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Oxytocin supplementation alleviates age-related insulin resistance through down regulation of pro-inflammatory cytokine gene expression.

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Abstract

Insulin resistance has long been recognized as a characteristic of aging. Many studies reported a beneficial role of oxytocin in obesity and type 2 diabetes. The aim of this study is to investigate the role of oxytocin in age-related insulin resistance in Wistar rats. Forty male rats were divided into four equal groups: young group, oxytocin-treated young group, old group and oxytocin treated old group. Oxytocin treated groups received Intraperitoneal (Ip) injection of oxytocin in a dose of 3 mg/kg body weight for 5 days. Experimental procedures included measurement of body weight, Body Mass Index (BMI) and intraperitoneal glucose tolerance tests. HOMA-IR index was also calculated. Assays of serum triglycerides, total cholesterol, LDL-C and HDL-C as well as serum IL-1β, IL-6 and TNF-α were performed. Malondialdehyde (MDA) and mRNA of IL-6, TNF-α and IL-1β were measured in Soleus Muscle (SM) and EF homogenates. Compared to young rats, old rats exhibited significant increase in BMI and HOMA-IR as well as an abnormal glucose tolerance test. Serum triglycerides, IL-6 and TNF-α were significantly increased in old rats. Furthermore, the MDA and mRNA of TNF-α and IL-6 of both SM and EF homogenates were significantly higher in old rats compared to young controls. These results, however, were notably lower in oxytocin supplemented rats compared to non-treated age matched group. In conclusion, oxytocin improves age related insulin resistance by alleviating inflammation and oxidative status in insulin sensitive tissues without affecting body weight.

Keywords: Oxytocin, Insulin resistance, Aging, Proinflammatory cytokines, Antioxidant.

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Introduction

Type 2 diabetes mellitus is a metabolic disorder characterized by both impaired insulin action on target tissues and defective pancreatic beta-cell insulin secretion [1]. Traditional views of type 2 diabetes pathophysiology indicated peripheral insulin resistance, i.e., the inability of insulin-target tissues to respond properly to the hormone, as the main driver of altered glucose homeostasis [2].

Insulin resistance has been long recognized as a characteristic of aging in humans and rodents [3]. Hence, aging has been considered as one of the factors which accelerate the development and progression of metabolic syndrome [4]. Moreover, insulin resistance could increase with age in relation to several well-known age-related changes, including hormonal changes, increased oxidative stress and inflammation [5]. Chronic, low-grade, systemic inflammation is widely accepted as a significant risk factor underlying aging and age-related type 2 diabetes [6,7]. The increased production of Pro-inflammatory cytokines was shown to act in an autocrine or paracrine manner to induce insulin resistance in peripheral tissues and macrophages [8,9]. Coincidently, ROS generation as a result of oxidative stress has been implicated in the development of insulin resistance and type 2 diabetes [10].

Apart from the traditional role of oxytocin in the physiology of reproduction and lactation, the role of oxytocin in metabolic regulation started to attract attention. Oxytocin-deficient mice exhibited decreased insulin sensitivity and impaired glucose tolerance [11]. Oxytocin was found to reverse obesity as well as the related glucose and insulin disorders in mouse models with therapeutic significance in the treatment of obesity and insulin resistance. Oxytocin administration reduced obesity related diabetic changes ranging from insulin resistance, glucose intolerance, pancreatic islet hypertrophy and hepatic steatosis [12,13]. The anti-inflammatory and antioxidant effects of oxytocin were also reported to ameliorates the immediate myocardial injury in heart transplant through down-regulation of the myocardial inflammatory response, reactive oxygen species, and neutrophil-dependant myocardial apoptosis [14].

Despite the metabolic advantage of oxytocin in increasing insulin sensitivity and combating insulin resistance, together with the reports about age related decline of oxytocin activity at least in rats [15], no thorough studies have been reported

from the perspective of its possible effects in these conditions in aged rats. We postulate that oxytocin administration might improve the age-associated insulin resistance and enhance sensitivity of insulin dependent tissues. We also assume that oxytocin might be able to lower inflammatory and oxidative states of the insulin-sensitive tissues in aged rats. Hence, the aim of this study was to investigate the effects of oxytocin on age related insulin resistance in old rats. Moreover, we tested the anti-inflammatory and antioxidant effects of oxytocin as a possible mechanism for its action.

Materials and Methods

This randomized controlled study was conducted in the Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt, from June 1 to August 31, 2015, and was approved by the ethics committee of FMSU REC, Cairo, Egypt. The work was undertaken on 40 male Wistar rats. The rats were maintained under standard conditions of boarding. The investigation conformed to the guide for the care and use of laboratory animals published by the United States National Institutes of Health. The rats were fed standard diet prepared in our laboratory according to the normal nutritional dietary requirements.

Rats were weighed and divided into four equal group (n=10). Group I: young age group (2-3 Months old rats,), group II: old age group (22-24 months old), group III Oxytocin treated young rats, and finally group IV, oxytocin treated old rats. Oxytocin treated groups received intraperitoneal injection of oxytocin in a dose of 3 mg/ kg body weight Ip for 5 days. Control rats were injected with saline as a vehicle. The dose of oxytocin was chosen according to a pilot study in our laboratory and previous studies [13]. No known side effects of oxytocin were reported in the literature and oxytocin administration was considered safe with no reliable side effects for short term use in controlled research settings [16].

Experimental procedures

Glucose tolerance tests were conducted on overnight fasted rats. A blood sample was taken from the tail of each rat and was used to determine blood glucose and serum insulin levels. Rats were then given a bolus injection of glucose (2 g/kg) into the intra-peritoneal cavity. Blood glucose concentrations were measured in other samples from the rat's tail with a glucometer at 30, 60, 90 and 120 min. Insulin was measured using a rat insulin enzyme-linked immunosorbent assay kit (ALPCO Diagnostics Salem, NH). HOMA-IR test was used as a marker for insulin resistance using the equation previously described [17]. HOMA-IR=(fasting glucose × fasting insulin)/22.5. Insulin concentration is expressed in μU/L and glucose in mmol/L.

Rats were then weighed and anaesthetized with thiopental sodium 40 mg/kg intraperitoneally (Sandoz GmbH, Kundl-Austria). Body lengths were measured for later calculation of BMI using the formula: weight in kg/ body length in m^2 [18]. Blood samples were collected from the abdominal aorta, centrifuged, and the serum was stored at -20°C in separate aliquots for biochemical assays. From each rat the soleus muscle of one leg was carefully dissected from the surrounding tissue, stored at -80°C for determination of MDA, and for cytokine mRNA measurement. Epididymal pads of fat were removed, stored at -80°C for measurement of MDA and mRNA expressions of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α.

Biochemical analysis

The following analyses were carried out for cholesterol, triglycerides, and High Density Lipoprotein (HDL) using kits from Biodiagnostic, Egypt: low density lipoprotein (LDL) was calculated according to the formula described elsewhere [19]. The serum concentrations of IL-1β, IL-6 and TNF-α were determined by ELISA (Life Technologies, USA) using DS2 automated ELISA analyser (Dynex Technologies,USA), according to the manufacturer's instructions.

Measurement of tissue MDA

Soleus muscles were homogenized in cold KCl solution (1.5%); MDA levels were estimated by the double heating method [20]. The same steps were repeated to determine the MDA in adipose tissue.

Expression of mRNAs of genes in the soleus muscle and epididymal fat

RNA was extracted from the homogenized soleus muscle and epididymal fat. Real time quantitative fluorescence PCR with SYBR Green was used to measure expression of TNF-α, IL-1 and IL-6 mRNAs in the soleus muscle and epididymal fat with GAPDH as an internal reference. The gene sequences were identified in Gene Bank for the design of specific primers (Table 1). Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. Then, 4 μl of total RNA were subjected to reverse transcription with random primers, and M-MuLV reverse transcriptase (Fermentas, #EP0451, European Union) in 20 μl of reaction mixture at 37°C for 1 h and then at 95°C for 3 min using a PCR instrument, to convert RNA into complementary DNA (cDNA). Then, 5 μl of cDNA were added to the 50 μl reaction mixture, followed by amplification in an automatic quantitative fluorescent PCR instrument (Model 7500, Applied Biosystems, USA). The PCR conditions were: pre-denaturation at 93°C for 3 min, 40 cycles of denaturation at 93°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec. All gene expressions were normalized to GAPDH, which served as an internal control for the quality of isolated RNA from each homogenized muscle and adipose tissue samples. The data represented relative quantification, using the comparative delta-delta Ct method. The gene expression is reported as relative quantities in subjects with or without cardiovascular disease as related to an external reference sample and normalised to an endogenous reference.

Table 1. *Reverse transcription-polymerase chain reaction primer sequences results.*

	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)
TNF-α	GCATGATCCGCGACGTGGAA	AGATCCATGCCGTTGGCCAG
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
IL-1β Forward	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
GAPDH	CCTCTCTCTTGCTCTCAGTAT	GTATCCGTTGTGGATCTGACA

In young animals, no significant changes in oxytocin treated young rats as compared to young control regarding all the parameters studied. Both the initial and final body weights were significantly higher in control and oxytocin-treated old rats compared to control and oxytocin-treated young animals respectively (P<0.001 each). Moreover, the body weights at the end of the experimental period in all groups studied were not significantly different from their corresponding initial values. In the old groups, non-treated rats showed significantly higher BMI and HOMA-IR as compared to young controls (P<0.05) while their fasted blood glucose and serum insulin values were not significantly different. However, blood glucose levels during IP glucose tolerance test at 0.5, 1 h, 1.5 h and 2 hours after glucose challenge were significantly higher in old rats compared to young control rats (P<0.001). In contrast, oxytocin-treated old rats showed significantly lower HOMA-IR index (P<0.05) when compared to non-treated old rats. In addition, blood glucose level during IP glucose tolerance test showed significant decline in oxytocin treated old rats compared to their age matched control at time 0.5, 1, 1.5 and 2 hours (P< 0.001) as shown in Tables 2-4).

Compared to young controls, old rats showed significantly higher serum levels of triglycerides, IL-1β, IL-6 and TNF-α (P<0.001, P<0.05, P<0.05 and P<0.01, respectively). However, the changes in serum LDL-C, HDL-C and total cholesterol were statistically insignificant. On the contrary, oxytocin-treated old animals exhibited significantly lower serum levels of triglycerides as compared to age matched controls (P<0.05). Similarly, IL-1β, IL- 6 and TNF-α were significantly lower (P<0.05, P<0.05, P<0.01 respectively). However, the changes in LDL-C, HDL-C and total cholesterol were not significantly different in these groups (Table 5).

Table 2. *Mean+SEM of starting and final as well as the changes of body weight (gm) in control and oxytocin-treated young and old rats during the 5-days treatment period.*

	Young	Young OXY	Old	Old OXY
Starting BW	108.5+1.32	110+1.33	319*+2.01	321.8#+2.08
Final BW	112.2+1.44	113.2+1.56	322.1*+2.24	328.9#+2.01
The change	2.7+0.45	3.2+0.78	3.1+0.46	7.1+0.12

*P<0.001 significant from young group; #P<0.001 significant from young Oxy group.

Table 3. *Effect of Oxytocin (OXY) on the Body Mass Index (BMI), Blood Glucose, Serum insulin, HOMA-IR index, as well as, basal and insulin stimulated Glucose Uptake (GUP) in Soleus Muscle (SM) and Epididymal Fat (EF) in young and old rats (mean+SEM n=10).*

	Young	Young OXY	Old	Old OXY
BMI	4.55+0.04	4.53+0.04	5.42*+0.04	5.26+0.04
Fasting blood glucose (mg/dl)	80.5+0.94	79.7+0.78	84.5+0.53	81.4+0.48
Fasting insulin (μU/ml)	7+0.71	7.1+0.77	8.11+0.14	7.3+0.10
HOMA-IR	1.39+0.02	1.4+0.02	1.69*+0.03	1.46#+0.02

*P<0.05 significant from young group; #P<0.05 significant from old group.

Table 4. *Blood glucose during glucose tolerance test.*

Blood glucose mg/dl	0 h	0.5 h	1 h	1.5 h	2 h
Young	80.5 ± 0.94	138.4 ± 0.44	122.7 ± 0.58	92.2 ± 0.60	81.4 ± 1.1
Young OXY	79.7 ± 0.78	137.7 ± 0.50	123.2 ± 0.60	90.7 ± 0.75	79.3 ± 0.91
Old	84.5 ± 0.53	162.8* ± 1.00	152.7*±1.3	138.3* ± 0.85	128.7* ± 1.00
Old OXY	81.4 ± 0.48	141.3# ± 0.57	127.3# ± 0.92	93.1# ± 0.78	85.6# ± 0.66

*P<0.001 significant from young group; #P<0.001 significant from old group.

Table 5. Effect of Oxytocin (OXY) on the serum lipids (triglycerides, total cholesterol, LDL-C, HDL-C) and pro-inflammatory cytokines (IL-Iβ, IL-6 and TNF-α in young and old rats (mean+standard error of the mean, n=10).

	Young	Young OXY	Old	Old OXY
Triglycerides (mg/dl)	48.4 ± 0.26	49.3 ± 0.58	56.6*** ± 0.64	51.2# ± 0.38
LDL-C (mg/dl)	57.82 ± 1.41	39.34 ± 0.19	47.2 ± 1.73	40.5 ± 1.25
HDL-C (mg/dl)	46.5 ± 0.74	47.9 ± 0.77	42.5 ± 0.72	46.7 ± 0.72
Cholesterol (mg/dl)	94 ± 1.05	97 ± 1.75	101 ± 1.35	97.4 ± 1.21
Plasma IL-1β (pg/ml)	2.1 ± 0.1	2.12 ± 0.06	3.42* ± 0.09	2.60# ± 0.08
IL-6 (pg/ml)	2,13 ± 0.13	2.24 ± 0.13	3.99* ± 0.10	2.88# ± 0.07
TNF-α (pg/ml)	3.22 ± 0.06	3.16 ± 0.07	5.68 ** ± 0.19	3.48## ± 0.14

*P<0.05, **P<0.01, ***P<0.001 significant from young group, #P<0.05, ##P<0.01 significant from old group.

Old rats showed significantly higher MDA levels of soleus muscle and epididymal fat homogenates when compared with young controls (P<0.05 and P<0.01, respectively). Moreover, soleus muscle homogenates of old rats showed significantly higher mRNA of IL-1β and TNF-α compared to the young group (P<0.05 each). Similarly, significantly higher IL-6 and TNF-α (P<0.05 each) were found in epididymal fat homogenates of old rats. However, the higher levels of mRNA of IL-6 of soleus muscle and IL-1β of epididymal fat of old rats did not reach the level of significance when compared with the corresponding values of young controls (Table 6).

Expressing our data as Δ Ct oxytocin administration to old rats resulted in significantly lower soleus muscle content of MDA and mRNA of IL-1β and TNF-α as compared to young counterparts (P<0.05, P<0.001, and P<0.05, respectively). Similar significant reduction in epididymal fat content of MDA and mRNA of IL-1β and TNF-α was found with oxytocin supplementation (P<0.01, P<0.001 and P<0.05, respectively). However the mRNA of IL-6 of soleus muscle and IL-1β of epididymal fat did not differ significantly between oxytocin-treated and untreated old rats (Table 6).

Table 6. Effect of oxytocin treatment on tissue content of MDA and mRNA of IL-1β, PL-6 and TNF-α in young and old rats (mean+SEM n=10).

	Young	Young OXY	Old	Old OXY
SM homogenates				
MDA	17.1 ± 0.27	16.6 ± 0.30	21.3* ± 0.31	18.0# ± 0.21
IL-1β	1.34 ± 0.07	1.26 ± 0.07	2.13* ± 0.06	1.42## ± 0.02
IL-6	1.13 ± 0.04	1.16 ± 0.03	1.43 ± 0.04	1.26 ± 0.04
TNF-α	1.24 ± 0.04	1.21 ± 0.04	1.74* ± 0.04	1.38# ± 0.02
EF homogenates				
MDA	15.6 ± 0.27	14.7 ± 0.28	20.5** ± 0.37	16.3## ± 0.22
IL-1β	1.62 ± 0.05	1.55 ± 0.08	2.01 ± 0.05	1.73 ± 0.08
IL-6	1.40 ± 0.07	1.31 ± 0.05	2.15* ± 0.04	1.24### ± 0.06
TNF-α	1.21 ± 0.06	1.33 ± 0.05	1.94* ± 0.10	1.19# ± 0.07

*P<0.05 ***P<0.001 significant from young group, #P<0.05 ##P<0.01 significant from old group.

Correlation studies showed significant positive correlations between HOMA-IR index and the serum IL-1β, IL-6 and TNF-α (P<0.01, P<0.01 and P<0.001 respectively). Similarly, significant positive correlation were found with mRNA content of IL-1β, IL-6 and TNF-α in SM (P<0.001, P<0.02 and P<0.001) and in EF (P<0.01, P<0.01 and P<0.001) respectively. MDA content of SM and EF shows additional significant positive correlations with HOMA-IR index (P<0.02 and P<0.01) respectively (Table 7).

Table 7. Correlation coefficient (r) between HOMA-IR index and both the inflammatory and oxidative markers in all rat groups.

	IL-1β	IL-6	TNF-α	MDA
Serum	0.458411**	0.42538**	0.5464***	-
SM homogenates	0.523761***	0.397284*	0.61282***	0.410758*
EF homogenates	0.600123***	0.607116***	0.482804**	0.421169**

*P<0.02, **P<0.01, ***P<0.01 significance for the r value.

Discussion

The present study demonstrated age related decline in glucose tolerance reflected in an abnormal glucose tolerance test and significant elevation of HOMA-IR index. There were also significant increases in tissue and plasma content of pro-inflammatory cytokines and thiobarbituric acid reactive substances. The apparent insulin resistance and the associated inflammatory and oxidative patterns were improved in old rats upon systemic oxytocin administration.

The deterioration of insulin homeostasis in aged rats agrees with the previous studies [5,21]. The improvement of age related glucose tolerance upon oxytocin administration indicates involvement of oxytocin in peripheral metabolic functions of skeletal muscle and adipose tissue. Oxytocin enhancement of glucose disposal evidenced by glucose tolerance test reflects improved insulin sensitivity of oxytocin

supplemented old rats. These data are in accordance with others [22], and confirmed by the decrease in glucose tolerance and insulin sensitivity in oxytocin deficient mice [11]. On the other hand, contradicting reports about the effect of oxytocin may be attributed to the differences in the dose of oxytocin, animal species and the experimental model used in their study [23].

The effect of oxytocin on glucose tolerance and insulin resistance was previously attributed to the body weight lowering and antiobesity effect [22]. Surprisingly, we did not find any significant differences in the body weight or body mass index when oxytocin treated rats compared with age matched non-treated counterparts. The lack of body weight lowering effect was supported by other studies where exogenous oxytocin administration was not associated with significant changes in body weight of atherosclerotic mice [24] or hyperlipedimic rabbits [25] In addition, oxytocin showed its therapeutic effect against prediabetic or diabetic animal model regardless its effect on obesity [13]. Thus we could attribute the beneficial effect of oxytocin in aged rats in our study to an anti-obesity-independent mechanism. One of the mechanisms by which aging contributes to insulin resistance is chronic inflammation where accumulation of pro-inflammatory cytokines had a negative impact on insulin signaling during aging [5].

Our data showed significant elevation of proinflamatory cytokines both in plasma and in insulin sensitive tissues. We could thus attribute the state of age related insulin resistance to the state of chronic inflammation displayed in old rats. These inflammatory cytokines act in autocrine and paracrine manner to induce insulin resistance in peripheral tissue. Moreover, our results showed a highly significant positive correlation between insulin resistance index (HOMA-IR) and inflammatory markers, either their systemic concentration or their level of gene expression in adipose tissue or in the muscle (Table 7).

The beneficial effect of oxytocin could thus be related to the improvement of the negative impact of pro-inflammatory cytokines on insulin signaling during aging. Our data showed down regulation of the expression of pro-inflammatory genes in epididymal fat and skeletal muscle with oxytocin administration. Although our assumption regarding this molecular mechanism of oxytocin in insulin resistance is totally new, this view is supported by the findings in other similar situations. Previous reports had showed that oxytocin infusion in models of myocardial infarction improved function in the injured heart through reduction of inflammation [26,27]. Furthermore, chronic peripheral oxytocin administration inhibited inflammation and atherosclerotic lesion development where expression of adipokines from visceral adipose tissue was indicative of decreased adipose tissue inflammation [25]. Specifically, oxytocin infusion reduced the secretion of IL-6 from epidymal fat ex-vivo in APOE -/- mice [24].

We assume that TNF-α and other pro-inflammatory cytokines in could be the link between inflammation, aging and IR. The increased TNF-α stimulated inhibitory phosphorylation of serine residues of IRS-1 either directly [28] or through activation of IKKβ [29] or JNK [30] pathways. Specifically, phosphorylation of these residues impedes the normal association of IRS-1 with insulin receptors, thereby impairing the downstream of insulin signaling [31,32]. We could also extrapolate that the anti-inflammatory effect of oxytocin could disturb the IKKβ and JNK pathways inhibiting serine phosphorylation of IRS-1 and improving insulin signaling. Inhibition of JNK or IKKK [30,33] improved insulin sensitivity in various models of insulin resistance in association with reduced inhibitory serine phosphorylation of IRS-1 [30].

Furthermore, we have demonstrated a significant age-related increase in MDA reflecting increased free radical production in old rats, in accordance with previous studies [34]. The significant positive correlations between MDA content of epididymal fat and soleus muscle and HOMA-IR index in our study provide an index about the close association between reactive oxygen species and IR [35,36]. Initially, mutations and deletions which occur in DNA lead to impaired function of the respiratory chain and enhanced ROS production with subsequent accumulation of fatty acid metabolites [37]. Eventually, activation of protein kinase C leads to activation of IKK and JNK followed by impaired insulin signalling [38].

The significant decline of malondialdehyde content in insulin sensitive tissue of oxytocin-treated old rats addresses the antioxidant effect of oxytocin supplementation in combating insulin resistance. This finding agrees with the recent conclusions about the negative correlation between maternal levels of oxytocin and both the total oxidative status and the oxidative stress indices [39,40]. This amelioration of oxidative status converges with the anti-inflammatory results in inhibiting IKK and JNK pathways potentiating the insulin signaling mechanism.

In summary, the present study demonstrates that oxytocin supplementation attenuated the age-related inflammatory and oxidative messier in insulin sensitive tissues independent of its effects on body weight. It subsequently improved insulin resistance state and thus may provide a possible therapeutic intervention in the aging population.

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