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Insulin Down-Regulates Angiotensinogen Gene Expression and Angiotensinogen Secretion in Cultured Adipose Cells

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Adipose tissue is an important source of angiotensinogen (AT) after liver. Since an association exists between body mass index, hypertension, and insulin-resistance, the role of insulin on the regulation of AT gene expression and AT secretion was examined in cultured Ob1771 and 3T3-F442A adipose cells. Within a physiological range of concentrations (1–17 nM), insulin exerted a negative effect on the abundance of AT mRNA and the secretion of AT. Alterations of insulin-resistance by treatment of adipose cells with TNF-α or the thiazolidinedione BRL49653 led respectively to a decrease or an increase in the potency of insulin to down-regulate AT gene expression, whereas maximal inhibition by insulin increased from 30% in TNFα-treated cells to 60% in BRL49653-treated cells. These results suggest that a potential link between insulin resistance and high blood pressure may exist by means of increased AT secretion from adipose tissue, especially in obese subjects.

Key Words: **insulin; angiotensinogen; adipose cell; insulin-resistance.**

Angiotensinogen (AT), the unique substrate of renin in the renin-angiotensinogen system (RAS) and precursor of angiotensin II (A-II), plays an important role in the regulation of blood pressure, fluid and electrolyte homeostasis (1). The involvement of the RAS in hypertension and several cardiovascular diseases has been proposed and growing evidence suggests that local RAS may act as distinct systems from the plasma RAS (2, 3). AT is known to be mainly produced by liver (3) but AT mRNA is present in several tissues including brown and white adipose tissues (4–6). Recent studies have shown that adipose cells of 3T3-L1, 3T3-F442A and Ob1771 clonal lines synthesize and secrete AT (7–10), in agreement with the observation that only adipocytes appear to secrete AT in adipose tissue (11). Since epidemiological and physiological data implicate circulating angiotensinogen levels in the regulation of blood pressure (12–14), and since a possible involvement of increased plasma AT has been proposed in hypertension in obese patients (15), the hormonal control of angiotensinogen synthesis and secretion from adipocytes appears of interest. In contrast to liver cells which are the target of various hormones for the regulation of AT, adipose cells are only responsive to glucocorticoids which are able to up-regulate AT gene expression and AT secretion (11). In addition to glucocorticoids, a possible role of insulin can be postulated although a causal relationship between insulin and hypertension remains a controversial issue (16). In order to gain some insights on this point, a study of the insulin effect on the regulation of AT in adipose cells was initiated as (i) the excess of adipose tissue mass, in particular visceral fat accumulation every so often accompanied by insulin-resistance, is correlated with high blood pressure (17), (ii) the development of hypertension in spontaneously hypertensive rats is accompanied by an increase of cardiac and fat AT mRNA levels (18), and (iii) the adipose tissue appears to represent the main source of AT after liver (3). Our data presented herein indicate

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Abbreviations used: AT, angiotensinogen; A-II, angiotensin II; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6 dichloro-1-β-D ribofuranosyl benzimidazole; FBS, fetal bovine serum; GPDH, glycerol-3-phosphate dehydrogenase; RAS, renin-angiotensin system.

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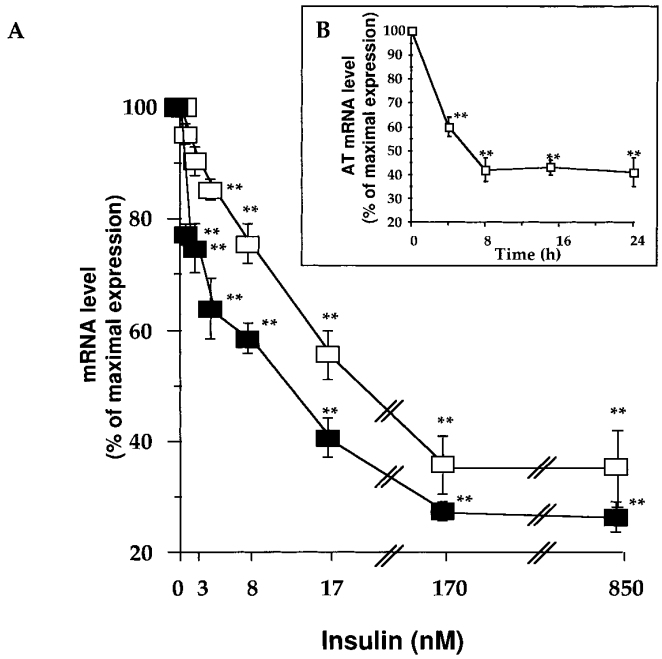

Insulin (nM)	AT secretion (% of maximal secretion)
0	100
3	94±6 *
30	77±9 *
300	65±10 **
850	55±2 **

FIG. 1. Kinetics of insulin action and dose-response relationship of insulin to AT mRNA content and AT secretion in Ob1771 adipose cells. 18 day post-confluent Ob1771 adipose cells were thoroughly washed to remove insulin as described in "Materials and Methods" and maintained in basal medium alone or containing increasing concentrations of insulin for 24 h (A) or exposed for different periods of time to 17 nM insulin (B). RNAs were extracted and analyzed by Northern-blot as described in "Materials and Methods." The signals were normalized to β-actin signals and are expressed as % of AT mRNA (□) and adipsin mRNA (■) content determined in the absence of insulin (A) or at time zero (B). The results are given as means ± S.E.M. for three independent experiments. (C) Culture media of adipose cells were collected at time 24 h and AT was determined as described in "Materials and Methods." The results are expressed as % of AT secretion determined in the absence of insulin. This basal AT secretion was 545 fmol/culture well. The results are given as means ± S.E.M. of four independent experiments. The values statistically different from the 100% mRNA or AT level measured in the absence of insulin are indicated by * ($p < 0.05$) or ** ($p < 0.01$).

that, in cultured adipose cells, insulin down-regulates AT mRNA content and AT secretion. Moreover insulin-resistance is associated with an increase in AT mRNA content whereas insulin-sensitivity is associated with a decrease in AT mRNA content.

MATERIALS AND METHODS

Cell culture. Culture conditions of Ob1771 and 3T3-F442A preadipose cells have been already described (19,20). Cells were plated in 60-mm dishes at 10^3 and 2×10^3 cells/cm^2 for 3T3-F442A and Ob1771 cells respectively and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), biotin, pantothenate and antibiotics as previously described (defined as standard medium). At confluence, 3T3-F442A cells were maintained in standard medium supplemented with 2 nM triiodothyronine (T_3) and 3 nM insulin (defined as differentiation medium) for fourteen days. Confluent Ob1771 cells were cultured in standard medium supplemented with 17 nM insulin, 2 nM T_3, 10 nM dexamethasone and 100 μM isobutylmethylxanthine (IBMX) during the first 3 days only to trigger differentiation. Ob1771 cells were then fed with standard medium supplemented with 17 nM insulin and 2 nM T_3 until day 16 or 18 after confluence Media were renewed every other day. Under these conditions, 80 % of the cell population appeared under the microscope as lipid-filled differentiated adipose cells for both clonal lines. Where indicated, insulin removal from differentiated cells was performed by rinsing the cells twice at 37°C (30 min each) with DMEM containing 8% FBS. Insulin-deprived cells were then maintained for 24 h in standard medium supplemented with 2 nM T_3 only (defined as basal medium) before insulin addition.

RNA analysis. Total RNA was prepared as described by Chomczynski and Sacchi (21). At least two dishes were pooled for each condition. Northern-blot analyses were performed as described previously (10).

Determination of AT protein in conditioned media. Differentiated Ob1771 or 3T3-F442A cells were maintained in basal medium in the absence of insulin for 24 h as described above. Subsequently, cells were rinsed twice with H16/F12 (1/1; v/v) serum-free medium (30 min each) and maintained in H16/F12 medium supplemented with 0.2 nM T_3, 20 nM sodium selenite, 10 μg/ml aprotinin and 100 μM ascorbate, in the absence or the presence of insulin. Unless otherwise stated, media were collected 24 h later and analyzed for AT as described previously (10, 11). The use of serum-free conditions was required owing to serum interference in AT determinations (11).

Statistical analysis. All statistical comparisons were performed by one-way analysis of variance (ANOVA) using the StatView software package. Statistical difference between two groups was sought by the Fisher protected least-squares difference test.

Materials. Culture media were obtained from Gibco (Cergy-Pontoise, France). Fetal bovine serum was a product of Seromed

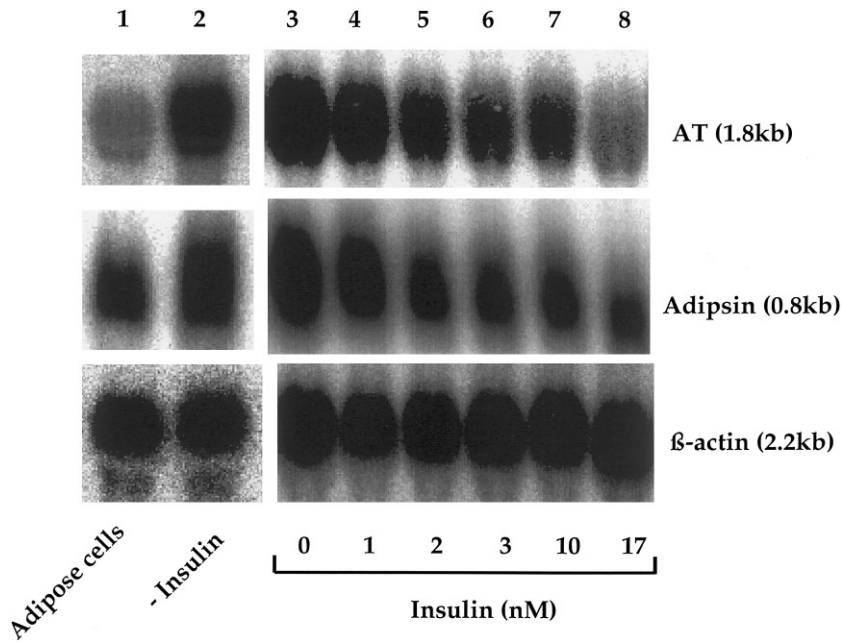

FIG. 2. Northern-blot analysis of AT mRNA content of 3T3-F442A adipose cells after insulin removal followed by insulin readdition. Lane 1: adipose cells were obtained after exposure of confluent 3T3-F442A cells for 14 days to the differentiation medium; lane 2: same cells as in lane 1 but deprived of insulin for 24 h (see " Materials and Methods " for details); lanes 3 to 8: same cells as in lane 2 but reexposed or not for 24 h to insulin at varying concentrations as indicated. RNAs were extracted and analyzed by Northern-blot as described in "Materials and Methods."

(Berlin, Germany). (α-^{32}P)dCTP, random priming kit and Hybon membranes were from Amersham (Les Ulis, France). Enzymes for nucleic acid manipulations were from Eurogentec (Seraing, Belgium). BRL49653 was obtained from SmithKline Beecham Pharmaceuticals (Welwyn, UK). The products used for AT radioimmunoassays were purchased from Amersham (Les Ulis, France) except the angiotensin I antibody which was obtained from ICN (Orsay, France). Recombinant mouse TNF-α as well as other chemical products, including the RNA synthesis inhibitor 5,6 dichloro-1-β-D ribofuranosyl benzimidazole (DRB) were obtained from Sigma Chimie (Saint-Quentin, France). The source of the various cDNA probes used in this study is indicated in the "Acknowledgments" section.

RESULTS

Inhibitory effect of insulin on AT gene expression and AT secretion. The effect of insulin on the steady-state level of AT mRNA was first examined in 18-day post-confluent differentiated Ob1771 cells maintained in standard medium supplemented with 2 nM T_3 and various concentrations of insulin. As shown in Fig. 1A, a 24-h exposure to insulin led to a dose-dependent decrease of AT mRNA. This decrease, although less pronounced, was similar to that of another differentiation-related mRNA, namely adipsin, which has been previously reported to be negatively regulated by insulin in 3T3-F442A in an opposite manner to that of glycerol-3-phosphate dehydrogenase (GPDH) mRNA (19). The effect of insulin occurred with an EC_{50} value of ~15 nM. The effect on AT mRNA was maximal after 8 h of exposure to 17 nM insulin (Fig. 1B). Interestingly, the amount of AT protein secreted by Ob1771 cells maintained during 24 h in the presence of increasing concentrations of the hormone was reproducibly found to be decreased dose-dependently (Fig. 1C). To see whether these observations were not confined to a particular cell line, further experiments were carried-out with cells of the 3T3-F442A clonal line. Following complete adipose conversion in the differentiation medium, insulin removal led within 24 h to a large increase in AT mRNA content (Fig. 2, lanes 1 and 2). Readdition of increasing concentrations of insulin to insulin-deprived cells showed the reversibility of the phenomenon which was dose-dependent, and the inhibitory effect of insulin was then observed within a physiological range of hormone concentrations (1–17 nM) (Fig. 2, lanes 3–8 and Fig. 3A). As expected (19), a similar inhibitory effect of insulin could be observed on the expression of the adipsin gene. For both genes, insulin inhibition was partial (from 60% for AT gene to 80% for adipsin gene, Fig. 3A). The effect of insulin occurred with an EC_{50} value of ~0.8 nM for the adipsin and AT genes and was maximal within 10 h of hormone exposure (Fig. 3B). This concentration is in agreement with the affinity of the insulin receptor reported in these cells (22). Although of lower magnitude, a reproducible inhibitory effect of insulin was observed for AT secretion at a time where the decrease was found to be maximal, i. e. at

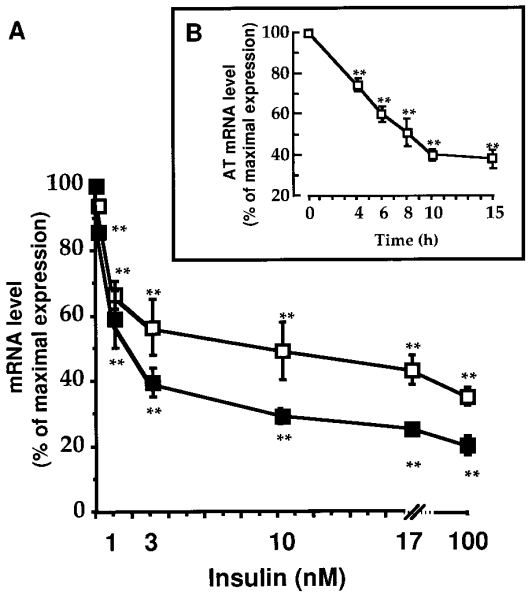

FIG. 3. Kinetics of insulin action and dose-response relationship of insulin to AT mRNA content in 3T3-F442A adipose cells. 3T3-F442A adipose cells were obtained and treated as in Fig. 2. They were exposed to increasing concentrations of insulin for 24 h (A) or exposed for different periods of time to 3 nM insulin (B). RNAs were extracted and analyzed by Northern-blot as described in "Materials and Methods." The signals were normalized to β-actin signals and are expressed as % of AT mRNA (□) and adipsin mRNA (■) content determined in the absence of insulin (A) or at time zero (B). The results are given as means ± S.E.M. for three independent experiments. The values statistically different from the 100% AT mRNA level measured in the absence of insulin are indicated by * ($p < 0.05$) or ** ($p < 0.01$).

time 24h. Decreases of 15% and 30% were obtained at 1 nM and 3 nM insulin, respectively (not shown). It should be pointed out that, under the same conditions other genes are positively regulated by insulin: it is the case for GPDH (19, 23), the adipocyte fatty acid-binding protein (a-FABP) (24) as well as for type 1 plasminogen activator inhibitor (PAI-1) (Morange, P., Aubert, J. *et al.* to be published). It is worth noting that the insulin effect on the expression of AT gene, as previously reported for that of adipsin gene (19), was direct and not glucose-dependent since it occurred similarly in glucose-free medium (not shown). This is in contrast to the insulin stimulatory effect of the expression of the gene encoding for a-FABP in adipose cells which appeared glucose-dependent *via* endogenous fatty acid synthesis (24).

Effect of BRL49653 and TNF-α on insulin inhibition of the expression of AT and adipsin genes. Agents known to be involved in the control of insulin sensitivity of adipose cells were further used to examine their influence on the regulation of the expression of AT and adipsin genes. For that purpose, a thiazolidinedione BRL49653, known to be a potent antidiabetic compound *in vivo* and an insulin sensitizer *in vitro* (25–28) and TNF-α, known to interfere with insulin signal transduction (29) were selected to respectively increase and decrease insulin sensitivity in 3T3-F442A adipose cells. Optimal exposure times and concentrations of BRL49653 and TNF-α were chosen: (i) no fat cell recruitment occurred in the presence of 1 μM BRL49653, known as an adipogenic agent (30), as the whole population of cells was already differentiated and (ii) an exposure time of 6 h to 100 pM TNF-α proved to be not cytotoxic. It is important to note that the initial AT mRNA content was quite similar in cells pre-treated or not with TNF-α, excluding a positive effect of this cytokine on the transcription of the AT gene previously reported in liver cells (31). As shown in Fig. 4, a significant and opposite shift in the dose-response curves of the inhibitory effect by insulin on AT gene expression was observed compared to that obtained for untreated cells. Moreover a dramatic change in the maximal inhibitory effect of insulin was observed on the expression of the AT gene. When comparing thiazolidinedione-treated and TNF-α-treated cells, the EC_{50} value for inhibition increased approximately 3-fold (from ~0.5 to ~1.5 nM insulin) whereas the maximal inhibitory effect increased approximately 2-fold (from ~30 to ~60%) (Fig. 4A). Similar observations could be made regarding the regulation of the adipsin gene, with EC_{50} values increasing ~3-fold (from ~0.3 to ~1 nM insulin) accompanied by a 2.5-fold increase in the maximal inhibitory effect (from ~30 to ~80%) (Fig. 4B).

DISCUSSION

Since the characterization of RAS components in various tissues, few studies on the nutritional and hormonal control of AT gene expression and AT secretion have been performed in adipose cells. The occurrence of a nutritional regulation of AT has been described in rat adipose tissue (32), and recent evidence has illustrated a transcriptional control of AT gene expression by fatty acids in preadipose cells (10) as well as by a stable analogue of prostacyclin in preadipose and adipose cells (33), a regulation which implicates likely nuclear receptors of the peroxisome proliferator-activated receptors (PPARs) family. So far, in adipose cells, glucocorticoids appear as the only steroid hormones able to up-regulate AT gene expression and AT production (11). Altogether, the above results are in favor of fatty acids and metabolites as well as glucocorticoids playing a key role in enhancing angiotensinogen production from adipose cells. Quite to the contrary, the results presented herein give evidence that insulin down-regulates in cultured adipose cells the expression of the AT gene and the production of AT. Determining the production of AT is of utmost importance and more physiologically relevant since a lack of parallelism between AT mRNA content and AT secretion rate has already been observed (10). Therefore, it is not surpris-

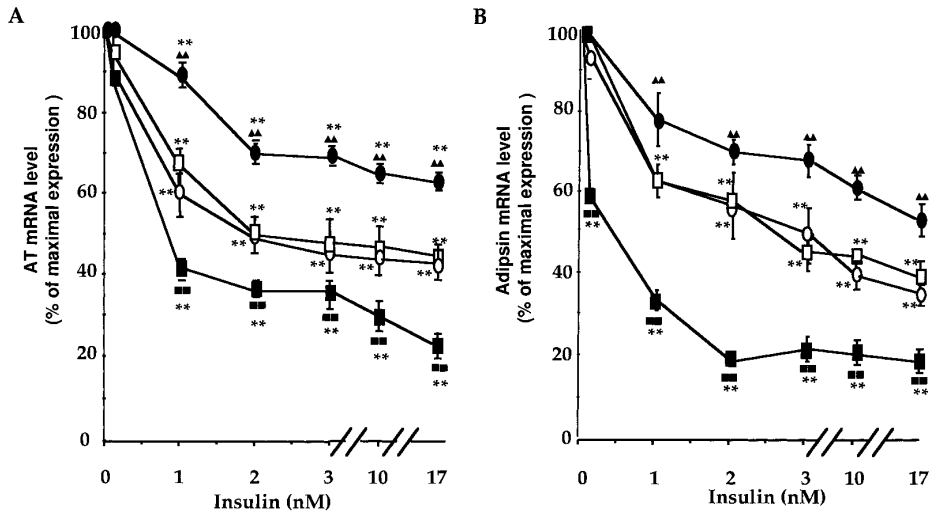

FIG. 4. Opposite regulation by thiazolidinedione BRL49653 and TNF-α of the inhibitory effect of insulin on AT mRNA and adipsin mRNA content. 3T3-F442A adipose cells were first treated to remove insulin as described in the legend to Fig. 1. Then they were exposed to increasing concentrations of insulin in the absence (□) or the presence of 1 μM BRL49653 for 24 h (■). In parallel, adipose cells remained either untreated (○) or were pretreated (●) for 6 h in basal medium supplemented with 100 pM recombinant mouse TNF-α. After removing TNF-α by two washes (30 min each) with DMEM containing 8% FBS, cells were exposed to increasing concentrations of insulin for 24 h. RNAs were extracted and analyzed by Northern-blot. The signals were normalized to β-actin signals and are expressed as % of the maximal expression of AT mRNA (A) or adipsin mRNA (B). The results are given as means ± S.E.M. of three independent experiments. The values statistically different from the 100% AT mRNA level measured in the absence of insulin are indicated by ■■ ($p < 0.01$). Statistical comparisons between values obtained for untreated (□, ○) versus treated cells (■, ●) were also performed and indicated by ▲▲ ($p < 0.01$ for TNFα) or by ■■ ($p < 0.01$ for BRL 49653).

ing that the magnitude of the insulin effect was different on AT mRNA content and AT secretion. In addition, the weaker effect of insulin on AT secretion compared to AT mRNA content might be explained by an opposite positive effect of the hormone on AT release by the cells, as observed for adipsin in 3T3-L1 adipocytes (34).

The down-regulation of AT takes place within a physiological range of insulin concentrations as previously described in rat hepatoma Reuber H35 cells (35). This phenomenon is reminiscent of the regulation of the expression of adipsin gene previously reported in 3T3-F442A adipose cells and which involves primarily a rapid inhibition of the transcriptional rate (19). Indeed, with respect to AT gene, no significant change in the half-life of AT mRNA (8 h ; Ref. 10) could be observed upon insulin exposure when using DRB as transcription inhibitor, allowing to exclude a destabilizing effect of the hormone on AT mRNA.

It is interesting to note that the expression of AT gene and the secretion of AT are not abolished in adipose cells even exposed to high insulin concentrations. To our knowledge, no insulin-response element and associated *trans*-acting factor(s) have been described up-to-now as to the promoter of the AT gene but it is likely that the transcriptional activity still observed under these conditions may implicate constitutive and differentiation-dependent factors able to bind to defined *cis*-acting sequences which have been previously described in AT gene (36–39).

Of particular interest is the fact that modulating in an opposite way the insulin responsiveness of adipose cells by a thiazolidinedione (BRL49653) or TNF-α leads respectively to an increase or a decrease in the potency and also to an increase or a decrease in the maximal capacity of insulin to down-regulate the expression of AT gene. These results suggest that, at any given concentration of insulin, insulin-resistance in adipose cells should be accompanied by higher amounts of secreted AT. The opposite effect of TNF-α and BRL49653 is of interest since on one hand TNF-α is recognized as playing *in vivo* a role in the development of insulin-resistance in muscle and adipose tissue (40–42). On the other hand, thiazolidinediones block in adipose cells the inhibitory effect of TNF-α on the expression of various lipid-related genes, *i.e.* lipoprotein lipase and a-FABP (43), and a central role of PPARγ2 in mediating TNF-α and BRL49653 effects has been proposed (44). Moreover, thiazolidinediones have been recently shown to block TNF-α-induced inhibition of insulin signaling through activation of the nuclear receptor PPARγ (45).

In aggregate, our data suggest that insulin-resistance increases AT production by adipocytes and that increasing insulin sensitivity favors inhibition of AT production by this hormone. At the present time, al-

though the association of insulin-resistance and/or hyperinsulinemia with high blood pressure has gained experimental evidence (16), the nature of the link has remained elusive in relation to an excess of adipose tissue mass, regarding particularly visceral fat (15–17). It is tempting to postulate that, *in vivo*, increased local production of AT in adipose tissue of obese patients suffering from NIDDM may contribute to hypertension often observed in these individuals.

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