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The influence of biochanin a consumption on c-CBL-associated protein level in adipose tissue of streptozotocine-nicotinamide induced diabetic rats

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ABSTRACT

Increased c-Cbl associated protein (CAP) level in adipose tissue can facilitate glucose uptake via GLUT 4 in adipocytes. Increased level of CAP results from activation of PPARγ transcription factor. Biochanin A, soy isoflavonoid, is a PPARγ agonist. Present study was designed to investigate the influence of Biochanin A supplementation on CAP gene expression and insulin resistance. 12-week-old male Wistar rats were usedin this study.Type 2 diabetes mellitus was induced by intraperitoneal injection of Streptozotocin followed by Nicotinamide. Present study was designed in 8 groups of 5 animals. Biochanin A was supplemented for a month at 4 different doses: 1 and 5 mg/kg; intraperitoneal, 10 and 20 mg/kg; orally. Fasting blood glucose, serum insulin level and HOMA index were examined. CAP level in adipose tissue was tested by immunoblotting analysis. Findings of this study indicated that administration of Biochanin A at doses of 5mg/kg; intraperitoneal, and 20mg/kg; orally, could increase the expression of CAP in adipose tissue significantly. Additionally, insulin resistance improved and fasting blood glucose level decreased considerably. According to the results, increased expression of CAP in adipocytes can be associated with improvement of insulin resistance.

Keywords: Biochanin A, GLUT 4, C-cbl associated protein, Adipose tissue, Insulin resistance

INTRODUCTION

There are 13 various glucose transporter (GLUT) proteins in different organs of body. They are on cell surfaces and uptake glucose and other sugars from blood circulation. Among them, GLUT 4, which is highly expressed in adipocytes and skeletal muscle cells, plays the most important role in glucose homeostasis. It uptakes glucose into adipocytes and skeletal muscle cells in the manner of insulin dependent[1].

Dysregulation of GLUT 4 translocation to the cell surfaces, usually leads to inability of skeletal muscle cells and adipocytes to uptake glucose. In the other words, insulin dependent glucose uptake disturbs that means insulin resistance; it is the main pathological cause of type 2 diabetes mellitus[2].

Diabetes mellitus is classified into type 1 diabetes mellitus and type 2 diabetes mellitus. Type 1 results from complete destruction of pancreatic β cells, and type 2 usually results from insulin resistance. According to above, type 1 diabetes should be treated by external insulin administration; while type 2 diabetes should be treated by drugs which can improve insulin resistance. The epidemiological studies indicate that diabetes mellitus will affect 439 million people all over the world in 2030; approximately 90% of these are type 2 diabetic patients[3].

The drugs which often use in type 2 diabetic patients have two major mechanisms; stimulation of insulin secretion from pancreatic β cells or facilitating GLUT 4 trafficking to the cell surfaces of skeletal muscle cells and adipocytes. It seems that the second mechanism is more important than the first one, because GLUT 4 is the last step in glucose uptake and decreases the blood glucose. In the other words, increased insulin level without GLUT 4 cannot be useful[1, 2].

c-Cbl- associated protein (CAP), also known as SORBS1, is an adaptor protein that exists in adipocytes and skeletal muscle cells and binds to the proto-oncogene c-Cbl. It contains three C-terminus SH3 binding domains and one sorbin-like region. CAP plays an important role in GLUT4 translocation to cell surfaces in a PI-3 kinase(phosphorylated inositol 3-kinase) independent pathway and facilitates glucose uptake. So it can be a therapeutic target in type 2 diabetes mellitus[4, 5].

Several studies reported that, peroxisome proliferator-activated receptor γ (PPARγ), a ligand inducible transcription factor whichis highly expressed in adipocytes, can increase the expression of CAP gene; because a PPARγ-responsive element (PPRE) was identified in promoter region of CAP gene. So it can be concluded that, PPARγ agonists can be useful in increasing the expression of CAP gene[3, 6, 7].

BiochaninA (BCA) is a natural isoflavonoid that can be found in soy, red clover and some legumes. However, the anti-diabetic effects of BCA has been approved its mechanism of action is still controversial[3, 8, 9]. Insulin mimetic effects, increasing of glucose uptake in peripheral tissues, stimulation of insulin secretion from beta pancreatic cells and regulating the key enzymes in carbohydrate metabolism are probable anti-diabetic mechanisms of BCA [9]. Some studies indicated that BCA is a PPARγ agonist[8, 10]. PPARγ plays an important role in glucose homeostasis; probably anti-diabetic effect of BCA is associated with PPARγ activation[11]. As a result, BCA as aPPARγ agonist, can activate PPARγ transcription factor which will lead to increased expression of CAP gene and CAP level eventually[6, 12].

According to the above facts, the present study was designed to examine the influence of BCA consumption on CAP level in adipose tissue of Streptozotocin-Nicotinamide (STZ-NCD) induced diabetic rats. Furthermore, body weight (BW), HOMA index, fasting blood glucose (FBG) and insulin level were tested.

MATERIALS AND METHODS

1-Experimental animals

12-Weeks old male Wistar rats (body weight of 200-230g) were purchased from central animal house of Hamadan University of Medical Sciences, Hamadan, Iran. Animals were kept in 12-Hour light/dark period at a stable temperature (21-23˚C) and 55%±10% relative humidity. Rats were nourished by standard chow and water during the experiment[13].The protocol for experiment on animals was approved by the Ethics committee of Hamadan University of Medical Sciences (Iran).

2-Diabetes mellitus induction in rats

For induction of diabetes mellitus, rats should be fasted overnight. Prior to 60mg/kg STZ (STZ, S130, Sigma) injection, NCD 120mg/kg (NCD, N3376, Sigma) was injected to prevent the complete destruction of pancreatic beta cells; partial destruction of pancreatic beta cells is close to human type 2 diabetes mellitus[14]. STZ was dissolved in 0.1Molar citrate sodium buffer (pH=4.7); and the solvent for NCD was normal physiological saline. The interval between two injection was 15 minutes and injection was performed intraperitoneally (IP). FBG level was measured seventy two hours after injection using a glucometer (Glucocard 01, Arkray, Japan).The FBG threshold to diagnose diabetic animals, was 126 mg/dl. Blood samples were obtained from tail vein[13, 15-17].

3-Research design

The present study was designed in eight groups of 5 animals. Dimethyl sulfoxide (DMSO) was used as BCA solvent (BCA, D2016, Sigma); 0.05%DMSO for oral consumption and 75%DMSO for IP injection. Oral consumption was performed using a gavage syringe. BCA at doses of 10 and 20mg/kg was administrated orally and at doses of 1 and 5mg/kg was injected IP. The used solvent (DMSO) at similar concentrations and similar ways was administered in the healthy- and diabetic- control groups.

A week after induction of diabetes, treatment was began and lasted for a month. FBG and BW were examined 3 times: before induction of diabetes, 7 days after induction of diabetes, and one month after treatment. Visceral adipose tissue samples were taken from animals. The samples were stored in -80˚C freezer. The serum was separated from blood samples and kept at -20˚C for insulin level testing[19, 20].

4- Serum insulin measurement

Rat insulin ELISA kit (ERINS, Thermo scientific) was used to measure the serum insulin level. Homeostatic model assessment insulin resistance (HOMA-IR) was measured as follows:

Insulin(μU/ml) × glucose(mg/dl) / 405[21].

5- Extraction of tissue protein

RIPA lysis buffer system (Sc-24948, Santa Cruz) was used for lysing the adipose tissue. 0.3 gr of adipose tissue was homogenized by adding 0.9 ml of RIPA buffer and 15μl protease inhibitor. Homogenates were centrifuged at 4˚C (12000 g, 20minutes). Fat layer was separated and the supernatants were stored at -20˚C. Bicinichoninic acid (Sc-202389, Santa Cruz) method was used to measure the protein concentration in extracted samples[4, 22].

6-Analysis of proteins by immunoblotting

Extracted proteins from different samples were subjected on 10%SDS-polyacrylamide gel electrophoresis (SDS-PAGE),and subsequently were blotted on nitrocellulose membrane. Blocking procedure was performed with 5% skim milk in TBST (0.1% Tween 20 in Tris buffered saline, pH=7.6) for 1-hour at room temperature with agitation. Blots were incubated overnight with primary antibodies against β-actin (1/500, Sc-130657, Santa Cruz) or CAP (1/200, Sc-25496, Santa Cruz) in TBST at 4˚C on a shaker. For reducing non-specific binding of primary antibodies, while agitation, membrane was washed in TBST for 30 minutes. Subsequently, immunoblots were exposed by 1/4000 dilution of horse-radish peroxidase (HRP) – conjugated secondary antibody (Sc – 2005, Santa Cruz) in TBST for 1-hour at room temperature on a shaker. After that, another washing protocol, as mentioned above, was performed. For detecting the bands, enhanced chemiluminescence (ECL) detection kit (Sc-2048, Santa Cruz), was used. Bands intensities were quantified by using Image J Software[21, 23].

7-Statistical analysis

The given data are presented as mean±SD. Statistical analysis were carried out by using one way ANOVA followed by post hoc Tukey´s test. The level of significance adopted was $p<0.05$.

RESULTS

In this study, the influence of BCA consumption on BW, FBG, serum insulin level, HOMA index and CAP level in rat adipose tissue was investigated. Table 1 and table 2 demonstrate the effect of BCA administration on animals' BW in STZ-NCD induced diabetic rats. It can be inferred from **table 1** that IP injection of BCA at dose of 5mg/kg, increased BW significantly compared to untreated diabetic rats ($p<0.001$) , but it did not reach to those of healthy rats ($p<0.001$). It should be noted that IP injection of BCA at dose of 1mg/kg had no considerable effect on weight gain when it was compared with that of diabetic control group ($p>0.05$). As is shown in **table 2**, oral administration of BCA at dose of 20 mg/kg could affect weight gain significantly compared to untreated diabetic rats ($p<0.001$). However, BW in animals which received 10mg/kg of BCA, did not change considerably compared to diabetic control animals ($p>0.05$).Obviously, there was considerable difference in BW between healthy and diabetic (treated and untreated) animals ($p<0.001$).

Table 3 and table 4 illustrate the influence of BCA supplementation on FBG, serum insulin level and HOMA index in STZ-NCD induced diabetic rats. In **table 3**, considerable reduction in FBG level of rats that received 5mg/kg of BCA, compared with untreated diabetic animals, is observed ($p<0.001$).However, there was no significant reduction in FBG level of rats which received 1mg/kg of BCA, compared to diabetic control rats ($p>0.05$). It can be inferred from table 3 that IP injection of BCA at both dose of 1 and 5 mg/kg, had no significant effect on serum insulin level compared to untreated diabetic animals ($p>0.05$), HOMA index improved significantly in animals that treated by 5mg/kg of BCA during the experiment compared to other diabetic animals that received 1mg/kg of BCA and untreated diabetic animals ($p<0.001$). Additionally, in table 3, notable differences are observed in FBG, serum insulin level and HOMA index between healthy and diabetic (treated and untreated) animals ($p<0.01$). It can be deduced from **table 4** that oral administration of BCA at dose of 20mg/kg had significant effect on FBG reduction

compared to untreated diabetic rats ($p<0.001$). There is no remarkable difference in FBG level between diabetic controland rats treated by 10mg/kg of BCA ($p>0.05$). Oral administration of BCA at both dose of 10 and 20 mg/kg could not affect the serum insulin level comparing to untreated diabetic rats ($p>0.05$). However, it should be noted that oral administration of BCA at dose of 20mg/kg, could improve HOMA index considerably compared to diabetic control animals ($p<0.001$). There was no significant difference in HOMA index between animals that were treated by 10mg/k of BCAand diabetic control rats ($p>0.05$). According to table 4, FBG, serum insulin level and HOMA index are significantly different in healthy rats compared to that of diabetic (treated and untreated) animals ($p<0.01$).

Fig.1 and Fig.2 exhibit the effect of BCA consumption on CAP level in adipose tissue of STZ-NCD induced diabetic animals. It can be inferred from **Fig.1** that IP injection of BCA at dose of 5mg/kg could increase the level of CAP in adipose tissue compared to diabetic control animals ($p<0.01$). There was no significant difference in CAP level between animals which received 1mg/kg of BCA, healthy and diabetic control animals ($p>0.05$). According to **Fig.2**, a considerable increase in CAP level of animals that received 20 mg/kg of BCA compared with diabetic control animals, is observed ($p<0.001$). As is shown in Fig.2, induction of diabetes could not affect the CAP level in adipose tissue; in the other words, treated animals by 10 mg/kg of BCA, healthy, and diabetic control animals hadsimilar CAP level ($p>0.05$).

DISCUSSION

The previous studies indicated that increased CAP level in adipose tissue accompanies with insulin resistance improvement [4, 6, 24-27]. When insulin binds to its receptor on the surfaces of adipocytes, an adaptor protein called APS, facilitates the interaction between c-Cbl/CAP complex and insulin receptor. This interaction leads to phosphorylation of c-Cbl in tyrosine residue and binding the c-Cbl/CAP complex to a lipid raft protein called Flotillin. After that, c-Cbl phosphorylates the CrkII/C3G complex in tyrosine residue. C3G protein is a guanine nucleotide exchange factor that activates TC10 protein, which is a member of Rho protein family. ActivatedTC10 facilitates the GLUT4 translocating to cell surface. Obviously, by increased translocation of GLUT4 to cell surfaces, glucose uptake will increase and it means improvement of insulin resistance. In the other words, CAP improves insulin resistance in an alternate pathway of insulin signaling. As a result, increasing CAP level in adipose tissue can be a therapeutic target in type 2 diabetes patients[4, 25-27].

Consistent with the previous studies, findings of our study illustrated that increased CAP level in adipose tissue is accompanied by reduction of FBG level and improvement of insulin resistance. Therefore it can be concluded that BCA, as a PPARγ agonist, can be an effective agent in control of type 2 diabetes mellitus by increasing the expression of CAP gene and CAP level subsequently in adipose tissue.

Gupte et al. demonstrated that insulin dependent c-Cbl/CAP signaling, is an alternate pathway that helps the PI-3kinase pathway to uptake glucose [4]. Baumann et al. indicated that, there is a PPRE in promoter region of CAP gene [6].Thirone et al. reported that CAP contributes to the regulation of insulin dependent glucose uptake in adipose tissue of insulin resistant animals [27]. Azizi et al. showed that BCA has antidiabetic and antilipidemic effects in STZ-induced diabetic rats[9]. Wang et al. illustrated that BCA is a PPARγ agonist and PPARγ plays an important role in glucose homeostasis in adipose tissue [12]. Harini et al. reported that BCA consumption in diabetic rats led to reduction of FBG level [8].

According to the findings of the present study, it can be concluded that BCA supplementation in both injectable form and oral consumption can be useful in control of type 2 diabetes mellitus. It should be noted that, most effective dose, best administration form and the side effects of BCA are the important challenges to approve the BCA an appropriate drug in type 2 diabetic patients. Our study is a novel experiment that presented a mechanism for BCA in improving insulin resistance via an alternate insulin dependent pathway.

Table 1: The influence of IP injection of BCA on animals' body weight

	Healthy Control (g)	Diabetic Control (g)	Diabetic+ 1mg/kgBCA (g)	Diabetic+ 5mg/kgBCA (g)
Pre treatment day 0	221.56±17.1	218.43±15.3	223.32±19	220.14±18.3
7 days after diabetes induction	228.34±21.2	198.62±22.3	201.54±24.5	203.31±19.6
One month after treatment	270.62±17.3	170.32±16.4^{a*}	172.25±20.2^{a*}	240.3±23.4^{a*,b*,c*}

*Mean±SD body weight in healthy control (n=5) , diabetic control (n=5) and BCA-treated rats (1 and 5mg/kg; n=5). a: compared with healthy control; b:compared with diabetic control; c:compared with diabetic rats treated by 1mg/kg BCA. *: p<0.001.*

Table 2:The influence of oral consumption of BCA on animals' body weight

	Healthy Control (g)	Diabetic Control (g)	Diabetic+ 10mg/kgBCA (g)	Diabetic+ 20mg/kg BCA (g)
Pre treatment day 0	222.43±22.1	224.58±19.7	223.73±18.3	221.53±23.4
7 days after diabetes induction	235.74±24.3	212.13±23.6	215.73±25.2	214.34±22.3
One month after treatment	278.27±22.2	176.41±19.8^{a*}	172.87±22.4a*	250.37±20.8^{a*,b*,c*}

*Mean±SD body weight in healthy control (n=5), diabetic control (n=5) and BCA-treated rats (10 and 20mg/kg; n=5). a:compared with healthy control; b:compared with diabetic control; c:compared with diabetic rats treated by 1mg/kg BCA. *: p<0.001.*

Table 3: The effect of IP injection of BCA on FBG, insulin and HOMA index in studied groups

	Healthy control	Diabetic control	Diabetic+1mg/kgBCA	Diabetic+ 5mg/kgBCA
Pre treatment day0 FBG (mg/dl)	78.3±8.3	80.21±9.2	75.42±9.5	79.63±7.9
7 days after diabetes induction FBG (mg/dl)	79.1±8.6	193.54±14.3	187.92±17.2	190.86±18.1
One month after treatment FBG (mg/dl)	76.3±7.9	200.34±17.8^{a*}	194.31±16.9^{a*}	142.8±17.9^{a*,b*,c*}
Insulin (µU/ml)	13.3±0.97	9.4±0.76^{a*}	9.6±0.93^{a*}	9.5±0.82^{a*}
HOMA-IR	2.7±0.61	4.8±0.61^{a*}	4.69±0.77^{a*}	3.1±0.71$^{a\#,b*,c*}$

*Mean±SD FBG, insulin and HOMA-IR in healthy control (n=5) , diabetic control (n=5) and BCA-treated rats (1 and 5 mg/kg; n=5). a: compared with healthy control; b:compared with diabetic control; c:compared with diabetic rats treated by 1mg/kg BCA. #: p<0.01, *: p<0.001.*

Table 4: The effect of oral consumption of BCA on FBG, insulin and HOMA index in studied groups

	Healthy Control	Diabetic Control	Diabetic+ 10mg/kgBCA	Diabetic+ 20mg/kgBCA
Pre treatment day0 FBG (mg/dl)	82.34±9.2	79.56±8.3	85.71±6.7	80.63±12.1
7 days after diabetes induction FBG (mg/dl)	83.1±9.5	183.71±22.4	190.64±20.7	188.71±24.6
One month after treatment FBG (mg/dl)	80.91±9.8	188.3±25.3^{a*}	183.47±21.9^{a*}	150.34±26.2^{a*,b*,c*}
Insulin (µU/ml)	12.9±0.46	8.9±0.7^{a*}	9.1±0.68^{a*}	8.8±0.81^{a*}
HOMA-IR	2.73±0.42	3.96±0.74^{a*}	4.12±0.63^{a*}	3.26±0.72$^{a\#,b*,c*}$

*Mean±SD FBG, insulin and HOMA-IR in healthy control (n=5), diabetic control (n=5) and BCA-treated rats (10 and 20 mg/kg; n=5). a: compared with healthy control; b:compared with diabetic control; c:compared with diabetic rats treated by 10mg/kg BCA. #: p<0.01, *: p<0.001.*

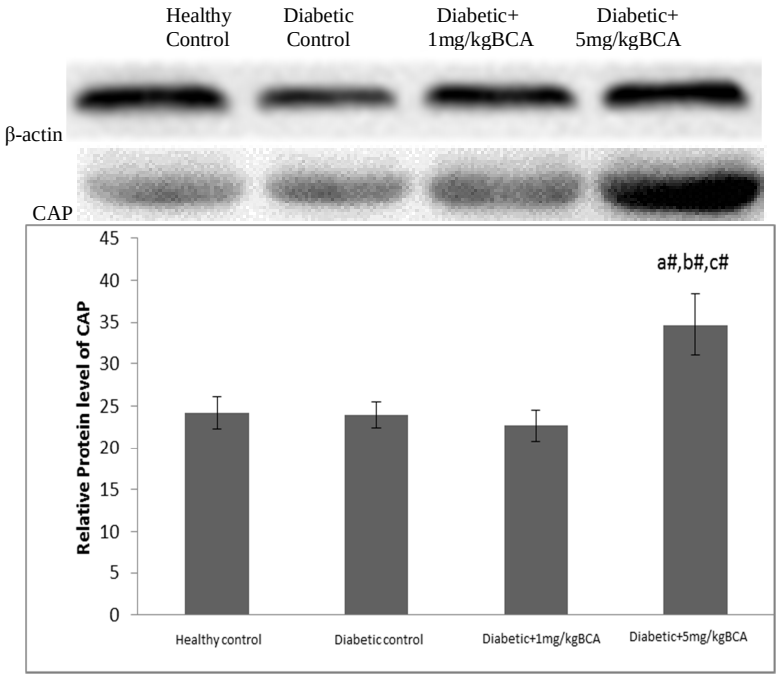

Fig.1. Results of western blot analysis of CAP in adipose tissue. Protein levels are expressed in arbitrary units after densitometric analysis. Bars represent the mean±SD. a: compared with healthy control; b: compared with diabetic control; c: compared with diabetic rats treated by 1mg/kg BCA. #: p<0.01

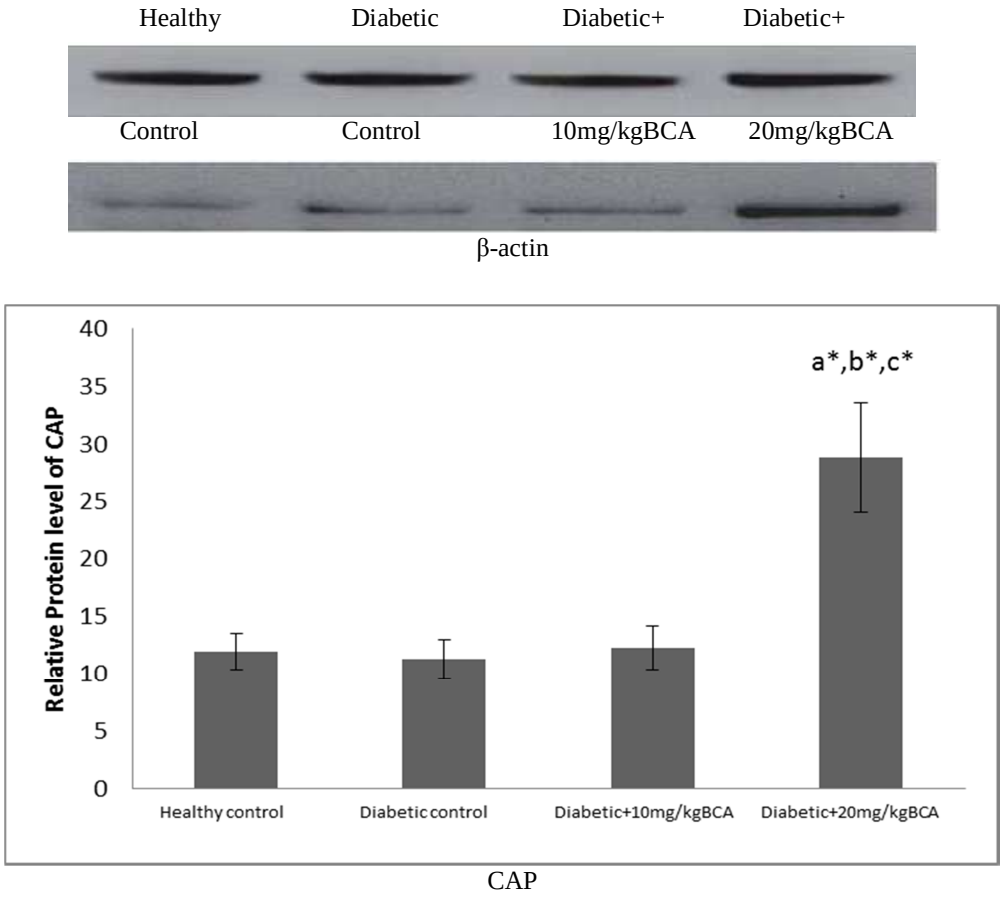

Fig.2Results of western blot analysis of CAP in adipose tissue. Protein levels are expressed in arbitrary units after densitometric analysis. *Bars represent the mean±SD. a: compared with healthy control; b: compared with diabetic control; c: compared with diabetic rats treated by 10mg/kg BCA. *: p<0.001.*

CONCLUSION

Results of the present study showed that BCA supplementation can increase the expression of CAP gene and CAP level subsequently in adipose tissue in a dose dependent manner. Additionally, reduced FBG level and improved HOMA-IR are other important findings of our study. Finally, BCA can be an appropriate drug for type 2 diabetic patients, because CAP facilitates the translocation of GLUT4 to cell surfaces, which is the most important step in improving insulin resistance.

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