit et al., 2007; Goldberger et al., 2006). An increase in HR during exercise results from the suppression of vagal tone at moderate exercise intensities and from both vagal withdrawal and sympathetic activation at higher intensities (Robinson et al., 1966). NPY3–36 may inhibit the presynaptic release of CATs from sympathetic nerves, but also may inhibit acetylcholine release and, consequently,

Table 3
Resting pre-exercise, end-exercise, and end-recovery physiological values for the moderate- and heavy-intensity exercises for the placebo and saxagliptin groups.

		Moderate-intensity exercise			Heavy-intensity exercise		
		Pre-exercise	End-exercise	End-recovery	Pre-exercise	End-exercise	End-recovery
Ventilation	Placebo	10.0 ± 0.6	48.4 ± 11.5	9.9 ± 3.0	11.1 ± 0.2	70.3 ± 16.7	9.6 ± 2.8
($L.min^{-1}$)	Saxagliptin	9.8 ± 0.7	48.0 ± 9.9	8.4 ± 1.5	12.9 ± 1.0	66.7 ± 13.6	9.1 ± 2.0
VO_2	Placebo	3.9 ± 0.2	25.7 ± 5.4	4.6 ± 0.8	5.3 ± 0.2	35.9 ± 8.2	4.8 ± 1.3
($mL.kg^{-1}.min^{-1}$)	Saxagliptin	4.7 ± 0.3	26.4 ± 6.4	4.0 ± 1.0	6.2 ± 0.6	35.0 ± 9.0	4.4 ± 1.5
VCO_2	Placebo	243 ± 12	1715 ± 380	283 ± 59	331 ± 9	2473 ± 487	279 ± 87
($mL.min^{-1}$)	Saxagliptin	287 ± 23	1698 ± 317	261 ± 63	368 ± 39	2421 ± 487	258 ± 87
$PetO_2$	Placebo	105.6 ± 0.2	97.5 ± 3.7	98.0 ± 7.6	100.2 ± 0.8	99.4 ± 3.3	96.7 ± 3.3
(mmHg)	Saxagliptin	97.7 ± 1.1	97.3 ± 3.8	100.8 ± 3.6	100.1 ± 1.0	99.7 ± 3.6	98.0 ± 5.0
$PetCO_2$	Placebo	30.2 ± 0.1	39.8 ± 3.7	36.2 ± 4.2	33.5 ± 0.3	39.5 ± 3.2	37.0 ± 3.1
(mmHg)	Saxagliptin	35.3 ± 0.8	39.4 ± 4.0	36.2 ± 2.6	34.0 ± 0.7	39.5 ± 3.1	35.8 ± 4.4
BP Syst	Placebo	122 ± 22	115 ± 21	103 ± 16	108 ± 13	126 ± 26	110 ± 4
(mmHg)	Saxagliptin	101 ± 11	101 ± 12	116 ± 15	111 ± 7	119 ± 18	108 ± 13
BP Dias	Placebo	59 ± 11	53 ± 8	54 ± 6	54 ± 13	57 ± 13	56 ± 8
(mmHg)	Saxagliptin	52 ± 3	48 ± 3	59 ± 11	64 ± 10	62 ± 16	52 ± 8

vagal bradycardia in the human heart by cardiac parasympathetic neurons (Schwertfeger et al., 2004). It has been proposed that NPY released from sympathetic nerves during exercise attenuates evoked cardiac vagal action for a prolonged period after exercise ends, but this hypothesis relies only on immunoreactive NPY quantification without investigations that could draw clear mechanistic conclusions (Ulman et al., 1997).

Inhibiting DPP4 significantly reduced the cleavage of NPY1–36 to NPY3–36 without modifying NPY1–36 concentrations nor affecting hemodynamic parameters. Conversely, it dramatically reduced NPY3–36 concentrations. The lack of a significant increase in the NPY1–36 concentration with DPP4 inhibition during heavy intensity exercise is mediated by an unknown compensatory mechanism that does not involve CATs, as these concentrations were not affected by saxagliptin. This also suggests that an alternative pathway exists for the clearance of NPY1–36, or that a counter-mechanism results in a decrease in absolute NPY1–36 release during exercise to prevent overstimulation in the context of a relative increase in the NPY1–36 concentration compared to NPY3–36. In this context, hemodynamic parameters (HR, blood pressure, VCO_2, VO_2, VE, RMSSD) were not affected by saxagliptin treatment.

5. Conclusion

We have shown that the secretion of NPY1–36 is dependent on the intensity of exercise, together with CATs, without a potentiating effect on cardiovascular performance in healthy athletes; the long duration of NPY immunoreactivity after exercise is mostly related to NPY3–36. We observed that DPP4 inhibition does not alter the vagal reactivation during the post-exercise recovery period. We also showed that, during intense exercise, DPP4 inhibition decreases NPY3–36 concentrations without increasing NPY1–36 concentrations. Further investigation is needed to understand this unknown regulatory mechanism, which is not related to NE as these concentrations remained unchanged during DPP4 inhibition.

CRediT authorship contribution statement

Philippe J. Eugster: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Nicolas Bourdillon:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Céline Vocat:** Investigation. **Gregoire Wuerzner:** Supervision. **Toan Nguyen:** Investigation. **Grégoire P. Millet:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Eric Grouzmann:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

None.

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