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BROWNING FORMATION MARKERS OF SUBCUTANEOUS ADIPOSE TISSUE IN RELATION TO RESTING ENERGY EXPENDITURE, PHYSICAL ACTIVITY AND DIET IN HUMANS

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ABSTRACT

Background: Regular exercise and diet may contribute to white adipose tissue (WAT) conversion into brown adipose-like phenotype that may increase resting energy expenditure (REE) leading to weight loss. We examined the relationship between REE, physical activity (PA) participation and diet with browning formation markers of subcutaneous WAT in healthy men. **Materials and methods:** We assessed REE, diet and body composition of 32 healthy men [age (years): 36.06±7.36, body mass index (BMI): 27.06±4.62 (kg/m^2)]. Participants also underwent measurements of PA (MET/minute/week) using the International Physical Activity Questionnaire, while they undertook a subcutaneous fat biopsy from the abdominal region to assess the mRNA of uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα), and peroxisome proliferator-activated receptor gamma (PPARγ). **Results:** We found no associations between UCP1, PGC-1α, PPARα and PPARγ mRNAs with REE, PA levels and diet ($p>0.05$). However, PGC-1α, PPARα and PPARγ mRNAs were more expressed in individuals displaying moderate than low PA levels ($p<0.05$). Furthermore, PGC-1α, PPARα and PPARγ mRNAs were negatively correlated with fat mass percentage ($p<0.05$). PGC-1α and PPARα mRNAs were also negatively correlated with BMI, while PGC-1α mRNA was inversely associated with waist-to-hip ratio ($p<0.05$). **Conclusions:** REE, PA levels and diet are not associated with browning formation indices of subcutaneous adipose tissue in healthy adult men.

KEY WORDS: uncoupling protein 1; brown-like adipocytes; exercise; IPAQ; nutrition

LIST OF NON-STANDARD ABBREVIATIONS

BAT: Brown adipose tissue

BMI: Body mass index

FNDC5: Fibronectin type III domain-containing protein 5

IPAQ: International Physical Activity Questionnaire

MET: Metabolic equivalent

PA: Physical activity

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPARα: Peroxisome proliferator activated receptor alpha

PPARγ: Peroxisome proliferator activated receptor gamma

REE: Resting energy expenditure

UCP1: Uncoupling protein 1

WAT: White adipose tissue

WHR: Waist-to-hip ratio

Introduction

Brown adipose tissue (BAT) in humans is characterized by increased mitochondrial content and an increased ability to uncouple cellular respiration via the action of uncoupling protein 1 (UCP1) in response to cold exposure [1]. This mechanism appears to be important, since UCP1-mediated thermogenesis may represent up to 30% of resting energy expenditure (REE) in adult humans [2]. Animal studies have shown that increased BAT activity reduces weight gain, improves insulin sensitivity, lowers free fatty acid levels in serum, and reduces the risk for type 2 diabetes and other metabolic disorders [3-7]. Interestingly, some studies show that regular exercise training may increase BAT activity in animals [8-12], while evidence in humans suggests that participation in habitual physical activity (PA) is associated with BAT activity in supraclavicular and spinal areas [13]. Finally, a low protein diet [14, 15] and high-fat diet [16-19] in animals, may increase BAT activity due to diet-induced thermogenesis that occurs via UCP1 activity [20].

White adipocytes also express UCP1, which indicates a brown adipose-like phenotype that may be been linked to obesity resistance in animals [3, 5, 21]. Several animal studies have also shown that exercise may initiate the conversion of subcutaneous white adipose tissue (WAT) into brown adipose-like phenotype indicated by the presence of UCP1 and several other genes [22-25]. For instance, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is expressed in WAT [26], and peroxisome proliferator activated receptor gamma (PPARγ), can stimulate genes that are involved in the differentiation of brown fat cells [27, 28]. Furthermore, *in vitro* studies reported that PPARγ enhances UCP1 mRNA in both BAT [29] and WAT [30].

It was also hypothesized that a physical exercise mechanism can increase REE leading to body weight loss, through the induction of a brown adipose-like phenotype in WAT [31]. More specifically, PGC-1α upregulates the production of the fibronectin type III domain-containing protein 5 (FNDC5) in skeletal muscle [31]. FNDC5 is then cleaved and releases into the bloodstream a product called Irisin [31]. Irisin may bind to the surface of white adipocytes changing their phenotype into brown-like adipocytes [31]. This hypothesis was confirmed by the presence of UCP1 within WAT that may occur, at least in part, by the action of peroxisome proliferator activated receptor alpha (PPARα) [31], a transcription factor having a key role in the browning process of animal WAT [32]. Furthermore, regular exercise training increases PPARγ mRNA in WAT of mice [33, 34]. However, evidence in humans showed that regular exercise has no effect on UCP1 mRNA in WAT [35]. Given that humans display white adipocytes that may contain UCP1 [36] – the only known contributor to initiate BAT activity [1] – the presence of UCP1 in WAT cells indicates a brown adipose-like phenotype [37].

Nutrition may also play role in the presence of UCP1 in WAT. For instance, a ketogenic diet (high-fat, adequate-protein and low-carbohydrate) may increase UCP1 expression in epididymal WAT of mice independently of PA levels [38]. Overall, the actual *in vitro* and *in vivo* evidence is inadequate or incomplete to support the browning formation of WAT in response to regular exercise and nutrition. Regarding exercise, this is mainly due to different types of animals and different exercise interventions used, while relevant data from human studies are very limited. Similarly, evidence from human studies to examine the effects of diet on browning formation of WAT barely exists. Therefore, the aim of the current study was to

examine the relationship between REE, PA participation and diet with browning formation markers of subcutaneous WAT in healthy men.

Materials and methods

Participants and procedure

The study conformed to the standards set by the Declaration of Helsinki and was approved by the Ethics Committee of the University of Thessaly (protocol no. 698/2013). The inclusion criteria were: healthy adult men, non-smokers, no chronic disease and/or being under medication treatment. Thirty-two healthy men [age (years): 36.06±7.36, body mass index (BMI): 27.06±4.62 (kg/m^2)] were recruited by advertisements in a local newspaper in Trikala, Thessaly, Greece and the data collection started in July 2013 and ended in June 2014. The characteristics of the participants are shown in Table 1.

To assess energy and nutrient intake of the participants, we retrieved diet recalls from two weekdays and one weekend day that were randomly selected within a week prior to the measurements. On the day of the measurements, the participants visited the laboratory between 07:00 and 09:00 am and completed the "usual week" short form of the International Physical Activity Questionnaire (IPAQ) that has been validated for healthy Greek adults [39]. Subsequently, participants underwent the following anthropometry measurements: body height was measured using a Seca (Hamburg, Germany) stadiometer, body weight was measured using a precision scale (KERN & Sohn GmbH, Version 5.3, Germany), while waist-to-hip ratio (WHR) was assessed using a tape measure. Percent body fat and fat-free mass were measured via bioelectrical impedance using a body composition monitor (Fresenius

Medical Care AG & Co. KGaA D-61346 Bad Hamburg, Germany). Finally, the participants underwent an assessment of REE by indirect calorimetry that was followed by a subcutaneous fat biopsy.

Assessment of resting energy expenditure

Participants were instructed to transport themselves to the research laboratory in a vehicle on the day of the measurement. REE assessments were conducted between 07:00 to 09:00 am following a 12-hour fast, while participants refrained from exercise, alcohol, and passive smoking during the 72 hours prior to the measurements [40, 41]. REE was measured using an automated gas analyser (Vmax, CareFusion, USA) to record respiratory variables every 20 seconds in a supine position for 30 minutes within a quiet room maintained at 22-24°C. From the 30 minutes of the collected data, the first and last five minutes were removed [41]. The remaining 20 minutes of the collected data were averaged to obtain the final REE (kcal) value [41]. Respiratory gas measurements were extracted using the Weir equation [42] to convert VO2 and VCO2 values to REE (kcal) values.

Assessment of physical activity (PA) levels

IPAQ is self-reported and was completed by each participant at the laboratory. Detailed explanations were provided to the participants to ensure the clarity of the questionnaire while an investigator was continuously available for further clarification. The obtained data were transformed into weekly metabolic equivalent (MET-minute/week) based on low, moderate, and vigorous PA intensity, number of days/week and minutes/day of their PA participation following IPAQ guidelines [43]. More specifically, the following equations were used: a) Walking (Low level) = 3.3

MET*minutes of walking*days of activity, b) Moderate intensity level = 4 METs *minutes of walking*days of activity, and c) High (vigorous) intensity level = 8 MET*minutes of walking*days of activity. The total MET-minute/week for each participant was calculated according to the equation: MET-minute/week = Walking (MET*min*days) + Moderate (MET*min*days) + High [(vigorous) (MET*min*days)] [43].

The PA categories were grouped based on the number of days/week, minutes/day, and number of MET-minute/week of PA [43]. Specifically, the PA level of the participants that did not meet the criteria for "moderate" and/or "high" levels of PA was classified as "low". The PA level of the participants who reported: a) three or more days of vigorous-intensity activity of at least 20 minutes/day, or b) five or more days of moderate-intensity activity and/or walking of at least 30 minutes/day, or c) five or more days of any combination of walking, moderate-intensity or vigorous-intensity activities with a minimum total PA of at least 600 MET-minute/week, was classified as "moderate" [43]. Finally, the PA level of the participants who reported: a) vigorous-intensity activity on at least three days/week with a minimum total PA of at least 1500 MET-minute/week, or b) seven days/week of any combination of low, moderate, or vigorous intensity activities with a minimum total PA of at least 3000 MET-minute/week was classified as "high" [43].

Subcutaneous fat biopsies

All biopsies were executed by an experienced surgeon following a previous methodology [44] based on a standardised non-diathermy method (Chachopoulos et. al. 2017, unpublished data). The participants underwent a subcutaneous fat biopsy after at least a 12-hour fast [45]. Each participant was positioned on a surgical bed in

a supine position. The site of the incision was disinfected and a 10 ml of 2% xylocaine (no adrenaline) was injected in the region of the incision for local anaesthesia. An incision was made until the adipose tissue was revealed that was approximately 3-5 cm to the left or right of the navel while the incision length was approximately 2-2.5 cm. Subsequently, the subcutaneous tissue was removed with operating scissors and when the adipose tissue became visible nearly 500 mg of adipose tissue was captured and removed. The collected adipose tissue sample was immediately immersed in liquid nitrogen at -190°C. For the final deposition, the samples were placed in Eppendorf tubes and they were deposited in a freezer at -80°C until analysis.

Gene expression analysis

The analysis of gene expression has been done by an investigator who was blinded to the aim of the study. Total RNA was extracted from adipose tissue biopsies using RNeasy Lipid Tissue mini kit (QIAGEN) following the manufacturer's protocol. First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega). Quantitative real time polymerase chain reaction for the UCP1, PGC-1α, PPARα, and PPARγ genes was performed using Sybr Green fluorophore. The change in fluorescence at every cycle was monitored and a threshold cycle above background for each reaction was calculated. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out in at least duplicated for every sample. 18S rRNA gene was constantly expressed under all experimental conditions and was then used as a reference gene for normalization [46].

Assessment of diet

The participants were contacted by telephone – at 22:00 pm, after their last meal – on three separate randomly-selected days (two weekdays, one weekend day) during a one-week period for the completion of a 3-day diet record. All food and beverages consumed on the day of contact were recorded. All diet records were analysed by a trained investigator using the software Nutritionist Pro, Version 5.4.0, Axxya Systems (Redmond, WA, USA). This software has been previously used for research purposes [47, 48]. For the analysis, a search was conducted in Nutritionist Pro for each food and beverage consumed. Details regarding the amount and preparation of each food and/or beverage were also included. Once all the relevant information regarding the food or beverage was entered the software, a corresponding list of macro and micronutrient content was provided and subsequently saved. This process was repeated for all food and beverages listed on the diet record. If a food or beverage was not found within the Nutritionist Pro database, the investigator manually entered the macro and micronutrient content of that food and saved it to the database for future use. The feedback provided by the software for each food or beverage used in this study included the total energy intake (kcal), total weight of food (gr), protein (gr), carbohydrate (gr), total fat (gr), sugar (gr) and caffeine (mg).

Statistical analysis

Following previous methodology, we removed the mean values (i.e. outliers) of UCP1, PGC-1α, PPARα and PPARγ mRNAs that were at a distance of more than two standard deviations from the mean of the distribution [13, 49]. This analysis resulted to the removal of two data points of PGC-1α, one of PPARα and two of

PPARγ, while UCP1 values displayed no outliers. Non-parametric tests were used throughout. We examined associations between UCP1, PGC-1α, PPARα, and PPARγ mRNAs with REE, PA (MET-minute/week), energy intake (kcal), total weight of food (gr), protein (gr), carbohydrate (gr), total fat (gr), sugar (gr) and caffeine (mg) as well as age, BMI, WHR, fat mass percentage, fat free mass (kg), using Spearman correlation coefficient. We used Mann-Whitney U tests to assess differences of UCP1, PGC-1α, PPARα and PPARγ mRNAs due to variation in PA levels (low/moderate) and Kruskal-Wallis analysis of variance with post hoc Mann-Whitney U tests to assess differences of BMI categories (normal/overweight/obese). All analyses were conducted with SPSS (version 22; SPSS Inc., Chicago, IL, USA) and a $p \leq 0.05$ level of significance.

Results

We found no associations between the mRNAs of UCP1, PGC-1α, PPARα and PPARγ genes with REE and PA levels ($p>0.05$). Mann–Whitney U tests showed that PGC-1α ($z=-2.468$, $p=0.01$), PPARα ($z=-2.093$, $p=0.04$) and PPARγ ($z=-1.998$, $p=0.05$) mRNAs were more expressed in individuals displaying moderate than low PA levels (Figure 1), while the IPAQ data analysis revealed that none of the participants displayed high PA levels. Finally, we found no associations between UCP1, PGC-1α, PPARα and PPARγ mRNAs with age, energy intake (kcal), total weight of food (gr), protein (gr), carbohydrate (gr), total fat (gr), sugar (gr) and caffeine (mg).

The PGC-1α mRNA was negatively correlated with fat mass percentage (rho=-0.69, $p=0.001$) (Figure 2A), BMI (rho=-0.78, $p=0.001$) (Figure 2B), and WHR (rho=-0.62, $p=0.001$) (Figure 2C). Similarly, the PPARα mRNA was negatively

associated with fat mass percentage (rho=-0.45, p=0.01) (Figure 3A) and BMI (rho=-0.45, p=0.01) (Figure 3B), while the PPARγ mRNA was negatively correlated with BMI (rho=-0.40, p=0.03) (Figure 4). Additionally, individuals with normal BMI (19-24.9 kg/cm^2) revealed increased PGC-1α mRNA (z=-2.276, p=0.02) compared to overweight individuals (BMI 25-29.9 kg/m^2) (Figure 5). Also, individuals with normal BMI revealed increased PGC-1α mRNA (z=-3.220, p=0.001), PPARα mRNA (z=-1.987, p=0.05) and PPARγ mRNA (z=-2.167, p=0.03) compared to obese individuals (BMI ≥30 kg/m^2) (Figure 5). Finally, overweight individuals (BMI=24.9-29.9 kg/cm^2) revealed increased PGC-1α mRNA (z=-2.325, p=0.02) compared to obese individuals (BMI ≥30 kg/m^2) (Figure 5).

Discussion

The aim of the current study was to examine the relationship between REE, PA participation and diet with browning formation markers of subcutaneous WAT in healthy men. We found no associations between UCP1, PGC-1α, PPARα and PPARγ mRNAs and REE. In contrast to previous evidence showing that UCP1 in WAT was positively correlated with REE and negatively with BMI and weight loss [31], the current study could not confirm any link between UCP1 mRNA in WAT and metabolism indices (i.e. REE, BMI, WHR, fat mass and fat-free mass). Considering the recent finding that UCP1 mRNA in WAT is negatively associated with weight loss in healthy women [50] we could hypothesize the opposite; that is, increased REE and body weight loss could affect UCP1 in WAT. Nevertheless, this remains to be elucidated. Previous data also showed that PPARγ promotes brown adipose-like phenotype of WAT [23, 30] and it would be expected to be positively associated with REE since increased REE may be the result of browning formation in WAT [51-53].

However, the PPARγ activation in BAT may promote REE via fatty acid oxidation, although, this process may be suppressed in WAT because of the repression of PPARγ by the steroid receptor coactivator-2, which increases triglyceride accumulation and decreases free fatty acids in WAT [54]. This may explain, at least in part, the findings in the current study.

Based on a recent suggestion that regular exercise training may transform WAT cells into brown-like adipocytes [31, 55], we would expect to observe that individuals with increased PA levels would display increased UCP1 mRNA – the direct marker of browning formation of WAT [37]. However, we found no association between UCP1 mRNA and PA levels. This finding is in line with the only controlled trial published to date reporting a non-significant increase of UCP1 mRNA in subcutaneous WAT of healthy adults in response to a 12-week exercise training intervention [35]. Furthermore, a recent single group design study showed no change in the gene expression of browning markers (including UCP1) in WAT of healthy women after a 16-week exercise training intervention [50].

As previously suggested, PGC-1α, PPARα and PPARγ mRNA can affect UCP1 to cause browning formation in WAT [30, 31, 56]. We found no associations between the mRNAs of PGC-1α, PPARα and PPARγ genes with PA levels. However, we observed that individuals with moderate PA levels express more PGC-1α, PPARα, and PPARγ mRNAs than individuals with low PA levels. PGC-1α is increased in WAT in response to exercise in mice [23, 57]. In humans however, PGC-1α mRNA did not change after an 8-week resistance training intervention [58]. PPARα was suggested to increase UCP1 mRNA in WAT in response to exercise [31] while PPARγ of subcutaneous fat of mice was not altered in response to an 8-

week exercise programme [59] or it was increased in response to chronic exercise in mice [33].

Collectively, activation of PGC-1α, PPARα and PPARγ genes could indicate browning formation of WAT through the increase of UCP1 expression [31, 32, 60]. Nevertheless, even though we observed that PGC-1α, PPARα and PPARγ mRNAs are more expressed in individuals that display moderate than low PA levels, this was not accompanied by a relevant association with UCP1 mRNA. Also, we found no positive associations between PGC-1α, PPARα, and PPARγ mRNAs with REE. Increased REE may result from browning formation in WAT [51-53] while PA levels are positively associated with REE in humans [61]. Notably, we did not detect an association between PA levels and REE in the current study. Furthermore, we found that none of the genes examined in the current study are associated with the diet of our participants. In this regard, a previous animal study showed that a ketogenic diet (high-fat, adequate-protein and low-carbohydrate) could increase UCP1 in WAT [38]. Our participants did not follow a ketogenic diet, which may explain our findings.

The inverse association of PGC-1α mRNA with fat mass, BMI and WHR, indicates low demand of energy in WAT given that PGC-1α increases fatty acid oxidation in mice [62, 63], which designates increased demands of energy. An inverse association between PPARα mRNA with fat mass and BMI, was also found. PPARα is activated under conditions of energy deprivation [64] and its activation promotes the catabolism of fatty acid beta oxidation [65]. Given that WAT is mainly responsible for energy storage [66], the inverse associations of PPARα mRNA with fat mass and BMI indicate increased energy storage in WAT. Furthermore, PPARγ mRNA was inversely associated with BMI in the current study. PPARγ increases fatty acids uptake in WAT via the improvement of insulin sensitivity [67], while it

increases glucose uptake in WAT [68]. Given that free fatty acids are positively correlated with increased adiposity in adults [69] the observed inverse association of PPARγ mRNA with BMI in the current study seems believable.

It is reasonable to assume that the present results may have been influenced by methodological limitations such as the lack of prior power calculation to determine an appropriate sample size. However, this was not possible given the lack of similar design studies that could be utilised to perform sample size calculations. Therefore, a post-measurement power calculation was conducted using an online software (DSS Research) to test >95% statistical power. This revealed 100% of power based on the UCP1 mRNA value (0.27±0.15) we detected in our study and expected UCP1 mRNA value (0.00010±0.0003) from a previous controlled trial that examined the effect of exercise on UCP1 mRNA of subcutaneous WAT in humans [35]. The study only measured mRNAs of the genes examined. The quantity of mRNA indicates the transcription levels of a specific gene which could be associated to protein concentrations. However, it is well known that protein concentrations cannot be predicted from mRNA levels [70-72] as one molecule of mRNA may be used several times to encode the same protein [70-72]. Therefore, an analysis of the protein concentrations should be performed in the future to assess whether these proteins are involved in the browning formation of WAT in humans.

UCP1 mRNA is stimulated by thyroid hormones such as triiodothyronine [73]. Unfortunately, we were unable to measure thyroid hormones in our study to determine any potential relationship with UCP1 mRNA. Even though the thyroid hormones were not measured, our participants had no clinical symptoms or characteristics of overt hypothyroidism. Also, hypothyroidism is more common in women than in men (~7 fold) [74]. Given that our participants were only men, we are

confident that their thyroid status could not affect UCP1 mRNA. Additionally, it is important to note that our participants were not highly active, as we did not detect individuals with high PA levels. Furthermore, even though a 3-day diet recall is considered an optimal tool to assess diet [75], it may also underestimate the energy intake [76]. Finally, our findings are different from those in animal studies, which can be primarily explained by the different conditions that our participants were exposed compared to animals (i.e. PA levels, diet).

CONCLUSIONS

We conclude that REE, PA levels and diet are not associated with browning formation markers of subcutaneous adipose tissue in healthy men. This reflects the conditions that our participants were exposed regarding the PA levels and diet. UCP1 mRNA in WAT is not linked to body weight and composition (i.e. BMI, WHR, fat mass). PGC-1α, PPARα and PPARγ mRNAs are negatively associated with increased fat mass accumulation parameters (BMI, WHR and fat mass) suggesting a positive association of PGC-1α, PPARα and PPARγ with lipid catabolism in WAT. The mechanisms of the latter associations remain to be determined.

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TABLES

Table 1: Characteristics of the participants

	Mean ± Standard deviation
Age	36.06±7.36
Waist to hip ratio	0.91±0.07
Body mass index	27.60±4.62
Fat mass %	28.32±8.57
Fat-free mass (kg)	52.90±5.02
MET/minute/week	914±736

MET: Metabolic equivalent

FIGURES

Figure 1: UCP1, PGC-1α, PPARα and PPARγ mRNA expressions of subcutaneous white adipocytes in healthy men due to variation of low and moderate physical activity levels

*Significant differences between low and moderate levels of PGC-1α (p=0.01), PPARα (p=0.04) and PPARγ (p=0.05) mRNA expressions. UCP1: Uncoupling protein 1; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARα: peroxisome proliferator activated receptor alpha; PPARγ: peroxisome proliferator activated receptor gamma.

Figure 2: Associations of fat mass, body mass index and waist to hip ratio with PGC-1α mRNA expression of subcutaneous white adipocytes in healthy men

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

Figure 3: Associations of fat mass and body mass index with PPARα mRNA expression of subcutaneous white adipocytes in healthy men

PPARα: peroxisome proliferator activated receptor alpha

Figure 4: Associations of body mass index with PPARγ mRNA of subcutaneous white adipocytes in healthy men

PPARγ: peroxisome proliferator activated receptor gamma

Figure 5: UCP1, PGC-1α, PPARα and PPARγ mRNA expressions of subcutaneous white adipocytes in healthy men due to variation of body mass index

*Significant differences between normal and overweight individuals of PGC-1α mRNA (p=0.02)
Significant differences between normal and obese individuals of PGC-1α mRNA (p=0.001), PPARα mRNA (p=0.05) and PPARγ mRNA (p=0.03).
£ Significant differences between overweight and obese individuals of PGC-1α mRNA (p=0.02)
UCP1: Uncoupling protein 1; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARα: peroxisome proliferator activated receptor alpha; PPARγ: peroxisome proliferator activated receptor gamma