Increased cGMP promotes healthy expansion and browning of white adipose tissue

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ABSTRACT **With more than half a billion individuals affected worldwide, obesity has reached pandemic proportions. Development of "brown-like" or "brite" adipocytes within white adipose tissue (WAT) has potential antiobesity and insulin-sensitizing effects. We investigated the role of cyclic GMP (cGMP) signaling, focusing on cGMP-dependent protein kinase I (PKGI) in WAT. PKGI is expressed in murine WAT, primary adipocytes, and 3T3-L1. Treatment of adipocytes with cGMP resulted in increased adipogenesis, with a 54% increase in expression of peroxisome proliferator-activated receptor-γ. Lentiviral overexpression of PKGI further increased adipogenesis, whereas loss of PKGI significantly reduced adipogenic differentiation. In addition to adipogenic effects, PKGI had an antihypertrophic and anti-inflammatory effect *via* RhoA phosphorylation and reduction of proinflammatory adipokine expression. Moreover, PKGI induced a 4.3-fold increase in abundance of UCP-1 and the development of a brown-like thermogenic program in primary adipocytes. Notably, treatment of C57BL/6 mice with phosphodiesterase inhibitor sildenafil (12 mg/kg/d) for 7 d caused 4.6-fold increase in uncoupling protein-1 expression and promoted establishment of a brown fat cell-like phenotype ("browning") of WAT *in vivo*. Taken together, PKGI is a key regulator of cell size, adipokine secretion and browning of white fat depots and thus could be a valuable target in developing novel treatments for obesity.—Mitschke, M. M., Hoffmann, L. S., Gnad, T., Scholz, D., Kruithoff, K., Mayer, P., Haas, B., Sassmann, A., Alexander Pfeifer, A., Kilić, A. Increased cGMP promotes healthy expansion and browning of white adipose tissue. *FASEB J.* 27, 1621–1630 (2013). www.fasebj.org**

Key Words: beige adipocytes · adipokines · obesity

Two distinct types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), are present in mammals. Besides being specialized in triglyceride (TG) storage, WAT has an important endocrine function (1). In contrast, BAT is a thermogenic tissue that dissipates energy in the form of heat by metabolizing fatty acids (2). BAT thermogenesis depends on high mitochondrial content and expression of uncoupling protein 1 (UCP-1), which functions as a mitochondrial proton leak, thereby generating heat instead of ATP (3). Active BAT in neonates is present in the interscapular region but has also been detected in human adults in the neck and paravertebral areas (refs. in ref. 4). In addition to these discrete brown fat depots, disseminated adipocytes with typical features of brown adipocytes (BAs), so called "brite" (brown-in-white) or "beige" cells (refs. in ref. 4), have been found in WAT after cold exposure or chronic treatment with β-adrenergic agonists or peroxisome proliferator-activated receptor-γ (PPARγ) agonists (5–7). Beige cells are developmentally distinct from classical BAs derived from myogenic *Myf5*-positive progenitors (7–9). In contrast, development of beige cells, also known as "brown-

Abbreviations: 5-aza-dC, 5-aza-deoxycytidine; aP2, adipocyte protein 2; BA, brown adipocyte; BAT, brown adipose tissue; cAMP, cyclic adenosine-3′,5′-monophosphate; CCL, chemokine (C-C motif) ligand; cGMP, cyclic guanosine-3′,5′-monophosphate; Cre, cre recombinase; Dexa, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; H&E, hematoxylin and eosin; IBMX, 3-isobutyl-1-methylxanthine; IHC, immunohistochemistry; LV-cntr, control lentivirus; LV-Cre, cre recombinase lentivirus; LV-PKGI, PKGI lentivirus; MCP1, monocyte chemoattractant protein 1; mtDNA, mitochondrial DNA; NP, natriuretic peptide; PDE, phosphodiesterase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, PPARγ coactivator 1α; PKG, protein kinase G; PPARγ, peroxisome proliferator-activated receptor γ; PRDM16, PR domain containing 16 zinc finger transcription factor; qPCR, quantitative PCR; RhoA, Rho kinase A; ROCK, Rho-associated protein kinase; SCAD, short-chain acyl-CoA dehydrogenase; T_3, triiodothyronine; TG, triglyceride; TNFα, tumor necrosis factor α; UCP-1, uncoupling protein 1; WA, white adipocyte; WAT, white adipose tissue; WATi, inguinal white adipose tissue; WATg, gonadal white adipose tissue; WT, wild type

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ing," involves either phenotypic "transdifferentiation" of existing white adipocytes (WAs; ref. 10) or *de novo* differentiation of bipotential white/beige progenitors (9, 11), depending on the fat depot.

The second messenger cyclic guanosine-3′,5′-monophosphate (cGMP) plays a crucial role in cardiovascular homeostasis (12). Recent studies indicate that cGMP is also involved in energy metabolism (13–16); however, the exact mechanisms are still unclear. The physiological effects of cGMP can be mediated through cyclic nucleotide-gated ion channels, cGMP-regulated phosphodiesterases (PDEs), and cGMP-dependent protein kinase Gs (PKGs) (12). In mammalian cells, PKGs are expressed as two different isoforms (PKGI and PKGII; refs. 17–19).

PKGI has been shown to be required for BA differentiation and for enhancement of BAT development *in vivo* (20, 21). Previous studies indicated a potential role for cGMP/PKGI in WAs (13–16). Interestingly, it has been recently shown that natriuretic peptides (NPs) can also induce browning of WAT (22). However, the role of PKGI in adipocytes is still under debate due to problems with the specificity of PKGI inhibitors (23, 24). Moreover, the very existence of PKGI in adipose tissue was recently questioned (25). Here, we analyzed the role of PKGI in WA differentiation, applying gain- and loss-of-function models in the adipocyte cell line 3T3-L1 and primary adipocytes isolated from murine WAT. In addition, we also investigated the potential role of the PDE inhibitor sildenafil in the establishment of the brown fat cell-like phenotype of WAT.

MATERIALS AND METHODS

Animals and *in vivo* study

Homozygous $PKGI^{fl/fl}$ mice (20) were housed under normal, pathogen-free conditions at 23 ± 1°C and were allowed free access to standard chow and water. $PKGI^{fl/fl}$ mice were crossed with *Adipoq* $creER(T)^2$ mice (generous gift from Stefan Offermanns, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany) to obtain adipocyte specific PKGI knockout. Ten-week-old animals were injected with 1 mg tamoxifen/d for 5 consecutive days. Male C57BL/6N mice [wild type (WT), 10 wk of age, 6 mice/group] were treated for 7 d with 0.9% saline or sildenafil (12 mg/kg/d; Sigma-Aldrich, Steinheim, Germany; ref. 26). After treatment with sildenafil, fat and lean mass were measured in body composition analyzer LF50H (Bruker, Bremen, Germany), and tissues were rapidly isolated, frozen and stored at −80°C. Experiments were conducted in accordance with the German legislation on protection of animals and the U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation of primary cells, cell culture, and virus infection

Preadipocytes were isolated from inguinal WAT (WATi) and gonadal WAT (WATg) of 8- to 12-wk-old WT and $PKGI^{fl/fl}$ mice. Fat pads were digested in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Darmstadt, Germany) containing 0.5% BSA (Sigma-Aldrich) and collagenase type II (1.5 mg/ml; Worthington, Lakewood, NJ, USA). Floating mature adipocyte fraction was used for analysis of PKGI expression and the remaining part was centrifuged at 1000 rpm for 10 min. Pellet was resuspended in DMEM supplemented with 10% FBS and 1% P/S (growth medium) and passed through a 100-μm nylon mesh. Cells (4×10^5) were seeded on 6-well plates and grown at 37°C and 5% CO_2. Cells were differentiated 2 d after reaching confluence (d 0) with induction medium [DMEM with 5% FBS, 1% P/S, 0.25 μM dexamethasone (Dexa), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 nM triiodothyronine (T_3), 50 mg/ml L-ascorbate, 1 mM D-biotin, 17 mM panthothenate, 0.5 mM metformin, and 0.172 μM insulin; all from Sigma-Aldrich]. Medium was exchanged every second day for 7 d. After induction, cells were maintained in differentiation medium (induction medium without IBMX and Dexa) for an additional 4 d. 3T3-L1 cells (from American Type Culture Collection, Rockville, MD, USA) were maintained in DMEM containing 10% FBS and 1% P/S (growth medium). For differentiation, 2 d after confluence (d 0) 3T3-L1 cells were induced with growth medium containing 1 μM Dexa, 0.5 mM IBMX, and 0.172 μM insulin (induction medium 3T3). At 48 h after induction, cells were maintained in medium containing 10% FBS and 0.172 μM insulin (differentiation medium 3T3) until differentiated. 8-pCPT-cGMP (cGMP; 200 μM; Biolog, Bremen, Germany) was given to activate PKGI, starting 48 h before induction. For browning experiments, primary WAs were induced for 6 d with 0.5 mM metformin (Sigma-Aldrich) in presence and absence of 200 μM cGMP or 10 μM sildenafil. RNA was collected at d 6 of experiments. Lentiviral vectors expressing full-length PKGI lentivirus (LV-PKGI) and empty control lentivirus (LV-cntr) were previously described (20). For conditional PKGI knockout, $PKGI^{fl/fl}$ cells were infected with cre recombinase lentivirus (LV-Cre) recombinase. Production of recombinant lentivirus and infection of cells were performed as described previously (27).

3T3-L1 cells were treated with 10 μM Y-27632 (Merck Chemicals, Schwalbach, Germany) from d 0 to 3 or 7, and 10 ng/ml tumor necrosis factor α (TNF-α; Sigma-Aldrich) was added for 24 h.

Oil Red O staining

To visualize lipid accumulation, paraformaldehyde (4% PFA)-fixed cells were stained with Oil Red O solution (3 mg/ml in isopropyl alcohol; Sigma-Aldrich) for 4 h at room temperature and rinsed 3 times with water.

TG measurement

For determination of total TG levels, an enzymatic colorimetric method was used, as described previously (20).

Analysis of mRNA abundance

Total RNA was extracted from cells using InnuSolv (AnalytikJena, Jena, Germany). cDNA was reverse-transcribed from 1 μg RNA using the Transcriptor First Strand synthesis kit (Roche, Mannheim, Germany) with random hexamer primers. quantitative PCR (qPCR) was performed as described previously (20).

Flow cytometric analysis and real-time qPCR of mitochondrial DNA (mtDNA)

3T3-L1 adipocytes were incubated in culture medium supplemented with 50 nM MitoTracker Green FM (Life Technolo-

gies) for 45 min at 37°C. Cells were trypsinized, washed with PBS, and resuspended in PBS. Fluorescence was analyzed by flow cytometry on a FACS Calibur (BD Biosciences, Heidelberg, Germany) as described previously (20). Genomic DNA was isolated from 3T3-L1 cells, and qPCR for mitochondrial markers (*mt-Nd1*, *mt-Co1*, and *mt-CytB*) was performed as described previously (20).

Western blotting

Protein preparation and Western blotting were performed as described previously (20). The following antibodies were used: antibodies against adipocyte protein 2 [aP2; also know as fatty acid-binding protein 4 (FABP4)], PPARγ, PPARγ coactivator 1α (PGC-1α), Rho kinase A (RhoA), and pRhoA (Ser-188) (all Santa Cruz Biotechnology, Heidelberg, Germany); UCP-1 and actin (Sigma-Aldrich); tubulin (Dianova, Hamburg, Germany); pNF-κB p65, NF-κB p65, pIKK, and IKK (Cell Signaling, Danvers, MA, USA), GAPDH (Epitomics, Burlingame, CA, USA), and PKGI (generated in our laboratory). All bands were quantified by densitometric analysis using the QuantityOne software (Bio-Rad Laboratories, München, Germany).

Immunohistochemistry (IHC)

Paraffin-embedded BAT, WATi, and WATg sections were either stained with hematoxylin and eosin (H&E) or used for IHC as described previously (20). Primary antibody (against PKGI or UCP-1) was applied overnight at room temperature. After being washed with 1% BSA-TBS, biotin-conjugated secondary antibody against rabbit (Dianova) was applied for 1 h at room temperature and developed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Values are presented as means ± SEM. Statistical differences between 2 groups were determined by unpaired *t* test. Comparisons among several groups were performed by 1-way ANOVA followed by Student-Newman-Keuls *post hoc* test to calculate the relevant *P* values. For the *in vivo* part of the data, we performed power analysis using GPower3 freeware for calculation (28).

RESULTS

PKGI is expressed in adipose tissue and in adipocytes during differentiation

We first analyzed expression of PKGI in murine WAT and BAT. Using a PKGI-specific antibody and Western blotting, we detected PKGI in both WATg and WATi, as well as in BAT (**Fig. 1*A***). Because there are different cell types in fat tissue, we isolated mature fat cells from WAT, where we also demonstrated expression of PKGI (Fig. 1*A*). Using immunohistochemical staining, we detected PKGI in tissue sections of WATg, WATi, and BAT (Fig. 1*B*). Global *Prkg1*-knockout mice display a severe phenotype due to vascular and intestinal dysfunctions and die soon after birth (29); therefore, we used a genetic model of inducible deletion of *Prkg1* in adipose tissue. To obtain adipocyte-specific *Prkg1* knockout, we crossed mice carrying 2 floxed *Prkg1* alleles ($PKGI^{fl/fl}$; ref. 20) with *Adipoq* creER(T)^2 mice (30). Staining for PKGI was strongly reduced in adipocytes of different fat depots of *Adipoq* cre $PKGI^{fl/fl}$ mice treated with tamoxifen. In contrast, PKGI staining of vessel walls was not affected (Fig. 1*B*).

We also analyzed PKGI expression in 3T3-L1 cells and in primary adipocytes isolated from murine WATg and WATi. Cells were differentiated using specific protocols (Supplemental Fig. S1*A*, *B*), and PKGI was detected during *in vitro* differentiation in primary WAs and, albeit at lower levels, in 3T3-L1 preadipocytes (Fig. 1*C*).

PKGI enhances adipogenesis in 3T3-L1 cells

To test the function of PKGI in WA differentiation, we first analyzed the effect of cGMP in WT 3T3-L1 adi-

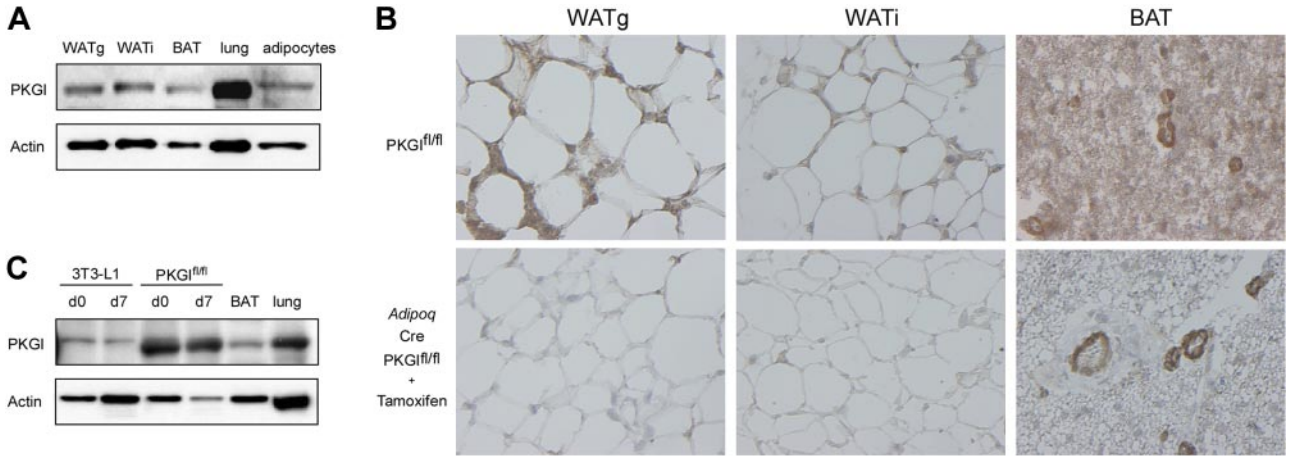

Figure 1. PKGI is expressed in adipose tissue. *A*) Western blot analysis of PKGI expression in WATg, WATi, BAT, lung, and mature adipocytes isolated from WAT. *B*) Representative PKGI IHC stainings (brown stain) in sections of WATg, WATi, and BAT from $PKGI^{fl/fl}$ mice and *Adipoq* creER(T)^2 $PKGI^{fl/fl}$ mice treated with tamoxifen. *C*) Protein levels of PKGI in 3T3-L1 and primary adipocytes at d 0 and 7 of differentiation protocol, compared with BAT and lung levels from WT mice.

pocytes and 3T3-L1 cells transduced with LV-PKGI (Supplemental Fig. S1*C*, *D*). Treatment with 8-pCPT-cGMP (cGMP) from d 2 to 7 resulted in enhanced adipose differentiation, as indicated by Oil Red O staining at d 7 (**Fig. 2*A*, *B***). Overexpression of PKGI and its activation by cGMP further increased the number of Oil Red O-positive cells (Fig. 2*A*, *B*). A positive effect of cGMP treatment on cellular lipid accumulation during differentiation was also confirmed by analysis of cellular TG content at d 7 (Fig. 2*C*). To further understand these findings on molecular basis, we investigated abundance of the adipogenic master transcription factor PPARγ and one of its target genes, *aP2*. In line with the increase in lipid accumulation and TG content, levels of PPARγ and aP2 in LV-PKGI-transduced 3T3-L1 adipocytes treated with cGMP were elevated by 217 and 235%, respectively, as compared with both untreated and cGMP-treated WT adipocytes (Fig. 2*D*).

PKGI is crucial for adipogenesis in primary white preadipocytes

Next, we examined adipogenesis in a loss-of-function model using primary cells isolated from murine WAT. We isolated preadipocytes from $PKGI^{fl/fl}$ mice (20) and deleted *Prkg1* in these preadipocytes ($PKGI^{0/0}$) *in vitro* using LV-Cre (Supplemental Fig. S1*E*, *F*). Differentiation of $PKGI^{fl/fl}$ preadipocytes (Supplemental Fig. S1*B*) resulted in a marked lipid accumulation, as evidenced by Oil Red O staining at d 11 (**Fig. 3*A*, *B***). In contrast, $PKGI^{0/0}$ cells were only poorly differentiated and displayed reduced Oil Red O staining (Fig. 3*A*, *B*). Analysis of TG content revealed a significant decrease of lipid content in $PKGI^{0/0}$ (Fig. 3*C*) as compared with $PKGI^{fl/fl}$ adipocytes. Western blot analysis of WA at d 7 showed a significantly lower expression of PPARγ and aP2 in $PKGI^{0/0}$ as compared with $PKGI^{fl/fl}$ cells (Fig. 3*D*). Apart from aP2 (which is highly regulated by PPARγ), mRNA levels of PPARγ downstream targets phosphoenolpyruvate carboxykinase (*Pepck*), *Perilipin*, and short-chain acyl-CoA dehydrogenase (*Scad*) were also significantly reduced in $PKGI^{0/0}$ cells (Fig. 3*E*). Mitochondrial biogenesis was impaired in $PKGI^{0/0}$ cells, as seen in lower protein levels of cytochrome *c* (Fig. 3*F*). In addition, levels of UCP-1 mRNA were significantly decreased in $PKGI^{0/0}$ cells (Fig. 3*G*), indicating impaired potential for browning.

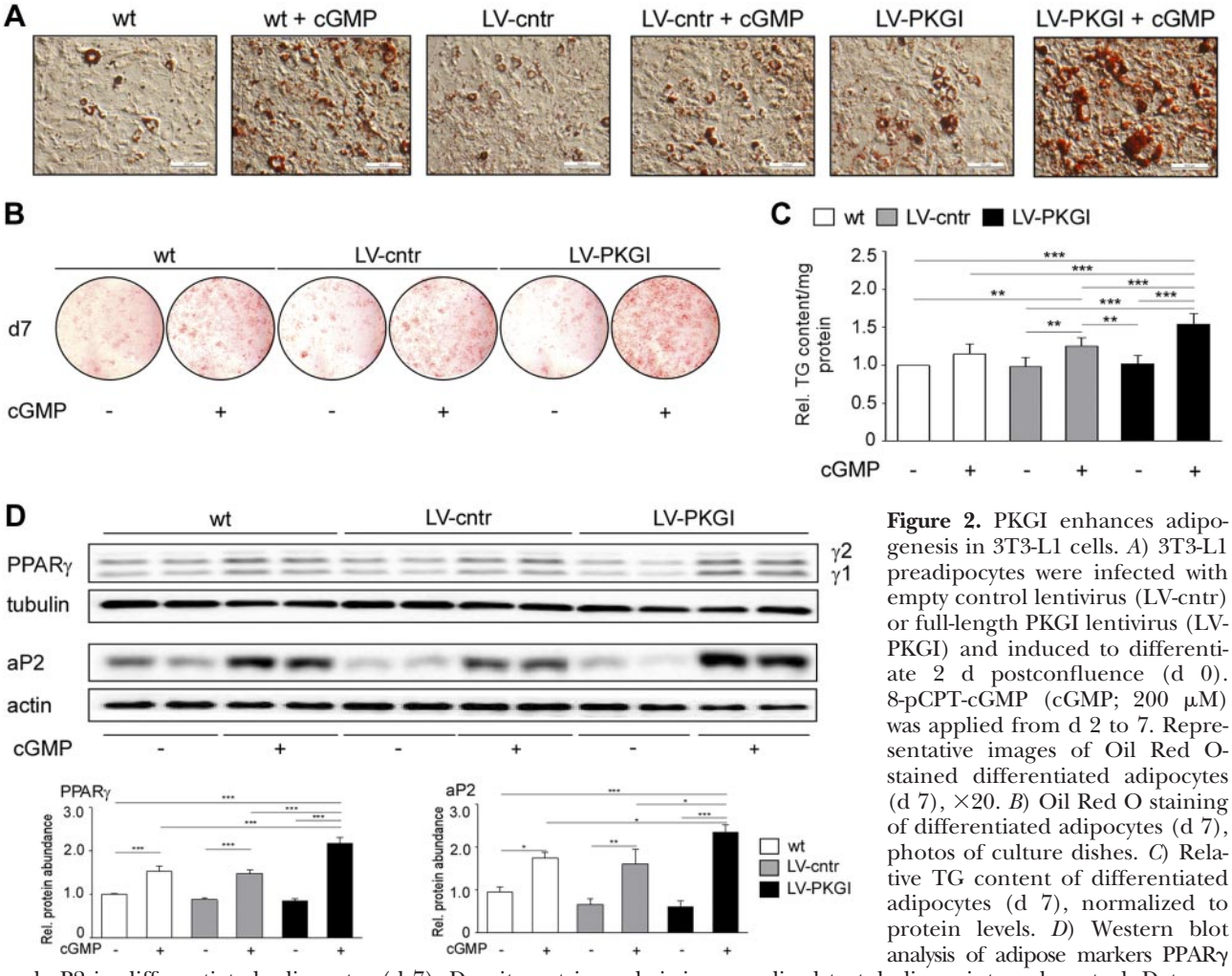

Figure 2. PKGI enhances adipogenesis in 3T3-L1 cells. *A*) 3T3-L1 preadipocytes were infected with empty control lentivirus (LV-cntr) or full-length PKGI lentivirus (LV-PKGI) and induced to differentiate 2 d postconfluence (d 0). 8-pCPT-cGMP (cGMP; 200 μM) was applied from d 2 to 7. Representative images of Oil Red O-stained differentiated adipocytes (d 7), ×20. *B*) Oil Red O staining of differentiated adipocytes (d 7), photos of culture dishes. *C*) Relative TG content of differentiated adipocytes (d 7), normalized to protein levels. *D*) Western blot analysis of adipose markers PPARγ and aP2 in differentiated adipocytes (d 7). Densitometric analysis is normalized to tubulin as internal control. Data are represented as means ± SEM $n \geq 3$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

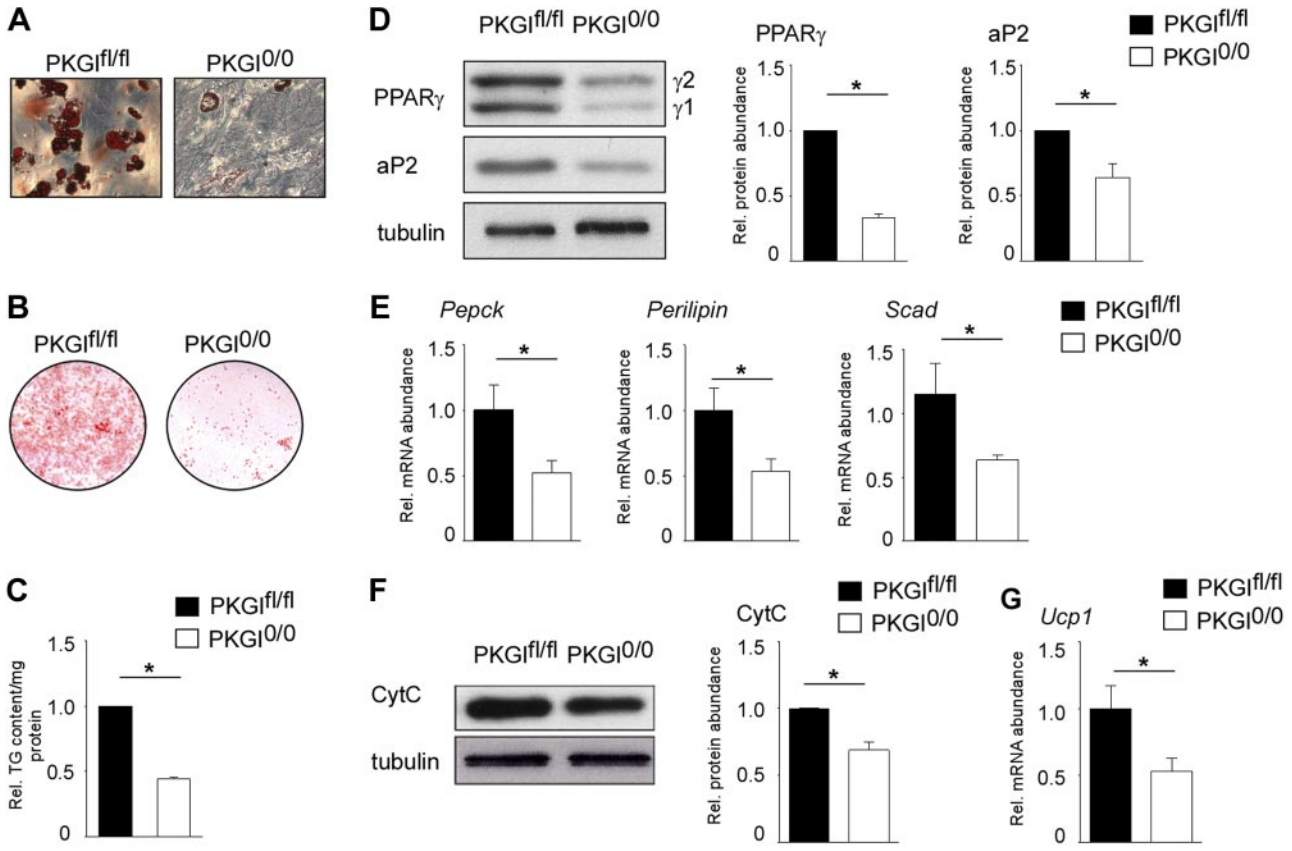

Figure 3. Ablation of PKGI diminishes adipogenesis in primary murine WAs. *A*) Primary PKGI-floxed white preadipocytes ($PKGI^{fl/fl}$) were infected with LV-Cre for conditional PKGI knockout ($PKGI^{0/0}$) and differentiated. Representative images of Oil Red O-stained differentiated adipocytes (d 11), ×20. *B*) Oil Red O staining of fully differentiated adipocytes (d 11), culture dishes. *C*) Relative TG content of differentiated adipocytes (d 11), normalized to protein levels. *D*) Western blot analysis of adipose markers PPARγ and aP2 in differentiated adipocytes (d 7). *E*) mRNA expression levels of *Pepck, Perilipin*, and *Scad* in differentiated WAs (d 7). *F*) Western blot analysis of cytochrome *c* (CytC) as a marker of mitochondrial number in differentiated adipocytes (d 7). *G*) mRNA levels of UCP-1 in differentiated WAs (d 7). Densitometric analysis is normalized to tubulin as internal control. Data are presented as means ± SEM; $n \geq 3$. $^*P < 0.05$.

PKGI influences mitochondrial biogenesis and the thermogenic potential of WAs

Adaptive thermogenesis is a unique feature of BA and requires expression of the mitochondrial UCP-1 (2). Basal expression of UCP-1 and PGC-1α is negligible in white fat (2). We have previously shown that activation of PKGI leads to significant increase of *Ucp1* mRNA in BA (20). Recent data showed browning of WAs treated with NPs (22). Therefore, we investigated effects of cGMP on browning of primary murine adipocytes isolated from WATi. After 6 d treatment with cGMP, primary WAs were shifted to a brown-like phenotype (**Fig. 4*A***), as shown by significantly increased up-regulation of *Ucp1* by 4.3-fold and of transcriptional coregulators *Pgc1*α and PR domain containing 16 zinc finger transcription factor (*Prdm16*) by 2.3- and 2.5-fold, respectively. In addition, treatment of WAs with sildenafil caused an increase in expression of *Ucp1*, *Pgc1*α, and *Prdm16* by 4.6-, 3.2-, and 1.5-fold, respectively, which was comparable to the cGMP effect *in vitro* (Fig. 4*B*). Interestingly, in contrast to primary WAs, treatment with cGMP did not increase *Ucp1* expression in 3T3-L1 cells (Supplemental Fig. S2*A*). A recent report (31) implicated differences in UCP-1 promoter methylation between white (3T3-L1) and brown (HIB-1B) adipocyte cell lines. To investigate whether cGMP plays a role in

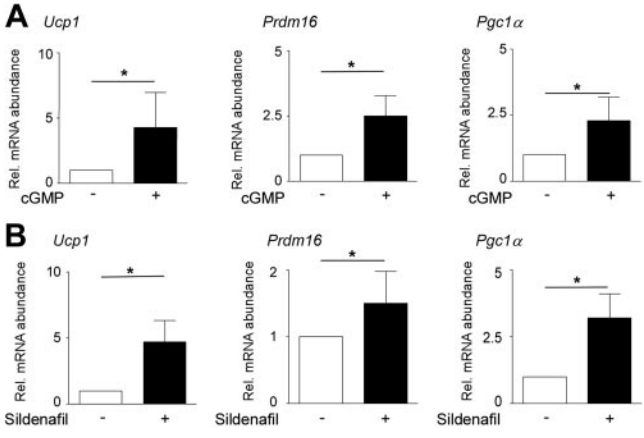

Figure 4. PKGI promotes browning of primary murine WAs. *A, B*) mRNA levels of *Ucp1*, *Prdm16*, and *Pgc1*α in WAs isolated from WATi, treated as indicated with 200 μM 8-pCPT-cGMP (cGMP; *A*) or with 10 μM sildenafil for 6 d (*B*). Data are presented as means ± SEM; $n \geq 5$. $^*P < 0.05$.

3T3-L1 browning after UCP-1 promoter demethylation, cells were treated for 24 h with demethylating agent 5-aza-deoxycytidine (5-aza-dC). Indeed, treatment of 3T3-L1 cells with 5-aza-dC increased basal *Ucp1* mRNA levels, which were even further augmented in demethylated cells expressing high levels of PKGI (LV-PKGI) treated with cGMP (Supplemental Fig. S2*A*). Because of the cGMP effect on mitochondrial content in BAs, we examined mitochondrial biogenesis in 3T3-L1 cells treated with cGMP. MitoTracker fluorescence analysis was performed in control cells and preadipocytes infected with LV-PKGI treated with or without cGMP for 2 d. Clearly, cGMP-treated LV-PKGI cells displayed a significant enhancement of MitoTracker fluorescence (Supplemental Fig. S2*B*). Increased mitochondrial biogenesis in PKGI-overexpressing cells was further confirmed by qPCR of genes encoded by mtDNA: cytochrome *c* oxidase subunit 1 (*mt-Co1*), cytochrome *b* (*mt-CytB*), and NADH dehydrogenase-1 (*mt-Nd1*; Supplemental Fig. S2*C*).

PKGI promotes adipogenesis and regulates adipokine expression *via* inhibition of RhoA

Among different substrates of PKGI (12, 19, 32), the small GTPase RhoA is of special interest. We and others have shown that RhoA is phosphorylated by PKGI at Ser-188 in different cell types (HeLa, VSCM, and BA), which, in turn, prevents activation of its downstream effector Rho-associated protein kinase (ROCK; refs. 20, 33, 34). Furthermore, it was demonstrated that inhibitors of ROCK promote adipogenesis in 3T3-L1 cells (35). However, the effect of PKGI on RhoA has not been studied in WAs. To investigate the interaction of PKGI and RhoA in these cells, we analyzed RhoA Ser-188 phosphorylation. We found a significant increase in pRhoA Ser-188 after cGMP stimulation in WAs isolated from $PKGI^{fl/fl}$ mice (**Fig. 5*A***). *In vitro* deletion of *Prkg1* with Cre reduced pRhoA Ser-188, and this could not be increased by the addition of cGMP (Fig. 5*A*).

RhoA/ROCK activity increases with diet-induced obesity due to mechanical stress and plays an important role in regulation of adipocyte size (36). Treatment of WT 3T3-L1 cells from d 4 to 12 with cGMP exerted an antihypertrophic effect, as measured by adipocyte size (Fig. 5*B*), which paralleled the phosphorylation of RhoA (Fig. 5*A*). Another important function of RhoA is regulation of cytokine release from adipose tissue (36). Considering the inhibitory effect of PKGI on RhoA/ROCK signaling, we investigated whether cGMP/PKGI also regulates expression of selected adipokines. Expression of adiponectin was increased by cGMP treatment in 3T3-L1 cells (Fig. 5*C*). This effect was more pronounced in cells overexpressing PKGI (Fig. 5*C*). In

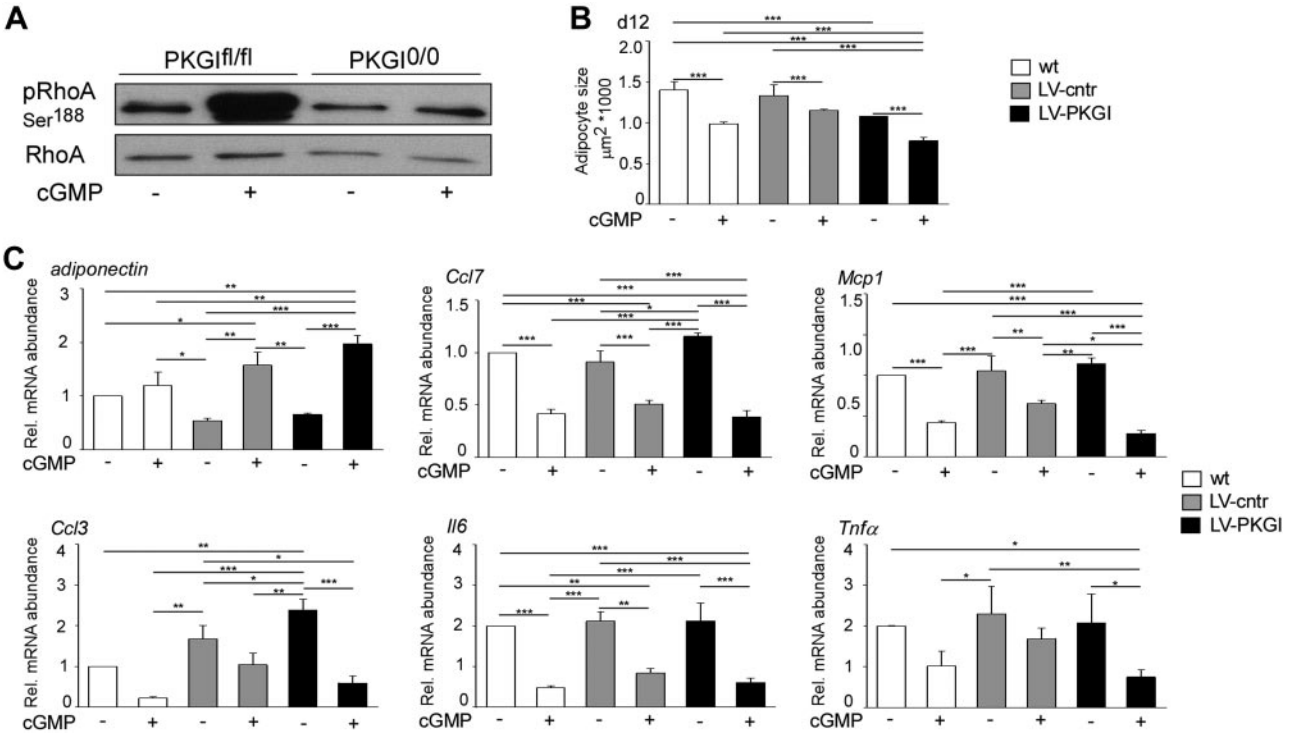

Figure 5. PKGI inhibits RhoA signaling and causes antihypertrophic and anti-inflammatory effects in WAs. *A*) Western blot analysis of phosphorylation of RhoA on Ser-188 in primary WAs from $PKGI^{fl/fl}$ and $PKGI^{0/0}$ mice. *B*) 3T3-L1 cells were differentiated *in vitro* with or without prior infection with LV-cntr or LV-PKGI and treated with cGMP (d 4 to 12) as indicated. cGMP inhibits adipocyte hypertrophy as assessed by measuring adipocyte surface area. *C*) qPCR analysis of the adipocytokines *adiponectin, Ccl7, Mcp1, Ccl3, Il6* and *Tnfa* in differentiated cells. Treatment with 200 µM 8-pCPT-cGMP (cGMP) from d 2 to 7 as indicated. Data are represented as means ± SEM; $n = 4$ for cell size; $n = 4–8$ for adipokines. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

contrast to adiponectin, the abundance of monocyte chemoattractant protein 1 (*Mcp1*) and chemokine (C-C motif) ligand 7 (*Ccl7*), *Ccl3*, *Il6*, and *Tnfa*, which are up-regulated in obesity and contribute to macrophage infiltration into adipose tissue (37), was decreased in 3T3-L1 cells when stimulated with cGMP compared with untreated WT cells (Fig. 5*C*). A further decrease of *Mcp1*, *Ccl7*, and *Tnfa* expression was detected in LV-PKGI-transduced adipocytes treated with cGMP. Recent data by Tateya *et al.* (38) implicated cGMP signaling in reduction of NF-κB signaling in both hepatocytes and macrophages. We also investigated whether cGMP *via* RhoA can inhibit NF-κB signaling. Therefore, we investigated phosphorylation of NF-κB p65 and mRNA expression levels of *Il6* and *Mcp1* in WT 3T3-L1 cells in the presence and absence of Rho signaling inhibition (39). Addition of TNF-α (10 ng/ml) increased NF-κB signaling (as seen by increased phosphorylation of NF-κB p65; Supplemental Fig. S3*A*). Pretreatment of cells with ROCK inhibitor Y-27632 (10 μM) significantly reduced activation of NF-κB signaling pathway (Supplemental Fig. S3*A*). Same effects were seen for *Il-6* and *Mcp1* mRNA expression (Supplemental Fig. S3*B*).

These data suggest that cGMP/PKGI plays a role in remodeling and immunoregulation of adipocytes by decreasing adipocyte size and secretion of adipokines *via* inhibition of RhoA.

Increased cGMP stimulates browning *in vivo*

As shown in Fig. 4, both cGMP and sildenafil clearly promote browning of WAs *in vitro*. It has been shown by Ayala *et al.* (26) that 12 wk sildenafil treatment of WT mice fed high-fat diet reduces body weight and improves energy balance. To investigate whether sildenafil, *via* increasing cGMP levels, might induce browning *in vivo*, 10-wk-old C57BL/6N mice were treated with sildenafil for 7 d. We choose short-term sildenafil treatment to delineate starting molecular changes, bypassing the effects of sildenafil on body weight. Indeed, short term treatment did not change body weight (**Fig. 6*A***). Body composition of mice was also not altered in sildenafil-treated mice compared with WT mice (Fig. 6*B*). Next, we focused on WATi analysis, because it has been shown that WATi has a high capacity for browning (40). The H&E staining of WATi from sildenafil-treated mice showed an appearance of multilocular adipocytes within white fat, suggesting that sildenafil induces BAT-like remodeling of WATi (Fig. 6*C*). We therefore analyzed the expression of UCP-1. WATi of sildenafil-treated mice showed an increased staining of UCP-1, demonstrating the presence of beige adipocytes (Fig. 6*C*). Moreover, not only IHC but also Western blot analysis showed an increase in UCP-1 by 4.0-fold and PGC-1α expression by 1.4-fold (Fig. 6*D*). Interestingly, expression of both UCP-1 and PGC-1α was not significantly changed in BAT after sildenafil treatment (Supplemental Fig. S3*C*).

DISCUSSION

The outstanding role of the cGMP signaling pathway for the homeostasis in the cardiovascular system is well described (12). Several studies started to unravel the role of cGMP signaling in cellular metabolism and whole-body energy balance: cGMP has been shown to regulate glucagon secretion (41), mitochondrial biogenesis (20, 21, 42), and development of classical BAT (20, 21, 42). In addition, previous research demonstrated that the ANP/NPR-A/cGMP pathway promotes adipogenesis in WAs (15). Moreover, it has recently been shown that cardiac NPs promote browning of WAT (22). Several mechanisms by which cGMP might regulate white fat cells can be envisioned: cGMP effects could be mediated by PDEs, including PDE5 and PDE3B, *via* regulation of cGMP and/or cyclic adeno-

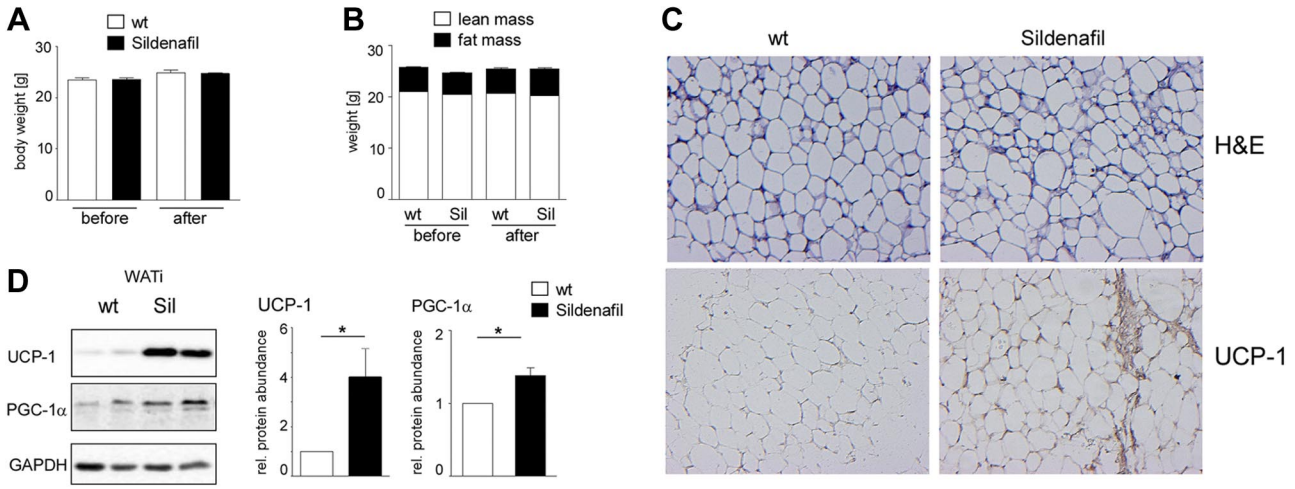

Figure 6. Sildenafil induces browning of WATi. *A*, *B*) C57BL/6N mice were treated for 7 d with 0.9% NaCl or with sildenafil (12 mg/kg/d). Neither body weight (*A*) nor body composition (*B*) changed during this short treatment. *C*) Representative images of H&E and UCP-1 IHC staining in sections of WATi of WT and sildenafil-treated mice. *D*) Western blot analyses of UCP-1 and PGC-1α were performed in WATi. GAPDH was used as loading control. Data are presented as means ± SEM $n = 6$. $^*P < 0.05$.

sine-3′,5′-monophosphate (cAMP) levels (43, 44). An increase in cAMP can stimulate protein kinase A, but high cAMP concentrations might also cross-activate PKG (45). PKGI mediates many effects of cGMP in various tissues, but the role of PKGI in adipocytes remains controversial. Based on experiments with pharmacological inhibitors of PKG in 3T3-L1 cells (16, 22, 46), it was suggested that PKGI might mediate the cGMP effects in adipocytes. However, the efficacy and specificity of PKG inhibitors was questioned by several studies (23, 24). Intriguingly, it was recently claimed that PKGI is absent in murine fat tissue (25).

Here, we clearly show that PKGI is expressed in 3T3-L1 cells and primary preadipocytes as well as in differentiated WAs. Western blotting and IHC unequivocally demonstrated expression of PKGI in WAT and mature adipocytes of WT mice. Using the conditional *Prgk1* mouse model, we could demonstrate the specificity of the histological data and unequivocally prove the presence of PKGI in different WAT depots and BAT. Inducible deletion of *Prgk1* in mice significantly reduced expression of PKGI in adipocytes, while expression in other cell types (VSMCs) was not affected. Using lentiviral overexpression of PKGI, we observed that PKGI enhances adipogenesis in 3T3-L1 preadipocytes, whereas ablation of *Prgk1* in preadipocytes isolated from $PKGI^{fl/fl}$ mice resulted in abrogation of adipogenesis.

Nisoli *et al.* (42) have shown that NO induces mitochondrial biogenesis *via* cGMP in a broad spectrum of cells. Interestingly, an increase in mitochondrial biogenesis after treatment with lipoamide was shown to be mediated *via* stimulation of eNOS expression in 3T3-L1 cells (46). Here, we demonstrate that PKGI mediates cGMP-induced mitochondrial biogenesis and browning in WAs *in vitro*. An important distinction between BAs and WAs is the presence of the UCP-1 (2). Mice expressing UCP-1 in adipose tissue at 2–10% of levels observed in BAT are resistant to genetic and diet-induced obesity (47). Therefore, strategies aiming to modulate UCP-1 expression in WAT may open a novel therapeutic approach for obesity. Given the recently discovered role of NO/cGMP/PKGI signaling in the development of the thermogenic program in BAT (20, 21, 42, 48), we asked whether PKGI might be involved in the development of beige adipocytes. BAs originate from progenitors with myogenic characteristics and express the transcriptional coregulator PRDM16 (7, 49). PRDM16 controls development of BAs in brown and white fat depots (49). Beige adipocytes are molecularly and developmentally distinct from BAs but share crucial features (4). Beige cells express PGC-1α and exhibit a high mitochondrial content; they also express UCP-1 and are capable of norepinephrine-mediated thermogenesis (4). The molecular mechanism for UCP-1 expression exclusively in BAT but not in WAT remains unclear, but it could be either due to tissue-specific expression of genes involved in mitochondrial biogenesis or methylation of the CRE motif in the *Ucp1* promoter, which suppresses *Ucp1* expression (50). Shore *et al.* (31) reported that WAT and BAT indeed differ in *Ucp1* promoter methylation status and that treatment with methyl transferase inhibitor increases *Ucp1* expression in 3T3-L1 preadipocytes. Interestingly, as shown in this study, primary WAs can be turned into the beige adipocytes after cGMP treatment without addition of demethylating agent.

cGMP/PKGI are established as antihypertrophic factors in cardiomyocytes (12). In the present study, we demonstrated that cGMP treatment *in vitro* exerts an antihypertrophic effect on WAs. Hara *et al.* (36) demonstrated that Rho/ROCK signaling leads to the hypertrophic adipocyte phenotype and, consequently, inflammatory changes in adipose tissue. However, the upstream signals that control RhoA activity in WAT were not investigated (32). PKGI activation by cGMP increased phosphorylation of RhoA at Ser-188 in primary WAs, which implies that cGMP *via* PKGI exerts antihypertrophic effects in white fat through inhibition of the RhoA/ROCK pathway.

WAs not only serve as largest energy reserve in mammals, they also have an endocrine function by secreting adipokines, which are also regulated by the RhoA signaling pathway. These adipocyte-derived cytokines are involved in inflammatory processes, glucose metabolism, and cardiovascular regulation and can further contribute to the increased risk of obesity-related diseases (51, 52). Unlike other adipokines, plasma level of adiponectin is reduced in obese individuals (53). Conflicting reports on the role of NO in regulation of adiponectin expression were published. It has been shown that NO directly down-regulates gene expression and secretion of adiponectin in 3T3-L1 adipocytes *via* cGMP-independent mechanism (54). However, recent reports demonstrated that NO and NPs significantly enhance adiponectin expression *via* cGMP (21, 55, 56). In this context, note that inhibition of RhoA *in vivo* resulted in an increase in adiponectin expression (36). Cytokines, like TNF-α, promote adipokine expression *via* NF-κB in 3T3-L1 and Kupffer cells (38, 39). To mimic inflammatory processes *in vitro*, we treated 3T3-L1 cells with TNF-α for 24 h. TNF-α treatment stimulated NF-κB signaling and increased expression of *Il6* and *Mcp1*. The effects of TNF-α on pp65, *Il6*, and *Mcp1* were reduced by pretreatment with ROCK inhibitor. Our study clearly shows that activation of PKGI in adipocytes increases adiponectin expression and, in parallel, decreases gene expression of proinflammatory cytokines (*Mcp1*, *Ccl3*, *Ccl7*, *Il6*, and *Tnfa*). The exact mechanism of how cGMP/PKGI regulates proinflammatory cytokines is still not completely understood; however, our data, together with recent publications by Hara *et al.* (36) and Tateya *et al.* (38) are pointing toward existence of cGMP/PKGI/RhoA/NF-κB signaling in WAs, which could make cGMP regulation in fat an extremely interesting therapeutical target.

In summary, increasing cGMP in WAT might be used to keep adipocyte size, inflammatory response, and adipocytokine secretion in a healthy range despite

increased energy intake; in other words, achieving a healthy expansion of WAT (1).

In light of our *in vitro* data and recent understanding of emergence of BAs in WAT (browning), we investigated the role of cGMP/PKGI *in vivo* in mice treated with sildenafil for 7 d. Ayala *et al.* (26) showed that chronic inhibition of cGMP hydrolysis improves energy balance and enhances *in vivo* insulin action in a mouse model of diet-induced insulin resistance. This could not be explained *via* changes in thermogenic pathways, because the researchers could not demonstrate any difference in UCP-1 expression in BAT of sildenafil-treated mice. However, WAT was not analyzed in this study (26). Here, we show that even short-term treatment with sildenafil significantly increased both UCP-1 and PGC-1α protein expression in WATi. Our data are in agreement with a recent report of Bordicchia *et al.* (22) in which short-term treatment with B-type natriuretic peptide causes browning of WAT. Increased browning of WAT could lead to increased energy expenditure and weight loss. Interestingly, 1 wk treatment of mice with sildenafil is sufficient to start a molecular and cellular switch toward a brown-like phenotype.

cGMP/PKGI-induced browning of WAT together with the antihypertrophic and anti-inflammatory effects could potentially shine a light for an antiobesity use of this well-known PDE inhibitor and other drugs that increase cGMP levels. FJ

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