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Expression of cGMP-dependent protein kinase type I in mature white adipocytes

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ABSTRACT

The presence of cGMP-dependent protein kinase I (cGKI) in murine adipocytes has been questioned, although cGKI was implicated in the thermogenic program of fat cells (FCs) and to exert anti-hypertrophic/-inflammatory effects in white adipose tissue. Herein, cGKI was detected in adipocytes from control mice, whereas FCs from global cGKI knockouts ($cGKI^{-/-}$) and cGKIα rescue (αRM) mice remained cGKI-negative. cGKI mutants exhibit decreased adipocyte size, plasma leptin levels and reduced body-weights as compared to litter-matched controls. Low abundance of adiponectin in WAT and plasma of αRM animals together with previously confirmed high IL-6 levels indicate a low-grade inflammation. However, αRMs were protected from streptozotocin-induced hyperglycemia. Our results suggest that cGMP/cGKI affects both glucose and FC homeostasis in more complex mode than previously thought.

1. Introduction

High levels of circulating pro-inflammatory molecules together with excessive lipid accumulation in white adipose tissue (WAT) and in non-adipose tissues and organs are major causes of insulin resistance and type-2 diabetes [1,2]. In contrast, brown adipose tissue (BAT) has favorable effects on metabolism [3]. Promotion of BAT energy expenditure and/or expansion of brown-like fat cells are predicted to be beneficial in treatment of metabolic disorders. Indeed, WAT-specific expression of the usually highly BAT restricted uncoupling protein 1 (UCP-1) was sufficient to increase non-shivering thermogenesis in transgenic mice and thereby to prevent obesity [4].

Accumulating evidence suggest that a natriuretic peptide (NP)/cyclic guanosine monophosphate (cGMP) pathway plays an equivalent, but independent role for lipolysis and adipogenesis as the sympathetic nerve system, which signals via β-adrenoreceptors and cAMP to promote brown fat cell activity and "browning" of WAT [5–12]. Infusion of NP or a targeted inactivation of the NP clearance receptor (a negative regulator of NP signaling) stimulated the expression of thermogenic markers such as UCP-1 in mitochondria-enriched BAT and in WAT. This resulted in marked increases in energy expenditure and favorable effects on body weight (BW) [10].

As in many types of cells, cGMP in fat cells most likely exert its effects via activation of cGMP-dependent protein kinase I (cGKI) [13,14]. Indeed, an over-expression of a constitutively active cGKI in many tissues including the adipose organ protected female, but not male mice against diet induced obesity [15]. Analysis of gene-targeted cGKI mouse models and cultured pre-adipocytes implied that adipogenic effects of cGMP rely on a functional cGKI [8,9,12]. The data suggest that cGMP/cGKI fulfill important roles in adipose tissues i.e. on fat cell size, adipokine levels, inflammation and browning of WAT depots. However, NO/cGMP might as well affect mitochondriogenesis [16,17] by cGKI-independent mechanisms i.e. via cross-talk through phosphodiesterases which may affect cAMP signaling [18,19] and at least one report has also questioned the presence of cGKI in murine adipocytes [20].

We studied WAT expression of cGKI in global cGKI knockouts and gene-targeted cGKIα rescue mice (referred to as αRM) [21] in comparison to their respective age- and littermate controls to resolve the confusion on the presence of cGKI in murine white fat cells. Moreover, in αRM mice elevated levels of IL-6 [12,20,22] and low levels of the anti-inflammatory factor adiponectin [20]

Abbreviations: cGKI, cGMP-dependent protein kinase I; NPs, natriuretic peptides; cGMP, cyclic guanosine-3′,5′-monophosphate; NO, nitric oxide; WAT, white adipose tissue; BAT, brown adipose tissue; SM, smooth muscle; UCP-1, uncoupling protein 1; cAMP, cyclic adenosine-3′,5′-monophosphate; BW, body weight; FFA, free fatty acids; FC, fat cell; STZ, streptozotocin; APN, adiponectin; HSL, hormone-sensitive lipase; IL-6, interleukin-6.

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were reported. Adiponectin concentration is decreased in obesity [23,24] and low adiponectin with high IL-6 levels coincide with insulin resistance [25,26] in murine models of altered insulin sensitivity [27–29]. Therefore, one would predict a mild insulin resistance in the cGKI mutant mouse lines due to the persistent low-grade systemic inflammation. However, together with these diabetes-associated changes in inflammatory signaling we observed improved glucose tolerance, reduced total fat mass [30], significantly smaller fat cells, reduced plasma leptin levels and strong protection from insulin resistance following streptozotocin-injections in gene-targeted cGKI animals. These findings suggest that cGMP and cGKI might affect glucose and fat cell homeostasis in a more complex mode than previously thought.

2. Materials and methods

2.1. Experimental animals

The generation of the global cGKI knockout mice (genotype: $cGKI^{-/-}$) and the αRM mice (genotype: $Sm22\alpha^{cGKI\alpha/+}$; $cGKI^{-/-}$) has been described previously [21,30]. All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München and had free access to water and standard chow. The local government's Committee on Animal Care and Welfare München approved the experimental procedures. For *in vivo* experiments, male gene-targeted $cGKI^{-/-}$ and αRM were used and compared to their age- and littermate controls on a 129SV background.

2.2. Western blot analysis

Freshly isolated inguinal WAT was immediately frozen in liquid nitrogen, and then homogenized in 200 µl protein lysis buffer (20 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2.5 mM EDTA; 0.2 mM PMSF; protease inhibitor cocktail (one tablet per 50 ml buffer) (Roche)). Separation of proteins by gel electrophoresis was done according to a previously published protocol using 12% SDS gels [31]. For immunodetection primary rabbit anti-cGKI (dilution 1:200) [32], rabbit anti-HSL (dilution 1:500) (Cell signalling), rabbit anti-adiponectin (dilution 1:500) (Cell signalling) were used. Equal loading was verified with anti-β-actin antibodies (dilution 1:20.000) (Sigma–Aldrich).

2.3. Immunofluorescence on inguinal WAT paraffin sections

Immunodetection was performed on 8-µm serial sections. Primary antibody/antigene complexes were detected with secondary antibodies conjugated to fluorescent dyes (dilution 1:200 (Invitrogen). Primary antibodies used were specific for cGKI (dilution 1:50) [32], adiponectin (dilution 1:50) (Cell Signalling) and HSL (dilution 1:50) (Cell Signalling).

2.4. Sectional planimetry of adipocyte size

Digital images of hematoxylin and eosin stained WAT sections were analyzed by UTHSCSA ImageTool, version 3.0 by tracing the perimeter of the adipocytes.

2.5. Streptozotocin treatment

Intraperitoneal injections of multiple low doses of STZ (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) were performed in 4 h fasted, eight-week-old male αRM and control mice at 5 consecutive days according to a protocol approved by the Animal Models of Diabetic Complications Consortium (published at http://www.diacomp.org).

2.6. Oral glucose-tolerance tests

Mice were fasted over-night before oral glucose challenge (2 g/kg body weight). Animals' glucose homeostasis was monitored for 2 h as described [30] 4 weeks after the first day of the STZ treatment. Blood samples were collected via the tail vein and blood glucose levels were measured using an Ascenscia Elite Sensor (Bayer).

2.7. Determination of plasma adiponectin and leptin levels

Plasma levels of adiponectin were measured using commercially available adiponectin ELISA kits (ENZO Life Science) from blood samples collected by final heart punctuation of fasted and deeply anesthetized mice. Plasma leptin levels were determined using the 96-well fluorescent Milliplex immunoassay in 10 µl of plasma collected upon over-night fasting via the tail vein.

3. Results

3.1. cGKI is present in the adipose vasculature and in fat cells of murine WAT

On a cellular level, cGKI was present in mature adipocytes of control mice, whereas inguinal WAT sections of global $cGKI^{-/-}$ knockouts and αRM animals remained cGKI-negative (Fig. 1A). High levels of cGKI were detected in the adipose vasculature of control mice and, as expected, in αRM animals. We observed a significant reduction in white fat cell size in the gene-targeted cGKI animals (Fig. 1B), which is in good agreement with previous findings in these mouse models i.e. lower BW, less total fat mass and reduced levels of plasma free fatty acids (FFA) [30]. The expression pattern was confirmed by Western blots that demonstrated the presence of the cGKI protein in lysates obtained from inguinal WAT depots of control mice, whereas cGKI remained undetectable in protein preparations from global $cGKI^{-/-}$ WAT (Fig. 2B) confirming results of Pfeifer and his co-workers [8,12]. Furthermore, the cGKI present in mature white murine fat cells is not a degradation product of the native enzyme, because it has an identical molecular weight as the canonical cGKI protein found in smooth muscle (Fig. 2B).

3.2. Adiponectin and leptin plasma levels are reduced in gene-targeted cGKI mice

It was shown that cultured adipocytes stimulated with cGMP-analogues up-regulate the mRNA for the insulin sensitising, anti-inflammatory and anti-apoptotic adipokine adiponectin [12]. In line with this report, we now find low abundance of adiponectin in WAT sections and protein lysates of cGKI gene-targeted mice (Fig. 2A and B), whereas the level of hormone-sensitive lipase did not differ between the various genotypes. Low WAT adiponectin was paralleled by reduced circulating adiponectin levels in αRM mice in comparison to age- and litter-matched controls (Fig. 2C). Moreover, our present analysis of the adipose-tissue derived hormones revealed significantly reduced plasma levels for leptin (Fig. 2D), a reliable marker of body-mass, in the lean αRM model [33].

3.3. Protection from hyperglycemia following streptozotocin-injections to αRM mice

We previously noticed in the cGKI gene-targeted animals (i) an improved glucose clearance upon both oral and intra-peritoneal glucose tolerance tests with no difference in insulin secretion, (ii) low total body fat mass, (iii) reduced plasma FFA and (iv) an

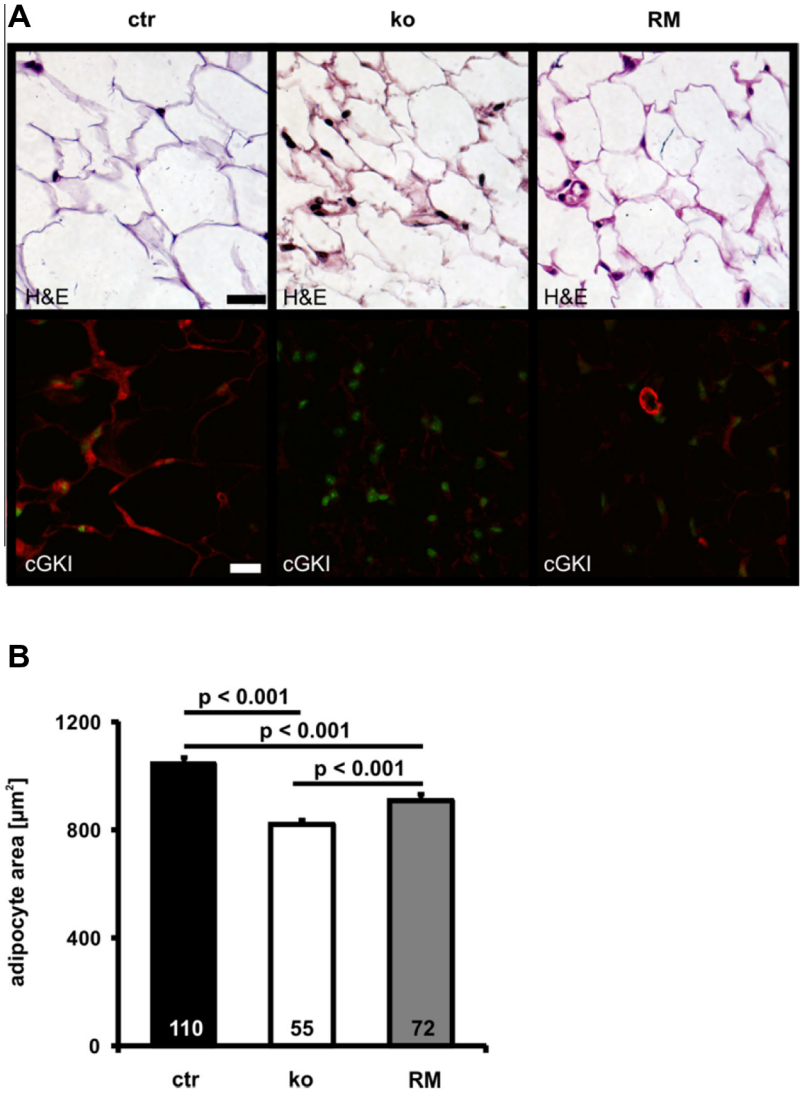

Fig. 1. Expression pattern of cGKI in WAT with planimetric assessment of fat cell size (A). Representative hematoxylin and eosin stain (H&E, upper panels) of WAT paraffin sections isolated from control (ctr), cGKI$^{-/-}$ (ko), and αRM (RM) animals. Using a specific cGKI antibody [32], the immunodetection on WAT sections revealed high levels of cGKI (red) in vessels of control, and as anticipated from the rescue strategy [21], in the vasculature of αRM WAT. Importantly, cGKI was detected only in control adipocytes, whereas cGKI$^{-/-}$ and αRM fat cells remained cGKI negative. DAPI was used to visualize the nuclei (green). Scale bars = 20 μm. (B) Planimetry was performed on H&E stained WAT sections as described in the Section 2. Individual adipocyte size was determined by defining the perimeter of 55–110 cells. In total, 3–4 different sections from $n = 3$ animals per genotype were analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase in the insulin-dependent glucose transporter 4 mRNA in skeletal muscle preparations [30]. These findings are supported by the very low plasma leptin levels (Fig. 2D) and smaller white fat cell size (Fig. 1B) as consistent markers for the lean phenotype of this mouse model. To test whether the improved glycemic control of αRM mice under "normal conditions" would be disturbed in an experimentally induced diabetes model of pancreatic inflammation, we studied glucose tolerance after multiple injections of low dose streptozotocin (STZ), which reportedly causes an almost complete β-cell loss within 2–3 weeks in mice. Oral gavage of 2 g/kg glucose induced a pronounced hyperglycemia in STZ treated control mice (Fig. 2E). Unexpectedly, plasma glucose levels of STZ treated αRM animals were significantly lower at all time-points examined (Fig. 2E) and within the range of αRMs that did not receive STZ [30].

4. Discussion

Our present results strongly support the finding [8,12] that the cGKI protein is expressed in adipocytes and that its deletion has a significant impact on WAT-derived hormones. This report further raises the possibility that WAT cGKI has an important function in the synthesis/secretion of adiponectin and leptin. Indeed, recent findings by Withers and co-workers imply that fat cell cGKI might be involved in the release of adipocyte-derived factors such as adiponectin [34]. Altered adiponectin levels in gene-targeted cGKI mice have been documented by us (present study) and others [20] and these results agree with the report that an inverse correlation exists between adiponectin and fat mass [35]. Furthermore, hypoadiponectinemia has been associated with elevated levels of inflammatory markers such as C-reactive protein and IL-6 [35,36]. Elevated plasma IL-6 levels that might stem from dysfunctional cGMP/cGKI in fat cells and/or hepatic stellate cells were reported by several studies in the αRM mouse model [12,20,22]. This is interesting, because IL-6 may act as a negative regulator of fat cell-derived hormones such as adiponectin and *vice versa* IL-6 expression can be negatively regulated by adiponectin [37,38]. Together these data support the notion that the adipose tissue is contributing to a low-grade systemic inflammation in the cGKI gene-targeted mouse models.

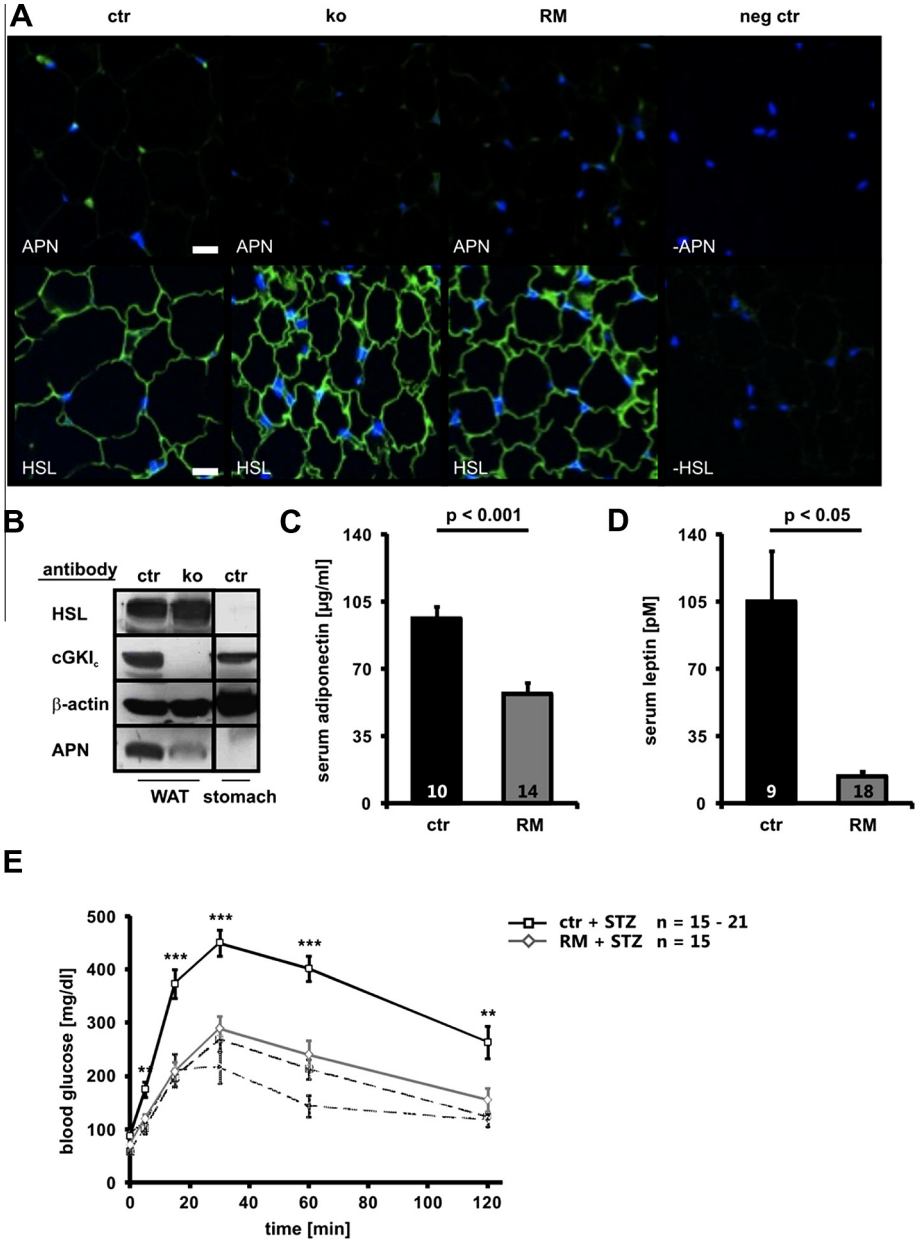

Fig. 2. Abundance of the adipose-tissue derived hormones adiponectin and leptin, and glycemic control upon injection of multiple-low doses of streptozotocin in cGKI mutant mice. (A) Immunodetection of adiponectin (APN, upper panels) and the white fat cell marker hormone-sensitive lipase (HSL, lower panels) in WAT of control (ctr), cGKI$^{-/-}$ (ko), and αRM (RM) mice. In parallel, negative control stainings (neg ctr) under the same experimental conditions without primary antibodies were performed. Scale bars = 20 µm. (B) Western blot analysis of WAT protein lysates derived from control (ctr) and cGKI$^{-/-}$ (ko) animals. cGKI was detected in the lysates from ctr but not from cGKI$^{-/-}$ mice. APN was present in ctr but only at low abundance in cGKI$^{-/-}$ WAT confirming the results of Fig. 2A. Simultaneous detection of β-actin and HSL demonstrated equal loading of the gel. A Western blot of lysates of wild type murine stomach is shown for comparison using the same antibodies. Lysates were positive for cGKI and negative for HSL and APN. 50 µg protein was loaded per lane. (C) Plasma adiponectin levels determined in control (n = 10) and age- and litter-matched αRM (n = 14) animals. (D) Plasma leptin levels of over-night fasted control mice (n = 9) and αRM mutants (n = 18) from the same litters. (E) Time courses of blood glucose shown for control (n = 15-21 animals per time point) and αRM (n = 15 animals per time point) mice 4 weeks after multiple low doses of streptozotocin (STZ). The oral glucose tolerance test (oGTT) was performed in fasted mice that received 2 mg glucose per g body weight ($^{**}P < 0.01$ and $^{***}P < 0.001$ indicate a significant difference between both groups at the respective time-points). oGTT data from over-night fasted (but not STZ treated) control (upper dashed line) and αRM animals (lower dashed line) are shown to allow comparison of the STZ effect in these mouse lines. The baseline oGTT data are reproduced from Leiss et al. 2011 [30].

A similar scenario i.e. a cGMP/cGKI-dependent control over the release of factors from a cellular compartment has been recently suggested for the synthesis/secretion of C-natriuretic peptide from endothelial cells [39]. However, we want to point out that the presented study shows preliminary data with regard to the mouse models used, because either conventional cGKI$^{-/-}$ or αRM mice were studied. These mice have multiple defects that are not related to the fat cell mass [21,22,30,31,39–42]. Therefore, we cannot delineate whether or not the deletion of cGKI in fat cells was the essential cause of the reported disturbance in some fat cell parameters *in vivo*. It is very tempting to speculate that low cGKI signaling in adipocytes confers protection against excessive fat accumulation but more sophisticated approaches are necessary to clarify whether the observed changes indeed result from a lack of cGKI in murine WAT. Mitschke et al. [12] have recently generated an inducible adipocyte-specific cGKI mouse model, but unfortunately the *in vivo* phenotype(s) of the conditional mutants were not reported, so far. Since cGKI appears to be essential for the

differentiation of BAT [8,12], one would expect therefore that the cGKI mutant mice produce less BAT which status has been associated with obesity [1]. However, with respect to body-weight and relative fat mass an opposite behavior has been observed in the cGKI gene-target mice. The αRM mice are lean, have low levels of leptin and adiponectin, live up to a year, and are apparently "resistant" to STZ-induced diabetes mellitus indicating that cGKI, at least in mice that are fed a normal diet, affects both glucose and fat cell homeostasis.

It was also reported that the sleep and waking profile of αRM mice is shifted to more activity during daytime, when mice typically rest, and to more sleep at night [43]. These phase shifts did not affect the total food consumption over 24 h, however during the day-phase an increase in energy expenditure that might contribute to the lean phenotype of αRM mouse model was noted [20].

Together, the "anti-diabetic" and "anti-obese" properties of modified cGKI signaling in mice could be conferred either directly from its role in WAT (and BAT) or indirectly by complex i.e. opposing mechanisms that may include cGKI (dys-)/function in skeletal muscle and liver [20], pancreas [30] and distinct central nuclei in the hypothalamus [44] with the latter one being essential regulators of energy homeostasis, feeding behavior and satiety. At least so far, a whole brain-specific inactivation of cGKI could not easily phenocopy the metabolic features of αRM mice *in vivo* [20].

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Conflict of interest

None declared.

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