



Research Article

Insulin Receptor and Glucose Transporter-4 Expression in the Skeletal Muscle of Chronically Stressed Rats

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ABSTRACT

Background: Stress defined as a disruption in the normal homeostatic functions of an organism caused by stressor (a physiological or psychological challenge) is an unavoidable normal component of life. Previous studies suggest that stress hormones have acute adverse effects on glycaemic control. The aim of this study was to assess the effect of chronic psychological and physical stress on the expression of insulin receptor and GLUT4 transporters in male Sprague-Dawley rats. **Methods:** Male rats (12 weeks old) were randomly distributed into 3 groups: control, water avoidance stress (WAS), forced swimming stress (FSS). The stress procedures were performed between 9:00 and 11:00 am to minimize the effect of circadian rhythm and lasted for 28 consecutive days. Levels of insulin and corticosterone in the blood were determined using enzyme-linked immunosorbent assay. Glucose metabolism was assessed by glucose tolerance test (GTT) and insulin tolerance test (ITT), and expression of insulin receptor (INSR) and glucose transporter-4 (GLUT4) in skeletal muscle. **Results:** The FSS rats had decreased food intake as well as final body weight and without adverse changes in GTT, stress worsened insulin sensitivity in FSS rats and increased insulin in the blood. Stress also increased corticosterone, decreased INSR and GLUT4 in the skeletal muscle of both groups. **Conclusion:** Chronic stress evokes insulin insensitivity and impairs glucose metabolism through the down-regulation of INSR and GLUT4 in skeletal muscles.

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INTRODUCTION

Stress defined as a disruption in the normal homeostatic functions of an organism caused by stressor (a physiological or psychological challenge) is an unavoidable normal component of life. Animals like other forms of life have developed mechanisms to cope with stress, in order to maintain homeostasis and survive. Stress stimulates several adaptive hormonal responses, prominent among which are the secretion of catecholamines from the adrenal medulla, corticosteroids from the adrenal cortex, and

adrenocorticotropin from the anterior pituitary (Carrasco *et al.*, 2003). In fact, the sympato-adrenal and hypothalamic-pituitary-adrenocortical systems have complex interactions to maintain the internal environment during exposure of organism to a wide variety of stressors (Kevetnansky *et al.*, 1993). The effects of “stress” on energy metabolism are complex and may depend on the nature of the stressor (ie, positive versus negative stressors, heterotypic [different types] versus homotypic [same types] repeated stressors, psychological versus metabolic stressors), and may differ from the short-term effects of stress hormones. Previous studies suggest that stress hormones have acute adverse effects on glycaemic control (Raikkonen *et al.*, 1996; Strommer *et al.*, 1998; Soop *et al.*, 2001) however; long-term repeated homotypic stressors may have opposing effects. For instance, repeated immobilization stress in rat increases hepatic glucose uptake (Zhou *et al.*, 2001), improved haemoglobin A1c and glucose tolerance, whereas short-

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term immobilization transiently increased blood glucose (Kai *et al.*, 2000). In addition, chronic immobilization stress resulted in improved glucose tolerance and insulin secretion in rat (Thiago *et al.*, 2013).

Insulin biological actions are essential for glucose level control (Pereira *et al.*, 2003). Insulin resistance is defined by decreased sensitivity or loss of its metabolic response. Insulin resistance can be generated by alterations in the number of insulin receptors and in insulin signalling (Jellinger, 2007) and several researchers have observed that insulin resistance may be linked to abnormalities in glucose transporter4 receptor (GLUT4). Insulin plays a key role in glucose homeostasis as it regulates the disposal and storage of glucose by stimulating the uptake of glucose into the muscle and fat. Insulin activates the insulin receptor (INSR), which phosphorylates and recruits different substrate adaptors such as the insulin receptors substrate family of proteins. This leads to an increase in the quantum of glucose transporter (GLUT4) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissue, and therefore to an increase in the uptake of glucose from blood into these tissues. Despite existing evidence, the exact role of INSR and GLUT4, especially under chronic stress condition, on glucose homeostasis is still unclear. In this study, we examined the effect of chronic psychological and physical stress on glucose tolerance and insulin sensitivity in male Sprague-Dawley rats. More importantly, the expression of insulin receptor and GLUT4 transporters were assessed.

MATERIALS AND METHODS

Animals

Male rats (12 weeks old) were obtained from Animal House of the College of Medicine, University of Lagos and housed 6 per cage under controlled conditions for the light/dark cycle, temperature, and humidity. The animals were kept in the same animal facility for at least 1 week before the experiments. Rats were fed a standard chow diet and water *ad libitum*. All experiments and procedures were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Research Council, and was approved by the Ethics Committee of the College of Medicine of the University of Lagos.

Stress protocols

To acclimatize the rats to manipulation by humans, all rats (stressed and controls) were handled daily for one week. The animals were divided into three groups, consisting of 6 animals in each group. Group one served as the control and no stress or treatment was

applied to the rats in this group while group 2 and 3 served as the forced swimming stress (FSS) and water avoidance stress (WAS) respectively. These test stress procedures were performed between 0900 and 1100 h to minimize the effect of circadian rhythm and lasted for 28 consecutive days. Control rats were still handled and weighed daily but remained in their home cages.

Forced Swimming Test

Physical stress was induced according to a previous method (Anand *et al.*, 2010) and amended. Briefly, the rats were individually placed inside a 25 cm PVC cylinder (with a 14 cm diameter) containing 20 cm of water that was maintained at $24 \pm 2^\circ\text{C}$ and were forced to swim for 10 min. Animal judged to be immobile (when it ceased struggling and remained floating motionless in water, making only movements necessary to keep its head above water) were removed.

Water Avoidance Test

The test apparatus consisted of a PVC tank (45 cm length x 25 cm width x 25 cm height) with a block (10 x 8 x 8 cm) affixed to the centre of the floor. The tank was filled with fresh room temperature water ($24 \pm 2^\circ\text{C}$) to within 1 cm of the top of the block. The animals were placed on the block for a period of 1 h daily. This well characterized test represents a potent psychological stressor with large elevations of ACTH and corticosterone within 30 min (Million *et al.*, 1999).

Assessment of food intake and body weight

Food intake was measured daily between 09:00 and 10:00 throughout the experiment by measuring the difference between the amount of feed put in the cage and the remaining amount. The weight of the animals was measured once a week during the experimental period by a digital scale (Ohaus Scout Pro, Pine Brook, New Jersey, USA).

Glucose tolerance and insulin tolerance tests

A separate group of stressed rats was used to perform glucose tolerance (GTT) and insulin tolerance tests (ITT). These tests were conducted on day 7, 14 and 28 of the experimental period. Rats that were used for the glucose tolerance test (GTT) were fasted overnight (16 h). Basal levels of glucose (0 min) were measured from the tail vein, followed by oral administration of glucose (200 mg/kg). Blood glucose levels were measured at 30, 60, 120 and 180 min using the portable glucose meter (Accu-Chek glucose meter, Roche Diagnostics, Germany) using tail vein blood.

Insulin tolerance test is a simple method to measure insulin resistance that has a good correlation with glucose clamp studies (Sin *et al* 1996). Rats that were subjected for insulin tolerance test (ITT) were fasted for 4 hours. Basal blood glucose levels (0min) were

Table 1: Effects of chronic psychological and physical stress on food intake and body weight in rats.

Parameters	Timeline	Control	WAS	FSS
Food Intake (g)	Day 7	142.29±2.96	125.71±6.81*	135.86±5.62*
	Day 14	153.50±9.56	152.00±2.87	128.67±8.37*#
	Day 21	153.67±4.84	159.00±2.74	141.00±3.66*#
	Day 28	155.00±4.23	156.20±3.52	130.80±4.49*#
Body Weight (g)	Baseline	157.17±8.97	158.00±5.53	150.17±5.31
	Week 1	184.83±10.33	172.83±7.27	171.17±5.23
	Week 2	195.17±10.91	181.50±7.64	175.17±6.02*
	Week 3	218.67±13.02	208.17±10.97	192.67±7.30*
	Week 4	219.33±12.08	204.33±8.55	185.67±8.30*#

Data expressed as mean±SEM (n=6). *P<0.05 vs control and # P<0.05 vs WAS.

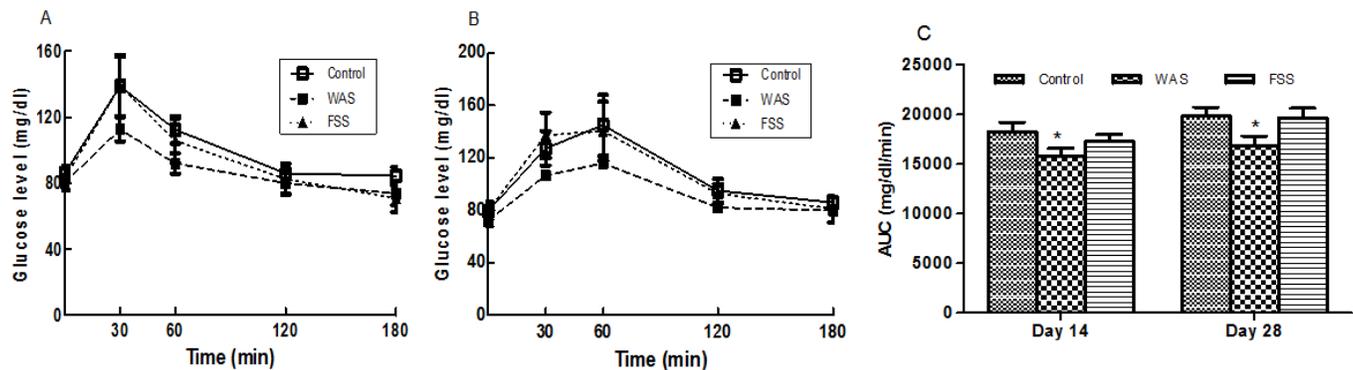


Fig. 1. Effects of a chronic psychological and physical stress on glucose tolerance. “A” and “B” are glucose responses during OGTT after 14 and 28 days of stress; “C” is the area under curve of the blood glucose for the OGTT (A) and ITT (B). Data are expressed as means ± SEM (n=6).

measured followed by injection of insulin (2 U/kg) into the peritoneum, and blood glucose levels were measured at 15, 30, 60, and 120 min using the same portable glucometer as in the GTT above. Total area under the curves (AUC) in response to glucose or insulin administration was calculated using GraphPad Prism Software (GraphPad Software, San Diego California, USA)

Expression of corticosterone, insulin, insulin receptors and GLUT4

After light ether anaesthesia, blood samples were taken following over-night (16 h) fasting, 1ml blood was collected in an Eppendorf tube containing 5 µl heparin (5000 IU/ml) (Chalkley *et al.*, 2002), and centrifuged at 3000 rpm for 5 min at 4°C (Tolcikis *et al.*, 1995). Plasma was removed and kept at -20 °C for measuring the corticosterone, insulin, insulin receptors and GLUT4 concentrations. These parameters were determined using enzyme immunoassay (EIA) kit (Elabscience Biotechnology Co., China). The procedure specified in the manufacturer’s manual for the kits were followed. A 96-well microtitre plate was used to conduct the analysis. Intra- and inter-assay coefficients of variations for corticosterone and insulin

measurements were 2.04% and 9.88%, 10.6% and 10.8%, 7.1% and 6.5%, respectively.

Statistical analysis

GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA was used for all statistical analysis. All data are presented as mean±SEM. One-way analysis of variance (ANOVA) were performed and followed by Tukey test. A *p* value below 0.05 was considered to be statistically significant.

RESULTS

Food intake, body weight and weight gain

As indicated in Table 1, the food intake of the WAS and FSS rats were significantly lower (*p*<0.05) compared with control rats during the first seven days of the experimental period. However the FSS rats showed a significant decrease (*p*<0.05) in food intake during the 28-day experimental period compared with WAS and control rats. There was no significant difference (*p*<0.05) in the food intake between WAS and control rats after the first week of experiment. The FSS rats showed significant reduction (*p*<0.05) in the overall body weight at day 14, 21 and 28 compared

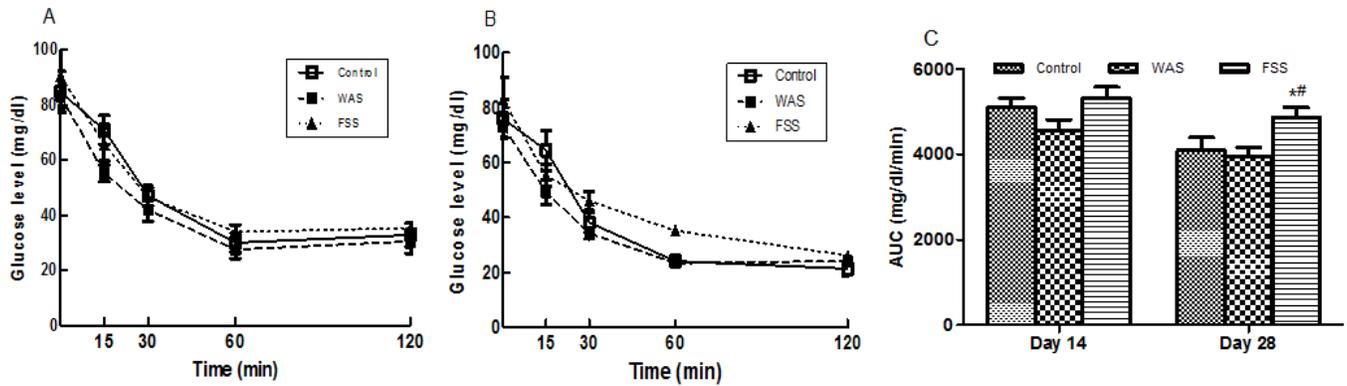


Fig. 2: Effect of chronic psychological and physical stress on insulin sensitivity. “A” and “B” are glucose responses during ITT after 14 and 28 days of stress; “C” is the area under curve of the blood glucose for the ITT (A) and ITT (B). Data are expressed as means \pm SEM (n=6). *P<0.05 vs control and # P<0.05 vs WAS.

with control rats. However, there was no significant difference ($p>0.05$) in the body weight of FSS rats.

Glucose tolerance

After 14 days of exposure to the stress paradigm, WAS rats had improved glycaemic control compared with control (unstressed) rats as demonstrated by both the glucose tolerance curve (Fig. 1a) and significantly higher glucose AUC ($p<0.05$, Fig. 1c). Before the glucose load, there was no difference in the baseline blood glucose levels among the experimental groups of rats. The glucose challenge of 2g/kg dramatically raised the blood glucose level of in all groups at 30 min but it was remarkably lower in the WAS rats compared with control rats. Glucose tolerance was comparable between the FSS and control rats.

After 28 days of exposure to stress, WAS rats again demonstrated improved glycaemic control compared with control (unstressed) rats as evidenced by the glucose tolerance curve (Fig. 1b) and a significantly higher glucose AUC ($p<0.05$, Fig. 1c). There was no difference in the baseline (time-point 0 min) blood glucose levels among the experimental groups of rats. The glucose challenge of 2g/kg dramatically raised the blood glucose level of in all groups at 30 min and peaked at 60 min but the increase was markedly lower in the WAS rats compared with control rats. The blood glucose level of the FSS rats was comparable with control values.

Insulin tolerance

Glucose responses during ITT after 14 days of stress paradigm in the control and stressed rats are presented in Figure 2a. Fifteen minutes after an intra-peritoneal injection of insulin, there was a significant decrease in the blood glucose level in all groups of rats. The blood glucose levels in all groups decreased continuously and the lowest level was attained at the 60 min time interval. The WAS rats produced a greater degree of

fall in their blood glucose level as compared with the control group. After 28 days, WAS and FSS rats showed a significant decrease in the glucose level as

compared with unstressed control rats indicating improved insulin sensitivity (Fig. 2b). This observation was supported by the AUC data shown in Figure 2. The AUC_{ITT} for the FSS groups were significantly higher than the control rats.

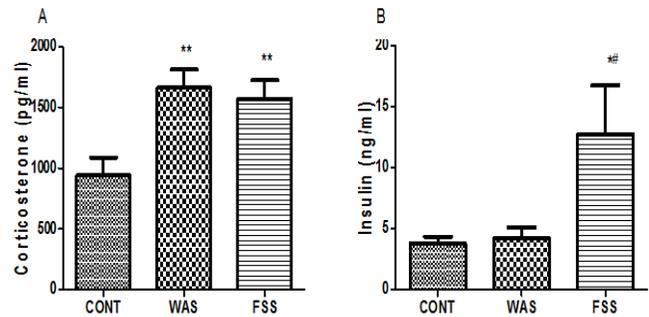


Fig. 3: Serum concentration of corticosterone (A) and insulin (B) in control, WAS and FSS rats. Data are expressed as means \pm SEM (n=6). *P<0.05 vs control and # P<0.05 vs WAS.

Effect of chronic psychological stress on corticosterone and insulin level

As depicted in Fig.3a, the concentration of corticosterone was significantly increased ($p<0.05$) indicating elevated stress level in FSS and WAS compared with unstressed control rats. Rats chronically exposed to FSS showed a significantly increase ($p<0.05$) in serum insulin concentration compared with the unstressed control. However, there was no significant difference in the insulin level in the WAS rats compared with the control rats (Fig 3b).

Effect of chronic psychological stress on the expression of insulin receptor and GLUT4

The expression of INSR was not significantly different ($p>0.05$) in both stressed groups of rats compared with

the control (Fig.4a). However, the expression of GLUT4 was significantly lower in the FSS and WAS rats compared to the control rats, although the reduction was more markedly lower in the FSS rats when compared with WAS rats (Fig. 4b).

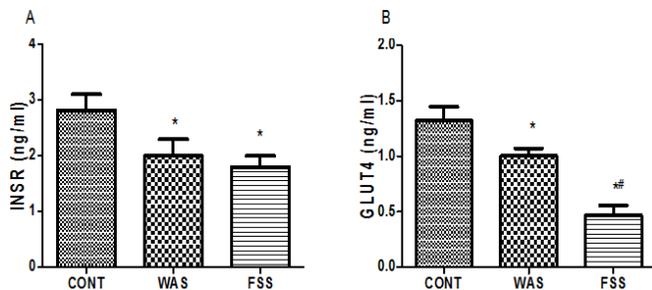


Fig. 4. Expression of insulin receptor (A) and GLUT4 (B) in the gastrocnemius muscle of control, WAS and FSS rats. Results are expressed as mean \pm SEM (n=6). *P<0.05 vs control and # P<0.05 vs WAS.

DISCUSSION

The present study was designed to study the effect of chronic physical and psychological stress on glycaemic control and expression of INSR and GLUT4 receptor in Sprague-Dawley rats. We employed the forced swimming and water avoidance tests to induce respectively a state of physical and psychological stress in the rats. The results demonstrated the development of insulin insensitivity with impairment of glucose metabolism through the down-regulation of INSR and GLUT4 expression under chronic physical stress condition.

As observed in this study, physical stress caused a significant decrease in the food intake of FSS rats while the WAS rats had a significant reduction only in the first 7 days of exposure to the psychological stressor. The significant decrease in the food intake can be said to be responsible for the observed decrease in the body weight of the FSS rats. Stress-induced reduction in food intake has been reported to cause a decrease in weight gain (Neyanataru *et al.*, 2005; Zardooz *et al.*, 2006). Catecholamines and glucocorticoid which are key stress hormones stimulate protein breakdown while the stress activated hormone sensitive lipase degrades triglycerides in the adipose tissue (Konstandi *et al.*, 2013) thereby negatively impacting on body weight. Given that the link between stress, food intake and weight loss (or gain) has been used as a marker to assess the severity of the stress model (Nirupama *et al.*, 2012), it is evident from our data that FSS appears to be more severe than WAS as a model of stress and also adversely affected absolute body weight and / or weight gain.

Stress is suggested to deteriorate glycaemic and promotes metabolic dysfunction such as glucose intolerance, insulin resistance, hypertension,

hyperglycemia and increased leptin (Aikens *et al.*, 1997; Levine *et al.*, 2006; Adam *et al.*, 2007). Prior findings indicate that chronic physical stress in rats impairs glucose tolerance and carbohydrate metabolism (Zardooz *et al.*, 2006a). In the present study, chronic physical stress prominently aggravates glycaemic control in the FSS rats. The increased glucose levels observed during chronic physical stress may be due to the hyperglycaemic effect of catecholamines and glucocorticoids, released respectively by the activation of the sympho-adreno-medullary and pituitary-adreno-cortical systems (De Boer *et al.*, 1990; Surmit *et al.*, 1992). Meanwhile, data from the chronic psychologically-stressed WAS rats showed opposite results as the animals failed to develop glucose intolerance and insulin insensitivity. Corroborating our findings, Rostamkhani *et al.*, (2012) demonstrated the effects habituating effects of chronic psychological stress on plasma glucose level in rats. Their study demonstrated an increase in plasma glucose concentration on day 15, but not on day 30 as compared to day 1. This observation suggests that increasing days of exposure to the psychological stress could lead to adapted response with plasma glucose level similar to the control. Taken together, the present findings of ours where glucose control was adversely affected under chronic physical stress condition but relatively maintained under psychological stress highlight the complex nature of stress on glucose metabolism whereby the nature of the stress applied leads to different metabolic responses.

The hypothalamic-pituitary-adrenal(HPA) axis is highly responsive to physical and psychological stress (Heim *et al.*, 2008; Guerry *et al.*, 2011) and is well known to play a role in the development of insulin resistance (Li *et al.*, 2009; Ursache *et al.*, 2012). In the present work, corticosterone levels were significantly increased following chronic exposure to both stress types. Exposure to stress levels of corticosterone produces physiological responses that are characteristic of insulin resistance such as hyperinsulinaemia (Vaughan *et al.*, 2015). Our data indicate an increase in the insulin level in the FSS rats, suggesting that it may contribute to the development of insulin resistance following chronic physical stress. Conversely, we did not find an increase in insulin level following psychological stress in the WAS rats (at least at the time point tested), as the animals also failed to develop insulin resistance. The present findings support prior report by Zardooz *et al.* (2006b) stating that chronic psychological stress significantly lower plasma insulin levels and increases the responsiveness of pancreatic β cells to glucose. These authors also reasoned that low insulin levels of the stressed animals may be due to reasons other than the reduction of insulin releasing capacity of pancreatic β cells.

Different pathways of carbohydrate metabolism are reportedly altered under stressful conditions (Postic *et al.*, 2004). We focused on the skeletal muscle to analyse the mechanism of systemic insulin resistance caused by physical stress because the skeletal muscle is responsible for 80% of whole body insulin stimulated glucose metabolism (Shulman *et al.*, 1990). As previously described, down-regulation of INSR and GLUT4 expressions in insulin-sensitive tissues mediates insulin resistance (Ruan *et al.*, 2002; Nieto-Vazquez *et al.*, 2008). In this study, we also recorded similarly reduced expression of INSR and GLUT4 and decline systemic insulin sensitivity in rats exposed to both chronic physical and psychological stress. Alteration of insulin signalling pathways is well known to be associated with glucose dysregulation. To conclude, our study demonstrates that chronic stress evoked insulin insensitivity and impairs glucose metabolism through the down-regulation of INSR and GLUT4 in skeletal muscles of Sprague-Dawley rats. It will be interesting to understand the mechanism(s) underlying/associated with the different metabolic responses from various stress modes and/or duration of exposure in future studies.

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