Novel adipocyte aminopeptidases are selectively upregulated by insulin in healthy and obese rats

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Abstract

The lack of a complete assembly of the sensitivity of subcellular aminopeptidase (AP) activities to insulin in different pathophysiological conditions has hampered the complete view of the adipocyte metabolic pathways and its implications in these conditions. Here we investigated the influence of insulin on basic AP (APB), neutral puromycin-sensitive AP (PSA), and neutral puromycin-insensitive AP (APM) in high and low density microsomal and plasma membrane fractions from adipocytes of healthy and obese rats. Catalytic activities of these enzymes were fluorometrically monitoring in these fractions with or without insulin stimulus. Canonical traffic such as insulin-regulated AP was not detected for these novel adipocyte APs in healthy and obese rats. However, insulin increased APM in low density microsomal and plasma membrane fractions from healthy rats, APB in high density microsomal fraction from obese rats and PSA in plasma membrane fraction from healthy rats. A new concept of intracellular compartment-dependent upregulation of AP enzyme activities by insulin emerges from these data. This relatively selective regulation has pathophysiological significance, since these enzymes are well known to act as catalysts and receptor of peptides directly related to energy metabolism. Overall, the regulation of each one of these enzyme activities reflects certain dysfunction in obese individuals.

Key Words
- physiology
- protein biochemistry
- adipocyte
- insulin
- obesity

Introduction

EC 3.4.11.3 protein (IRAP, insulin-regulated aminopeptidase), also known as vp165, GP160 (Keller 2004), placental leucine aminopeptidase (AP) (Mizutani et al. 2011), cystyl AP, oxytocinase (Rogi et al. 1996), and angiotensin (ANG) IV receptor (Albiston et al. 2010), has an important interaction with subcellular distribution of GLUT4 in adipocytes (Jordens et al. 2010). IRAP is well-recognized as a companion of glucose transporter GLUT4 and a key regulator of the insulin-regulated pathway. Insulin-dependent glucose uptake is regulated by translocation of GLUT4 from intracellular compartments to the plasma membrane (Keller et al. 2002). In adipocytes and muscle cells, IRAP traffics together with GLUT4 to the specialized compartments (Jordens et al. 2010). Phosphorylation of AS160 protein, the Akt substrate regulating GLUT4 translocation, is required for insulin-stimulated translocation of GLUT4 to the plasma membrane in adipocytes. However, AS160 is recruited to membranes by an IRAP-independent mechanism. Furthermore, although IRAP knockdown has significant effects on GLUT4 traffic, GLUT4 knockdown does not affect IRAP trafficking, demonstrating that IRAP traffics independently of...
GLUT4 (Jordens et al. 2010). On the other hand, the deficiency of IRAP acting as ANG IV receptor is known to suppress the plasminogen activator inhibitor type 1 (PAI-1) expression in adipocytes. This deficiency also upregulates the uncoupling protein-1-mediated thermogenesis in brown adipose tissue and it increases energy expenditure to prevent the development of obesity. These facts have suggested a therapeutic potential for IRAP/ANG IV receptor blockade in diet-induced obesity (Niwa et al. 2015). EC 3.4.11.3 has preference for substrates containing the N-terminal Cys (Alponti et al. 2015), such as vasopressin and oxytocin, but also acts on ANG III, Lys-bradykinin, and bradykinin (Keller 2004, Ruster & Wolf 2013), all of which with important interaction with the energy metabolism. ANG II, ANG III, and ANG IV promote the production and release of PAI-1 by adipocytes and contribute to the typical damage of fibrinolytic system in obese individuals (Skurk et al. 2013). Lys-bradykinin and bradykinin are kininogen-derived peptides with potent vasoactive, proinflammatory, and pro-angiogenic actions (Bhoola et al. 1992, Moraes et al. 2014). Vasopressin plays an antilipolytic role in adipocyte (Hiroyama et al. 2007), while oxytocin is known as an anorexigenic agent, reducing meal size and intake of highly palatable foods, such as sucrose (Maejima et al. 2011), and increasing the latency to the first meal (Blevins & Ho 2013). EC 3.4.11.3 is the only AP in adipocytes (Keller et al. 1995, Keller 2004, Jordens et al. 2010) known to be translocated between high density microsomal fraction (HDM) and low density microsomal (LDM) fractions toward the plasma membrane (MF) under insulin stimulus, therefore originating the canonical concept of IRAP (Keller et al. 1995, 2002, Keller 2004, Jordens et al. 2010). In obese individuals this traffic occurs independently of insulin stimulation (Alponti et al. 2015). Recently, other zinc-dependent AP enzymes were identified in adipocytes, such as dipeptidyl peptidase IV (DPPIV; Kirino et al. 2009), which was also associated with obesity (Sell et al. 2013), as well basic AP (APB, EC 3.4.11.6) and puromycin-sensitive (PSA, EC 3.4.11.14) and puromycin-insensitive (APM, EC 3.4.11.2) neutral AP (Alponti & Silveira 2015). Adipocyte PSA activity in LDM has reported to be higher in obese than in healthy rats, suggesting its relationship with increased proteasome activity in obese rats (Alponti & Silveira 2015). The extracellular/intraluminal portion of EC 3.4.11.3 shares homology with catalytic domains of EC 3.4.11.6, EC 3.4.11.14, and EC 3.4.11.2 (Keller 2004, Albiston et al. 2007). These four enzymes also have at least ANG III as a common substrate (Wright & Harding 1995, 1997, Prieto et al. 2003, Martinez-Martos et al. 2011). As occurs for IRAP, the subcellular levels of APB, APM, and PSA activities in HDM, LDM, and MF from adipocytes can influence their pathophysiological roles depending on the functionality of each subcellular compartment and the availability of each one of these compartments (Albiston et al. 2008). On the other hand, those APs can be influenced by insulin and insulin-related metabolic challenges, such as obesity. It is well known that obesity causes alterations on adipose tissue distribution and function and that it causes broad effects on cytokine, chemokine, and hormone expression, on lipid storage, and on the composition of adipose-resident immune cell populations with consequences to insulin sensitivity (Makki et al. 2013). However, there is no data about the influence of insulin on the hydrolytic activity of these novel adipocyte APs from healthy and obese rats.

The present study investigates the dynamics of APB, APM, and PSA hydrolytic activities under insulin stimulus in HDM, LDM, and MF from adipocytes of healthy and monosodium glutamate obese rats. The study is a first step for understanding the pathophysiological roles of these new adipocyte APs. It aims to clarify whether these enzymes are modulated by insulin in certain subcellular compartments. Since these enzymes play an important role as catalysts and receptor of peptides directly related to energy metabolism, this knowledge is important to understand the mechanism of diseases related to imbalance in insulin action, such as diabetes mellitus and obesity, both with high incidence among the general population and causing huge impact on public health.

**Materials and methods**

**Animals and treatments**

Immediately after birth, male Wistar rats were housed with a lactating female in a polypropylene box (inside length × width × height = 56 cm × 35 cm × 19 cm) within a ventilated container (Alesco Ind. Com Ltda, Monte Mor, Brazil) under controlled temperature (24 ± 2 °C), relative humidity (65 ± 1%), and 12 h light:12 h darkness cycle (lights are turned on at 0006 h). Twenty-four hours after birth, the animals were subdivided into two groups, treated according to the methodology described by Alponti & Silveira (2010), as follows: i-MSG: received a daily s.c. bolus injection of 1-glutamic acid monosodium salt (Sigma) in saline 0.9% (w/v) (4 mg/g body mass), in the cervical region, between 0730 and 0900 h of light period (1330–1500 h), at a maximum volume of 0.2 ml, until they were 10 days old; i-C (control): received the...
same volume of saline 0.9% (w/v) s.c., in the same scheme as described above. On 22nd day the animals were weaned and the female was removed from the cage. On 90th day, obesity was evaluated by Lee index, calculated by body mass (g) \(0.33 / \text{naso-anal length (cm)}\) (Nakagawa et al. 2000, Kaufhold et al. 2002). All experimental groups had access to the same animal food and allowed to feed \textit{ad libitum}: Nuvilab CR-11 (Sogorb, Sao Paulo, Brazil) with the following composition: 22% protein (w/w), 55% carbohydrate (w/w), 4% lipids (w/w), and 10% vitamins and minerals (w/w) (total of 3 kcal/g). Animals were allowed to drink water \textit{ad libitum}.

The conducts and procedures involving animal experiments were approved by the Butantan Institute Committee for Ethics in Animal Experiments (License number CEUAIB 684/2009) in compliance with the recommendations of the National Council for the Control of Animal Experimentation of Brazil. All efforts were made to minimize suffering.

**Adipocyte isolation**

The animals were euthanized by decapitation under anesthesia by the i.p. administration of 0.2 ml/100 g body mass of a 3:2:4 mixture of ketamine hydrochloride solution (100 mg/ml), hydrochloride xylazine solution (100 mg/ml), and distilled water. After euthanasia in an appropriate room, isolated from other animals, retroperitoneal fat pad was removed through the manual dissection and then washed with saline 0.9% (w/v). Total mass (g) of fat pad was measured and 3 g were separated and submitted to collagenase digestion as previously described (Alponti et al. 2015, Alponti & Silveira 2015). Briefly, this quantity of fat pad was added to 9 ml of DMEM (Cultilab, Campinas, Brazil) containing 25 mM HEPES (pH 7.5), 4% BSA (w/v) (Sigma) and 45 mg of collagenase (Sigma) and then filtered through a nylon mesh. This filtrate was centrifuged at 8 C for 1 h under gentle shaking. Subsequently, this mixture was washed with eight volumes of buffered washing solution (115 mM NaCl, 0.8 mM MgSO\(_4\).7H\(_2\)O, 5.3 mM KCl, 1.4 mM CaCl\(_2\).2H\(_2\)O, 0.89 mM NaH\(_2\)PO\(_4\).H\(_2\)O, 25 mM HEPES, 1 mM Na pyruvate, 145 mM BSA; pH 7.4, at 25°C), and then filtered through a nylon mesh. This filtrate was centrifuged at 4 g for 1 min at 25 °C. The pellet containing vascular stroma (capillaries, endothelial cells, mast cells, macrophages, and epithelial cells) was removed by suction and discarded, while the supernatant, containing the suspension of adipocytes, was washed and centrifuged again at the same conditions for three times more. The resultant suspension of isolated adipocytes was microscopically observed to verify the absence of vascular stroma.

**Incubation of isolated adipocytes with insulin**

KRBH–BSA buffer (121 mM NaCl, 1.2 mM MgSO\(_4\), 4.9 mM KCl, 2.4 mM NaH\(_2\)PO\(_4\), 0.33 mM CaCl\(_2\), 20 mM HEPES, pH 7.4, 4% BSA (w/v), 5 mM glucose) was added to adipocytes suspension obtained from each animal, at a ratio of 3 ml of buffer for each gram of adipose tissue that originated the suspension. Subsequently, saline or \(10^{-6}\) M insulin (Sigma) at a maximum volume of 60 μl were added and then the mixture was incubated for 15 min at 37 °C under gentle agitation. Insulin was dissolved in water and diluted in KRBH–BSA buffer. After the incubation period, the mixture was immediately used in the next procedure.

**Obtaining HDM and LDM fractions and plasma membrane**

Adipocyte fractionation was performed as previously described (Alponti et al. 2015, Alponti & Silveira 2015).

**Protein**

Protein content (HDM, LDM, and MF) was measured in triplicate at 630 nm in a Bio-Tek FL600FA microplate fluorescence/absorbance reader (BioTek, Winooski, VT, USA) by Bradford method (Bradford 1976) with a Bio-Rad protein assay kit (Bio-Rad) using BSA dissolved in the same sample diluent as standard.

**Peptidase activities**

APB, APM, and PSA were quantified based on the amount of β-naphthylamide released (Gasparello-Clemente et al. 2003) as the result of incubation (180 min, 37 °C), in 96-well flat-bottom microplates, of adipocyte fractions (HDM, LDM, and MF), in triplicate, respectively with prewarmed solution of 4 mM l-Arg-β-naphthylamide (Sigma) (solubilized in H\(_2\)O) in phosphate buffer, 0.05 M, pH 6.5, containing BSA 0.1 mg/ml, 150 mM NaCl and 0.02 mM puromycin (for APB), 2.5 mM l-α-Ala-β-naphthylamide (Sigma) (solubilized in 0.012 N HCl) in phosphate buffer, 0.05 M, pH 7.4, containing BSA 0.1 mg/ml, 1 mM \(N\)\(-\)dithiothreitol (DTT) (Sigma) in the presence of 0.02 μM puromycin (for APM), and 0.5 mM l-α-Ala-β-naphthylamide (Sigma) (solubilized in 0.012 N HCl) in phosphate buffer, 0.05 M, pH 7.4, containing BSA 0.1 mg/ml, 1 mM DTT, in the presence and absence of 0.02 μM puromycin, with the resulting values obtained in
the presence of puromycin subtracted from those obtained in the absence of puromycin (for PSA). The β-naphthylamine content was estimated fluorometrically (with a Bio-Tek FL600FA microplate fluorescence reader) at 415 nm emission wavelength and 335 nm excitation wavelength. The fluorescence value obtained at zero time (blank) was subtracted, and the relative fluorescence was then converted to picomoles of β-naphthylamine by comparison with the standard curve of β-naphthylamine dissolved in the same diluent used in the incubation (Sigma). APB, APM, and PSA activities were expressed as picomoles of hydrolyzed substrate per minute per milligram of protein. The existence of a linear relationship between hydrolysis time and protein content in the fluorometric assay was a prerequisite.

Data analysis

The quantitative data were shown as mean ± S.E.M. and statistically analyzed using the software GraphPad InStat (La Jolla, CA, USA). Regression analysis was performed in order to obtain standard curves of protein and β-naphthylamine. ANOVA, followed by Tukey test, was used to compare and determine differences among the groups. To calculate mean ± S.E.M., the median of each measurement in triplicate were used. The dispersion between replicates was < 5%. A minimum critical level P < 0.05 was set for significant differences among values expressed as mean ± S.E.M.

Results

Modulation of APB, APM, and PSA activities by insulin

Figure 1 shows that APB activity was higher in HDM from obese rats after incubation with insulin (560.9 ± 75.25, n = 8) when compared with healthy (149.9 ± 38.3, n = 10) and obese (185.2 ± 25.25, n = 10) rats under baseline conditions. There was no change of APB in LDM and MF between healthy and obese rats in baseline conditions or under insulin stimulation.

Figure 2 shows that in LDM, APM was higher in healthy rats under insulin stimulus (4534 ± 1616, n = 8) than in healthy (1184 ± 194.4, n = 10) or obese (792 ± 168.5, n = 12) rats under baseline conditions. In MF, APM was higher in healthy rats under insulin stimulus (6469 ± 1606, n = 8) than in obese rats in baseline conditions (353 ± 105, n = 10). In HDM, APM did not differ between healthy and obese rats in baseline conditions or under insulin stimulus.

Figure 3 shows that in LDM and HDM, PSA of healthy and obese rats in baseline conditions or under insulin stimulus presented similar levels. In MF, PSA was very higher in healthy rats under insulin stimulus (433 ± 170.3, n = 10) than in obese rats in baseline conditions (42.2 ± 24.2, n = 16).

APB, APM, and PSA activities in HDM, LDM, and MF, in the absence and presence of insulin

ANOVA was performed among HDM vs LDM vs MF for each enzyme activity in baseline conditions (APB-C-s, P = 0.2681; APB-MSG-s, P = 0.1303; APM-C-s, P = 0.3121; APM-MSG-s, P = 0.0040 for HDM > LDM and HDM > MF; PSA-C-s, P = 0.1183; PSA-MSG-s, P = 0.1830) and under insulin stimulus (APB-C-i, P = 0.0938; APB-MSG-i, P = 0.3009; APM-C-i, P = 0.3373; APM-MSG-i, P = 0.3042; PSA-C-i, P = 0.9422; PSA-MSG-i, P = 0.2468), showing the absence of simultaneous decrease or increase of all enzyme activities in HDM respectively in correspondence to a similar increase or decrease in LDM or MF, except for APM in MSG, which was prominent in HDM under basal conditions.

Figure 1

Comparison of APB activity in HDM, LDM, and MF fractions from isolated adipocytes of retroperitoneal fat pad of healthy (C) and obese (MSG) rats in baseline conditions (s) or under insulin stimulus (i). Values are mean ± S.E.M. from triplicates of n animals (n in parenthesis over the bars). ANOVA, C:s × C:i × MSG-s × MSG-i, HDM, P = 0.0033; LDM, P = 0.0843; MF, P = 0.0773, multiple comparison Tukey’s test (HDM: C:s × MSG-i, P < 0.01, MSG-s × MSG-i, P < 0.05, different letters indicate significant differences among different groups in the same fraction).
conditions. Also, simultaneous decrease or increase of all enzyme activities in LDM was not detected respectively in correspondence to a similar increase or decrease in MF.

Discussion

Several studies have pointed out the excess of visceral fat as the main problem related to obesity and metabolic syndrome (Wajchenberg et al. 2002, Chan et al. 2004, Després & Lemieux 2006). The increase of the absolute mass and energy’s demand of retroperitoneal fat pad should contribute to the later establishment of type 2 diabetes mellitus in obesity induced by MSG (Alponti et al. 2008) and thus this visceral depot was investigated here. MSG model of obesity promotes lesions to CNS, specifically in the arcuate nucleus and lateral hypothalamus. This type of lesion also occurs in chronic human obesity (Moraes et al. 2009, Purkayasta & Cai 2013). Another advantage of this model is its independence of dietary components, differently of diet-induced obesity, when hyperphagia is obtained by using some unhealthy components with high palatability (Hariri & Thibault 2010). Also, unlike knockout models, the MSG one makes it possible to evaluate the ontogenetic influence in the development of obesity (Eisener-Dorman et al. 2009). In addition to increased mass of retroperitoneal fat pad (Alponti & Silveira 2010), MSG obese animals are normophagic (Nardelli et al. 2011), normoglycemic (Alponti & Silveira 2010), hyperinsulinemic (Nardelli et al. 2011), and hyperleptinemic (Perello et al. 2004).

Growing evidences also exist about the involvement of APB, APM, and PSA in the energy metabolism. Mapping of APM in the hypothalamus and hippocampus of food deprived and obese rats showed the regulation of this protein under these metabolic challenges (Alponti et al. 2011). APB and neutral AP in peripheral tissues and in CNS were previously reported to be altered in diabetes mellitus induced by streptozotocin (Zambotti-Villela et al. 2007, Zambotti-Villela et al. 2008). Although it should be predictable that changes in adipocyte subcellular distribution or regulation could occur between healthy and obese subjects, or between basal condition and insulin

Figure 2
Comparison of neutral APM activity in HDM, LDM, and MF fractions from isolated adipocytes of retroperitoneal fat pad of healthy (C) and obese (MSG) rats in baseline conditions (s) or under insulin stimulus (i). Values are mean ± S.E.M. from triplicates of n animals (n in parenthesis over the bars). ANOVA, C-s×C-i×MSG-s×MSG-i: HDM, P = 0.1291; LDM, P = 0.0150; MF, P = 0.0062, multiple comparison Tukey’ test (LDM: C-s×C-i, P < 0.05; C-i×MSG-s, P < 0.05; MF: C-i×MSG-s, P < 0.01, different letters indicate significant differences among different groups in the same fraction).

Figure 3
Comparison of neutral PSA activity in HDM, LDM, and MF fractions from isolated adipocytes of retroperitoneal fat pad of healthy (C) and obese (MSG) rats in baseline conditions (s) or under insulin stimulus (i). Values are mean ± S.E.M. from triplicates of n animals (n in parenthesis over the bars). ANOVA, C-s×C-i×MSG-s×MSG-i: HDM, P = 0.0710; LDM, P = 0.6127; MF, P = 0.0131; multiple comparison Tukey’ test (MF: C-i×MSG-s, P < 0.05, different letters indicate significant differences among different groups in the same fraction).
treatment, these predictions had not been shown until now, except for IRAP (Keller et al. 1995, 2002, Keller 2004, Jordens et al. 2010). Stimulation with 10^{-6} M insulin, as used in this study, is known to promote \textit{in vitro} translocation of IRAP between HDM and LDM toward MF in adipocytes (Ross et al. 1996, Demaegdt et al. 2008). Obviously, the enzymatic activities of adipocytes fractionated immediately after removal from the animal and those who have suffered incubation at 37°C in artificial medium could not be compared due to rheological effects related to altered environmental medium and thermolability features of each enzyme during incubation. In adipocytes fractionated immediately after removal from obese rats, APM prevails over APB and PSA in LDM, while in HDM and MF, APB, APM, and PSA are similarly distributed (Alponti & Silveira 2015). However, after \textit{in vitro} incubation under the conditions of the present study, APM prevails over APB and PSA in HDM, LDM and MF from adipocytes of obese rats. Meanwhile, these changes due to \textit{in vitro} conditions are not relevant for the purpose of the present study, since comparisons between activities in the absence and presence of insulin stimulation in healthy and obese animals were made under the same controlled conditions. Each subcellular compartment under study has peculiar features. The disruption of adipocytes by homogenization originates microsomal vesicles formed from fragments of the endoplasmic reticulum. HDM corresponds to rough microsomes that have ribosomes adhered to their outer surface, while LDM corresponds to smooth microsomes. The inner medium of rough microsomes is biochemically equivalent to the endoplasmic reticulum lumen, while LDM is derived from smooth portions of the endoplasmic reticulum, vesicles fragments of the Golgi apparatus, endosomes, and other sources, but without ribosomes (Alponti & Silveira 2015). Nowadays, it is known that intracellular peptides generated by the proteasome and oligopeptidases are functional and may be natural modulators of protein interactions within cells, acting in signal transduction and to improve insulin resistance in mice fed a high-caloric diet (Berti et al. 2012, Russo et al. 2014). Therefore, the presence of AP activities in these adipocyte fractions permits to infer their pathophysiological role in protein synthesis, maturation, and targeting to plasma membrane. Here, the comparison of the same enzymatic activity among different subcellular fractions under the same stimulus and in certain animal condition shows that insulin did not promote translocation in any AP activities under study. Translocation could be inferred only if simultaneous decrease of enzyme activity in the compartment of origin had been found in correspondence to a similar increase in the compartment of arrival either in retrograde or anterograde sense. APM activity is upregulated by insulin only in LDM of healthy rats, despite of a same trend observed in HDM and MF of healthy rats and in LDM and MF of obese rats, suggesting that upregulation of APM by insulin is part of the normal functionality of the machinery for the maturation of newly synthesized proteins and peptides that can be directed to MF or to extracellular environment. PSA activity tends to be upregulated by insulin in HDM and MF of healthy rats and in LDM and MF of obese rats, suggesting its minor involvement than that of APB and APM, respectively in the synthesis and maturation of newly synthesized proteins and peptides under insulin stimulus, which is independent of obesity. Furthermore, selective regulation on AP activities was demonstrated and each one reflects certain dysfunction in obese animals. Prominent APM in HDM suggests an increased activity of synthesis and/or post-translational processing of APM and/or that APM has a role in these pathways of other proteins and peptides in obese rats under basal conditions. We show that APB activity is upregulated by insulin only in HDM of obese rats, despite of a same trend observed in HDM of healthy rats and in LDM and MF of obese and healthy rats, reinforcing the hypothesis of an exacerbate and deleterious role of insulin to stimulate the process of synthesis of new proteins and peptides in obesity (Loh et al. 1988, Yasothornsrikul et al. 1998). Higher PSA and APM activities in MF of healthy rats under insulin stimulus than in obese ones in baseline conditions were also detected in the present study, suggesting insulin-stimulated action of these enzymes on extracellular peptides such as ANG III (Wright & Harding 1995, 1997, Prieto et al. 2003, Martinez-Martos et al. 2011) in healthy rats and its failure in obese ones where ANG III is preserved with consequent damage to the fibrinolytic system (Skurk et al. 2001).

In conclusion, this study demonstrated for the first time that insulin promotes intracellular compartment-dependent upregulation of APB, APM, and PSA activities in adipocytes and does not promote traffic of these activities between HDM and LDM fractions toward the plasma membrane as it occurs for IRAP. In fact, insulin upregulates these novel APs without promoting anterograde intracellular traffic. This regulation is relatively selective, since it is prominent in different enzyme activities and in different compartments between healthy and obese rats. Overall, this regulation process on each one of these enzyme activities reflects certain dysfunction in obese rats.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
P F S conceived, P L A and R F A carried out experiments, P F S and R F A analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions. All authors have read and agree with the manuscript as written.

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References
Albiston AL, Peck GR, Yeatman HR, Fernando RN, Ye S & Chai SY 2007 Pharmacology and Physiology at Laboratory of Pharmacology for technical and agree with the manuscript as written.


