Adrenomedullin improves cardiac function and prevents renal damage in streptozotocin-induced diabetic rats

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Dobrzynski, Eric, David Montanari, Jun Agata, Juhong Zhu, Julie Chao, and Lee Chao. Adrenomedullin improves cardiac function and prevents renal damage in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab 283: E1291–E1298, 2002.—Adrenomedullin (AM) is a potent vasodilating peptide and is involved in cardiovascular and renal disease. In the present study, we investigated the role of AM in cardiac and renal function in streptozotocin (STZ)-induced diabetic rats. A single tail-vein injection of adenoviral vectors harboring the human AM gene (Ad.CMV-AM) was administered to the rats 1-wk post-STZ treatment (65 mg/kg iv). Immunoreactive human AM was detected in the plasma and urine of STZ-diabetic rats treated with Ad.CMV-AM. Morphological and chemical examination showed that AM gene delivery significantly reduced glycogen accumulation within the hearts of STZ-diabetic rats. AM gene delivery improved cardiac function compared with STZ-diabetic rats injected with control virus, as observed by decreased left ventricular end-diastolic pressure, increased cardiac output, cardiac index, and heart rate. AM gene transfer significantly increased left ventricular long axis (11.69 ± 0.46 vs. 10.31 ± 0.70 mm, n = 10, P < 0.05) and rate of pressure rise and fall (+6,090.1 ± 597.3 vs. +4,648.5 ± 807.1 mmHg/s, –4,902.6 ± 644.2 vs. –3,915.5 ± 805.8 mmHg/s, n = 11, P < 0.05). AM also significantly attenuated renal glycogen accumulation and tubular damage in STZ-diabetic rats as well as increased urinary cAMP and cGMP levels, along with increased cardiac cAMP and Akt phosphorylation. We also observed that delivery of the AM gene caused an increase in body weight along with phospho-Akt and membrane-bound GLUT4 levels in skeletal muscle. These results suggest that AM plays a protective role in hyperglycemia-induced glycogen accumulation and cardiac and renal dysfunction via Akt signal transduction pathways.

strepotozotocin; adrenomedullin; gene delivery; adenovirus

ADRENOMEDULLIN (AM) is a potent 52-amino acid vasodilator originally isolated from tissue extracts of human pheochromocytoma (20). AM has been detected in a variety of organs, such as the adrenal gland, kidney, heart, lung, spleen, and brain, and has also been found to be secreted from endothelial and vascular smooth muscle cells (17, 31). AM is involved in a variety of biological activities, including vasodilation, diuresis, and inhibition of aldosterone secretion (6, 20). Plasma AM levels are increased in patients with cardiac hypertrophy, heart failure, renal dysfunction, and hyperglycemia (10, 19, 26). AM has been reported to act in an autocrine/paracrine fashion to prevent cardiovascular and renal damage (33). Recently, AM has been shown to have a key role in cardiovascular development. AM knockout (−/−) mice were embryonically lethal by midgestation due to cardiovascular abnormalities (1). These results suggest a possible role for AM in the regulation of cardiac and renal function (17).

AM's effect on cardiac function has been suggested to result from the activation of an autonomic baroreflex response, which increases the heart rate to counteract the hypotensive effects of AM (8, 22). However, observations in sheep and rat papillary muscle suggest that AM has direct inotropic effects (18, 28). Even with conflicting reports of AM's effects on cardiac function, there are studies showing direct effects on cardiac cells. AM is not only produced in cardiomyocytes but is capable of binding to and triggering physiological responses of cardiomyocytes (33). AM has been reported to stimulate cAMP and cGMP levels and to reduce extracellular matrix formation along with DNA and protein synthesis in cardiomyocytes (25, 31, 33). Recently, AM has been linked to embryonic cardiovascular development (1).

Diabetics suffer from vessel damage leading to both vision and circulation problems and are three to eight times more likely to suffer from abnormal cardiac function, termed “diabetic cardiomyopathy.” Diabetic cardiomyopathy ranges in severity from mild dysfunction to failure (29). Rats treated with streptozotocin (STZ) not only develop hypoinsulinemia but also cardiomyopathy, polydypsia, hyperglycemia, and glucosuria (7, 34). Previous studies showed that hearts and kidneys from STZ-treated rats contain abnormally high levels of glycogen (3). Glycogen accumulation may be a contributing factor to the development of cardiac and renal dysfunction. Nishimatsu et al. (27) recently reported that AM is capable of directly activating Akt via phos-

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phatidylinositol 3-kinase (PI 3-kinase) in rat aorta. Akt activation inhibits apoptosis and enhances the survival of cardiomyocytes. Phosphorylated Akt can also stimulate cellular glucose usage and stimulate glucose transporter 4 (GLUT4) to the membrane in skeletal muscle (21, 23). In this study, we investigated the potential protective role and mechanism of action of AM in hyperglycemia-induced cardiac and renal damage.

MATERIALS AND METHODS

Administration of STZ. STZ (Sigma Chemical) was dissolved in ice-cold 0.05 M citrate buffer (pH 4.5). Two-week-old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were injected with 65 mg/kg STZ via the tail vein.

Replication-deficient adenoviral vectors and animal preparations. Adenoviral vectors harboring the human AM with the 4F2 enhancer (Ad.CMV-AM) or luciferase cDNA (Ad.CMV-Luc) under the control of the cytomegalovirus enhancer/promoter (CMV) were constructed and prepared as previously described (16). Experimental rats received 1.2 × 10¹⁰ plaque formation units, or pfu, of Ad.CMV-AM or Ad.CMV-Luc 1 wk post-STZ treatment. Animal care for all rats in this study conformed to the rules set forth by National Institutes of Health (NIH) guidelines.

Plasma and urine collection and analysis of physiological parameters. Rat plasma was collected at various time points throughout the experiment. Blood glucose levels were measured 7 days post-STZ treatment and at the end of the experiment (OneTouch Profile, Johnson and Johnson, Milpitas, CA). Twenty-four-hour urine was collected 7 days after adenoviral vector-mediated gene delivery. Animals were allowed free access to food and water during the collection period. Urine was collected and centrifuged at 1,000 g for 10 min to remove particles, and the volume was recorded. Urine and plasma samples were stored at −20°C for biochemical analysis.

RIA for human AM, cAMP, insulin, and cGMP. Immunoreactive human AM was determined in plasma by an RIA for human AM by use of rabbit anti-human AM 1–52 antisera (Peninsula Laboratories, San Carlos, CA), as previously described (2). Plasma insulin levels were measured by a commercially available rat insulin kit according to the manufacturer's instructions (Linco, St. Charles, MO). Urinary and cardiac cAMP and gGMP levels were also determined by RIA (11, 14).

Morphological and histological investigation. Paraffin-embedded tissues were cut at 4 μm and stained with Periodic acid Schiff (PAS). Stained sections were analyzed microscopically and morphometrically. PAS-stained heart sections were examined for glycogen content within the cellular compartment. PAS-stained kidney sections were also evaluated and graded on a scale of 1 to 5, where 5 represented the greatest damage. The detailed scale follows: 1 = normal tissue; 2 = some outer medullary tubule damage; 3 = damage to outer medullary tubules, as well as at least one-third of the cortical distal tubules; 4 = >50% of distal tubule damage; and 5 = majority of distal tubules disrupted as well as at least one-third of outer medullary tubules and cortical distal tubules. The tissue was minced, placed in RIPA buffer (1× PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor cocktail), and subjected to two 15-s polytron cycles on ice. Skeletal muscle membrane fractions were isolated, as previously described (37). Briefly, skeletal muscle was minced and homogenized, and the supernatant was collected by centrifugation at 9,000 g for 20 min. The supernatant was then centrifuged at 180,000 g for 90 min. The pellet was resuspended and loaded onto a 10–30% (wt/wt) continuous sucrose gradient and centrifuged at 48,000 rpm for 55 min. Protein concentrations were determined by Lowry's method.

Analogs containing equal amounts of protein (50–100 μg) were subjected to SDS-PAGE and blotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were incubated with specific antibodies against phosphorylated Akt (pAkt), Akt (Cell Signaling Technology, Beverly, MA), PKC-β2 (Santa Cruz Biotechnology, Santa Cruz, CA), and GLUT4 (East Acres Biologicals, Southbridge, MA). Blots were developed using an enhanced chemiluminescence method (Amersham Pharmacia Biotech). Blots were exposed to autoradiography films at varying times to ensure that the film was not overexposed. Autoradiography films were scanned into Photoshop (Adobe), and densitometric measurements were obtained using the NIH image software package.

Statistical analysis. Results are expressed as means ± SE. Comparisons among groups were made by ANOVA followed by Fisher's protected least significant difference test or by an unpaired Student's t-test. Differences were considered significant at P < 0.05.

RESULTS

Diabetic state and blood glucose levels. Blood glucose levels were measured at various time points through-
out the experiment. Five days post-STZ treatment, blood glucose levels of all treated animals reached 436.2 ± 12.7 mg/dl (n = 10). At the end of the experiment, there were no significant differences in blood glucose levels in treated STZ-diabetic rats injected with the AM or luciferase gene (447.0 ± 64.0 vs. 417.3 ± 65.8 mg/dl, n = 10; Table 1). All animals treated with STZ developed hypoinsulinemia (Table 1).

Table 1. Physiological parameters of STZ-diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ-Luc</th>
<th>STZ-AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW, g</td>
<td>1.05 ± 0.02</td>
<td>0.69 ± 0.07*</td>
<td>0.86 ± 0.08†</td>
</tr>
<tr>
<td>LV, g</td>
<td>0.82 ± 0.02</td>
<td>0.55 ± 0.05*</td>
<td>0.66 ± 0.07†</td>
</tr>
<tr>
<td>LVLA, mm</td>
<td>12.60 ± 0.54</td>
<td>10.31 ± 0.70*</td>
<td>11.69 ± 0.46†</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>356 ± 8</td>
<td>233 ± 24</td>
<td>253 ± 16†</td>
</tr>
<tr>
<td>HW/BW, g/100 g body wt</td>
<td>0.29 ± 0.01</td>
<td>0.29 ± 0.02</td>
<td>0.33 ± 0.02†</td>
</tr>
<tr>
<td>LV/BW, g/100 g body wt</td>
<td>0.23 ± 0.09</td>
<td>0.24 ± 0.02</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>82.7 ± 9.4</td>
<td>63.6 ± 11.3*</td>
<td>79.5 ± 16.5†</td>
</tr>
<tr>
<td>Cardiac cAMP, nmol/µg protein</td>
<td>4.30 ± 1.83</td>
<td>4.94 ± 1.51</td>
<td>6.30 ± 1.34†</td>
</tr>
<tr>
<td>Urinary cAMP, nmol-100 g body wt^-1 day^-1</td>
<td>37.5 ± 3.5</td>
<td>39.3 ± 3.3</td>
<td>55.2 ± 2.9†</td>
</tr>
<tr>
<td>Urinary cGMP, nmol-100 g body wt^-1 day^-1</td>
<td>2.9 ± 0.4</td>
<td>5.1 ± 0.2</td>
<td>7.7 ± 0.8†</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>353 ± 8</td>
<td>245 ± 7*</td>
<td>268 ± 8†</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>85.3 ± 4.7</td>
<td>447.0 ± 64.0*</td>
<td>417.3 ± 65.8*</td>
</tr>
<tr>
<td>Blood insulin, ng/ml</td>
<td>0.33 ± 0.15</td>
<td>0.16 ± 0.04*</td>
<td>0.18 ± 0.09*</td>
</tr>
</tbody>
</table>

Values for each group are means ± SE (n = 6–10). Control animals are normal and did not receive streptozotocin (STZ). STZ-Luc and STZ-AM, STZ-diabetic rats injected with the luciferase or adrenomedullin gene, respectively. HW, heart weight; LV, left ventricular weight; LVLA, left ventricular long axis; BG, blood glucose; CO, cardiac output. Statistical significance between groups was determined by ANOVA. *P < 0.05 vs. Control; † P < 0.05 vs. STZ.

Fig. 1. Effect of human adrenomedullin (AM) or luciferase (Luc) gene delivery on glycogen accumulation in the heart of streptozotocin (STZ)-diabetic rats. Representative histological sections of rat hearts at 14 days after gene delivery, subjected to Periodic acid Schiff (PAS) staining (magnification ×125). A: typical left ventricle sections from control (a), STZ-Luc (b), and STZ-AM (c) animals. Note the marked reduction of PAS-positive staining in the AM-treated STZ-diabetic group. B: chemical quantification of glycogen levels present in cardiac tissue. Values are means ± SE (n = 8).
Expression of human AM in STZ-induced diabetic rats. Immunoreactive human AM levels in rats receiving AM gene delivery were measured by a specific RIA for human AM. Human AM levels in the plasma 6 days after gene delivery reached 78.9 ± 81.7 ng/ml, and urine levels 7 days after gene delivery reached 15.9 ± 6.8 ng/ml (n = 7). Linear displacement curves of serial dilutions of urine and plasma from rats injected with the human AM gene displayed parallelism to the human AM standard curve, indicating their immunological identity (data not shown).

Effect of adenoviral-mediated gene delivery of human AM on body weight of STZ-induced diabetic rats. AM gene delivery improved the body weight of STZ-diabetic rats. Normally, rats gain ~5 g body wt⁻¹·day⁻¹, but after STZ treatment the animals neither gained nor lost weight. By the end of the experiment, the STZ-diabetic rats treated with Ad.CMV-AM developed a slight but significant increase in body weight compared with the STZ-diabetic rats treated with Ad.CMV-Luc (Table 1, n = 10, P < 0.05).

Effects of human AM gene delivery on morphology and tissue glycogen content in STZ-induced diabetic rats. Morphological evaluation of cardiac tissue revealed a beneficial effect of AM gene delivery. PAS stains sugar moieties bright red, clearly distinguishing cardiac and renal tissues of STZ-diabetic control rats from STZ-diabetic rats treated with human AM (Figs. 1, A–C, and 2, A–C). AM gene delivery resulted in reduction of glycogen accumulation in the heart and kidney. A chemical glycogen assay was used to quantify tissue glycogen levels. Cardiac glycogen levels were significantly reduced in the STZ-diabetic animals receiving AM gene delivery compared with the control STZ animals injected with the luciferase gene (6.14 ± 1.56 vs. 9.51 ± 3.25 mg/g tissue, n = 5, P < 0.05; Fig. 1D). Similarly, kidney glycogen levels were also significantly reduced by AM gene delivery compared with the luciferase control diabetic rats (1.26 ± 0.27 vs. 1.73 ± 0.32 mg/g tissue, n = 4, P < 0.05; Fig. 2D). Scaled kidney damage was also significantly reduced in AM-treated STZ-diabetic rats (3.30 ± 0.20 vs. 4.20 ± 0.37 scale, n = 5, P < 0.05; Fig. 2E). However, glycogen levels within the skeletal muscle were not affected (0.5 ± 0.1 vs. 0.4 ± 0.1 mg/g tissue, n = 4).

Effect of human AM gene delivery on cardiac function of STZ-diabetic rats. Three weeks post-STZ treatment, which correlates to 2 wk after gene delivery, animals were subjected to cardiac function evaluation. AM gene delivery was observed to provide an overall protective effect on the function of diabetic hearts. Figure 3 (top) depicts typical maximal rate of left ventricular (LV) pressure rise and fall (± ΔP/Δt) in waveforms from each of the experimental groups. The ± ΔP/Δt was signifi-
cantly improved in the STZ-diabetic rats injected with human AM compared with the STZ-diabetic rats injected with the luciferase gene (4,581.8 ± 810.9 vs. 6,150.1 ± 1,750.2 mmHg/sec, n = 11, P < 0.05; Fig. 3, bottom). An index of congestive heart failure, left ventricular end-diastolic pressure, also showed significant improvement in the STZ-diabetic rats treated with the human AM gene compared with the STZ-diabetic rats treated with the luciferase gene (6.15 ± 0.82 vs. 8.82 ± 2.47 mmHg, n = 11, P < 0.01, Fig. 4). Table 1 shows that AM protects against cardiac dysfunction of STZ-diabetic rats via improvement in cardiac output, cardiac index, and left ventricle long axis.

Effect of human AM gene delivery on cAMP and cGMP levels. AM gene delivery significantly increased cardiac cAMP levels in STZ-diabetic rats compared with control rats (6.30 ± 0.34 vs. 4.94 ± 1.51 nmol/µg protein, n = 6 and 4, P < 0.05). In addition, urinary cAMP (55.2 ± 2.9 vs. 39.3 ± 3.3 nmol·100 g body wt⁻¹·day⁻¹; n = 6 and 4, P < 0.05) and cGMP levels were also increased in the AM-treated STZ-diabetic rats (7.65 ± 0.79 vs. 5.1 ± 0.23 nmol·100 g body wt⁻¹·day⁻¹; n = 4, P < 0.05, Table 1).

Alteration of cardiac Akt. Figure 5 shows the effect of AM gene delivery on phosphorylated Akt (pAkt) and total Akt (tAkt) in the heart. Densitometric analysis revealed that STZ-diabetic rats treated with AM had a significantly increased pAkt-to-tAkt ratio compared with STZ-diabetic rats treated with luciferase.
(0.385 ± 0.105 vs. 0.130 ± 0.027 arbitrary units, n = 3, P < 0.05).

Membrane-bound GLUT4 in skeletal muscle. Figure 6 shows the effect of AM gene delivery on skeletal muscle tissue preparations. Figure 6A shows an increase in the pAkt-to-tAkt ratio in the skeletal muscle of AM-treated STZ-diabetic rats (0.675 ± 0.027 vs. 0.132 ± 0.013 arbitrary units, n = 2, P < 0.05). Membrane fractions of skeletal muscle were probed with a specific antibody for GLUT4 (Fig. 6B). Densitometric analysis showed a significant increase in GLUT4 present in the membrane of skeletal muscle tissues from STZ-diabetic rats treated with the AM gene compared with STZ-diabetic rats treated with the luciferase gene (390.8 ± 113.2 vs. 226.9 ± 54.7 arbitrary units, n = 3, P < 0.05). However, membrane-bound PKC-β2 identified by Western blotting revealed no differences between AM-treated and control STZ-diabetic rats (data not shown).

DISCUSSION

The STZ-diabetic model has proved useful in a variety of research studies for the characterization of treatments associated with diabetes in humans. Since 1971, STZ has been used to create experimental diabetic animal models. STZ causes hyperglycemia by specifically inducing DNA strand breaks in pancreatic islet β-cells and stimulates nuclear poly(ADP-ribose) synthetase, thus depleting intracellular NAD⁺ and NADP⁺ levels. Reduction of intracellular NAD⁺ and NADP⁺ inhibits proinsulin synthesis, leading to a diabetic state (38). It has been suggested (32) that the production of activated oxygen species (superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen) has a major role in the development of STZ-induced diabetes.

In this study, we have shown that AM gene delivery is capable of improving the cardiac function of STZ-diabetic rats. Immunoreactive human AM levels were observed in both urine and plasma of STZ-diabetic rats receiving an AM-harboring adenovirus, indicating that human AM was being produced and secreted from both the liver and the kidney. In previous studies we observed AM mRNA expression in the liver, heart, and kidney, along with a reduction in blood pressure in hypertensive rats after intravenous injection of Ad.CMV-AM (36). The STZ-treated rats did not develop hypertension, and AM gene delivery did not produce a significant reduction in blood pressure. The use of metabolic cages failed to allow detection of a diuretic effect in the STZ-diabetic rats receiving the human AM transgene. This may indicate either that the expression level of recombinant human AM achieved in this study was not high enough to produce an effect, or more likely that AM’s diuretic effect was masked due to the massive urinary excretion from STZ-diabetic rats (>100 ml/24 h).

AM has also been suggested to have a major role in cardiac and renal function in addition to blood pressure regulation (26). Morphological evaluation and chemical assays demonstrated that somatic human AM gene delivery is capable of preventing cardiac glycogen ac-
cumulation in STZ-diabetic rats, suggesting increased glucose utilization. Extensive cardiac glycogen levels could be attributed to cardiovascular dysfunction and the development of cardiomyopathy. Although an increase in PKC-β2 activity has been linked to diabetic cardiomyopathy (35), we found that AM has no effect on membrane-bound PKC-β2 in STZ-diabetic rats (data not shown). Reduction of cardiac damage was observed as improved ±AP/Δp, LVEDP, LV weight, heart weight, and LV long axis of the STZ-diabetic rats treated with human AM. These observations are consistent with the notion that AM provides cardiovascular protection.

AM’s biological effects on vasodilation and natrurexis have been shown to be mediated by both cAMP and nitric oxide-cGMP signaling pathways (15, 32). We observed an increase in both cardiac and renal (or urinary) cAMP levels, along with increased urinary cGMP levels, in the STZ-diabetic rats receiving AM gene delivery. In addition, AM increases phosphorylation of cardiac Akt. These results suggest that AM’s protective effects are mediated, at least partially, through the PI 3-kinase-Akt pathway, along with cAMP and cGMP second messenger cascades (13, 15, 27). Akt is known to inhibit apoptosis and glycogen synthase kinase (GSK)3 while stimulating GLUT4 translocation and phosphofructokinase 2 (PFK-2) (9). These known Akt activities could help explain the observed protective effects of AM gene delivery in STZ-induced diabetic rats. Interaction with GSK3, PFK-2, and GLUT4 could lead to increased utilization of glucose, leading to a reduction in glycogen accumulation and thus improvement in body weight, cardiac function, renal damage, and overall health of the animal. The cAMP second messenger cascade could also be involved in the reduction of glycogen accumulation. Increased cAMP levels can activate protein kinases, leading to inactivation of glycogen synthase and thus inhibition of glycogen production (12). These same pathways could also be active in skeletal muscle, leading to the observed increased membrane-bound GLUT4 via the Akt pathway, and thus improved glucose utilization and body weights of AM-treated STZ-diabetic rats.

Adenoviral gene delivery is capable of producing high levels of the transgene; however, due to the lack of viral genome integration, transgene expression is only temporary. Adenoviral vectors can also stimulate host immune responses, leading to inflammation, loss of infected host cells, and thus transgene expression (4). To minimize the host immune and inflammatory responses, and the possibility of readministration without the production of notable host immune response, it is essential to develop improved viral vectors for prolonged transgene expression. Improved delivery vectors like adeno-associated viral vectors would make a prolonged experimental time line possible, which could potentially improve the elucidation of AM effects.

In this study, we have demonstrated that adenovi-mediated gene delivery of human AM not only provided protection from cardiac dysfunction but also prevented body weight loss and kidney tubule damage in STZ-diabetic rats. The observed beneficial effects may result from AM’s ability to counteract deleterious effects due to a diabetic state and suggest a potential therapeutic use for AM and/or its signal transduction pathway in cardiovascular and renal diseases, especially cardiomyopathy.

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