Control of glycogen synthase through ADIPOR1-AMPK pathway in renal distal tubules of normal and diabetic rats

Philippe G. Cammisotto, Irene Londono, Diane Gingras, and Moïse Bendayan

Department of Pathology and Cell Biology, University of Montreal, Montreal, Quebec, Canada

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Cammisotto PG, Londono I, Gingras D, Bendayan M. Control of glycogen synthase through ADIPOR1-AMPK pathway in renal distal tubules of normal and diabetic rats. Am J Physiol Renal Physiol 294: F881–F889, 2008. First published February 6, 2008; doi:10.1152/ajprenal.00373.2007.—Diabetic nephropathies are characterized by glycogen accumulation in distal tubular cells, which eventually leads to their apoptosis. The present study aims to determine whether adiponectin and AMPK are involved in the regulation of glycogen synthase (GS) in these structures. Western blots of isolated distal tubules revealed the presence of adiponectin receptor ADIPOR1, catalytic AMPK subunits α1 and α2, their phosphorylated active forms, and the glycogen-binding AMPK subunit β2. ADIPOR2 was not detected. Expression levels of ADIPOR1, AMPKα1, AMPKα2, and AMPKβ2 were increased in streptozotocin-treated diabetic rats, whereas phosphorylated active AMPK levels were strongly decreased. Immunohistochemistry revealed the presence of ADIPOR1 on the luminal portion of distal tubules and thick ascending limb cells. Catalytic subunits α1 and α2, their phosphorylated active forms, and the glycogen-binding subunit β2 were also found in the same cells, confirming immunoblot results. In vitro, 5-aminimidazole-4-carboxamide-1-β-d-ribofurano-side (AICAR; 2 mM) and globular adiponectin (10 µg/ml) activated catalytic AMPK in distal tubules isolated from kidneys of normal rats but much more weakly in those from diabetic rats. GS inhibition paralleled AMPK activation in both groups of animals: active GS levels were low in control animals and elevated in diabetic ones. Finally, glucose-6-phosphate, an allosteric activator of GS, was also increased in diabetic rats. These results demonstrate that in distal tubular cells, adiponectin through luminal ADIPOR1 activates AMPK, leading to the inhibition of GS. During hyperglycemia, this regulation is altered, which may explain, at least in part, the accumulation of large glycogen deposits.

Adiponectin receptors; AMP-activated protein kinase; diabetes

GLYCOGEN NEPHROSIS (also known as Armanni-Ebstein lesions) is a feature of type 1 diabetes in human and rodents (1, 34, 49). In hyperglycemic conditions, cells of the distal tubule and thick ascending limb (TAL) absorb high amounts of glucose that accumulate into glycogen deposits (1, 3, 17, 34). Large glycogen accumulations disrupt cell function and alter expression and distribution of integrins with loss of basal infoldings and apical microvilli. Cells eventually undergo caspase-mediated apoptosis and detach (1, 26).

AMP-activated protein kinase (AMPK) is a master metabolic and antiapoptotic coordinator (33, 35, 41, 53). It is a heterotrimeric complex formed by a catalytic (α) and two regulatory (β and γ) subunits (44). Catalytic AMPK subunits are activated by phosphorylation within a conserved sequence common to both isoforms α1 and α2 (9, 15). Once activated, they shutdown metabolic pathways consuming ATP (fatty acid and glycogen synthesis) and upregulate ATP-generating pathways (glycolysis and β-oxidation), therefore restoring energy levels (4, 5, 6). Inhibition of glycogen synthesis occurs through phosphorylation of glycogen synthase (GS) after activation of catalytic AMPK (11). The AMPK β-subunit, which possesses a glycogen binding site, is required for such regulation (32).

Adiponectin (also known as ACRP30 or adipoQ) is a major activator of AMPK. Its role in the control of glycogen synthesis in skeletal muscle and liver (4, 7, 8, 50) and in the regulation of inflammation and cellular apoptosis (33, 41) has been extensively studied. It is secreted essentially by white adipocytes (18, 39) and circulates in blood under various polymeric forms, ranging from globular (trimer) to high-molecular-weight structures (HMW) (13, 28). Globular adiponectin has a high affinity for receptor ADIPOR1, which activates AMPK, whereas HMW forms preferentially bind receptor ADIPOR2, leading to increased activity of peroxisome proliferator-activated receptor (PPAR)-α signaling pathways (51, 52).

Numerous correlation analyses carried out on patients with diabetic type 1 nephropathies revealed a link between renal tubular injuries and increases in plasma adiponectin levels (24, 37, 38). These increases have been considered as an attempt to limit renal microvascular damages and inflammatory changes in kidney (24, 38). Adiponectin receptors ADIPOR1 and ADIPOR2 mRNA as well as AMPK mRNA are expressed in rat and human renal tissues (44, 52); however, their localization and roles in renal physiology have been scarcely investigated. The aim of the present study was to assess whether adiponectin receptors and AMPK subunits are present in distal tubules and TAL and whether they participate in the control of glycogen metabolism. We found that adiponectin receptors ADIPOR1 and AMPK are located in these structures. In control animals, globular adiponectin potently activated AMPK, leading to the inhibition of GS. In hyperglycemic conditions, ADIPOR1 and AMPK signaling was altered, as was their regulation of GS, which may explain the large accumulations of glycogen.

MATERIALS AND METHODS

Chemicals. The following antibodies were purchased: anti-ADIPOR1 (Affinity BioReagents, Golden, CO), anti-ADIPOR2 (Alph Diagnostics, San Antonio, TX), anti-AMPKß2 (H75) and anti-leptin receptor (K20) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-AMPKα (Thr172), anti-GS, and anti-phospho-GS (Ser441) (Cell Signaling, Boston, MA), anti-Tamm-Horsfall antigen (Cedarlane, Hornby, ON, Canada), and anti-AMPKα1 and anti-AMPKα2 (Bethyl Labora-

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Adiponectin receptors. Distal tubules and TAL are particularly targeted in diabetic tubulopathy due to the accumulation of intracellular glycogen (1,3, 17, 34). Adiponectin, through its...
receptors, is a major regulator of glycogen synthesis (7, 8, 50). To establish the presence of adiponectin receptors in distal tubules and TAL, we carried out Western blotting and immunohistochemistry on isolated distal tubules and TAL homogenates and on sections of renal tissue, respectively. Enrichment and purity of the distal tubule suspension was confirmed by morphological examination and by dot-blot analysis, using the antibody against the Tamm-Horsfall antigen (results not shown) (43). Electron microscopy further confirmed the typical morphology of the isolated distal tubules and TAL in the preparation (results not shown). Homogenates of isolated tubules from control and diabetic animals were resolved on a 10% acrylamide gel as described in MATERIALS AND METHODS. ADIPOR1 was revealed as a single band of 42 kDa in both groups of animals (Fig. 1A). ADIPOR2 was not found in renal tubules; however, it was present as a band of 34 kDa in a sample of rat liver tissue taken as a positive control (results not shown) (52). Quantitation of band densities showed that levels of ADIPOR1 were significantly higher in isolated tubules of diabetic rats (15.5 ± 3.10 as normalized to β-actin) compared with controls (0.86 ± 0.26 as normalized to β-actin) (Fig. 1A). Immunohistochemistry revealed that ADIPOR1 was localized to the apical portion of distal tubular and TAL cells in tissues of normal animals (Fig. 1, B and C). Plasma membranes at the base of the cells were devoid of staining. In tissues of diabetics animals, most of the tubules were altered with the appearance of Clear cells (1). These Clear cells display characteristic clear, empty cytoplasmic spaces left by the extraction of the large glycogen deposits. The Clear cells displayed strong stainings for ADIPOR1 at the level of the apical and basolateral regions as well as in the cytoplasm (Fig. 1C). A few tubules were not affected to the same degree with glycogen deposition, and their staining was not as intense. Although Yamauchi et al. (52) have reported, using molecular biology, the presence of ADIPOR2 in total renal homogenate, we were unable to detect ADIPOR2 in our isolated tubule preparations from either group. With immunocytochemistry, ADIPOR2 was only found in glomeruli and capillary endothelial cells, not in TAL and distal tubules (results not shown). Controls performed by omitting primary antibodies in the histochemical protocol displayed no staining (Fig. 1D).

AMPK subunits α1 and α2. AMPK is the main second messenger of ADIPOR1. To assess its presence in distal tubules and TAL, we carried out detection of the catalytic AMPK subunits α1 and α2. Both isoforms were found in isolated tubule homogenates as bands of 64 kDa, as previously reported for total kidney homogenates (Fig. 2, A and D) (12). Tubules from diabetic animals displayed a much enhanced expression for both isoforms α1 (32.2 ± 6.75 as normalized to β-actin) and α2 (67.4 ± 12.4 as normalized to β-actin) compared with tubules of control animals (3.64 ± 1.3 and 19.1 ± 6.74 as normalized to β-actin, respectively) (Fig. 2, A and D). Light microscopy showed that AMPKα1 and AMPKα2 are present in distal tubules and TAL of control animals at the level of the apical membrane and cytoplasm (Fig. 2, B and E). In diabetic animals, Clear cells also displayed a positive staining for AMPKα1 and AMPKα2 in the peripheral rim of cytoplasm (Fig. 2, C and F). Intensities of the staining for AMPKα1 and AMPKα2 appeared rather similar in tissues from normal and diabetic animals. Controls without primary antibodies confirmed the specificity of the staining.
**Glycogen-binding AMPK subunit β₂**. The AMPK subunit β₂ possesses a glycogen-binding site, which is involved in the control of glycogen synthesis (32). Western blots on isolated tubules confirmed the presence of this isoform at 34 kDa (Fig. 3A), with the β₁-isoforn not being expressed in renal tissue (12). Expression of the β₂-subunit in tissues of diabetic animals was twice that of normal ones (35.9 ± 3.59 vs. 19.5 ± 4.10 as normalized to β-actin) (Fig. 3B). Immunohistochemistry revealed an abundant staining of distal tubule and TAL cells from both normal (B and E) and diabetic animals (C and F). Clear cells in tubules (asterisks) of diabetic animals are stained for AMPKα₁ (C) and AMPKα₂ (F) in the apical portion (arrows) as well as basolateral plasma membranes (arrowheads). Bars, 50 μm.

**In vitro activation of AMPK by AICAR and globular adiponectin**. We subsequently assessed whether globular adiponectin and AICAR activate catalytic AMPK in isolated distal tubules and TAL. Globular adiponectin is the high-affinity ligand for ADIPOR1 (52). AICAR is an adenosine analog that is taken up into cells through adenosine transporters and is then phosphorylated intracellularly to form ZMP, which is an AMP analog. This ZMP allosterically increases AMPK activity independently from plasma membrane receptors (45) and reduces its rate of dephosphorylation (46). In vitro incubations were carried out in parallel on renal tubules isolated from control and diabetic animals. Total amounts of AMPKα₁ and AMPKα₂ were not affected by AICAR or adiponectin in our conditions for both groups (Fig. 5). Western blots revealed that AICAR (2 mM) and globular adiponectin (10 μg/ml) potently stimulated phosphorylation of the catalytic AMPK subunits in tubules of control animals (basal: 24.1 ± 5.37; AICAR, 66.9 ± 0.205 ± 0.068 for diabetic animals). Immunostaining was strong in the cytoplasm of distal tubules and TAL cells of normal animal tissues (Fig. 4B), whereas that of Clear cells present in tissues of diabetic animals was markedly reduced (Fig. 4C).
12.2; and adiponectin, 76.1 ± 8.90; as normalized to total catalytic AMPK) (Fig. 5). In tubules of diabetic animals, levels of phosphorylated AMPK, both basal and stimulated, were much lower (basal, 0.724 ± 0.167; AICAR, 1.38 ± 0.137; and adiponectin, 1.55 ± 0.285; as normalized to total catalytic AMPK) (Fig. 5), demonstrating significant decreases in activities and stimulation of catalytic AMPK in hyperglycemic conditions despite the higher pool of catalytic AMPK. Indeed, levels of basal AMPK in diabetic conditions are ~30 times lower than those in control conditions.

**Inhibition of GS by globular adiponectin and AICAR.** Once activated, catalytic AMPK activates glycogen synthase kinase-3 (GSK-3), which in turn phosphorylates GS on Ser641 (11). To determine whether the low activity of catalytic AMPK in renal tubules of diabetic animals leads to a decrease of inhibited GS, we incubated tubules isolated from control and diabetic rats with AICAR and globular adiponectin. Western blots revealed that the pool of glycogen synthase (phosphorylated and nonphosphorylated forms) was three times as high in tubules isolated from diabetic rats (21.5 ± 4.63 as normalized to β-actin) (+203%) as in tubules from control rats (7.1 ± 2.93 as normalized to β-actin). Phosphorylated inactive GS was also found to be increased in tissues from diabetic animals (8.20 ± 1.41 vs. 5.11 ± 1.08 as normalized to β-actin; +96%) (Fig. 6). Nevertheless, the ratio of inactive GS to total GS was much lower in the diabetic group (Fig. 7). When incubated with AICAR (2 mM) and globular adiponectin (10 μg/ml), tubules isolated from control rats displayed a potent increase in GS phosphorylation (basal, 18.9 ± 4.47; AICAR, 95.2 ± 26.7; and adiponectin, 162.1 ± 33.9; as normalized to total GS) (Fig. 7). The phosphorylation of GS was much weaker in tubules from diabetic animals than for controls (basal, 12 ± 2.3; AICAR, 21.7 ± 3.67; and adiponectin, 22.9 ± 3.06; as normalized to total GS). Total GS levels were not affected by the incubation conditions (results not shown). These results demonstrate that functional GS (expressed as the ratio of phosphorylated to total GS) is higher in diabetic condition, with regulation by AMPK severely impaired. Finally, glucose-6-phosphate, the allosteric activator of GS (31), was also found in higher concentrations in tubules from diabetic rats compared with controls (88.5 ± 24 vs. 154 ± 31.6 nmol/mg protein) (Fig. 8). Together, these results demonstrate that GS activity is higher in tubules of diabetic rats and that its inhibition by AMPK is impaired. All this contributes to the accumulation of glycogen in renal Clear cells of diabetic animals.

**DISCUSSION**

Control of GS by adiponectin and AMPK in skeletal muscles and liver is well established (11, 32). We presently report that a similar situation occurs in renal distal tubules, where globular adiponectin through ADIPOR1 activates AMPK, leading to the inhibition of GS. This control is impaired in diabetes, indicating that it might contribute to glycogen nephrosis.

As shown by immunohistochemistry and Western blotting, ADIPOR1 and the α1-, α2-, and β2-subunits of AMPK were localized to distal tubules and TAL. ADIPOR1 was present on
the luminal membrane of tubular cells, but not on the basolateral membrane. ADIPOR2 was not detected in distal tubules and TAL. On the other hand, we confirmed that leptin receptors, which are also potent activators of AMPK, were only present in the renal medulla (results not shown) and not in distal tubules and TAL. The expression of T-cadherin, another protein that binds the HMW isoform of adiponectin, is limited to the cardiovascular and nervous systems and is absent in kidney (19). Therefore, our results suggest that low-molecular-weight globular adiponectin originating from glomerular filtration is the major activator of AMPK through luminal ADIPOR1 receptors in distal tubules and TAL. In accordance, we did find globular adiponectin in samples of rat urine (results not shown); other groups have also reported its presence in urine from healthy and diabetic patients (24, 42). The absence of adiponectin receptors on the basolateral plasma membrane of distal tubule and TAL cells of normal animals suggests that circulating adiponectin does not participate in AMPK activation in distal tubule cells. In muscle cells, ADIPOR1 is more important than ADIPOR2 in the regulation of glucose metabolism (21, 51), and our results seem to favor similar mechanisms in kidney distal tubules.

ADIPOR1 and AMPK subunits display similar distributions in tissues of control and diabetic animals. However, ADIPOR1, AMPKα1, and AMPKα2 expression is greatly enhanced in tubules from diabetic animals, whereas phosphorylated AMPK levels are decreased. Insulin deficiency is known to increase expression of adiponectin receptor in muscle, whereas hyperinsulinemia decreases it (21, 47). Increases in AMPKα1 and AMPKα2 expression in kidney homogenates from streptozotocin-treated mice have also been reported (25, 48). Mechanisms similar to those reported for adiponectin receptor may explain these enhances (21, 47). Along the same line, insulin has been shown to modulate AMPK activity, and its absence in diabetic condition may interfere with catalytic AMPK synthesis (25, 48). It is also well known that prolonged hyperglycemia does affect cell signaling and gene expression (22, 25). Moreover, these increases in ADIPOR1 and catalytic AMPK may be an attempt by cells to compensate for the development of adiponectin resistance (4, 47). Finally, decreased levels of activated-AMPKα have been reported in diabetes-induced re-

Fig. 4. Phosphorylated activated AMPK (Pi-AMPKα) in renal tubules (A). Actin is shown as a loading control. Band intensities expressed in reference to actin (means ± SE; n = 4) reveal significant differences between tissues of CTL and STZ animals (**P < 0.01). Immunoperoxidase staining for phosphorylated AMPK is present in the cytoplasm and apical membrane of distal tubule and TAL cells in control animals (arrows, B). Staining is weak or even absent in Clear cells (asterisks) of diabetic animals (C). Bars, 50 μm.

Fig. 5. In vitro phosphorylation of catalytic AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and globular adiponectin in isolated renal tubules from CTL and STZ rats. Activation of AMPK (Pi-AMPKα) by AICAR (2 mM) or globular adiponectin (AdipoN; 10 μg/ml) was revealed by Western blot and quantified. Results are expressed in reference to total catalytic AMPK (means ± SE; n = 4). For both groups of animals, Pi-AMPK levels increased significantly in the presence of AICAR and adiponectin (**P < 0.01). Levels of basal AMPK relative to Pi-AMPK were drastically reduced in tissues of diabetic animals.
nal hypertrophy (25) and is similar to the adiponectin resistance present in other tissues (4).

In vitro incubation of isolated distal tubules and TAL with globular adiponectin and AICAR potently stimulates phosphorylation of catalytic AMPK in a way similar to other tissues (11, 41, 45). Interestingly, in tubules from diabetic animals, basal and stimulated levels of active AMPKα were six times lower than controls, despite the strong increase in expression of total catalytic AMPK α1- and α2-subunits. Several hypotheses may explain this decrease in AMPK activation. First, tubules made of glycogen-filled Clear cells may be unresponsive to adiponectin and AICAR stimulation; the small increase that we observed may just arise from those tubular cells not yet loaded with glycogen but still present in the tubules. Second, glucose may stimulate AMPK dephosphorylation, as proposed by Lee et al. (25). High concentrations of glucose have been shown to activate the phosphatase PP2A, which dephosphorylates AMPK in pancreatic β-cells and other tissues (9, 22). In addition, PP2A prevents association between AMPK subunits (14). Third, in the diabetic condition, the large volume of intracellular glycogen may cause the sequestration of AMPK, making it unavailable for its receptors (32). Indeed, AMPK β2-subunit possesses a glycogen-binding site, which could render the trimeric AMPK unavailable for activation by receptors (12, 32, 50). These hypotheses do not rule out each other and do not rule out other mechanisms interfering with ADIPOR1 signaling, which includes SOCS proteins (suppressor of cytokine signaling 3), the phosphatase PTP-1B (phospho-tyrosine phosphatase 1B), or an inhibition of LKB-1, the upstream kinase of AMPK that controls Thr172 phosphorylation (23, 46). Finally, it was recently reported that ketoacidosis influences AMPK and decreases its activity in heart muscle (30). Our animals do present strong ketonuria; however, interestingly, Zucker fa/fa rats, which are hyperglycemic without ketoacidosis (29), present numerous renal tubular Clear cells as well as decreased phosphorylated AMPK levels similar to those observed in our streptozotocin-treated rats. Further studies are needed to determine the exact mechanism responsible for the disruption of AMPK activity.

Once activated, AMPK leads to the phosphorylation of GS, resulting in its inactivation (11). In isolated tubules, expression of total GS is three times higher in tissues of diabetic animals than in those of control ones. On the other hand, the inactive phosphorylated form is twofold higher in tubules of diabetic animals. Yet, the ratio of inactive to total GS was lower in diabetic animals, which indicates greater GS activity. In vitro, adiponectin and AICAR potently stimulated phosphorylation of GS in tubules from controls, whereas they only weakly did so in those from diabetic animals, probably reflecting the activity of catalytic AMPK in each respective group. To be emphasized is the fact that glycogen phosphorylase kinase, a
key enzyme for the breakdown of glycogen, is not controlled by AMPK (2).

Other mechanisms regulating GS activity must be taken into account. Concentrations of the allosteric activator of GS (31), the glucose-6-phosphate, are higher in tubules from diabetic animals and may contribute to increase GS activity. Moreover, luminal glucose is absorbed through the apical membrane of distal and TAL cells by glucose/sodium transporters and pumped toward the basal extracellular space via GLUT 1 and GLUT 4 (16). AMPK regulates the translocation of glucose transporters (GLUT family) from the cytosol to the membrane in muscle (4, 42); the low AMPK activity in distal tubules of diabetic rats may thus contribute to the decreased translocation of GLUT transporters to the basal membrane, leading to glucose accumulation inside cells and stimulation of glycogen synthesis (16).

In summary, we have demonstrated that global adiponectin, through the luminal receptor ADIPOR1, potently activates catalytic AMPK in renal distal tubule and TAL cells, similarly to situations in other tissues. In diabetes, this activation is reduced despite an increase in the expression of catalytic AMPK subunits. Given that AMPK blocks glycogen synthesis, its depressed activity may explain, at least in part, the accumulation of glycogen and subsequent apoptosis observed in renal tubular cells in hyperglycemic conditions. However, we should keep in mind that regulation of AMPK and GS activities are complex, and other intracellular pathways and factors may also be involved.

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REFERENCES

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