Adiponectin Enhances Calcium Dependency of Mouse Bladder Contraction Mediated by Protein Kinase Cα Expression

Koji Nobe, Akiko Fuji, Kiyomi Saito, Takaharu Negoro, Yoshio Ogawa, Yasuko Nakano, Terumasa Hashimoto, and Kazuo Honda

Departments of Pharmacology (K.N., A.F., T.H., K.H.), Clinical and Molecular Pharmacokinetics/Pharmacodynamics (K.S.), and Pharmacogenomics (T.N., Y.N.), School of Pharmacy, Showa University, Tokyo, Japan; and Department of Urology, Showa University Hospital, Shinagawa-ku, Tokyo, Japan (Y.O.)

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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 (C57BlI 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 C57BlI 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 C57BlI 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 Ca2+–replaced conditions was significantly different between Adip-Sen and C57BlI mice (94.1 and 66.3% of normal contraction, respectively; n = 5). A parameter of Ca2+–sensitivity, the relation between intracellular Ca2+ concentration and contraction, was increased in the Adip-Sen mouse, compared with that in the C57BlI mouse. This Ca2+ dependency in the Adip-Sen mouse was reduced by a protein kinase C (PKC) inhibitor, but not by a Pho kinase inhibitor. Expression of the calcium-dependent isoform of PKC, PKCα, was increased in the Adip-Sen mouse bladder, and CCh-induced phosphorylation of PKCα was also enhanced, compared with those in the C57BlI mouse. In conclusion, adiponectin is associated with bladder smooth muscle contraction, which involves an increase in Ca2+ dependency of contraction mediated by PKCα 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; [Ca2+]i, intracellular calcium concentration; CCh, carbachol; G66976, 12-(2-Cyanoethyl)-6,7,12-trihydroxy-13-methyl-5-oxo-5H-indol[2,3-a]pyrrolo[3,4-c]carbazole; MLC, myosin light chain; PKC, protein kinase C; PL, phospholipids; p-PKCα, phosphorylated-PKCα; PSS, physiological salt solution; Y27632, (R)-(+)-trans-4-[(4-pyridyl)-4-(1-aminoethyl)-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, triglycérol, 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 rinsed in physiological salt solution (PSS). Subsequently, fat and connective tissues were removed from bladder tissue strips using cotton micro-scissors under stereoscopic microscopy. Urothelium was also removed. PSS, which was supplemented with 118 mM NaCl, 5.5 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 1.4 mM NaH2PO4, 21.4 mM NaHCO3, and 11.1 mM glucose, was aerated with 95% O2 and 5% CO2 at 37°C. Measurement of Isometric Force Development and Intracellular Calcium Concentration ([Ca2+]i). Each tissue was positioned vertically in a temperature-controlled 5-ml organ bath. One end of the tissue was connected to a strain gauge transducer (Type T-7-8-240; Orientec, Tokyo, Japan) to monitor contractile responses. Measurements were made under normal PSS and calcium-free (no CaCl2 added to PSS; [Ca2+]i-free PSS) 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-(1-aminoethyl)-cyclohexanecarboxamide 2HCl (Y27632; Wako Chemical Co., Japan) to determine 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 mouse 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 C57Bl mouse was similar to the response in the Adip-Sen mouse resulted in a suppression of this response. In the presence of 30 μM CCh, only 5.9% of the normal response was detected.

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 mouse 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 C57Bl mouse was similar to the response in the Adip-Sen mouse 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)-6,7,12-triahydroy-13-ethyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (G6976; 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 G6976 also slightly reduced force development in the C57Bl mouse (Fig. 3B). However, the inhibitory effect of G6976 in Adip-Sen mice was significantly increased (P < 0.01). In the presence of 1 μM G6976, 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>
<thead>
<tr>
<th>Mice</th>
<th>N</th>
<th>Body Weight</th>
<th>Adiponectin</th>
<th>Glucose</th>
<th>t-Cho</th>
<th>PL</th>
<th>FFA</th>
<th>Bladder Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl</td>
<td>5</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>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>
</table>

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, PKCα mRNA levels were also measured (Fig. 4C). Relative PKCα 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 PKCα 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-PKCα (p-PKCα) levels in C57Bl and Adip-Sen mice were not observed. However, a 30 μM CCh-induced increase in p-PKCα 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 PKCα-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 adiponectin affects bladder smooth muscle contraction, which is mediated by an increase in calcium dependency. A relation between \( \text{Ca}^{2+} \), 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 \( \text{Ca}^{2+} \) levels were similar. This indicates an increase in calcium sensitivity of the Adip-Sen mouse bladder contraction. Of interest, 50 mM KCl induced h 

This indicates an increase in calcium sensitivity of the Adip-Sen mouse, when CCh-induced \( \text{Ca}^{2+} \) levels are shown (B, lower panel). Relative expression levels of PKCa mRNA (C) and phosphorylated-PKC\( \alpha \) (p-PKC\( \alpha \)) levels were measured as described in Materials and Methods. A typical image of p-PKC\( \alpha \) levels is shown (inset; 1: unstimulated C57Bl mouse, 2: CCh-treated C57Bl mouse, 3: unstimulated Adip-Sen mouse, 4: CCh-treated Adip-Sen mouse). Each value represents the mean ± S.E.M. of at least five independent determinations. *P < 0.01; **P < 0.01, compared with values in the C57Bl mouse and PKCa levels, respectively.

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 Go6976 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, \( \beta \) and \( \gamma \)), calcium-independent PKC (PKCd, \( \mu \), and \( \theta \)), and atypical PKC isoforms (Salamanca and Khalil, 2005). In the current study, in bladder smooth muscle tissue, PKCa, \( \beta \), \( \mu \), and \( \theta \) 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 PKCan 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-PKCa 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, might play a role in bladder contraction. In many types of smooth muscle tissue, 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 is unknown. However, it has been reported that adiponectin stimulates the adiponectin-specific receptor (Adip-R) (Yamauchi and Kadowaki, 2008). This receptor is distributed in many types of tissue (Trujillo and Scherer, 2005), and we have also detected Adip-R mRNA in...
Adiponectin plays a role as a regulatory factor of bladder contraction. This involves enhancement of calcium sensitivity of the contraction, mediated by both expression and activation of PKCa.

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Authorship Contributions
Participated in research design: Nobe, Ogawa, Honda. Conducted experiments: Nobe, Negoro. Contributed new reagents or analytic tools: Fuji, Saito. Performed data analysis: Fuji, Hashimoto. Wrote or contributed to the writing of the manuscript: Nobe, Nakano, Honda.

References


Address correspondence to: Koji Nobe, PhD, Department of Pharmacology, School of Pharmacy, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan. E-mail: kojinobe@pharm.showa-u.ac.jp