Adiponectin Enhances Calcium Dependency of Mouse Bladder Contraction Mediated by Protein Kinase Ca\textsubscript{\alpha} Expression

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ABSTRACT

Adiponectin is an adipose tissue–secreted protein and is a multifunctional adipocytokine. However, the association of adiponectin with bladder contraction has not been investigated. In this study, the adiponectin-sense transgenic mouse (Adip-Sen mouse; age, 16–24 weeks; male) and age-matched controls (C57Bl mouse) were studied. The Adip-Sen mouse showed a significant increase in plasma adiponectin levels (56.2%; C57Bl mouse) were studied. The Adip-Sen mouse showed a significant increase in plasma adiponectin levels (56.2%; P < 0.01), compared with those in the C57Bl mouse, without affecting other lipid parameters. Isometric force development in bladder smooth muscle tissues were detected using an organ-bath system. Although carbachol (CCh)–induced (0.1–100 μM) time- and dose-dependent contractions in Adip-Sen mouse bladder were slightly enhanced, compared with those in the C57Bl mouse during a low range (0.3–1.0 μM) of CCh, differences could not be detected with other CCh concentrations. However, the reduction in contraction under Ca\textsuperscript{2+}–replaced conditions was significantly different between Adip-Sen and C57Bl mice (94.1 and 66.3% of normal contraction, respectively; n = 5). A parameter of Ca\textsuperscript{2+} sensitivity, the relation between intracellular Ca\textsuperscript{2+} concentration and contraction, was increased in the Adip-Sen mouse, compared with that in the C57Bl mouse. This Ca\textsuperscript{2+} dependency in the Adip-Sen mouse was reduced by a protein kinase C (PKC) inhibitor, but not by a Rho kinase inhibitor. Expression of the calcium-dependent isoform of PKC, PKC\textsubscript{\alpha}, was increased in the Adip-Sen mouse bladder, and CCh–induced phosphorylation of PKC\textsubscript{\alpha} was also enhanced, compared with those in the C57Bl mouse. In conclusion, adiponectin is associated with bladder smooth muscle contraction, which involves an increase in Ca\textsuperscript{2+} dependency of contraction mediated by PKC\textsubscript{\alpha} expression.

Introduction

Adiponectin is an adipocyte-secreted hormone and is present in the circulation of healthy humans at high concentrations (Goldstein and Scalia, 2004). Unlike most other adipocytokines, adiponectin levels decrease in individuals with obesity (Stumvoll et al., 2002), and adiponectin levels are further reduced in type II diabetes (Hotta et al., 2000). Because adiponectin plays a role in increases in glucose incorporation and insulin sensitization, it is thought that adiponectin is an endogenous antidiabetic factor (Kadowaki and Yamauchi, 2005). Moreover, it has been suggested that adiponectin is associated with coronary artery disease, stroke, nonalcoholic steatohepatitis, and several types of cancers (Lam and Xu, 2005; Trujillo and Scherer, 2005; Wang et al., 2007). In some of these adiponectin-related diseases, dysfunction of the urinary system is recognized as a complication. For example, alterations of urinary bladder smooth muscle tissue are found in diabetes, hypertension, and hyperlipidemia. It has also been reported that adiponectin affects vascular smooth muscle contraction (Ding et al., 2012). Therefore, we hypothesized that blood adiponectin levels are associated with bladder smooth muscle contractions. However, the association of adiponectin with urinary systems has not been investigated. To understand this association, we hypothesized that the role of adiponectin can be clearly defined under adiponectin-enhanced conditions. In 2006, we established an adiponectin-sense-transgenic (Adip-Sen) mouse (Saito et al., 2006). We found that plasma adiponectin levels were significantly increased in the Adip-Sen mouse (56.2%), compared with those in the wild-type mouse, suggesting a role of adiponectin in regulation of energy homeostasis.

This study aimed to determine whether there is an association of adiponectin with bladder smooth muscle contraction, which is an important function of the urinary system, with use of the Adip-Sen mouse. Mechanisms governing this association were also considered.

ABBREVIATIONS: Adip-R, adiponectin-specific receptor; Adip-Sen, adiponectin-sense-transgenic; A-kinase, cAMP dependent protein kinase; [Ca\textsuperscript{2+}], intracellular calcium concentration; CCh, carbachol; G66976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; MLC, myosin light chain; PKC, protein kinase C; PL, phospholipids; p-PKC\textsubscript{\alpha}, phosphorylated-PKC\textsubscript{\alpha}; PSS, physiological salt solution; Y27632, (R)-(+)–trans-N-(4-pyridyl)-4-(1-aminoethy)-cyclohexanecarboxamide 2HCl.
Materials and Methods

Generation and Maintenance of the Transgenic Mice. Male Adip-Sen mice (16–24 weeks) and age-matched control (C57Bl/6J) mice were prepared and maintained as described previously (Saito et al., 2006). Mice were housed at constant room temperature (20 ± 2°C) with 12-hour light and dark cycles. Mice were fed standard mouse chow, which included 4.5% fat (Oriental Yeast Corp., Tokyo, Japan). Food and water were available ad libitum, and mice grew satisfactorily. Animals were used for experiments at 16–24 weeks of age. This study was approved by the care and use of laboratory animals of the Japanese Pharmacological Society.

Blood Collection and Plasma Biochemical Assays. Blood samples were obtained from the inferior vena cava under ether anesthesia. The plasma supernatant was used for the detection of plasma glucose, phospholipids (PL), free fatty acids, triacylglycerol, and total cholesterol levels in clinical laboratory tests conducted by SRL Inc. (Tokyo, Japan).

Bladder Smooth Muscle Tissue Preparation. Mice were sacrificed by over-treatment of ether, and decapitation and bloodletting were then performed. The urinary bladder was isolated, and the tissue was minced with a scalpel. All tissues for analysis were then performed. The urinary bladder was isolated, and the tissue was minced with a scalpel. All tissues for analysis were then performed. Isometric force and intracellular Calcium Concentration ([Ca2+]i) were measured under normal PSS and calcium-free (no CaCl2) conditions. Bladder contractions were normalized to the cross-sectional area, as described previously (Nobe et al., 2009). Isometric force and [Ca2+]i were simultaneously measured using 4,5'-[3-[3-(4-acetoxy)benzofuran-5-yl]-6-[bis[2-(acetoxymethoxy)-2-oxoethyl]amino]benzofuran-5-yl]-ethoxy]-4-[2-[(acetoxy)ethoxy]-2-oxoethyl]aminolphenyl]-1-oxopropyl] piperazine-1-acetic acid (fura-PE3) 1000 dilution (polyclonal antibodies; Abcam Co., Cambridge, UK), followed by a horseradish peroxidase–conjugated secondary antibody (polyclonal IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Measurement of mRNA Levels. Messenger RNA levels in each bladder tissues sample were measured as reported in our previous study (Nobe et al., 2001).

Western Blot Analysis. Isolated fresh tissue samples were treated under various conditions, and then reactions were terminated using liquid nitrogen. Plasma membrane fractions for Western blot analysis were prepared as described previously (Nobe et al., 2008). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970), using a 12% polyacrylamide gel. Detection of each protein was performed in a similar manner as in our previous study (Nobe et al., 2010), with use of primary antibodies at 1:1000 dilution (polyclonal antibodies; Abcam Co., Cambridge, UK), followed by a horseradish peroxidase–conjugated secondary antibody (polyclonal IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Measurement of mRNA Levels. Messenger RNA levels in each bladder tissues sample were measured as reported in our previous study (Nobe et al., 2006).

Data Analysis. Values are presented as the mean ± S.E.M. obtained from at least five animals. Statistical differences (P < 0.01) for multiple comparisons were assessed using one-way analysis of variance for repeated measurements, followed by the Student-Newman-Keuls test (Y-Stat Program; Igaku Tosyo Shuppan Co., Ltd., Tokyo, Japan).

Results

Basic Characteristics of Adip-Sen Mice. Plasma adiponectin levels were significantly increased (56.2%) in Adip-Sen mice, compared with those in C57Bl mice at the age of 16–24 weeks, but body weight and the wet weight of bladder tissue were similar among the groups (Table 1). Moreover, plasma glucose, total cholesterol, PL, and free fatty acids levels were also similar among the groups.

Changes in Carbachol-Induced Bladder Force Development in Ca2+-Free PSS. Resting levels of isometric force in C57Bl and Adip-Sen mice bladders were 0.96 ± 0.05 and 1.11 ± 0.04 mN/mm2 (n = 5), respectively. KCl-induced (50 mM) sustained force development was also similar among the groups (unpublished data). Cumulative addition of 2-(aminocarboxyl)oxyl-N,N,N-trimethylammonium chloride [carbachol (CCh); Sigma-Aldrich, St. Louis, MO] induced significant increases in isometric force under normal calcium conditions. During 0.3–1 μM CCh stimulation, force responses in the Adip-Sen mouse were enhanced, compared with those in the C57Bl mouse, but increases in maximal response were not evident. Maximal force levels in C57Bl and Adip-Sen mice in the presence of 30 μM CCh were 4.37 ± 0.27 and 4.35 ± 0.14 mN/mm2 (n = 5), respectively.

To demonstrate an association between extracellular Ca2+ concentration and bladder contractility, force development was measured under Ca2+-free conditions (Fig. 1, A and B). Pretreatment of C57Bl mice with Ca2+-free PSS for 10 minutes significantly reduced the CCh-induced force development, compared with the response in normal PSS, and 33.8% of the normal response remained. However, the CCh-induced response in Ca2+-free PSS in Adip-Sen mice resulted in a suppression of this response. In the presence of 30 μM CCh, only 5.9% of the normal response was detected.

The relation between [Ca2+]i and force development was examined (Fig. 2). In fura-PE3–loaded tissue, changes in [Ca2+]i were expressed as R340/380. Resting and 50 mM KCl-treated R340/380 values were similar in C57Bl and Adip-Sen mice (unpublished data). Isometric force development elicited by CCh was simultaneously measured (Fig. 1). In the C57Bl mouse, a relationship was evident between R340/380 and force development. The slope of this relation was 1.04. In the Adip-Sen mouse, the CCh-induced increase in force development was similar to the response in the C57Bl mouse, but it was detected at a lower R340/380 than in the C57Bl mouse. Therefore, the slope of this relation in the Adip-Sen mouse was increased (2.57).

Effects of Rho Kinase and Protein Kinase C Inhibitors on CCh-Induced Force Development. To determine the associations of Rho kinase and PKC with CCh-induced bladder force development, we used (R)(+)- trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide 2HCl (Y27632, Wako Pure Chemical Co., Osaka, Japan) (Uehata et al., 1997), an inhibitor of Rho kinase, and 12-(2-Cyanoethyl)-6,7,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Gö6976; Sigma-Aldrich) (Martiny-Baron et al., 1993), an inhibitor of calcium-dependent PKC (Fig. 3). Pretreatment of the tissues with 1 μM Y27632 slightly reduced CCh-induced responses without affecting resting levels (Fig. 3A), but significant differences (P < 0.01) between Adip-Sen and C57Bl mice remained, which were similar to the responses in the absence of Y27632. Pretreatment with 1 μM Gö6976 also slightly reduced force development in the C57Bl mouse (Fig. 3B). However, the inhibitory effect of Gö6976 in Adip-Sen mice was significantly increased (P < 0.01). In the presence of 1 μM Gö6976, 30 μM CCh-induced responses in C57Bl and Adip-Sen mice remained at 85.2 and 53.1% of the control response, respectively.
Changes in PKC Isoforms in the Adip-Sen Mouse Bladder. To identify the distribution of PKC isoforms in mouse bladder smooth muscle, the expression of each isoform was assessed in C57Bl mice in the nonstimulated resting state (Fig. 4A). The calcium-dependent PKC isoforms PKCα and PKCβ were detected, and calcium-independent PKC isoforms, PKCμ, and PKCθ were also observed. The effect of PKCα and PKCβ expression on enhancement of adiponectin levels was assessed (Fig. 4B). In the Adip-Sen mouse, we observed a significant increase (119%; \( P < 0.01 \)) in PKCα levels, compared with those in the C57Bl mouse, but they were not affected by addition of 30 μM CCh at 37°C for 5 minutes. Adiponectin levels and CCh treatment did not alter PKCβ, PKCμ, and PKCθ levels in C57Bl and Adip-Sen mice.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>N</th>
<th>Body Weight</th>
<th>Adiponectin (µg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>t-Cho (mg/dl)</th>
<th>PL (mg/dl)</th>
<th>FFA (µg Eq/l)</th>
<th>Bladder Weight (mg)</th>
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</thead>
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<tr>
<td>C57Bl</td>
<td>5</td>
<td></td>
<td>28.5 ± 1.04</td>
<td>20.1 ± 0.55</td>
<td>86.7 ± 2.76</td>
<td>104.7 ± 5.63</td>
<td>206.2 ± 9.47</td>
<td>1005.4 ± 61.4</td>
<td>19.7 ± 1.03</td>
</tr>
<tr>
<td>Adip-Sen</td>
<td>5</td>
<td></td>
<td>27.6 ± 2.15</td>
<td>31.4 ± 0.61</td>
<td>89.4 ± 3.07</td>
<td>94.2 ± 5.97</td>
<td>205.8 ± 7.30</td>
<td>1041.6 ± 70.1</td>
<td>20.2 ± 1.46</td>
</tr>
</tbody>
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Adip-Sen, adiponectin-sense-transgenic; FFA, free fatty acids; PL, phospholipids; t-Cho, total cholesterol.

* \( P < 0.01 \), versus values in C57Bl mice.

**Fig. 1.** Effects of CCh on isometric force under normal and Ca²⁺-free conditions in the bladder of C57Bl and Adip-Sen mice. CCh-induced changes in isometric force (mN/mm²) were measured as described in Materials and Methods. Typical changes observed in bladder preparations isolated from C57Bl (A) and Adip-Sen (B) mice. Bladder tissues were preincubated in normal PSS (left panel) and Ca²⁺-free PSS (right panel) for 10 minutes, and then the indicated contractions of CCh were introduced. Concentration-response relationships for CCh-induced isometric force responses in the bladder of C57Bl (open symbols) and Adip-Sen (closed symbols) mice under normal (circles) and Ca²⁺-free (squares) conditions are indicated (C). Each value represents the mean ± S.E.M. of at least five independent determinations. *\( P < 0.01 \); #\( P < 0.01 \), compared with the values in the C57Bl mouse and responses in normal PSS, respectively.
To confirm an increase in protein levels in the Adip-Sen mouse, PKCa mRNA levels were also measured (Fig. 4C). Relative PKCa mRNA levels in C57Bl and Adip-Sen mice were 0.011 ± 0.001 (n = 5) and 0.045 ± 0.003 (n = 5), respectively (P < 0.01). Significant increases in both PKCa protein and mRNA expression were confirmed in the Adip-Sen mouse.

Activation of PKC involves auto-phosphorylation of PKC in many types of cells (Stempka et al., 1999; Bayer et al., 2003). In the nonstimulated resting state, phosphorylated-PKCa (p-PKCa) levels in C57Bl and Adip-Sen mice were not observed. However, a 30 μM CCh-induced increase in p-PKCa levels was detected only in the Adip-Sen mouse (Fig. 4D). This CCh-induced increase was 90.5% of resting levels (n = 5).

Discussion

The current study found an association of adiponectin with bladder smooth muscle contraction. This finding suggests that this association was involved in activation of PKCa-mediated calcium dependency of bladder smooth muscle contraction.

To investigate the association between adiponectin and bladder smooth muscle contraction, we developed the Adip-Sen mouse, because changes in bladder function(s) by adiponectin might be detectable under conditions of chronically increased adiponectin levels. In the Adip-Sen mouse, increases in adiponectin levels were confirmed without affecting body weight and other blood parameters (Table 1). These results are similar to our previous report (Saito et al., 2006). On the basis of these results, we consider that changes in the Adip-Sen mouse were caused by chronically increased adiponectin levels, and these changes were not attributable to secondary effects in these transgenic mice.

Dose-response curves of CCh stimulation in Adip-Sen and C57Bl mice were similar without 0.3–1 μM CCh stimulation (Fig. 1), but extracellular calcium dependency was significantly enhanced only in the Adip-Sen mouse. This result suggests that...
that adiponectin affects bladder smooth muscle contraction, which is mediated by an increase in calcium dependency. A relation between $[Ca^{2+}]_i$ and isometric force also supported this possibility (Fig. 2), because developed force levels in the Adip-Sen mouse were significantly increased, compared with those in the C57Bl mouse, when CCh-induced $[Ca^{2+}]_i$ levels were similar. This indicates an increase in calcium sensitivity of the Adip-Sen mouse bladder contraction. Of interest, 50 mM KCl induced h

In smooth muscle contraction, the Rho-Rho kinase pathway is one of the major signaling pathways. This pathway enhances calcium dependency mediated by inhibition of myosin light chain (MLC) phosphatase, and it induces accumulation of phosphorylated MLC (Hirano et al., 2004). Moreover, a role for PKC has also been proposed in smooth muscle contraction (Salamanca and Khalil, 2005). PKC enhances MLC kinase and other intracellular contractile factors. Associations of these pathways in bladder contraction have been previously reported (Yamaguchi, 2004; Durlu-Kandilci and Brading, 2006), and our results are consistent with these previous reports (Fig. 3). However, we found that inhibitory effects of Gö6976 were significantly enhanced only in the Adip-Sen mouse (Fig. 3B). These results indicate that the contribution of the PKC pathway in bladder contraction is enhanced in the Adip-Sen mouse. Therefore, we speculate that adiponectin regulates calcium dependency, which is mediated by activation of the PKC pathway.

We evaluated protein levels of PKC to examine the change in PKC with adiponectin-mediated increases in calcium dependency. It is generally accepted that PKC involves 10 or more isoforms, which involve calcium-dependent PKC (PKCa, β, and γ), calcium-independent PKC (PKCd, μ, and θ), and atypical PKC isoforms (Salamanca and Khalil, 2005). In the current study, in bladder smooth muscle tissue, PKCa, β, μ, and θ isoforms were detected (Fig. 4A). Among these isoforms, only PKCa was significantly enhanced, which depended on plasma adiponectin levels (Fig. 4B). These results indicate that calcium-dependent PKCa is chronically enhanced in the Adip-Sen mouse. Our results suggest that adiponectin enhanced PKCa expression and it increased calcium sensitivity of bladder contraction. Our findings of increased PKCa mRNA levels support this suggestion (Fig. 4C). To confirm that an increase in PKCa expression is associated with calcium dependency of Adip-Sen mouse bladder contraction, an important step of PKCa phosphorylation was investigated. The CCh-induced increase in p-PKCα levels was enhanced in the Adip-Sen mouse. Because phosphorylation is essential for PKCa activation (Stempka et al., 1999), this suggests that PKCa activity is enhanced in the Adip-Sen mouse. Both enhancement of PKCa expression and over-activation of PKCa might contribute to the increase in calcium sensitivity of bladder contraction. Myosin light chain phosphatase inhibitory protein, CPI-17, which is a downstream signaling pathway of PKCa activation (Stempka et al., 1999), this suggests that PKCa expression is associated with calcium dependency of Adip-Sen mouse bladder contraction. CPI-17 acts as an effector of PKC, and phosphorylation of CPI-17 contributes to an enhancement of the contraction mediated by inactivation of myosin light chain phosphatase (Hirano, 2007). In our preliminary trials, phosphorylated-CPI-17 levels in the Adip-Sen mouse bladder were enhanced, compared with those in the C57Bl mouse (unpublished data). Therefore, we considered that adiponectin-mediated alteration of bladder contraction might involve the pathway of PKCa and CPI-17.

The mechanisms involved in adiponectin-induced PKCa expression are not clearly understood. The relationship between adiponectin and PKCa expression was previously reported (Ekman et al., 2009; Nobe et al., 2009). However, changes in calcium dependency and its regulatory mechanism are not clearly understood. Therefore, we hypothesize that adiponectin is a regulatory factor of calcium sensitivity of bladder contraction.
CCh-induced force developments

(1) C57Bl

(2) Ca\(^{2+}\)-free + C57Bl

(3) Adip-Sen

(4) Ca\(^{2+}\)-free + Adip-Sen

Fig. 5. Adip-onectin alteration of CCh-induced bladder contraction in Ca\(^{2+}\)-free PSS in the Adip-Sen mouse.

C57Bl mouse bladder (unpublished data). Stimulation of Adip-R increases cAMP and activates CAMP-dependent protein kinase (A-kinase) (Ouchi et al., 2000). Activation of A-kinase contributes to both glucose incorporation and sensitization of insulin receptors (Ouedraogo et al., 2006; Wu et al., 2007). Moreover, cAMP regulates some gene expressions, which are mediated by cAMP-response element-binding protein (Paolillo et al., 1999; Cypess et al., 2011). An association of cAMP-response element-binding protein with PKC activity has been suggested. Therefore, we speculate that adiponectin-induced PKC\(\alpha\) expression in bladder smooth muscle contraction is also mediated by cAMP and/or A-kinase activation.

On the basis of our results, enhancement of force reduction in Ca\(^{2+}\)-free PSS in Adip-Sen mouse bladder can be interpreted as follows (Fig. 5). (1) Both Ca\(^{2+}\)-dependent (involving PKCa) and Ca\(^{2+}\)-independent (nPKCa and/or rho-rho kinase) pathways were involved in C57Bl mouse bladder contraction in normal PSS. These pathways contribute to CCh-induced force development as a normal contractile response. (2) In Ca\(^{2+}\)-free PSS, part of the contraction mediated by the Ca\(^{2+}\)-dependent pathway, was replaced in the C57Bl mouse bladder. Therefore, Ca\(^{2+}\)-independent pathway-associated contraction remained. (3) In Adip-Sen mouse bladder contraction, the developed force level was similar to the response in the C57Bl mouse. However, the contribution ratio of Ca\(^{2+}\)-dependent/Ca\(^{2+}\)-independent pathways in the Adip-Sen mouse was different from the ratio in the C57Bl mouse. In Adip-Sen mouse bladder contraction, contribution of the Ca\(^{2+}\)-dependent pathway was significantly enhanced by enhancement of PKCa expression. (4) Similar to the response in the C57Bl mouse bladder, part of the Ca\(^{2+}\)-dependent contraction in the Adip-Sen mouse bladder was suppressed in Ca\(^{2+}\)-free PSS. Because a major part of the contraction was suppressed, the total force level in the Adip-Sen mouse was significantly reduced, compared with that in the C57Bl mouse. We consider that the PKCa-mediated Ca\(^{2+}\)-dependent pathway plays a major role in changes in Adip-Sen mouse bladder contraction, but the association of the Ca\(^{2+}\)-independent pathway with these changes is unknown.


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