Adipose Tissue Renin–Angiotensin System

Local Renin Angiotensin Expression Regulates Human Mesenchymal Stem Cell Differentiation to Adipocytes

Kenichi Matsushita, Yaojiong Wu, Yoshihisa Okamoto, Richard E. Pratt, Victor J. Dzau

Abstract—Clinical and experimental evidence suggest that the renin–angiotensin system (RAS) plays a role in metabolic syndrome. Adipogenesis is suggested to modulate obesity and obesity-related consequences, such as metabolic syndrome. Although mesenchymal stem cells (MSCs) are a major source of adipocyte generation, the influence of RAS on MSC differentiation to adipocyte is unknown. We evaluated the expression of endogenous RAS in human MSCs during its differentiation to adipocytes and studied the effects of angiotensin II (Ang II), Ang II type 1 receptor blocker Valsartan, and type 2 (AT2) receptor blocker PD123319. Our data showed that differentiation was associated with an increase in cellular renin and AT2 receptor expression and a concomitant decrease in angiotensinogen and angiotensin-converting enzyme expression. The net effect is an increase in endogenous cellular angiotensin II production. Incubation with Ang II (exogenous) inhibited adipogenesis. Combined treatment of exogenous Ang II and Valsartan further inhibited adipogenesis, whereas combined treatment of Ang II and PD123319 completely abolished the inhibition of adipogenesis, suggesting an important role for the AT2 receptor. Blockade of endogenous angiotensin II effect by incubation with Valsartan alone inhibited adipogenesis, whereas PD123319 alone promoted adipogenesis, confirming the data using exogenous Ang II. The combination of Valsartan and PD123319 had no net effect. Our data demonstrate an important role of the expression of the local RAS in the regulation of human MSC differentiation to adipocytes. Elucidation of the molecular mechanism should provide important insight into the pathophysiology of the metabolic syndrome and the development of future therapeutics. (Hypertension. 2006;48:1095-1102.)

Key Words: renin–angiotensin system ■ angiotensin II ■ mesenchymal stem cells ■ gene expression ■ adipose tissue ■ obesity

Hypertension is frequently associated with diabetes or glucose intolerance, dyslipidemia, and obesity. This common condition is known as the metabolic syndrome.1 Patients with this syndrome have increased risk for cardiovascular disease. Several large clinical trials have shown that inhibitors of the renin–angiotensin–system (RAS) can significantly reduce the new onset of diabetes in patients with hypertension and other cardiovascular risk factors.2 These observations may have important clinical and therapeutic implications; however, the pathophysiological link between RAS and the metabolic syndrome is not well defined. Similarly, the mechanisms underlying RAS inhibitors’ prevention of new onset diabetes is unclear.

Obesity is one of the most important risk factors of new-onset diabetes.4 Increased adiposity predisposes individuals to both type 2 diabetes and metabolic syndrome. Severe obesity is associated with an increase in fat cell size in combination with increased fat cell number.5–7 New fat cells arise from a pre-existing pool of adipose stem cells irrespective of age.8,9 Interestingly, it has been reported that the adipocyte contains a complete RAS and that angiotensin influences preadipocyte maturation and differentiation.10 Recent studies showed that adipose stem cells are much like bone marrow–derived mesenchymal stem cells (MSCs).9,11,12 Furthermore, Liechty et al13 showed that human MSCs transplanted into fetal sheep marrow differentiated and incorporated into normal adult adipose tissue. Developmentally, MSCs are at the step between the undifferentiated multipotent embryonic stem cells and the adipose tissue-derived preadipocytes, and MSCs are a major cell source for adipogenesis.14–16 It has been speculated that MSCs play an important role in the maintenance of the mass and function of adult adipose tissue. Accordingly, in vitro differentiation of MSCs toward the adipogenic lineage provides a useful means for studying the regulation of the first steps of adipose cell development.

Although the causal link between the activity of the adipose RAS and maturation and function of adipose tissue is suggested, this link may be quite complex. Conflicting data exist as to the effects of angiotensin II (Ang II) type 1 (AT1) and type 2 (AT2) receptors on adipogenesis.10,17,18 Furthermore, the relation between RAS and MSC adipogenesis has not been examined. It is not known whether MSCs contain a RAS and whether angiotensin regulates the MSC differen-
ation and generation of adipose tissue. In this study, we hypothesize that local RAS plays an important role in the differentiation of MSCs to adipocytes. Our data demonstrate that endogenous (local) Ang II is increased in MSCs undergoing adipogenic differentiation and that Ang II activity at the AT\(_2\) receptor inhibits the differentiation of MSC to adipocytes, whereas Ang II activity at the AT\(_1\) receptor may stimulate it.

**Methods**

**Cell Culture and Oil Red O Staining**

Human MSCs (hMSCs) were obtained from Cambrex. Confluent cells were incubated in adipogenesis induction medium (DMEM supplemented with 10 μg/mL of insulin, 1 μmol/L of dexamethasone, 0.5 mmol/L of 3-isobutyl-1-methyl-xanthine [IBMX], 0.2 mmol/L of indomethacin, 10% FBS, 4 mmol/L of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) for 3 days. Next, the cells were incubated for 3 days in adipogenesis maintenance medium (DMEM supplemented with 10 μg/mL of insulin, 10% FBS, 4 mmol/L of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) and then switched to adipogenesis induction medium for 3 days. After a third exposure to adipogenesis maintenance medium, cells were harvested at day 18 after the initiation of differentiation. For Oil Red O staining, 0.36% Oil Red O solution (Adipogenesis Assay kit, Chemicon International, Inc) was used. Details are described in the online expanded materials and methods section available online at http://hyper.ahajournals.org.

**Pharmacological Treatment**

Ang II and AT\(_2\) receptor blocker PD123319 were purchased from Sigma. AT\(_1\) receptor blocker Valsartan was generously provided by Drs William Daley and Surai Shetty (Novartis Pharmaceuticals Corporation). For exogenous Ang II experiments, Ang II (1 μmol/L) or Valsartan (10 μmol/L) was added to the culture medium daily from day 0 to day 18. Treatment with Ang II combined with both PD123319 and Valsartan was also performed. For endogenous Ang II experiments, PD123319 (10 μmol/L) or Valsartan (10 μmol/L) was added to culture medium daily from day 0 to day 18. Treatment with PD123319 plus Valsartan was also examined.

**Quantitative RT-PCR**

The TaqMan probe primer system (Applied Biosystem) was used for quantitative RT-PCR. The primers and probes for angiotensinogen, renin, angiotensin-converting enzyme (ACE), AT\(_1\), AT\(_2\) receptor, peroxisome proliferator–activated receptor-γ (PPAR\(γ\)), fatty acid synthase (FAS), adiponectin, and the housekeeping gene GAPDH were adopted from literature. TaqMan PCR was performed using ABI Prism 7700 Sequence Detection System (Applied Biosystem). Target gene mRNA expression was normalized to GAPDH mRNA expression, and the relative amounts of all of the mRNAs were calculated using the comparative Ct (threshold cycle) method. Details are described in the online expanded Methods section.

**Ang II Enzyme Immunoassay**

We quantitated the Ang II levels released from cells into the culture medium. Ang II concentrations in conditioned medium samples were assayed with the commercially available enzyme immunoassay-based colormetric kit (Phoenix Pharmaceuticals) following the manufacturer’s protocol. The specificity of this assay is described in the online expanded Methods section.

**Lipid Droplet Accumulation**

At the end of the treatment period, cells were viewed in the Axiosvert 200 inverted microscope (Carl Zeiss). Images were captured with the AxiosCam MRC camera (Carl Zeiss). Lipid droplet area measurement was conducted using the IPLab image analysis software (Scanalytics).

**Determination of Adiponectin Protein in Conditioned Media**

Conditioned media from differentiated adipocytes with or without treatment were collected at day 18. Collected media were analyzed for secreted adiponectin protein by the commercially available ELISA kit (Phoenix Pharmaceuticals). The results are expressed as the percentage of adiponectin secretion determined at baseline (without treatment).

**Statistical Analysis**

All of the values are expressed as the mean±SEM. Student t test and 1-way ANOVA, followed by posthoc Fisher least significant difference test, were performed. Differences were considered to be significant when \(P<0.05\). All of the statistical procedures were done using the Statgraphics Plus Version 5.0 software (StatPoint).

**Results**

**Identification of Adipogenesis in hMSCs**

Adipogenesis of hMSCs was identified by histological analysis and quantitative RT-PCR for mRNA expression of adipocyte-related genes. Oil Red O was used to stain lipid droplets in mature adipocytes. MSC-derived adipocytes exhibited Oil Red O positive staining (Figure 1A and 1B). This process was also accompanied by the expression of adipogenesis target genes. PPAR\(γ\) is one of the important transcription factors that regulates adipocyte differentiation. FAS is considered as a central enzyme in de novo lipogenesis. MSC-derived adipocytes exhibited an increase in expression of PPAR\(γ\) (Figure 1C) and FAS (Figure 1D). Adiponectin is a hormone secreted from adipocytes. MSC-derived adipocytes also exhibited an increase (1924±403-fold) in expression of adiponectin (\(P<0.05\); n=7).

**RAS-Related Gene Expression and Ang II Production During Adipogenesis of hMSCs**

Regarding the components of RAS, MSC-derived adipocytes exhibited a decrease in angiotensinogen and ACE expression but an increase in renin and AT\(_2\) receptor expression. There was no significant difference in AT\(_1\) receptor expression (Figure 2A). The net effect of these changes was an increase in endogenous Ang II production. We measured the concentration of Ang II in the conditioned media of control hMSCs (day 18) and differentiated adipocytes (day 18). As shown in Figure 2B, the concentration of Ang II was significantly increased in the culture media of differentiated adipocytes as compared with that of the control hMSCs.

**Effects of Ang II on the Expression of Adipocyte-Related Genes**

To examine the effects of Ang II, we added 1 μmol/L of Ang II during the condition of differentiation. As shown in Figure 3, Ang II treatment significantly decreased PPAR\(γ\), FAS, and adiponectin mRNA levels. There was a dose effect of Ang II (online supplemental Figure 1A through IC, available at http://hyper.ahajournals.org). Consistent with cellular adiponectin mRNA expression levels, secreted adiponectin protein levels in conditioned media were lower in the Ang II treatment group (57.7±11.8% versus without treatment group; n=3; \(P<0.05\)). Tumor necrosis factor \(α\) and interleukin-6 are not specific for adipocytes, but they are known to influence adipocyte differentiation. MSC-derived adipocytes exhibited a
decrease in tumor necrosis factor α and interleukin-6 expression (online supplemental Figure IIA and IIB), but Ang II had no effect on the expression of both genes (online supplemental Figure IIC and IID).

Effects of Combined Treatments With Ang II and Receptor Blockers on the Expression of Adipocyte-Related Genes

To elucidate the role of Ang II receptor subtypes in mediating the inhibitory effect of Ang II, cells were treated with 1 μmol/L of Ang II in combination with 10 μmol/L of Valsartan or/and 10 μmol/L of PD123319 during differentiation. Valsartan is a specific AT1 receptor blocker, and PD123319 is a selective AT2 receptor blocker. As shown in Figure 3A through 3C, Ang II in combination with Valsartan further decreased the expression of adipocyte-related genes.

In response to treatment with Ang II in combination with both Valsartan and PD123319, the expression of adipocyte-related genes reverted to basal levels without treatment. Importantly, Ang II in combination with PD123319 completely abolished the decrease of adipocyte-related gene expression by Ang II (Figure 3), suggesting that the principal effect of Ang II on adipocyte-related gene expression is through the AT2 receptor. Secreted adiponectin protein levels in the Valsartan group and the Ang II plus PD123319 group showed the same trend (45.0±14.0% and 128.0±49.3% versus the without-treatment group, respectively; n=3).

Effects of Combined Treatments With Ang II and Receptor Blockers on Lipid Droplet Accumulation

We further quantitated the adipocyte differentiation by assessing lipid droplet accumulation using the IPLab image analysis software. This software distinguished the color of lipid droplets and calculated the area of lipid droplets. As shown in Figure 4, Ang II treatment decreased the lipid droplet accumulation. Ang II in combination with Valsartan further decreased the lipid droplets. By the treatment of Ang II in combination with both Valsartan and PD123319, the lipid droplet accumulation reverted to basal levels without treatment. Ang II in combination with PD123319 abolished the decrease of lipid droplet accumulation by Ang II (Figure 4).

Effects of Blockade of Endogenous (Local) Ang II in Adipogenesis of hMSCs

To elucidate the autocrine and paracrine effects of endogenous (local) Ang II and the role of its receptor subtypes, hMSCs were treated with 10 μmol/L of Valsartan or/and 10 μmol/L of PD123319 during the condition of differentiation. As shown in Figure 5A through 5C, Valsartan treatment decreased the expression of adipocyte-related genes. On the other hand, PD123319 treatment increased adipocyte-related gene expressions. There were no significant differences in adipocyte-related gene expressions between baseline and combined treatment of Valsartan with PD123319. Secreted adiponectin protein levels in the Valsartan group and PD123319 group showed the same trend (81.4±13.3% and 160.7±27.6% versus the without-treatment group, respectively; n=3). Regarding the lipid droplet accumulation, Valsartan treatment decreased the lipid droplets, whereas PD123319 treatment increased the lipid droplet accumulation to a higher level than baseline (Figure 6). There was no significant difference between the baseline and combined treatment of Valsartan with PD123319 (Figure 6).

Discussion

In this study, we have shown that hMSCs contain a local RAS. Endogenous Ang II production is increased in hMSCs undergoing adipocyte differentiation via increased local renin expression. Furthermore, we have demonstrated that Ang II inhibits adipocyte differentiation of hMSCs associated with an increase in the type 2 receptor. Our data would suggest that endogenous (local) Ang II secreted by hMSCs and differentiated adipocytes contributes to the modulation of adipogenesis of hMSCs.

We also studied the mechanism for increased local angiotensin production during adipogenesis. Among the compo-
The components of RAS, angiotensinogen and ACE gene expressions were downregulated, whereas renin and AT2 receptor expressions were upregulated during adipocyte differentiation. The net effect is that differentiated adipocytes increased Ang II production. Because both control hMSCs and differentiated adipocytes express high levels of angiotensinogen and ACE expression, our data would suggest that local renin is a key rate determinant of local Ang II in this system.

Studies related to the role of RAS in adipogenesis are limited and inconsistent. Ang II was reported to promote adipogenesis of rodent preadipocytes by AT2 receptor-mediated formation of prostacyclin.24,25 Angiotensinogen expression was also suggested to enhance adipogenesis in 3T3-L1 cells26 and transgenic mice.27 In contrast, Schling et al28 showed that Ang II reduced the adipogenesis of human preadipocytes. Janke et al10 also showed that Ang II inhibits adipogenesis of human preadipocytes via the AT1 receptor.

Figure 2. Involvement of RAS in the adipogenesis of hMSCs. (A) Expression of RAS-related genes of control hMSCs (day 18: C) and differentiated adipocytes (day 18: A). Total RNA was analyzed by quantitative RT-PCR for mRNA expression of angiotensinogen, renin, ACE, AT1 receptor, and AT2 receptor. Fold changes vs control hMSCs (C). Data are mean±SEM; n=7; *P<0.05 vs control hMSCs. n.s. indicates statistically nonsignificant. (B) Concentration of endogenous (local) Ang II in conditioned medium of control hMSCs (day 18) and differentiated adipocytes (diff. Adi.; day 18), measured using the enzyme immunoassay. Data are mean±SEM; n=8; *P<0.05 vs control hMSCs.

Figure 3. Effects of Ang II treatment with or without receptor blockers on the expression of adipocyte-related genes. One μmol/L of Ang II (All), 1 μmol/L of Ang II combined with 10 μmol/L of Varsaltan (All+Val), 1 μmol/L of Ang II combined with 10 μmol/L of PD123319 (All+PD123), or 1 μmol/L of Ang II combined with both 10 μmol/L of Varsaltan and 10 μmol/L of PD123319 (All+Val+PD123) were added to medium from the start of differentiation to day 18. Total RNA was analyzed by quantitative RT-PCR for mRNA expression of PPARγ (A), FAS (B), and adiponectin (C). Fold changes vs without-treatment group (No Tx). Data are mean±SEM; n=7 to 11; *P<0.05 vs without-treatment group. #P<0.05 vs All group. +P<0.05 vs All+Val group. n.s. indicates statistically nonsignificant.

Our observation that Ang II has inhibitory effects on adipogenesis is consistent with the latter reports. However, the effects of Ang II receptors differ from the report by Janke et al.10 These inconsistent data may reflect differences in species, cell types, and culture conditions. To explain the differences in our observation from that of Janke et al,10 methodologic issues deserve mention, as well as the difference in cell types. First, we used hMSCs instead of preadipocytes. Second, Janke et al10 used a hormonal mixture (insulin, triiodothyronine, and hydrocortisone) that did not include a xanthine derivative (IBMX). IBMX is a strong inducer of intracellular cAMP concentrations and is usually used in the induction of adipogenesis. However, IBMX could override the effects of subtle modulators of adipogenesis in...
some cases. Indeed, Schling et al.\textsuperscript{28} also reported that Ang II showed a distinct reduction in insulin-induced adipogenesis of human preadipocytes but only had a marginal effect on the adipogenesis induced by insulin, cortisol, and IBMX. In the present study, we used standard protocols, which contained IBMX. In this regard, the same methodologic issue might explain another controversy about the AT\textsubscript{2} receptor expression during differentiation of human preadipocytes.\textsuperscript{10,29} Janke et al.\textsuperscript{10} reported that AT\textsubscript{2} receptor expression was barely detectable, and there was no significant difference during adipogenesis, whereas AT\textsubscript{1} receptor expression was significantly upregulated during adipogenesis in human preadipocytes. On the other hand, Schling\textsuperscript{29} reported that AT\textsubscript{2} receptor expression was upregulated during adipogenesis, whereas AT\textsubscript{1} receptor expression was diminished during adipogenesis in human preadipocytes. Our data on AT\textsubscript{2} receptor expression from hMSCs to adipocytes are consistent with the latter report. We and Schling\textsuperscript{29} used 0.5 mmol/L of IBMX whereas Janke et al.\textsuperscript{10} did not use IBMX. Although the expression of angiotensin receptors on human preadipocytes is still controversial, our result clearly shows the upregulation of AT\textsubscript{2} receptor expression from hMSCs to adipocytes. Taken together, it is possible that the mechanisms of adipogenesis inhibitory effects of Ang II are different between preadipocytes and MSCs. The observation that AT\textsubscript{2} receptor expression is higher in more immature cells is compatible with the known

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**Figure 4.** Effects of combined treatments with Ang II and receptor blockers on lipid droplet accumulation. (A) Representative photos of each group using the IPlab image analysis software. (a) differentiated adipocytes without treatment, (b) treated with 1 \( \mu \text{mol/L} \) of Ang II, (c) treated with Ang II combined with Valsartan, (d) treated with Ang II combined with PD123319, and (e) treated with Ang II combined with both Valsartan and PD123319. Lipid droplet showed yellow color by the IPlab software. (B) Quantification of lipid droplet area. Relative ratio vs without-treatment (No Tx) group. Data are mean\( \pm \)SEM; \( n=20 \) to 48 fields from 5 to 12 independent experiments. \(*P<0.05\) vs without-treatment (No Tx) group. \#\( P<0.05\) vs All group. +\( P<0.05\) vs (All + Val) group. n.s. indicates statistically nonsignificant.
cytes to adipocytes that uses AT2 and AT1 receptors. Ang II regulation of adipogenesis from stem cells to preadipocytes could suggest the possibility of a 2-step process involved in this study.

null and AT1 receptor null MSCs. However, our work is on the nature of the AT2 receptor in developmental biology. Our data could suggest the possibility of a 2-step process involved in ang II regulation of adipogenesis from stem cells to preadipocytes to adipocytes that uses AT2 and AT1 receptors differentially.

We examined the relative contribution of the AT1 versus the AT2 receptor on MSC adipogenesis. Our data showed that exogenous ang II alone and the combination of ang II and Valsartan had inhibitory effects on adipogenesis, whereas the combination of ang II and PD123319 completely abolished the inhibitory effect of exogenous ang II. These results would suggest that the ang II effect is primarily through the AT1 receptor. Nevertheless, the data from the blockade of endogenous ang II would also suggest a role for the AT2 receptor. To elucidate the definitive roles of AT2 and AT1 receptors, these studies should be conducted on AT2 receptor null and AT1 receptor null MSCs. However, our work is on hMSCs, and, as such, we will not be able to perform these studies, because human Ang II receptor null cells do not exist. This is one of the limitations of this study.

It can be argued that Ang II and/or Valsartan reduced adipogenesis of hMSCs by its direct inhibitory effect on PPARγ expression. Recently, Di Filippo et al17 showed that the treatment of obese Zucker rats with irbesartan (another AT1 receptor blocker) reduced PPARγ immunostaining and the activity of the enzyme glycerol-3-phosphate dehydrogenase—accepted markers of adipocyte differentiation in adipose tissue. Their observation is in support of our results. PPARγ is expressed predominantly in adipose tissue, where it is known to play a critical role in adipogenesis and fat deposition.32 In general, failure to produce new adipocytes is considered to result in the increase in large insulin-resistant adipocytes and the predisposition to the development of diabetes and the metabolic syndrome.33 Thus, promoting new small insulin-sensitive adipocytes is thought to have antidiabetic effects. A recent report by Yvan-Charvet et al18 showed that AT1 receptor null mice fed a low-fat diet displayed an increased number of small adipocyte cells as compared with wild-type mice. We speculate that the increased small adipocyte might be recruited from MSCs. On the other hand, Rieuuset al34 showed that, by preventing adipocyte differentiation and lipid accumulation, a PPARγ-specific antagonist protects mice from high-fat diet-induced adipocyte hypertrophy and insulin resistance. In adipose tissue, the possibility exists that decreased PPARγ expression improved insulin sensitivity, as demonstrated in PPARγ-deficient mice35,36 or treatment with a PPARγ-specific antagonist.37 However, it must be acknowledged that conflicting data exist as to the effects of PPARγ activity, its agonist, and its antagonist on obesity and diabetes.32,34–38 Also, it is possible that Valsartan may directly stimulate PPARγ, independent of its AT1 receptor blocking action, as has been described with other AT1 receptor blockers in mouse 3T3-L1 preadipocytes and PC12W cells.39 In this study, we did not examine whether Valsartan directly stimulates PPARγ in hMSCs. Future experiments are necessary to understand the involvement of PPARγ in the ang II effect and its role in the mechanism(s) of the antidiabetic effect of Valsartan.

Finally, with respect to the contribution of MSC to adipogenesis in human adipose tissue, recent evidence shows that adult human adipose tissue contains stem cells, much like bone marrow stromal MSCs.9,11,12 In this study, we used human bone marrow MSCs. Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue. The remaining cells are various blood cells, fibroblasts, endothelial cells, pericytes, and adipose precursor cells of varying degrees of differentiation.40,41 Although it is difficult to know the exact ratio or percentage of cells in adipose tissue that are constituted by stem cells, Zuk et al42 attempted recently to address this question. According to their report, 300 cc of human adipose tissue obtained by suction-assisted liposuction yielded adipose stem cells of 2 to 6×10⁶ cells.8 This number is not insignificant. Although the role of MSCs in adult adipose tissue differentiation and generation is still unclear, we speculate that MSCs play an important role in the maintenance of the mass and function of adult adipose tissue.

![Figure 5. Effects of blockade of endogenous (local) Ang II on the expression of adipocyte-related genes. Temi μmol/L of Valsartan (Val), 10 μmol/L of PD123319 (PD123), or both 10 μmol/L of Valsartan and 10 μmol/L of PD123319 (Val+PD123) were added to medium from the start of differentiation to day 18. Total RNA was analyzed by quantitative RT-PCR for mRNA expression of PPARγ (A), FAS (B), and adiponectin (C). Fold changes vs without-treatment group (No Tx). Data are mean±SEM; n=5 to 10; *P<0.05 vs without-treatment (No Tx) group. #P<0.05 vs Val group. n.s. indicates statistically nonsignificant.](http://hyper.ahajournals.org/)

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This is one of the limitations of this study.
The present study demonstrates that the differentiation of hMSCs to adipocytes is inhibited by endogenous Ang II through the differential expression of local renin and AT₂ receptors. Such a role of local RAS might be physiologically important in the maintenance of the mass and function of adult adipose tissue. Thus, our results provide insight into the possible novel understanding of pathophysiology and therapy of obesity and obesity-related consequences, such as metabolic syndrome.

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**Disclosures**
V.J.D. has consultant/advisory board relationships with Novartis Pharmaceuticals Corporation, CV Therapeutics Inc, and Merck & Co, Inc. The remaining authors report no conflicts.

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