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Parathyroid Hormone Suppresses Insulin Signaling in Adipocytes

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Summary

Previous reports suggest that parathyroid hormone (PTH) is associated with insulin resistance. This research investigated the effects of PTH on insulin signaling in differentiated 3T3-L1 adipocytes. PTH (10 nM, 24 h) treatment induced a reduction in insulin-stimulated glucose uptake, AKT activity (phosphorylated AKT/total AKT protein expression) and a decrease in GLUT4 and IRS-1 protein expression compared to vehicle treated controls in differentiated adipocytes. PTH treatment also induced increased phosphorylation of IRS-1 on serine 307, which suppresses insulin signaling. In addition, treatment of cells with adenylyl cyclase inhibitor SQ52236 ameliorated the effects of PTH on insulin-stimulated glucose uptake, whereas inhibition of phospholipase C α (U73122) did not significantly alter the effects of PTH. Thus, PTH treatment of differentiated 3T3-L1 adipocytes suppresses insulin-stimulated glucose uptake and insulin signaling via cAMP pathway, potentially through the phosphorylation of IRS-1 at serine 307.

Keywords

insulin receptor; insulin receptor substrate-1; glucose; parathyroid hormone

Introduction

The physiological actions of parathyroid hormone (PTH) on bone health (Reeve, 1996) and regulation of blood calcium levels (Brown, 1991) have been extensively described. In this regard PTH is secreted by the parathyroid glands as a polypeptide containing 84 amino acids (Murray et al., 2005) and acts to regulate plasma calcium homeostasis (Brown, 1991). However, in addition to the classical actions of PTH on calcium metabolism, there is evidence showing that PTH is positively associated with the prevalence of diabetes. For example, an elevated level of PTH is associated with abnormal glucose metabolism (DeFronzo et al., 1983; Kumar et al., 1994). In an epidemiological study (n=1,071, 40–65 yrs), serum PTH was positively correlated with glucose level (Wareham et al., 1997). Further, plasma PTH level is inversely related to insulin sensitivity in 52 healthy subjects (Chiu et al., 2000). Likewise, a two- to four-fold higher prevalence of diabetes mellitus is observed in patients with high levels of PTH (Ljunghall et al., 1983). The relationship between PTH and insulin resistance is also supported by intervention studies as parathyroidectomy, as well as a reduction in serum PTH concentration in patients with high levels of PTH, served to normalize blood glucose levels (Akmal et al., 1985; Mak, 1985; Mak, 1983). Furthermore, when exogenous PTH is

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administered to laboratory animals there is an increase in plasma glucose concentrations and greater area under the curve (AUC) during glucose tolerance testing (Perna et al., 1990). These results suggest that PTH may interfere with either the ability of the pancreas to release insulin, the actions of insulin on glucose metabolism, or both.

Several studies have investigated the direct effects of PTH on insulin action in cultured cells. PTH treatment of osteoblast cell type, UMR 106-01, inhibited basal and insulin-stimulated glucose transport coincident with a reduction of glucose transporter 1 (GLUT1) mRNA (Thomas et al., 1995). In rat adipocytes, short term (1 h) PTH treatment decreased insulin stimulated glucose uptake by 30% and concomitantly enhanced phosphorylation of GLUT4 to reduce insulin-stimulated translocation of the transporter to the cell surface (Reusch, 1991). However the mechanism by which PTH suppresses glucose uptake has not been fully explored in differentiated adipocytes, highly insulin responsive cell types. Work described here tests the hypothesis that PTH acts in differentiated adipocytes to suppress insulin signaling via a cAMP mediated pathway.

Materials and Methods

Reagents and Chemicals

Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), TRIzol reagent, penicillin-streptomycin mixture, and 0.25% trypsin-EDTA were purchased from Gibco BRL Life Technologies (Rockville, MD). Calf serum was purchased from BioWhittaker (Walkersville, MD) and 2-deoxy- [3H]-glucose (specific activity: 9 μ Ci/mmol) was from Amersham (Piscataway, NJ). Human PTH-(1–84) was from Bachem (Torrance, CA). 2-deoxy-glucose, cytochalasin B, protease inhibitor cocktail, phosphatase inhibitor cocktail and RIPA buffer were from Sigma (St. Louis, MO). Insulin receptor antibody was from Santa Cruz (Santa Cruz, CA). Antibodies for IRS-1, phospho-IRS-1 (Ser 307), total AKT and phospho-AKT (Ser 473) were from Cell Signaling (Danvers, MA), GLUT1 and GLUT4 from Alpha Diagnostics (San Antonio, Texas). PCR Nucleotide Mix, RNasin Rnase Inhibitor, Oligo dT, Random Primers and MMLV reverse transcriptase were from Promega (Madison, WI). Inhibitors SQ52236 (adenyl cyclase) and U73122 (PLC) were obtained from Biomol (Plymouth Meeting, PA).

Cell Culture

Murine 3T3-L1 cells obtained from American Type Culture Collection (ATCC CL-173; Manassas, VA) were maintained in high glucose DMEM, 10% (vol/vol) calf serum with 100 U/ml penicillin, 100 μ g/ml streptomycin at 37°C in 95% air and 5% CO₂. Preadipocytes were induced to differentiate with 1 μ g/ml human insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 μ M dexamethasone in DMEM containing 10% FBS. After 2 days, the medium was replaced with DMEM containing 10% FBS, antibiotics and insulin and was renewed every two days. When more than 95% of the cells contained lipid droplets, the medium was changed with DMEM containing 10% FBS.

Determination of Insulin-Stimulated Glucose Uptake

Insulin-stimulated glucose uptake experiments were completed as described previously (Sweeney et al., 1999). Briefly, cells were grown in 12-well tissue culture plates and differentiated as described above. Cells were treated with PTH at the indicated times. Differentiated adipocytes were incubated in serum-free DMEM for 3 h prior to uptake experiments. Cell monolayers were washed twice with HEPES-buffered saline (140 mM NaCl, 20 mM Na-HEPES, 2.5 mM MgSO₄, 1 mM CaCl₂, 5 mM KCl, pH 7.4) and pretreated with insulin (20 min, 100 nM) followed by incubation for 10 min in HEPES-buffered saline (pH 7.4) containing 100 μ M unlabeled 2-deoxyglucose and 0.5 μ Ci/ml 2-deoxy- [3H]-glucose. The

reaction was terminated by washing three times with ice-cold 0.9% NaCl (w/v). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B. Cell associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting. Total cellular protein was determined by the bicinchoninic acid (BCA) Assay (Pierce, Rockford IL) (Smith, 1989).

mRNA Expression

Isolation of RNA was performed using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was further purified using RNeasy and RNase-free DNase kits (Qiagen, Valencia, CA). cDNA was prepared using oligoDT and random hexamer primers and the Omniscript cDNA kit from Qiagen, according to the manufacturers protocol. Primers used are shown in Table 2. RT-PCR was carried out using Brilliant SYBR Green QPCR Master Mix (Stratagene, Cedar Creek, TX) in at least triplicate using a thermocycler (Mx3000P, Stratagene) as follows: 95°C for 30 seconds, 55°C for 1 min, and 72°C for 30 to 40 seconds to achieve optimal amplification for each primer pair. Expression levels were determined by the ΔCt method using. Primer sequences for mouse insulin receptor (IR), insulin receptor substrate-1 (IRS-1), GLUT1, GLUT4, CAP and 18S are given in Table 1.

Western Blotting

Relative protein levels were assessed for insulin receptor, phospho-IRS-1, total IRS-1, GLUT1, GLUT4, phospho-AKT and total AKT. Fully differentiated 3T3-L1 cells were treated with PTH (10 nM) at indicated time points with or without insulin stimulation (100 nM, 20 min). Subsequently, cells were washed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1.0% Igepal CA-630, 0.5 % sodium deoxycholate and 0.1% sodium dodecyl sulfate, pH 8.0) with 1% protease inhibitor and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Protein concentration was determined by BCA assay. Equal protein levels were loaded in each lane of 5% (insulin receptor, phospho-IRS-1 and total IRS-1) or 10% (GLUT1, GLUT4, phospho-AKT and total AKT) SDS-polyacrylamide gels. Proteins were separated by electrophoresis and transferred to nitrocellulose membrane with Transblot apparatus (Bio-Rad Laboratories Inc., Hercules, California, USA). For immunoblotting, membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl) containing 0.1% Tween (TBS-T) for 1 h, washed twice with TBS-T, and incubated overnight with primary antibodies at 4°C in the blocking buffer. After washing, blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Bound antibody was visualized using Enhanced Chemiluminescence method, according to the manufacturer's instructions (ECL, Amersham). Density of immunoreactive bands was assessed by Scion Image (Frederick, Maryland); film exposure times were in the linear range of delectability. The results were expressed as fold change compared to vehicle.

To measure AKT activation, membranes were exposed to phospho-Akt (Ser473) antibody (Cell Signaling) followed by incubation with the secondary antibody. The blots were stripped with stripping solution (Chemicon) for 15 min at room temperature and reprobed with specific antibody to total AKT. To determine activation of IRS-1, blots were probed with 1:1000 dilution of phospho-IRS-1 (Ser307) specific antibody (Cell Signaling). After stripping, membranes were reprobed with total IRS-1 antibody. Data are expressed as the ratio of phospho-protein to total protein.

Statistical analysis

Data were analyzed by the ANOVA procedure using the Statistical Analysis System (SAS 9.1) software. Differences among groups were determined using Student-Newman-Keuls procedure at the $p < 0.05$ level. All data are presented as means $\pm$ SE.

Results

To investigate the impact of PTH on insulin sensitivity, insulin stimulated glucose uptake was determined following treatment of differentiated adipocytes with vehicle or PTH treatment. PTH decreased insulin-stimulated glucose uptake compared to controls in a time and dose-dependent manner with a significant reduction evident at 1 nM PTH (Figure 1). In contrast treatment of differentiated adipocytes with PTH alone did not alter basal glucose uptake (data not shown).

To assess the impact of PTH on insulin signaling, insulin-induced activation of AKT, a downstream target of insulin signaling, was determined. PTH treatment (10 nM) significantly reduced insulin-stimulated AKT activity (12%) in differentiated adipocytes compared to vehicle (Figure 2). The reduced activation of AKT by insulin following PTH treatment is consistent with the effects of PTH to reduce insulin-stimulated glucose uptake.

Exposure of differentiated 3T3-L1 adipocytes to PTH for 24 h significantly reduced mRNA and protein expression of IRS-1 (Figure 3). Although insulin receptor mRNA abundance was reduced, the abundance of insulin receptor α protein was not altered (Figure 3). In addition, the mRNA and protein abundance of GLUT4 was reduced following PTH compared to vehicle treatment (Figure 4). However, although GLUT 1 mRNA was reduced, protein levels were not altered with PTH treatment (Figure 4). In contrast, the abundance the c-Cbl-associated protein CAP mRNA, was not different with PTH treatment (0.98 ± 0.05) compared to control (1.00 ± 0.07). CAP serves as an adaptor protein recruited to the insulin receptor as a component of the pathway involved in the insulin-stimulated translocation of GLUT4 to the membrane (Kimura et al., 2001). These results support that the change in mRNA expression of insulin receptor, IRS-1, GLUT1 and GLUT4 was specific and not a general cellular effect.

The mechanism by which PTH treatment inhibits insulin-stimulated glucose uptake was explored by assessing the level of phosphorylation of IRS-1 serine 307 site, which inhibits IRS-1 activity. PTH treatment of differentiated adipocytes increased IRS-1 serine 307 phosphorylation:total IRS-1 compared to vehicle-treated control (Figure 5).

Finally, the role of PTH mediated cAMP or PLC activation on insulin stimulated glucose uptake was assessed. Cells were treated with cAMP inhibitor (SQ22536, 100 μ M) or PLC inhibitor (U73122, 10 μ M) in the presence and absence of PTH and insulin-stimulated glucose uptake assessed. The action of PTH to inhibit insulin stimulated glucose uptake was ablated when cells were incubated with SQ22536, whereas treatment with U73122 to inhibit of PLC was without effect (Figure 6). These results suggest that PTH mediates a reduction in insulin signaling via adenylate cyclase activity.

Discussion

Abnormal glucose metabolism (DeFronzo et al., 1983; Kumar et al., 1994) and a high prevalence of diabetes (Ljunghall et al., 1983) have been reported in patients with high blood levels of PTH (Wareham et al., 1997; Chiu et al., 2000; Prager et al., 1983). In the current work, our results demonstrate that PTH inhibits insulin signaling in adipocytes via adenylate cyclase and phosphorylation of IRS-1 on serine 307. These results support the clinical and epidemiological studies demonstrating an association between serum PTH levels and abnormal glucose metabolism or diabetes.

The results of the current study are consistent with previous studies showing that PTH leads to altered glucose homeostasis. PTH treatment leads to a biphasic response in rat osteogenic sarcoma UMR 106-01 cells such that insulin stimulated glucose uptake was increased within 1 h, but was suppressed after 16 h (Thomas et al., 1995). PTH treatment decreased insulin-

stimulated glucose uptake within 1 h in rat adipocytes (Reusch, 1991). Cumulatively, these results show that PTH inhibits insulin-stimulated glucose uptake similar to the results shown in the current study.

A downstream component of insulin signaling, the serine/threonine kinase AKT, plays a central role in the metabolic actions of insulin and is a marker for insulin signaling (Farese et al., 2005). For example, AKT activation is reduced in patients with type 2 diabetes (Krook et al., 1998) and overexpression of constitutively active AKT results in increased glucose uptake in 3T3-L1 adipocytes (Tanti et al., 1996). Our results demonstrate that PTH treatment of differentiated adipocytes decreased insulin-stimulated AKT activity at 24 h (Figure 3). These results are consistent with the effects of PTH on insulin-stimulated glucose uptake and support an effect of PTH to inhibit insulin signaling in adipocytes.

PTH treatment decreases mRNA levels of IR, IRS-1, GLUT1, and GLUT4. This effect was specific as the levels of CAP mRNA were not reduced. However, only the protein levels of IRS-1 and GLUT4 were reduced with PTH treatment. Therefore, it is unlikely that the decrease in GLUT1 mRNA plays a role in the decrease in insulin-stimulated glucose uptake. GLUT1 is primarily responsible for basal glucose uptake. The lack of effect of PTH treatment on basal glucose uptake is consistent with the lack of change in GLUT1 protein expression noted in our studies. It is possible that although the expression of IR α protein is not altered with PTH treatment, the expression of IR β may be altered and therefore may contribute to the reduced insulin signaling. The reduction of IRS-1 and GLUT4 protein expression suggest that these may play a role in the decrease in insulin-stimulated glucose uptake mediated by PTH treatment in adipocytes.

Serine phosphorylation of IRS-1 at residue 307 (pSer307 IRS-1) mediates TNF- α inhibition of insulin signaling (Lorenzo et al., 2008). Phosphorylation of IRS-1 at residue 307 inhibits IR tyrosine kinase activity (Aguirre et al., 2000; Aguirre et al., 2002; Pirola et al., 2004; Sun et al., 1992), which results in insulin resistance *in vivo* (Ishibashi et al., 2001) and *in vitro* (Will et al., 2002). Evidence supports that TNF- α mediates this phosphorylation event, at least in part, via activation of inhibitor κ B kinases (Lorenzo et al., 2008). Indeed, similar to PTH treatment of adipocytes in the current study, TNF α treatment decreased the concentration of IRS-1 and GLUT4 in adipocytes (Stephens et al., 1997; Wang et al., 1998) and induced serine phosphorylation of IRS-1 (Ruan et al., 2002a,b). PTH stimulated TNF-related activation-induced cytokine (TRANCE) expression in murine bone marrow cultures (Lee and Lorenzo, 1999) and caused dose and time-related increases in NF κ B DNA binding in Saos-2 human osteoblastic (hOB) cells similar as the TNF α -induced NF κ B activation (Ali et al., 1999). It is intriguing that both PTH and TNF are known to increase the expression of receptor activator of nuclear factor- κ B ligand (RANKL) (Dai et al., 2006), which can activate the nuclear factor κ B (Wong et al., 1998). Thus, because PTH and TNF α stimulate NF κ B activation, which is known to mediate the TNF α phosphorylation of serine 307 of IRS-1, they may function to induce serine phosphorylation of IRS-1 through similar molecular mechanisms. Further research is needed to delineate the molecular pathway by which PTH induces serine 307 phosphorylation of IRS-1.

Liganded PTH receptors activate G-proteins, leading to stimulation of adenylate cyclase and formation of cAMP (Tovey et al., 2006). Adenylate cyclase is the most commonly studied pathway for cellular responses to PTH, but liganded PTH receptors can also activate phospholipase C, protein kinase C, phospholipase D, and phospholipase A₂ (Tovey et al., 2006). In the current study, the addition of a cAMP inhibitor (SQ22536) blunted the PTH-induced decrease in glucose uptake whereas the addition of a PLC inhibitor (U73122) was without effect. Our result implicates a cAMP pathway mediating PTH effects in differentiated adipocytes to reduce insulin-stimulated glucose uptake and inhibit insulin signaling. Further

efforts to address a role of PTH-activated cAMP in insulin signaling will contribute to clarify the molecular mechanisms of PTH in insulin resistance.

Finally, the implications of a PTH mediated inhibition of insulin signaling in adipocytes on whole body insulin sensitivity have not been explored specifically. However, there is experimental evidence that links high levels of PTH with abnormal glucose metabolism (DeFronzo et al., 1983; Kumar et al., 1994) or increased risk for diabetes (Ljunghall et al., 1983). Interventions to normalize PTH in patients with elevated PTH levels also improved or normalized blood glucose levels (Akmal et al., 1985; Mak, 1985; Mak et al., 1983). Furthermore, an adipose specific reduction in GLUT4 has been shown to reduce whole body insulin sensitivity in a transgenic mouse model (Abel et al., 2000), demonstrating the impact of reduced glucose uptake in adipocytes on whole body insulin sensitivity. Our results suggest a mechanism by which PTH may impact overall body insulin resistance by reducing insulin signaling in the adipocytes. Further research is needed to determine the existence and implications of this mechanism of PTH action *in vivo*.

In summary, PTH decreases insulin-induced glucose transport in a dose-dependent and adenylate cyclase dependent manner, reducing protein expression of IRS-1 and GLUT4 and increasing the phosphorylation of IRS-1 on serine 307. Therefore, these cellular events in adipocytes may underlie the association of high serum levels of PTH with insulin resistance and incidence of diabetes.

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Figure 1. PTH Decreases Insulin-Stimulated Glucose Uptake in a Time and Dose Dependent Manner

Differentiated adipocytes were treated for the indicated times with 10 nM PTH (A) and at the indicated doses of PTH for 24 h (B). Insulin-stimulated (100 nM for 20 min) uptake of 2-deoxy- $[^3\text{H}]$ -glucose (100 μM) was determined in differentiated adipocytes (10 min). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B. Results for cytochalasin B samples was subtracted from each value, and results expressed (% of basal) as the percent of the mean of the same treatment (vehicle or PTH) without insulin stimulation (basal). The value of each bar represents the mean $\pm$ S.E (n = 4~6) from at least 3 experiments. Bars with an

asterisk (*) are different ($p < 0.05$) from vehicle control (panel A) and bars with different letters (panel B) differ among PTH treatment groups ($p < 0.05$).

Figure 2. PTH Treatment Suppresses Insulin-Stimulated AKT Activity

Differentiated adipocytes were treated with PTH (10 nM) for 24 h followed by insulin stimulation (100 nM) for 20 minutes. Following harvest, lysates were analyzed by Western blotting, probed for phospho-AKT, stripped and reprobed for total AKT. A representative blot of phospho-AKT and total AKT is shown in the upper panel (A) of vehicle treated (lanes 1 and 2) or PTH treated (lanes 3 and 4) of basal (lanes 1 and 3) or insulin stimulated (lanes 2 and 4). Density of signal was quantified (B) and expressed as insulin-stimulated phospho-AKT/total AKT followed by sample/vehicle (mean $\pm$ S.E). The experiments were repeated at least three times. Bars with asterisk (*) indicate differences ($p < 0.05$) from vehicle control.

Figure 3. PTH Treatment Effects on Expression of IR and IRS-1

Differentiated adipocytes were treated with PTH (10 nM) and protein expression by Western blotting for IR (A, representative blot and B, quantification) or IRS-1 (D, representative blot and E) or mRNA levels (C, IR and F, IRS-1) determined by real time RT PCR. Quantitative real-time PCR was normalized for all samples to 18S. Results are expressed as mean $\pm$ S.E (n = 8 ~ 10) from at least three experiments. Bars with asterisk (*) indicates significant difference from vehicle control, $p < 0.05$.

Figure 4. PTH Treatment Effects on Expression of GLUT1 and GLUT4

Differentiated adipocytes were treated with PTH (10 nM) and protein expression by Western blotting for GLUT1 (A, representative blot and B, quantification) or GLUT4 (D, representative blot and E) or mRNA levels (C, GLUT1 and F, GLUT4) determined by real time RT PCR. Quantitative real-time PCR was normalized for all samples to 18S. Results are expressed as mean $\pm$ S.E (n = 8 ~10) from at least three experiments. Bars with asterisk (*) indicates significant difference from vehicle control, p < 0.05.

Figure 5. PTH Treatment Decreased Phosphorylation of IRS-1 on Serine 307 Site

Differentiated adipocytes were treated with PTH (10 nM) for 24 h followed by insulin stimulation (100 nM) for 20 minutes. Cell lysates were probed for serine 307 phospho-IRS-1 (Ser 307 p-IRS-1) and blots were stripped and reprobed for total IRS-1 using Western blotting. A representative blot of Ser 307 p-IRS-1 and total IRS-1 is shown in the upper panel (A) of vehicle treated (lanes 1 and 2) or PTH treated (lanes 3 and 4) of basal (lanes 1 and 3) or insulin stimulated (lanes 2 and 4). Density of signal was quantified (B) and expressed as insulin-stimulated Ser 307 p-IRS-1/total IRS-1 followed by sample/vehicle (mean ± S.E, n=9). Bar with asterisk (*) indicates significant difference from vehicle control, $p < 0.05$.

Figure 6. PTH Treatment Suppression of Insulin Stimulated Glucose Uptake: Effects of Adenylate Cyclase and PLC Inhibitors

Differentiated adipocytes were treated with PTH (10 nM) for 24 h and either vehicle, SQ22536 (100 uM, adenylate cyclase inhibitor), or U73122 (10 uM, PLC α inhibitor). Insulin-stimulated (100 nM, 20 min) glucose uptake was determined in differentiated adipocytes employing 100 μ M unlabeled 2-deoxyglucose and 0.5 μ Ci/ml 2-deoxy- [3 H]-glucose (10 min). Nonspecific uptake was determined in the presence of 10 μ M cytochalasin B. The value of each bar represents the mean $\pm$ S.E (n = 4~6) from at least two experiments. Asterisk (*) indicates significant difference from vehicle control, p < 0.01.

Table 1

Primers for Real-time RT-PCR

Gene	Primers
IR	ATC CCG AAA GCG AAG ATC CCT TGA (F) GGT TCC TTT GGC TCT TGC CAC ATT (R)
IRS-1	CCC ACA GCA GAT CAT TAA CC (F) AGA GAC GAA GAT GCT GGT GC (R)
GLUT1	CAC TGT GGT GCT GTT TGT TGT (F) GGA AGC ACA TGC CCA CAA TGA AGT (R)
GLUT4	GGG TCC TTA CGT CTT CCT TCT (F) CCT CTG GTT TCA GGC ACT TT (R)
CAP	ACC ACC ATC GTC AAC CCT ACC ATT (F) ACC AGT CCT TCG ATC GCT CGG TAT (R)
18S	TTA GAG TGT TCA AAG CAG GCC CGA (F) TCT TGG CAA ATG CTT TCG CTC TGG (R)