

## Parathyroid hormone induces a browning program in human white adipocytes

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### Abstract

Energy dissipation through the promotion of brown adipose tissue (BAT) or browning of white adipose tissue has recently evolved as novel promising concept in the fight against metabolic disease. New evidence suggests that hormones can contribute to the thermogenic programming of adipocytes through paracrine or endocrine actions. Recent studies in rodents identified parathyroid hormone (PTH) and PTH-related peptide as mediators of energy wasting in cachexia models due to adipocyte browning. However, the effects of PTH on human adipocyte thermogenesis and metabolic activity are unknown. Here we isolated subcutaneous white adipocyte precursor cells (APCs) from human donors followed by stimulation with recombinant PTH. Our data show that acute and chronic PTH administration in primary *in vitro* differentiated human subcutaneous adipocytes induces a molecular thermogenic program with increased mitochondrial activity and oxidative respiratory capacity. PTH also enhances hormone sensitive lipase activity and lipolysis in human adipocytes which may contribute to the observed thermogenic effects. In summary, we demonstrate here that PTH is a novel mediator of human adipocyte browning, suggesting a hitherto unknown endocrine axis between the parathyroid gland and adipose tissue in humans.

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#### Author's Contributions

OCH, AF, FWK designed study, researched data and wrote manuscript. AD, KS, SBP, MB, LK, MV and AEB researched data and revised manuscript.

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## 1 Introduction

Brown adipose tissue (BAT) in contrast to white adipose tissue (WAT) dissipates significant amounts of chemical energy through uncoupled respiration. This process results in mitochondrial fatty acid oxidation and heat production (thermogenesis) 1, 2. Consequently, promotion of BAT function or transformation of white adipocytes into BAT-like or beige cells increases energy expenditure and counteracts weight gain in numerous experimental models. Particularly browning of WAT holds great promise as a novel anti-obesity concept given the large excess of white fat depots in obese individuals 3–6. The identification of factors controlling WAT browning in humans is imperative for a better understanding of the thermogenic processes occurring in human adipocytes and consequently for the development of pharmacologic approaches. There is accumulating evidence that a number of endocrine factors and hormones promote WAT thermogenesis and energy expenditure 7. Recently, parathyroid hormone (PTH) and tumor-derived PTH-related peptide have been linked to increased energy wasting due to WAT browning in mouse models of nephropathy and cancer cachexia 8, 9. In addition, humans with primary hyperparathyroidism had higher expression of classic brown fat marker genes in deep neck fat biopsies compared to patients with normal PTH levels 9. However, the direct thermogenic effects of PTH on human adipocytes and particularly the browning of white adipocytes have not been studied yet.

PTH signals through the G protein coupled PTH receptor (PTHr) that activates cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) 10. cAMP-mediated PKA activation also occurs in response to beta adrenergic stimulation which results in thermogenic gene expression 1. In fact, recent work in murine adipocytes has demonstrated that this shared signaling pathway mediates some of the thermogenic PTH effects seen in mice 8.

Together these preclinical data raised important questions about the role of PTH as an endocrine regulator of the transcriptional browning program in human WAT. Hence, we set out to study the thermogenic effects of PTH on primary *in vitro* differentiated human subcutaneous adipocytes.

## 2 Materials and Methods

### 2.1 Isolation and differentiation of human adipose precursor cells (hAPCs)

Abdominal subcutaneous adipose tissue was excised from healthy female patients undergoing abdominoplastic surgery between June 2017 and May 2018. All subjects provided written informed consent. This study was approved by the Ethics Committee of the Medical University of Vienna and was conducted in accordance with the principles of the Declaration of Helsinki (EK 1149/2011, EK 1032/2013). hAPCs were isolated and cultured as previously described 11. For each experiment cells from at least three donors were isolated. For every experiment the sample size was determined empirically based on preliminary experiments. Details in Supplementary Methods.

Confluent cells were induced using differentiation media supplemented with 0,85 $\mu$ M insulin, 2nM triiodothyronine, 5 $\mu$ M rosiglitazone, 0,5mM isobutylmethylxanthine, 1 $\mu$ M dexamethasone (all Sigma, St. Louis, MO, USA) for 2 days, followed by post-differentiation media supplemented with 0,85 $\mu$ M insulin, 2nM triiodothyronine, 5 $\mu$ M rosiglitazone (all Sigma). The post-differentiation media was changed every other day until day 6. Cells were stimulated with human recombinant parathyroid hormone (MyBioSource, San Diego, CA, USA), isoproterenol (Sigma) or vehicle on day 6 or during the entire adipogenic differentiation.

## 2.2 RNA preparation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Thermo Fischer Scientific, Carlsbad, CA, USA) and treated with DNase (Thermo Fischer Scientific, Carlsbad, CA, USA). RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Gene expression, normalized to 36B4, was analyzed by quantitative real-time RT-PCR (Sybr Green, Roche, Mannheim, Germany) using a QuantStudio 6 RealTime PCR System (Applied Biosystems). Primer sequences are available upon request.

## 2.3 Immunoblotting

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Standard western blotting techniques were performed using rabbit monoclonal antibodies against PTHR1 (ab189924, Abcam, Cambridge, UK) against GAPDH (2118, Cell Signaling, Danvers, MA, USA), HSL (4126, Cell Signaling), pHSL Ser660 (4107, Cell Signaling), and against  $\beta$ -actin (NB600-501, NovusBio, Littleton, CO, USA). Proteins were detected using the Fusion FX7 fluorescence and chemiluminescence system (Vilber Lourmat, Marne-la-Vallée, France).

## 2.4 Immunofluorescence

Cells cultured in chamber slides were fixed in 2% (vol/vol) paraformaldehyde. After permeabilization (1% Triton X) and blocking (3% BSA) cells were stained with a rabbit polyclonal antibody to UCP-1 (U6382 Sigma, 1:1000). Binding was visualized with a fluorescence secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes, Inc, Eugene, OR, USA) and analyzed on an Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) after DAPI counterstaining (Sigma).

## 2.5 Oxygen consumption assay

Mitochondrial function was assessed using the Seahorse XF Mito Stress Test kit (Seahorse Bioscience, North Billerica, MA, USA). Cellular oxygen consumption rate (OCR) was determined in differentiated human APCs following acute or chronic PTH stimulation using a modified protocol as previously described 12. Details in Supplementary Methods.

## 2.7 Statistical analyses

The statistical analysis was conducted with explorative data analysis and descriptive statistics using Prism 7 (GraphPad Software, La Jolla, CA, USA). More precisely, results are given as mean  $\pm$  standard error of the mean (SEM) and differences were analyzed using

either one-way ANOVA with Dunnett's post hoc test when comparing more than one treatment against the control group or unpaired two-tailed Student's t-test followed by Fisher's combination of probabilities for biological replicates for comparisons between two groups, after confirmation of normal distribution. Results shown are derived from biological replicates, not from technical replicates. A p-value  $\leq 0.05$  was considered statistically significant.

### 3 Results

In order to study the effects of PTH on human white adipocytes we isolated hAPCs from abdominal subcutaneous fat specimens obtained from three different donors. hAPCs were differentiated for six days using a standard adipogenic protocol. PTHR was readily expressed in fully differentiated cells (Supplementary Fig. 1A). First, a dose-response experiment was performed to assess the efficacy of PTH to induce a thermogenic gene program in human white adipocytes. Therefore differentiated hAPCs were stimulated with human recombinant PTH for six hours at various concentrations between 1pM to 100nM (Fig. 1A). 10nM of PTH resulted in the most potent induction of established thermogenic genes such as uncoupling protein-1 (UCP-1), type II iodothyronine deiodinase (DIO2), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) or potassium channel subfamily K member 3 (KCNK3) (Fig. 1A). Hence 10nM PTH was used in all other experiments. To study the effects of acute PTH administration on adipocyte thermogenesis in more detail, hAPCs from three different donors were fully differentiated followed by six hours of PTH stimulation. Both, 10nM and 100nM of PTH induced the expression of UCP-1 and other thermogenic genes in mature adipocytes (Fig. 1B). Notably, the effects of PTH on thermogenic gene expression exceeded those of the established beta-adrenergic agonist isoproterenol or were at least as pronounced. In order to study the functional consequences of the increased BAT gene signature in white adipocytes we analyzed the respiratory capacity in PTH-treated *in vitro* differentiated subcutaneous adipocytes using the *Seahorse*<sup>®</sup> technique. Indeed, 10nM of PTH enhanced uncoupled respiration resulting in increased maximum oxygen consumption rate (OCR) and non-ATP linked respiration (Fig. 1C,D). Given that free fatty acids (FFAs) derived from lipolysis are the primary substrate of oxidative phosphorylation in thermogenic cells we investigated potential PTH effects on lipolysis. Previous studies in mouse adipocytes had already suggested that PTH, similar to beta-adrenergic agonists, induces adipocyte lipolysis via PKA-mediated phosphorylation of hormone-sensitive lipase (HSL)<sup>13</sup>. Thus, we measured the concentrations of the lipolytic products, non-esterified fatty acids (NEFAs) and glycerol in the supernatant of PTH and/or isoproterenol stimulated in differentiated hAPCs. Both, PTH and isoproterenol markedly increased NEFA and glycerol release but the combination of PTH and isoproterenol had no additional effects (Supplementary Fig. 1B,C). In accordance with enhanced lipolysis, PTH also induced HSL activity as demonstrated by elevated pHSL/HSL protein ratios in differentiated adipocytes (Supplementary Fig. 1D,E).

Next, we investigated the effects of chronic PTH administration in an attempt to model a BAT-like phenotype in white hAPCs when applied throughout adipogenic differentiation. PTH-treated and control cells differentiated similarly with abundant lipid droplet formation and comparable expression of the adipogenic differentiation marker adipocyte protein 2

(aP2) by day 6 (Supplementary Fig. 1F,G). However, with chronic PTH stimulation, expression of UCP-1 and other thermogenic genes rose significantly in fully *in vitro* differentiated adipocytes (Fig. 2A). This was also confirmed at the protein level based on UCP-1 immunofluorescence staining (Fig. 2B). Gene expression of oxidative enzymes trended to be higher but not all reached statistical significance (Fig. 2C). These findings demonstrate that acute as well as chronic PTH administration confer a beige or BAT-like gene signature in human white adipocytes. To test whether these molecular changes had any functional consequences, we performed cellular respiration analysis. In response to chronic PTH administration maximum oxidative respiratory capacity increased significantly in accordance with a hypermetabolic cellular phenotype (Fig. 2D,E). Taken together, these data suggest PTH as a novel endocrine mediator of human adipocyte browning and increased metabolic activity.

## 4 Discussion

Activation of brown fat or reprogramming of WAT to acquire BAT-like function are effective measures to increase energy expenditure and mitigate obesity in various animal models 4, 5, 14. Accumulating evidence from clinical/translational studies suggests that also in humans, thermogenically active adipose tissue contributes to metabolic turnover, increased lipid clearance and improved glucose homeostasis 15–17. Recently, some endocrine factors including PTH have been added to the list of mediators regulating a thermogenic program in white or brown adipocytes. PTH is a peptide hormone that acts predominantly through the G-protein-coupled PTHR 1, which is primarily expressed in kidney and bone but also in adipose tissue 10. Recent work in mice has shown that both, PTH and PTH-related peptide induce browning of WAT with increased energy turnover and thereby promote muscle wasting and cachexia in models of kidney failure and lung cancer, respectively 8, 9. Here we extend these preclinical findings by demonstrating that human recombinant PTH elicits a robust thermogenic program in primary *in vitro* differentiated human subcutaneous adipocytes with increased mitochondrial respiration. These effects were present after acute as well as chronic PTH treatment, raising important questions about the physiologic relevance in patients with chronically elevated PTH concentrations. Kir and colleagues found increased expression of BAT marker genes in neck biopsies from patients with hyperparathyroidism compared to matched controls 9. Whether these molecular changes result in increased BAT activity and higher metabolic rate has yet to be investigated. Previous reports of patients with primary hyperparathyroidism are not suggestive of a healthier metabolic phenotype 18, 19. However, it is still possible that severe secondary hyperparathyroidism causes WAT browning and hypermetabolism, and contributes to the cachectic phenotype frequently seen in end stage renal disease patients.

Although very little is known about PTH signaling in adipose tissue, already more than 40 years ago it was reported that PTH induces lipolysis in rat and human fat explants with increased production of cAMP 20, 21. More recently, it was discovered that PTH promotes adipocyte lipolysis through cAMP-dependent activation of the PKA pathway, a downstream signal of the PTH receptor 13. Norepinephrine, the classic BAT activator, engages the same signaling pathway after activation of  $\beta$ -adrenergic receptors. Hence, it is conceivable that both, PTH and  $\beta$ -adrenergic agonists result in enhanced thermogenic gene expression by

employing the same molecular signals despite acting via different receptors. In fact, data presented here further substantiate this hypothesis given that both, PTH and isoproterenol independently increase HSL activity and lipolysis in human adipocytes. Since fatty acids serve as both, activators of UCP1 and fuel for thermogenesis 1 it is possible that PTH-dependent lipolysis contributes to the observed thermogenic effects.

In summary, we have demonstrated here that PTH is a potent transcriptional mediator of a thermogenic program in human white adipocytes and thereby evokes a lipolytic and hypermetabolic cellular phenotype. Our findings not only suggest a hitherto unknown endocrine parathyroid-adipose tissue axis in humans but significantly extend the limited knowledge of PTH action in human fat and may therefore have important clinical implications for conditions associated with altered PTH secretion.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>BAT</b>	brown adipose tissue
<b>WAT</b>	white adipose tissue
<b>PTH</b>	parathyroid hormone
<b>APC</b>	adipose precursor cells
<b>PTHrP</b>	parathyroid hormone related protein
<b>cAMP</b>	cyclic adenosine monophosphate
<b>PKA</b>	protein kinase A
<b>HSL</b>	hormone-sensitive lipase
<b>pHSL</b>	phosphorylated hormone-sensitive lipase
<b>UCP-1</b>	uncoupling protein 1
<b>OCR</b>	oxygen consumption rate
<b>DIO2</b>	type II iodothyronine deiodinase
<b>PGC1<math>\alpha</math></b>	peroxisome proliferator-activated receptor gamma coactivator 1-alpha

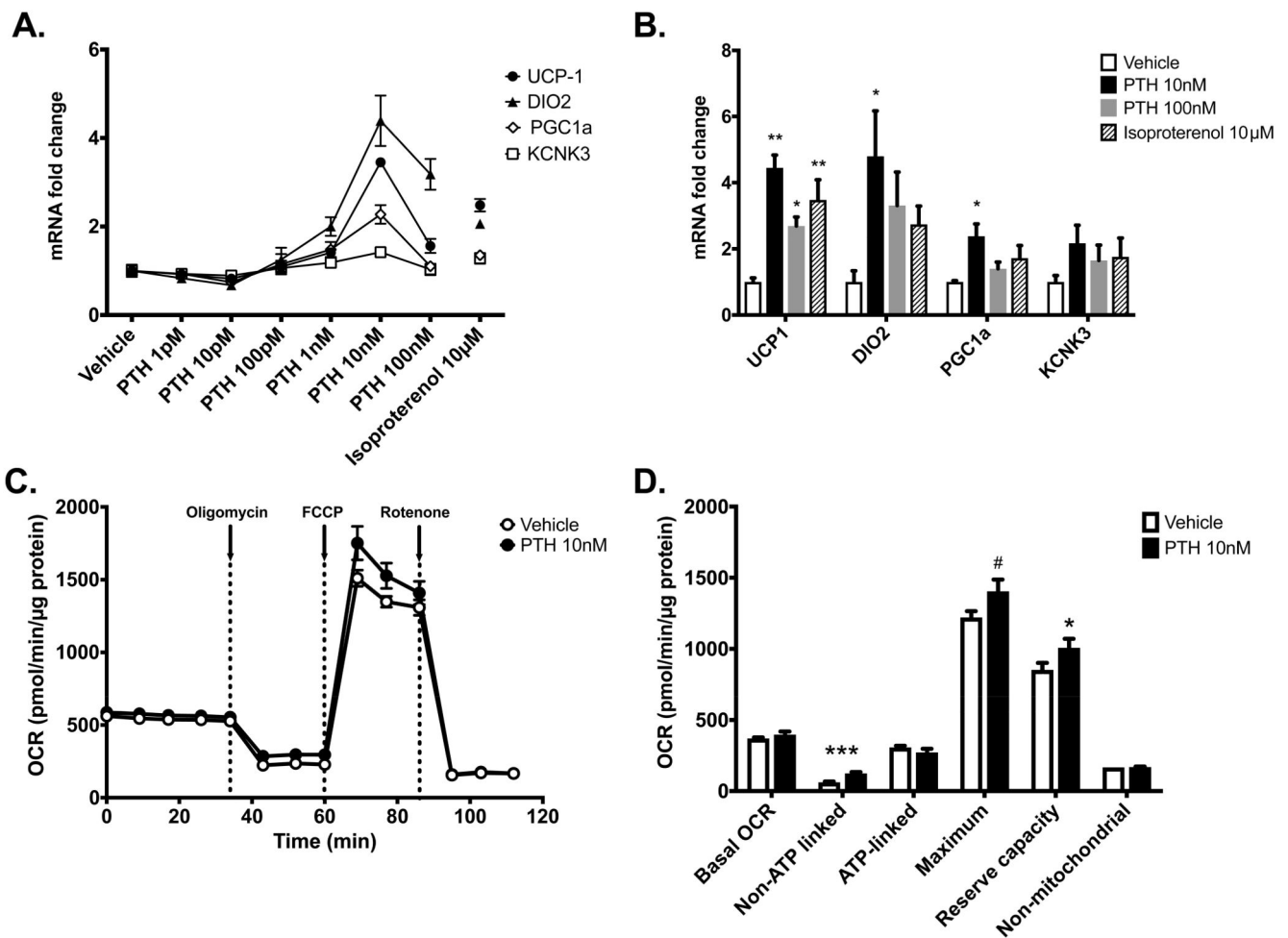
<b>KCNK3</b>	potassium channel subfamily K member 3
<b>FFA</b>	free fatty acids
<b>NEFA</b>	non-esterified fatty acids
<b>aP2</b>	adipocyte protein 2

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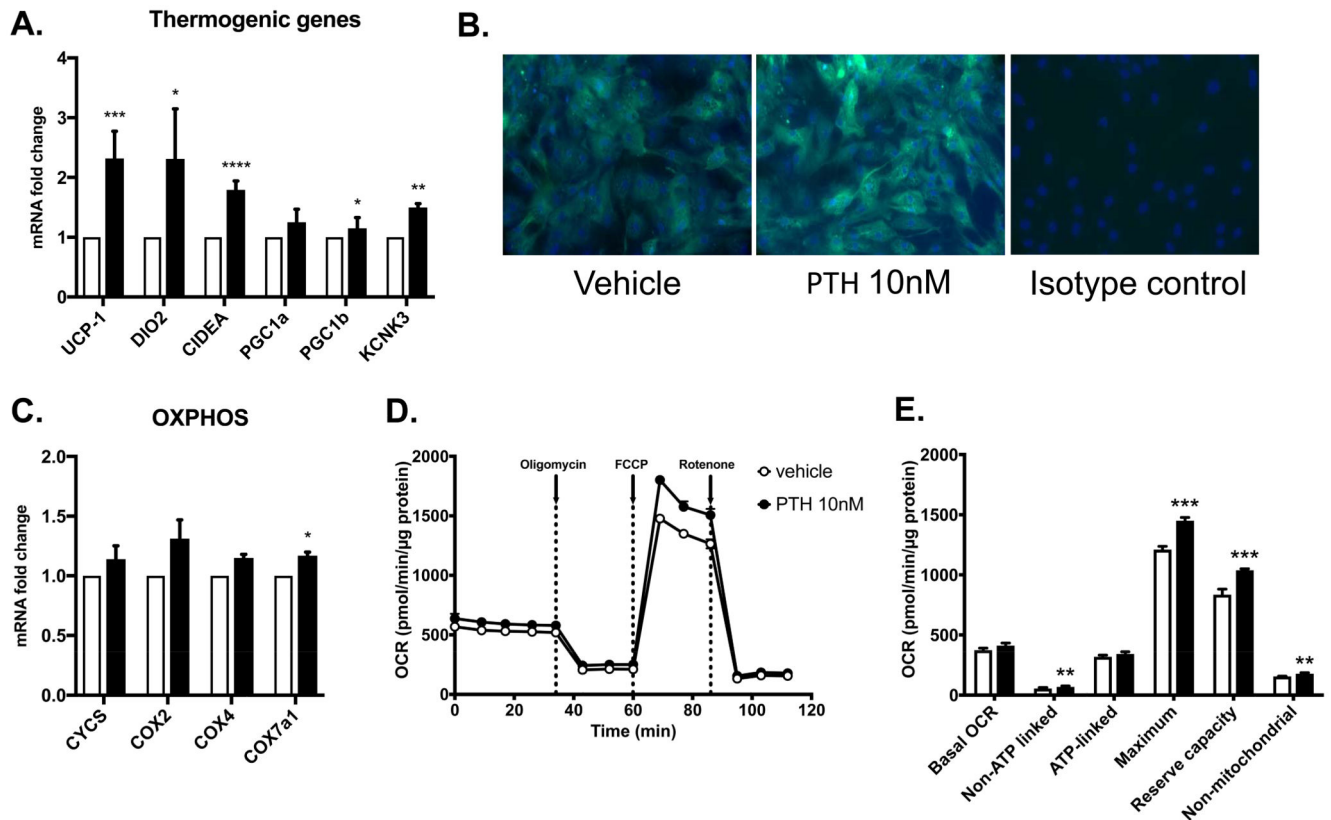
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**Figure 1. Acute PTH stimulation induces a thermogenic program in human white adipocytes.** PTH dose-response curve showing gene expression analysis of primary *in vitro* differentiated human adipocytes from a 34 year old woman (BMI 26,1 kg/m<sup>2</sup>) stimulated for 6h as indicated (A). Gene expression analysis in differentiated human adipocytes stimulated for 6h as indicated (n = 3 biological replicates from three different donors) (B). Oxygen consumption rate (OCR) in differentiated human adipocytes stimulated with 10nM PTH. Data is illustrated as real time replicate readings (n = 3 biological replicates from three different donors) (C) or as the group average for the given OCR variables (basal, non-ATP linked, ATP-linked, maximum, reserve capacity and non-mitochondrial respiration) calculated from the trace data (D). Data are given as mean ± SEM. Results were compared to vehicle. \*p < 0,05, \*\*p < 0,01, \*\*\*p < 0,001, #p = 0,056



**Figure 2. Chronic PTH treatment confers a BAT-like gene signature in differentiated human adipose precursor cells.**

Gene expression analysis of human adipose precursor cells (hAPCs) stimulated with PTH 10nM during adipogenic differentiation (n = 3 biological replicates from three different donors) (A). Representative UCP-1 immunofluorescence in fully differentiated APCs (B). Gene expression analysis of oxidative enzymes (n = 3 biological replicates from three different donors) (C). Oxygen consumption rate (OCR) of primary *in vitro* differentiated adipocytes stimulated with PTH 10nM. Data is illustrated as real time replicate readings, n = 3 biological replicates from three different donors (D) or as the group average for the given OCR variables (basal, non-ATP linked, ATP-linked, maximum, reserve capacity and non-mitochondrial respiration) calculated from the trace data for each sample (E). Data are given as mean ± SEM. Open bars represent vehicle, black bars represent PTH 10nM. Results were compared to vehicle. \*p < 0,05, \*\*p < 0,01, \*\*\* p < 0,001