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Restraint Stress Impairs Glucose Homeostasis Through Altered Insulin Signalling in Sprague-Dawley Rat

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Summary: The study investigated the potential alteration in the level of insulin and adiponectin, as well as the expression of insulin receptors (INSR) and glucose transporter 4 GLUT-4 in chronic restraint stress rats. Sprague-Dawley rats were randomly divided into two groups: the control group and stress group in which the rats were exposed to one of the four different restraint stressors; 1 h, twice daily for a period of 7 days (S7D), 14 days (S14D) and 28 days (S28D). Glucose tolerance and insulin sensitivity were evaluated following the final stress exposure. ELISA were performed to assess the level of insulin and adiponectin as well as expression of INSR and GLUT4 protein in skeletal muscle. Plasma corticosterone level was also determined as a marker of stress exposure. Restraint stress for 7 days caused transient glucose intolerance, while S14D rats demonstrated increased glucose intolerance and insulin insensitivity. However, restraint stress for 28 days had no effect on glucose tolerance, but did cause an increase in glucose response to insulin challenge. The serum level of adiponectin was significantly ($p< 0.05$) lower compared with the control value while insulin remained unchanged except at in S28D rats that had a significant ($p<0.05$) increase. The expression of INSR and GLUT4 receptors were significantly ($p< 0.05$) decreased in the skeletal muscle of restraint stress exposed rats. There was a significant ($p< 0.05$) increase in the plasma corticosterone level of the stress rats compared with their control counterparts. Restraint stress caused glucose intolerance and insulin insensitivity in male Sprague-Dawley rats, which becomes accommodated with prolonged exposure and was likely related to the blunted insulin signalling in skeletal muscle.

Keywords*:* Stress, Glucose tolerance, Insulin sensitivity, Glucose transporter-4, Corticosterone.

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INTRODUCTION

Stress may be defined as the state in which the brain interprets the quantity of stimulation as excessive or its quality as threatening (Chrousos, 1998). It is any condition that impairs the balance of the organism physiologically or psychologically. Exposure to stressors results in a series of important adaptive responses that enable an organism to cope with a changing environment (Sabban and Kvetnansky, 2001; Carrasco and Van de Kar, 2003). Prominent among the adaptive responses to stress are secretion of catecholamines from the adrenal medulla, corticosteroids from the adrenal cortex, and adrenocorticotropin from the anterior pituitary (Kevetnansky *et al*., 1993; Strommer *et al*., 1998). 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 (Strommer *et al*., 1998; Lay *et al*., 2014).

Stress primarily target the metabolic system and contributes significantly to the development of metabolic diseases (Soop *et al*., 2001; Lay *et al*., 2014). Indeed, a considerable amount of evidence from clinical and animal experiments has shown that stress reliably alter glucose metabolism resulting in hyperglycemia and has a role in the induction of insulin resistance in different tissues (Bonner-Weir *et al*., 1981; Chalkley *et al*., 2002). Elevated glucocorticoid and catecholamine levels antagonize the effects of insulin and also increase blood glucose concentration independent of their effects on insulin (Björntorp, 1997). Thus chronic over-secretion of these stress mediators may therefore contribute to the development of insulin resistance, overweight, and obesity (Vanltallie, 2002; Ozcan *et al*., 2004; Rozanski *et al*., 2005).

Although it has been shown that the expression of insulin receptors (INSR) and glucose transporter-4 (GLUT4) are disrupted in insulin resistant rodents (Pessin and Saltiel, 2000), these changes in rats exposed to restraint stress are scarcely available in literature. This study therefore investigated the effect of restraint stress on glucose homeostasis as well

insulin signalling factors such as INSR and GLUT4 in male Sprague-Dawley rat. Previous research done in our laboratory indicated a deterioration in insulin sensitivity in forced-swimming (physical stress) and water-avoidance (psychological stress) models of physical in rats (Morakinyo *et al*., 2016).

MATERIALS AND METHODS

Animals

Adult male rats (n=16; 12 weeks old) were obtained from Animal House of the College of Medicine, University of Lagos and housed 8 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 1 wk. Rats were divided into two groups, stressed and control (n=6/group). Rats of the stressed group were exposed to one of the four different restraint stressors; 1 h, twice daily for a period of 7 days (S7D), 14 days (S14D) and 28 days (S28D). The first exposure was between 09:00 and 12:00 h, and the second between 14:00 and 17:00 h (Toleikis and Godin, 1995). To minimize habituation, the sequence of the stressors was randomized for both the morning and afternoon sessions of the first week of exposure, and was repeated during the second week with the morning and afternoon sequences exchanged. The same procedure was done for the third and fourth week of the experiment. Control rats were weighed weekly and remained in their home cages throughout the experiment except when blood samples had to be taken. The stressors were as follows: (a) towel wrap secured with tape, (b) restraint in a plexy glass box (15×5 cm) with lid, (c) restraint in a polyvinyl chloride tube (L=15 cm, ID=4.5 cm) closed at either end, (d) immobilization on a board with tape (Vazquez-Vela *et al*., 2008). The animals exposed to stressors were returned to the animal facilities 15 min following stress exposure to minimize disturbance to the control group.

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

For the oral glucose tolerance test (OGTT), rats were fasted for 16 hr prior to the time the test commenced. Subsequently, a zero-time (baseline) blood sample was drawn and designated 0 min glucose level. Thereafter, each rat was given an oral glucose load of 2g/kg BW *(*Morakinyo *et al*., 2016*)* of glucose solution (D-Glucose: Sigma Cat. No. G-7528). Blood sample was drawn from tail vein after the glucose load at intervals of 30, 60, 120 and 180 min for measurement of glucose level. The glucose level was measured with a portable Accu-Chek glucose meter (Roche Diagnostics, Germany).

Rats that were used for insulin tolerance test (ITT) were fasted for 4 h. Basal blood glucose levels (0 min) were measured followed by injection of insulin (0.5 U/kg BW; Human Insulatard, Novo Nordisk) into the peritoneum, and blood glucose level was measured at 15, 30, 60, 90 and120 min by portable glucose meter using tail vein blood. Total area under the curves (AUC) in response to glucose or insulin administration was calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

Expression of corticosterone, insulin, insulin receptor, adiponectin 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 (Toleikis and Godin, 1995). Plasma was removed and kept at −20 °C for measuring the corticosterone, insulin and adiponectin. The skeletal tissue homogenate was used for the determination of insulin receptor and GLUT4 concentrations. The gastrocnemius muscle was homogenized in 9-volumes of ice-cold 0.1 mM phosphate buffer saline (pH 7.4) to prepare 10% homogenate. The homogenate was then centrifuged for 5 min at 5000×g to get the supernatant used for the measurement. 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.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) were performed and followed by Tukey test, $p > 0.05$ was considered to be statistically significant. Graph Pad Prism version

5.00 for Windows, GraphPad Software, San Diego California USA was used for all statistical analysis

RESULTS

Food intake, body weight and weight gain

Before evaluating the effect of restraint stress on food intake and mean body weight, we assessed the basal level of these parameters and found no significant difference in the experimental rat within our facility. However, food intake in the restraint-challenged rats were significantly lower than the control rat only in the S7D rats (Table 1). The mean body weight values of all stress groups were not significantly different from the control; however, the mean weight of animals in the S7D, S14D and S28D groups were 3.95, 6.19 and 6.65 % lower than that of the control group even though both stress and control groups of rats consumed the same amount of food (Table 1).

Glucose tolerance and Insulin sensitivity

Before glucose administration (0 min), both groups showed comparable FBG in S7D, S14D and S28D (Figure 1a-c). After glucose administration, blood glucose at 60 min was significantly higher in the S7D group compared with control rats but there was no significant difference at other time-points. The S14D rats exhibited decrease glucose tolerance as indicated by higher blood glucose levels during the glucose tolerance test and a higher AUC_{GTT} (Figure 1b). The glucose response in S28D rats was not significantly different form the control rats (Figure 1c).

The S14D rats showed decreased insulin sensitivity as demonstrated by a significantly lower timed blood-glucose levels (significant at all times post-glucose injection except 120 min) during the ITT as well as a higher AUC_{ITT} (Figure 2b) compared to the control. In S28D rats, insulin sensitivity was higher than control evidenced by lower glucose levels (significant at 60, 90 and 120 min post-insulin injection) and lower AUC_{ITT} (Figure 2c) compared to the control (Figure 2c). However, there was no significant difference in the time blood-glucose level in S7D rats pre- and post- insulin injection compared to the control rats (Figure 2a).

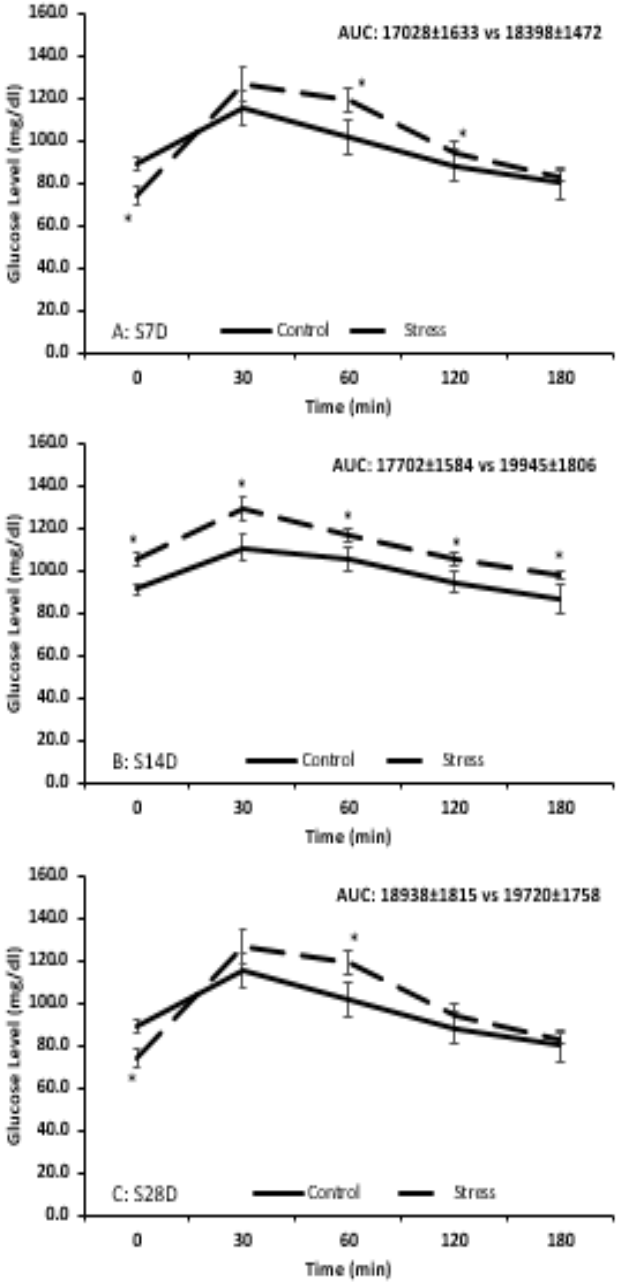

Figure 1. Effect of restraint stress on glucose tolerance at Days 7 (A), 14(B) and 28 (C). Insets data are the corresponding results of the OGTT as analysed by area under the curve (AUC_{GTT}). *p<0.05

Table 1: Effects of restraint stress on food intake, body weight and weight gain

Group	Days	Food Intake	Body Weight		%WG
			Initial	Final	
Control	**S7D**	82.14±4.59	173.67±10.04	182.33±7.31	4.99
	S14D	93.57±0.70	171.83±8.16	194.25±6.28	13.05
	S28D	135.28±5.23	175.50±12.63	211.50±10.04	20.51
Stress	**S7D**	72.85±3.20*	174.36±9.36	176.17±6.01	1.04
	S14D	90.14±1.83	172.81±7.05	184.67±4.81	6.86
	S28D	140.28±2.06	174.13±7.62	198.26±4.76	13.86

Data expressed as mean±SEM (n=6); *P<0.05 vs control; Initial = baseline / pre-stress exposure values; Final = post-stress values; %WG = % weight gain.

Table 2. Effect of restraint stress on corticosterone, insulin and adiponectin

Group	Stress	Corticosterone	Insulin	Adiponectin
S7D	-	711.43±30.64	4.24±1.29	25.64±2.72
	+	1266.31±72.09*	4.57±1.62	17.82±2.13*
S14D	-	698.15±33.27	5.06±1.12	28.27±3.14
	+	1194.56±80.18*	6.18±1.73	16.39±2.01*
S28D	-	676.60±35.03	4.48±1.08	29.73±4.21
	+	962.00±86.1*	6.51 ±1.01*	19.90±3.36

Data are expressed as mean ± SEM. *P < 0.05, compared with the control rats; n = 8. "+" indicates exposure to stress challenge, "–" indicates no exposure to stress challenge.

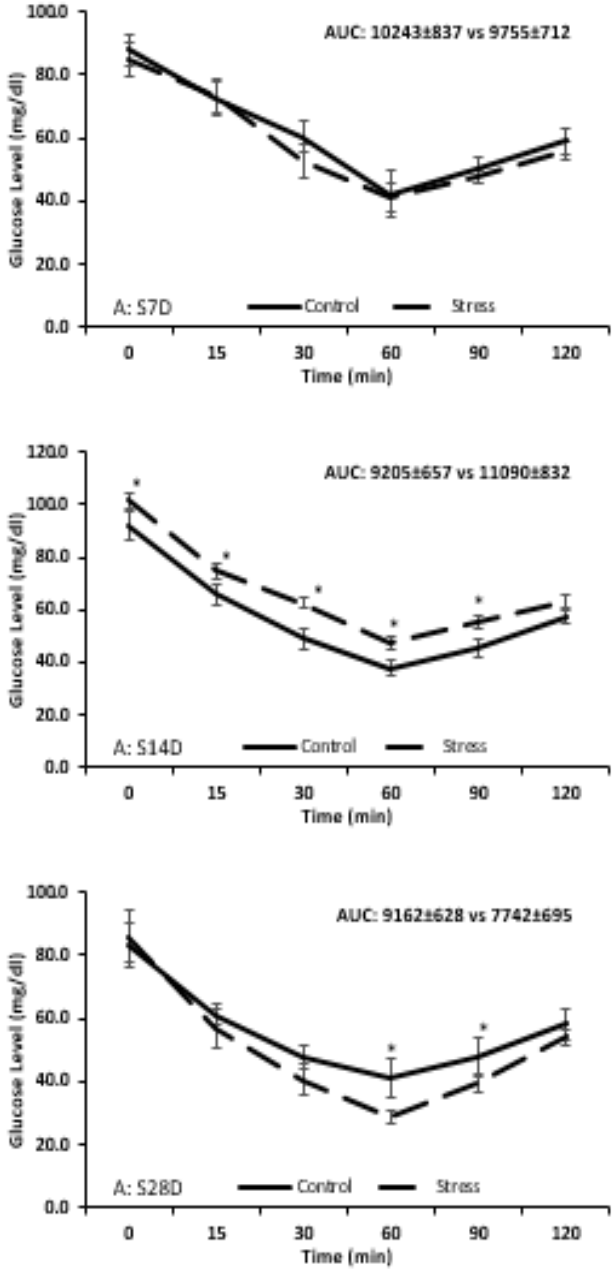

Figure 2. Effect of restraint stress on insulin sensitivity at Days 7 (A), 14(B) and 28 (C). Insets data are the corresponding results of the ITT as analysed by area under the curve (AUC_{ITT}). The values are presented as the means ± SEM of 6 rats per group. *p<0.05.

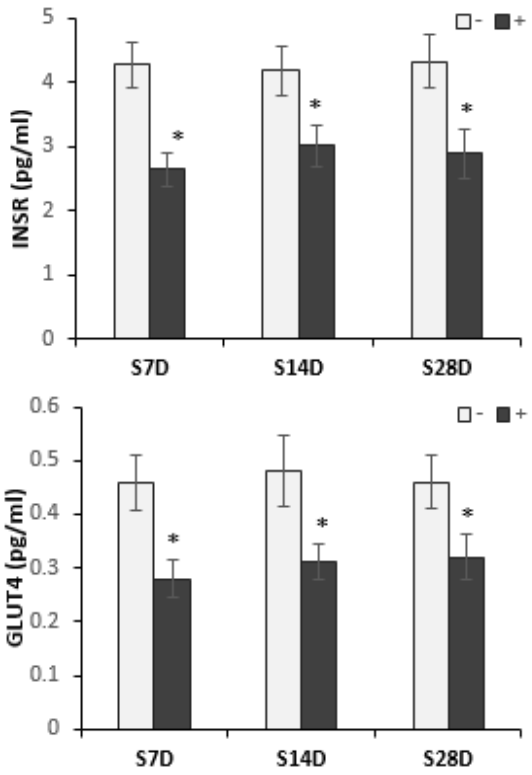

Figure 3: Quantitative analysis of INSR (a) and GLUT4 (b) expression in skeletal muscle. Data are expressed as mean ± SEM. *P < 0.05, compared with the control rats; n = 6

Corticosterone, insulin and adiponectin

The serum concentration of corticosterone, insulin and adiponectin were shown in Table 2. The results show a significant effect of restraint exposure on stress level in S7D, S14D and S28D rats compared with control groups. The corticosterone level in S7D was higher than S14D rats while S28D had a lower level of corticosterone as compared to the S14D rat, thus indicating habituation to the stress procedure. In addition, the expression of adiponectin, a known anti-inflammatory adipokine, was significantly reduced in all stressed groups except the S28D rats compared with their respective control group. However, serum insulin concentrations were not significantly different in all stress groups compared with the control after

repeated stress with the exception of S28D rats that had a significant ($p<0.05$) increase in insulin level.

INSR and GLUT4

We examined the mechanism underlying this effect on insulin tolerance in the stressed rats by measuring the expressions of INSR and GLUT4 in the skeletal muscle. Restraint stress increased the expression of GLUT4 but reduced INSR in S7D, S14D and S28D rats compared with the levels in control rats (Figure 3).

DISCUSSION

Any stimulus (stressor) that endangers the body's integrity or function results in a stress response, an adaptive response to solve stressful situation and determine new coping strategies (Landowski, 2007). For the present study on the potential effect and underlying mechanism of restraint stress on glucose homeostasis, the level of insulin and adiponectin, as well as the expression of INSR and GLUT4 in restraint-stress rats were investigated. The present results showed that the duration of exposure to restraint stress appears to produce different responses on glucose homeostasis. The results suggest that restraint-stress exposure (RSE) for 14-day produced greater glucose intolerance and insulin insensitivity compared with both 7-day and 28-day. In addition, the adiponectin level, as well as the expression of INSR and GLUT4 receptors in the skeletal muscle of the RSE rats were diminished relative to the control. However, prolonged exposure to restraint stress appears to cause a habituating effect in the S28D rats with muted / lower adverse responses in these endpoints.

Restraint stress can induce a complex stress reactions involving the hypothalamo-pituitary-adrenal (HPA) axis (Samson *et al*., 2007). Previous studies reported significant increases in plasma corticosterone levels after restraint-stress exposures (Pitman *et al*., 1988; Malisch *et al*., 2007). Serum corticosterone levels at the end of the exposure were significantly higher in all restraint-stress rats compared with controls, however, the severity appears to be diminished with increasing duration of stress exposure. This suggests that restraint induced stress reactions, and that the rats showed adaptation to the restraint after prolonged exposure.

Glucose homeostasis is critical for normal functioning of the central nervous system and cells which have an obligatory requirement for this metabolic substrate. The present findings indicated that restraint stress caused a significantly increase in the blood glucose level of rats in a time-coursed GTT. The mechanism by which stress raises the levels of glucose in these animals may be related to the possible enhanced activity of hypothalamic-pituitary adrenal axis during stress, resulting in increased secretion of adrenocorticotropic hormone (ACTH) and corticosteroids into the circulation. Release of ACTH in stress stimulates the adrenals to increase the production of catecholamines. These hormones mobilize stored carbohydrate reserves from the tissues which lead to elevated levels of blood glucose *(*Nade *et al*., 2009*)*. Another possible mechanism of hyperglycaemia is the blunted insulin signalling in the skeletal muscle observed in the restraint-stress rats.

Insulin resistance (insensitivity) is defined as an inadequate response by insulin target tissues, such as skeletal muscle, liver, and adipose tissue, to the physiologic effects of circulating insulin. Considering the results of the present study, glucose response to exogenous insulin administration was significantly lower in restraint-stress rats compared with their control counterpart. These findings are probably suggestive of insulin resistance in these animals and are consistent with similar reports of heightened insulin insensitivity under stressful conditions (Ceriello and Motz, 2004; Nakatani *et al*., 2005; Zardooz, 2006; Hentiksen *et al*., 2011). Insulin resistance has been induced by cortisol administration in rodents (van Donkelaar *et al*., 2014). In addition, sleep deprivation, a physiologic stressor has also been shown to increase cortisol concentrations and decrease insulin sensitivity in humans (McEwen, 2006; Donga *et al*., 2010). It is therefore plausible to conclude that stress-induced corticosteroid secretion has a negative consequence on insulin sensitivity. In another vein, decreased adiponectin levels as observed in this study could also impair systemic insulin sensitivity. Adiponectin is a novel adipocyte-specific protein that has been suggested to play a role in the development of insulin resistance. Reduced expression of adiponectin has been associated with some degree of insulin resistance (Wang *et al*., 2007). In order to assess whether defects in insulin-stimulated glucose transport activity was responsible for the stress-induced hyperglycaemia, we measured the expression of INSR and GLUT4 protein receptors in the skeletal muscles. The results showed that the expression of both receptors were significantly decreased compared with control rats. In skeletal muscle, activating the INSR leads to the translocation of GLUT4 from intracellular locations to the plasma membrane, where it facilitates the transport of glucose into the cell (Brewer *et al*., 2014). A major mechanism by which insulin signalling can be negatively regulated is via the alteration of INSR as well as GLUT4 activations. Since insulin-stimulated glucose uptake cannot function independently of the INSR, it is very likely that its reduced expression in this study account for the observed hyperglycaemia and insulin resistance. It thus seems to suggest that stress-induced disorder of glucose control could be

due to inadequate expression of INSR and GLUT4 protein receptors.

In summary, we found that exposure to restraint-stress was associated with glucose intolerance and insulin resistance in SD rats. Multiple insulin signalling receptors such as INSR and GLUT4 appear to be involved in the mechanism of stress-induced diabetogenic effects. However, these effects appear to become blunted with prolonged exposure to the stress factor.

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