

Is adrenomedullin a causal agent in some cases of type 2 diabetes?

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Abstract

The study of two populations with a recent onset of type 2 diabetes showed that a subset of the patients had higher levels of adrenomedullin (AM) than the rest of the diabetics. In this subset, physiological elevations of AM might have triggered the disease in predisposed individuals. Diabetics showed higher levels of AM than healthy controls. In addition, glycemia was measured in diabetic rats after injection of saline, AM, or antiAM antibody. AM elevated glycemia, whereas the antibody reduced circulating glucose to normal. These results suggest that manipulation of AM levels could represent a new approach in the management of diabetes for the appropriate individuals. Published by Elsevier Science Inc.

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder that affected 16 million people in the United States in 1995, according to estimates of the National Institute of Diabetes (NIDDKD, NIH). Management costs for diabetes in 1992 was \$92 billion [38]. The hallmark of diabetes, whether type 1 or type 2, is hyperglycemia. Clinical complications associated with diabetes, such are retinopathy, neuropathy, nephropathy, or atherosclerosis, are most likely the consequence of long-term hyperglycemia via both altered metabolic pathways and nonenzymatic glycation of proteins [48]. Under normal conditions, insulin released from the β cells of the pancreatic islets is constantly adjusted by neural [5] and humoral and nutritional [32] mechanisms, so that normoglycemia is maintained. However, in patients with diabetes, the regulation of blood glucose levels is impaired and the concentrations of circulating glucose are elevated.

Type 2 diabetes is the most frequent form of the disease [70–95% of all diabetic patients [38,48]], and is character-

ized by insufficient insulin secretion to compensate for insulin resistance in target tissues [46]. These two characteristics may coexist in subjects before developing diabetes, and these people are more vulnerable to factors that further reduce insulin output or insulin action [7]. Many steps in the stimulus-secretion coupling of glucose-induced insulin release have been implicated in the impaired insulin secretion in type 2 diabetes. These include the glucose transporter Glut 2, the glucokinase, the adenylate cyclase system, and various mitochondrial deficiencies, among others [26].

Adrenomedullin (AM) is a multifunctional peptide with many physiological actions, including hypotension [39], renal regulation [18], neurotransmission [2], growth [34, 47], and defense against microorganisms [45]. It has also been shown that AM and its related peptides are able to regulate the release of insulin [30] and other hormones, including catecholamines [21], adrenocorticotropin [41], aldosterone [20,49], and atrial natriuretic peptide [43]. All these functions are exerted through a specific membrane receptor [19] that activates adenylate cyclase and modulates Ca^{2+} flux in the target cells [44]. This receptor has been found in the rat pancreas through *in situ* hybridization and is expressed by the β cells of the islets of Langerhans [30]. For a recent review on AM, see Ref. [27].

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Two recent studies found that the levels of circulating AM were elevated in patients with type 2 diabetes when compared to normal controls [15,37]. The authors attributed this elevation to the vascular endothelial damage caused by advanced stages of diabetes [3]. We have recently demonstrated that AM is involved in the regulation of insulin secretion by producing an inhibitory tone in the islets [30], both in vitro and in the whole animal. Further, a specific monoclonal antibody directed against AM increased insulin secretion by neutralizing the intrinsically produced AM [30]. We have also found that AM-like immunoreactivity is expressed during pancreatic development [28,35]. These observations led us to hypothesize that AM may contribute to the impaired β -cell function during the development of type 2 diabetes. To test this hypothesis we used a two pronged approach: A) Measure blood levels of AM in two populations, one with a very recent onset of diabetes and the other at high risk for the disease, and B) Test the effects of AM on blood glucose levels in an animal model of prediabetes.

2. Methods

2.1. Patients

Two different populations with the common feature of developing hyperglycemia during a metabolic stress to β -cells were chosen. The first set of samples consisted of archival blood serum from 83 healthy adult male volunteers (age 18–45, body weight within 15% of ideal) that collaborated in a previous phase I clinical trial in which they were given a Beta-3 agonist (Lederle Compound BTA-243, protocol D97-P2-T1). As a result, the volunteers developed a transient disorder that mimicked noninsulin dependent diabetes mellitus, unlike the controls. The trial was a 14-day, randomized, double-blind, placebo-controlled, ascending safety, tolerance, and pharmacokinetic study of oral CL 238,796 versus placebo. The trial was conducted by Wyeth Ayerst Research (Radnor, PA USA). Blood serum from 28 untreated normal volunteers in the same age and weight range was collected for comparison purposes.

The second population was comprised of pregnant women of Latino origin receiving antepartum care at the Department of Obstetrics and Gynecology of the University of Southern California. Thirty patients met National Diabetes Data Group criteria for gestational diabetes mellitus (GDM), and 16 women had a normal 1-h screening test for GDM after 26 weeks gestation. The normal women were matched by age and trimester of pregnancy to the women with GDM. Serum from 11 matched nonpregnant women who had not suffered previous GDM was also analyzed.

None of the patients presented other clinical features at the time of the test. Special care was taken in excluding patients with infections or renal or blood pressure disorders,

as it has been reported that such conditions elevate circulating AM [9,16,17].

The procurement of the samples had I.R.B. approval by the Office of Human Subjects Research (NIH) and the Institutional Review Board of The University of Southern California School of Medicine.

2.2. HPLC characterization

First, 2.5 ml of normal human plasma obtained from the NIH Blood Bank was filtered and loaded onto a semi-preparative C18 column (DeltaPak, Millipore, 30 × 300 mm). Column retentate was selectively eluted over 90 min using a 5–60% acetonitrile gradient containing 0.1% trifluoroacetic acid and monitored at 210 and 280 nm (Beckman System Gold HPLC, San Ramon, CA USA). Twelve ml/min fractions were collected, freeze-dried, and analyzed by radioimmunoassay (RIA) as explained below. Synthetic AM was also run into the column and analyzed in the same way.

2.3. Radioimmunoassay

Quantification of AM was performed as previously described [29]. Serum samples were mixed with an equal volume of 0.1% alkaline-treated casein [24] in PBS, and extracted through C-18 Sep-Pak 400 mg cartridges (Waters Corp., Milford, MA USA). The proteins were eluted with acidified 80% isopropanol and the recovered volume freeze dried to eliminate the organic solvent. Extracts were reconstituted in 400 μ l RIA assay buffer (10 mM phosphate, 50 mM EDTA, 135 mM NaCl, 0.05% Triton X-100, 0.1% Tween 20, 1% BSA, 0.1% alkaline-treated casein, 20 mg/l phenol red, pH 7.5), spun at 14000 rpm for 10 min at 4°C to remove any solid matter, and three 100 μ l aliquots from each sample were separated for analysis. The RIA was performed using the Phoenix human AM RIA kit (Mountain View, CA USA) and following manufacturer's instructions. Briefly, 100 μ l of anti-AM antibody and 100 μ l of 125 I-AM were added to each sample and the mixture incubated at 4°C overnight. The following day, 500 μ l of goat anti rabbit antibody (1:150 in 6% PEG 8000) and 100 μ l of normal rabbit serum (1:100 in RIA buffer) were added and incubated for 1 h at 4°C. After centrifugation at 3750 rpm for 30 min at 4°C, the supernatant was discarded, and the radioactivity in the pellets measured in a 1277 Gammamaster instrument (Wallac, Gaithersburg, MD USA).

The radioactive counts were compared to a standard curve and the concentration of AM calculated by linear regression. Recovery in the assay was average 66%, and the variation between assays less than 10%. The displacement of tracer obtained by increasing volumes of serum extracts was parallel with that observed with the standard curve.

2.4. Glucose tolerance tests in rats

SHR/N-cp lean (+/+ or +/-cp) and corpulent (cp/cp) rats from the NIH colony were used in this study. The corpulent phenotype resembles the human noninsulin dependent diabetic syndrome and exhibits postprandial but not fasting hyperglycemia, age dependent loss of glucose tolerance, hyperlipidemia, glucosuria, and proteinuria [12]. Twelve animals were subjected to a glucose tolerance test as previously reported [30]. In brief, six lean and six obese 5 week-old male rats were fasted overnight and injected intraperitoneally (i.p.) with saline, AM (10 nanomols/100 g BW), and MoAb-G6 (1 mg/100 g BW) at weekly intervals. The monoclonal antibody MoAb-G6 has been previously characterized and has an established ability to block AM physiological effects in rat pancreas [30]. Five minutes after the injection, oral glucose was given (400 mg/100 g BW), and blood was collected from the tail vein 5 min before the injection (10 min before glucose gavage), just before the glucose load (0 min) and 30, 60, 120, 180, and 240 min after glucose load. Two lean and two corpulent rats were given an additional glucose tolerance test (saline injection) 1 week after the antibody injection to ensure that there was no change in glucose tolerance over the 3-week period. The glucose concentration was determined by the glucose oxidase method (Sigma) using a Centrifichem System (Union Carbide), following manufacturer's instructions.

2.5. Statistical analysis

The population data were analyzed statistically by analysis of variance using the general linear models procedure (Proc GLM) of SAS [42]. The data within population was further analyzed for the normalcy of distribution, skewness, kurtosis, and significant outliers using the univariate procedure of SAS (Proc Univariate). Outliers within a population were considered a significantly different subpopulation when the ranking indicated that a given individual was more than two standard deviations greater than the mean.

In the rat model, data were analyzed using the general linear models procedure (SAS). Incremental area under the curve was calculated and differences in least square means were compared for the three treatments. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of AM immunoreactivity

Synthetic AM was found to elute in fraction 49 from the C18 semipreparative column used. When human blood plasma was run through the same column a major immunoreactive peak was found in fraction 49 (Fig. 1), therefore demonstrating the specificity of our RIA assay.

3.2. Adrenomedullin levels in drug-induced diabetes

The normal control population ($n = 28$) had a circulating AM value of 15.79 ± 0.51 fmol/ml (mean \pm standard error), whereas the diabetic patients showed a content of 25.70 ± 1.24 fmol/ml ($P < 0.0001$). The univariate procedure was able to differentiate a subpopulation as outliers (6.0%, shadowed squares in Fig. 2). In this analysis the outlier population was demarcated by AM concentrations 50 fmols/ml or greater (more than two standard deviations apart from the mean). Some of these values reached up to 64.2 fmol/ml, which is more than four times the mean in the controls. Analysis excluding the outliers revealed a non-linear population distribution skewed toward high AM levels. When comparing the diabetic population without outliers with the normal controls, we still observed a statistically significant difference in mean AM serum concentrations ($P < 0.0001$).

3.3. Adrenomedullin levels in pregnancy and gestational diabetes

Pregnant women had significantly higher AM values than nonpregnant women ($P < 0.0001$; Fig. 3). Among pregnant women, there was no significant differences in mean AM values between women with or without GDM (33.35 ± 2.32 vs 29.66 ± 1.45 fmol/ml; $P > 0.05$). The distribution analysis identified two individuals (shadowed squares in Fig. 2) as outliers with higher concentrations of AM in their blood, reaching up to 69.3 fmol/ml.

3.4. Modification of the diabetic phenotype by AM and its antibody in rats

Plasma glucose response to oral glucose load after saline, AM and MoAb-G6 treatments are shown in Fig. 4. In lean as well as in corpulent rats, there was a significant increase in glucose at 30, 60, and 120 min after glucose load when rats were treated with AM as compared to saline-treated controls. Treatment with MoAb-G6 had no significant difference with saline in lean animals. However, in obese rats the antibody produced a significantly lower glucose response at 1, 3, and 4 h after glucose load. When the area under the curve was analyzed, significant differences were found in the lean rats among treatments with AM compared to either saline ($P < 0.03$) or MoAb-G6 ($P < 0.05$). In obese rats, the area under the curve for glucose in rats treated with AM was significantly higher than when treated with saline ($P < 0.02$) or MoAb-G6 ($P < 0.004$). No significant differences were observed in the area under the curve between saline and MoAb-G6 in either group ($P > 0.05$). An interesting observation is that, after treating the diabetic obese rats with the monoclonal antibody, the glucose concentration came back to basal levels after 3 h (thick arrow in Fig. 3B). This is in contrast with the other treat-

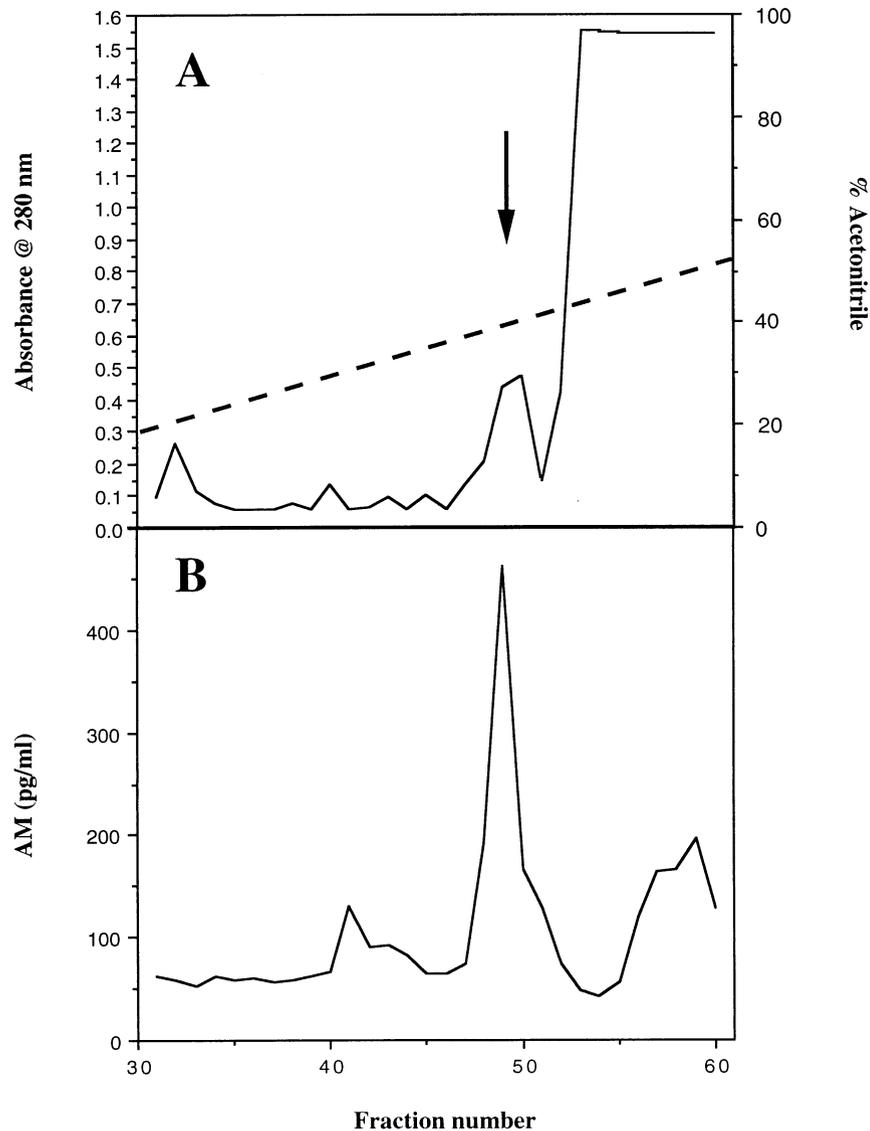


Fig. 1. HPLC profile of 2.5 ml of human plasma fractionated in a C18 semipreparative column (A), and AM contents of the plasma fractions as analyzed by RIA (B). Synthetic AM was run in the same way and was shown to elute in fraction 49 (arrow in B). The acetonitrile gradient is represented by a dashed line.

ments that showed postprandial hyperglycemia even at 4 h after glucose intake.

4. Discussion

In this study we have shown that two subpopulations can be partially distinguished by AM levels in patients who developed hyperglycemia under β -cell stress caused by either chemical treatment or pregnancy. The patients with a pharmacologically induced type 2 diabetes had higher levels of AM than the normal population, confirming previous observations [15,37]. Nevertheless, distribution analysis identified two subsets of diabetic individuals, one closer to the controls and another with high levels of AM. The source

of this excessive AM is still unclear. We cannot exclude the possibility of blood vessel damage that other authors postulated [15,37], but given the transient nature of beta-3 agonist-induced hyperglycemia, chronic endothelial damage that has been related to advanced stages of diabetes [4] seems unlikely. Elevations of AM levels have been also found in type 1 diabetic patients suffering from renal insufficiency with a disease duration of \sim 21 years, whereas in other type 1 patients with shorter disease duration, no statistical differences were found [11]. In these cases of prolonged disease, AM may come from damaged blood vessels, but this explanation is unlikely in the patients studied in the present report. Of interest, a recent report found that hyperglycemia was able to induce AM expression in cultured vascular smooth muscle cells by a mech-

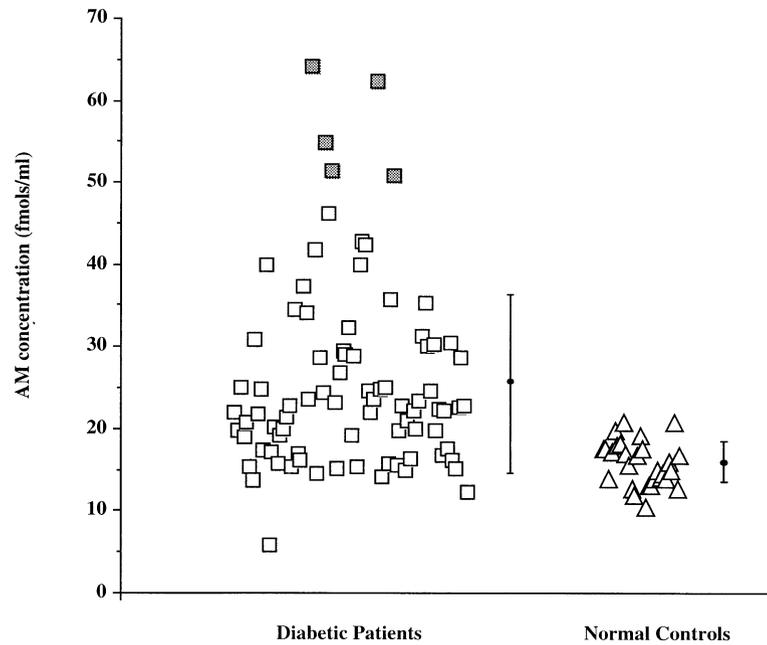


Fig. 2. AM concentration in blood serum from type 2 male diabetic patients (squares) and normal controls (triangles) in the first population used in this study as analyzed by radioimmunoassay. The horizontal axis represents the arbitrary order in the assay. AM concentrations are expressed as femtomoles per milliliter of blood serum. Bars indicate the mean and the standard deviation of the samples. Shaded squares represent outliers with AM concentrations higher than two standard deviations from the mean of the diabetic patients.

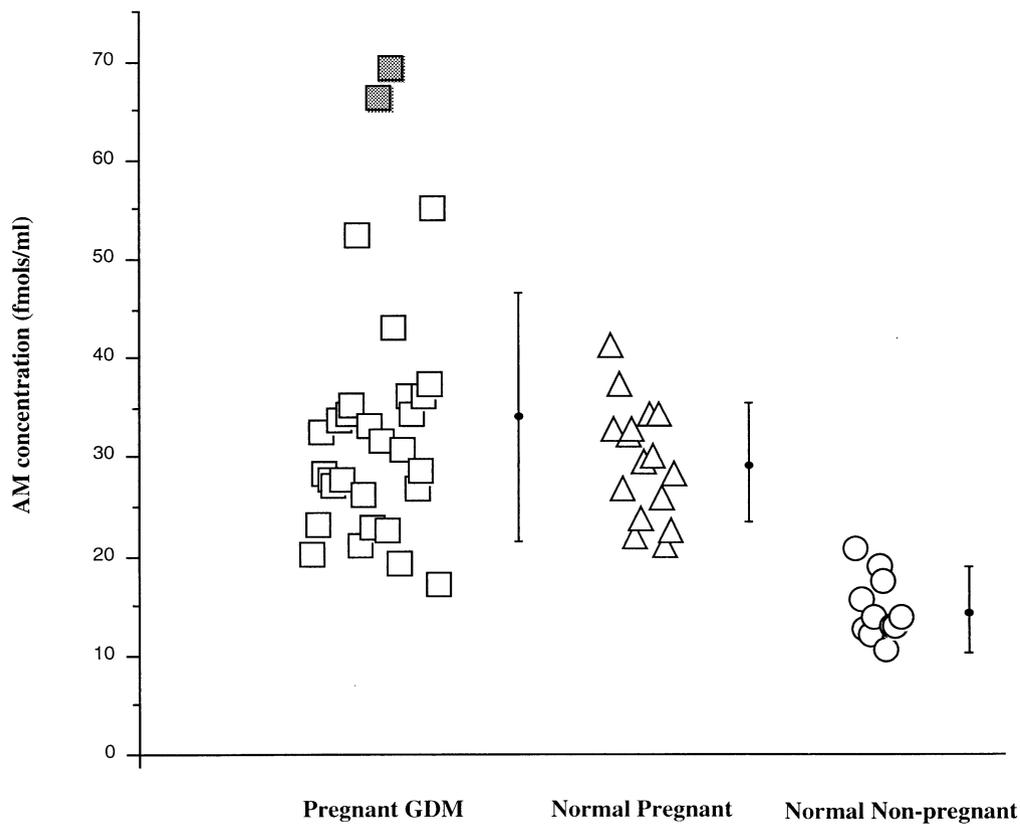


Fig. 3. Concentrations of AM in the blood of women of Latino origin. Squares represent pregnant patients with gestational diabetes, triangles represent age- and trimester-matched pregnant women without the disorder (normal pregnant controls), and the circles show the AM levels for normal volunteers (non-pregnant, non-diabetic females). The horizontal axis represents the arbitrary order in the assay. AM concentrations were assessed by radioimmunoassay and are expressed as femtomoles per milliliter of blood serum. Bars indicate the mean and the standard deviation of the samples. Shaded squares represent outliers with AM concentrations higher than two standard deviations from the mean of the diabetic patients.

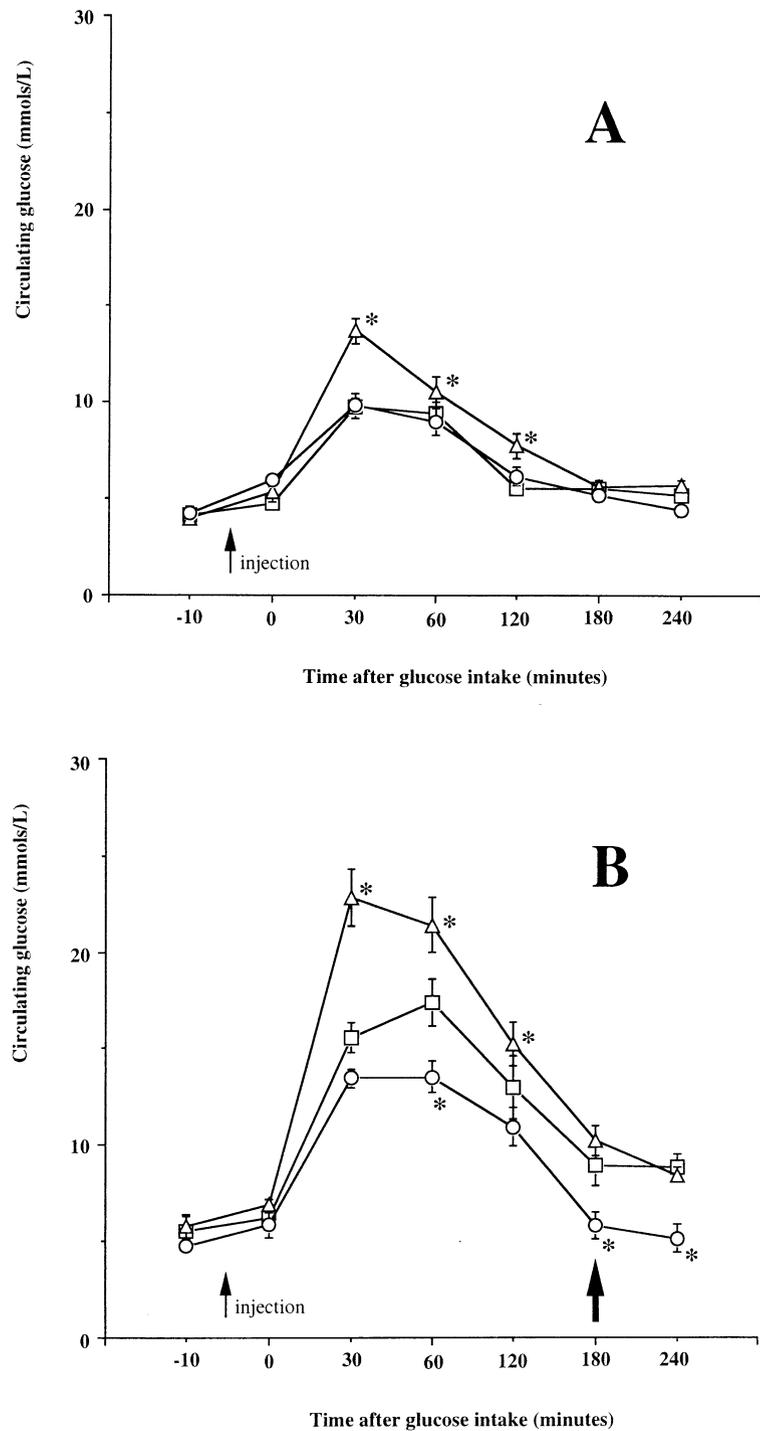


Fig. 4. Glucose tolerance test in lean (A) and obese diabetic (B) SHR/N-cp rats after injection of saline (squares), AM (triangles) and antiAM monoclonal antibody MoAb-G6 (circles). Asterisks represent points that were significantly different ($P < 0.05$) from the saline treatment. For details on the statistical analysis of the areas under the curve see the text. The thick arrow indicates the time point at which the antibody-treated diabetic rats recovered the basal levels of glucose. At this time point the other treatments present a clear postprandial hyperglycemia. Glucose concentrations are expressed as millimols of glucose per liter of blood serum. Blood samples were taken 10 min before glucose load (-10), immediately before [0], and 30, 60, 120, 180, and 240 min after glucose intake. Injection of the treatment solutions was given 5 min before glucose load (small arrows). Each point represents the average of six animals and their standard error.

anism dependent on protein kinase C [14]. The elevation of circulating AM could be previous to the diabetes onset or subsequent to it, but in either case it could have

diagnostic and/or prognostic value because of AM's ability to exacerbate the diabetic phenotype by inhibiting insulin release [30].

In contrast to the beta-3 agonist studies, we did not see a significant difference in AM levels between pregnant women with or without GDM, although four women in the GDM group had higher AM levels than any normal pregnant woman. A significant increase on AM concentration was shown in pregnant women as compared with matched nonpregnant controls. These results corroborate previous observations of elevations of AM during pregnancy [10,23]. The increase in AM during pregnancy is not surprising because this peptide is present in high amounts in the placenta [35], the amniotic fluid [25], and the developing fetus [35], and has been repeatedly related to many growth processes [29,34,47].

The pathogenesis of GDM appears to involve poor β -cell compensation for the insulin resistance of pregnancy [6]. Most women do not have diabetes immediately after pregnancy, but 20–50% develop diabetes later in life [22,33,40]. The fact that a large majority of women with GDM have a quantitative defect in insulin secretion during pregnancy [6] whereas only a small fraction have elevated AM levels indicates that AM is not a common contributor to the β -cell defect in GDM. However, it may contribute to the impaired β -cell compensation for insulin resistance in a subset of women with high AM levels. Long term follow-up for development of diabetes would help to clarify the contribution of AM to β -cell dysfunction.

The hypothesis of AM being the cause in some cases or, at least, an important factor leading to the hyperglycemia observed in type 2 diabetes was supported by the data accrued from the rat model. Intraperitoneal injections of AM induced in both lean and obese (diabetic) rats an increase in circulating glucose that was consistent with our previous results in regular nondiabetic rats [30]. Even more interesting was the observation of the monoclonal antibody causing a decrease in glycemia in diabetic rats, together with an improvement in postprandial recovery. Since this monoclonal antibody against AM has been previously characterized and shown not to crossreact with any other peptide hormone [30], these results clearly implicate AM in the maintenance of hyperglycemia. These observations were made after an acute injection of either AM or its antibody. Future experiments based on chronic exposure to either compound may further our knowledge on this process. As in any in vivo experiment, we must be cautious in interpreting the data. Although a direct action on insulin secretion seems to be the more plausible explanation based on previous results [30], we cannot exclude indirect effects through interactions with the cardiovascular [39], renal [18], or digestive [31] systems that might confound the direct action on the β cells.

The contribution of AM to β -cell dysfunction and hyperglycemia merits further investigation, as it is possible to envision new therapeutic approaches for the control of glycemia in diabetic patients using suppressors of AM. So far the only antagonist for AM that has been demonstrated to be efficient in regulating pancreatic function is the monoclonal

antibody used in this study. The difficulties of using such reagents for human therapy are well known [36], especially because this antibody is an IgA [30]. Nevertheless, new peptide antagonists are being tested in other systems and, for instance, the carboxy end of the AM molecule (AM_{22–52}) and an internal fragment (AM_{16–31}) seem to counteract AM effects [1,8]. In addition, the fragment proAM_{153–185} displays opposite actions to AM in blood flow regulation [13]. The development of new and more efficient antagonists to AM may be the basis for a new generation of drugs for the treatment of type 2 diabetes, especially for those patients displaying very high concentrations of this peptide.

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