1. Introduction

The incidence of diabetes mellitus is increasing worldwide from 30 million in 1985 to 382 million in 2014, with trends indicating a continued future rise (Wild et al., 2004; Leon and Maddox, 2015). This hypertensive state is closely linked to the development of diabetic nephropathy; that leads to structural changes, increased filtration pressure and microalbuminuria with compensatory activation of the renin-angiotensin system (Wolf and Ziyadeh, 1997). This renal damage may be linked to the cardiovascular autonomic neuropathy that is common in diabetic patients (Rolim et al., 2008) and is likely related with increased sympathetic activity (Esler, 2015). As the autonomic nervous system is responsible for maintaining various cardiovascular parameters such as the activity of the sinus node, end diastolic volume, end systolic volume, and systemic vascular resistance, its dysfunction can lead to arterial stiffness, left ventricular hypertrophy and ventricular diastolic dysfunction (Prince et al., 2010). The link between the renin-angiotensin system and the autonomic dysfunction has been reinforced by the observation that the use of angiotensin converting enzyme inhibitors and angiotensin receptor blockers help to slow the progression of cardiovascular autonomic neuropathy in diabetic patients (Vinik and Ziegler, 2007).
From these studies, it seems that the link between the sympathetic nervous system and the renin-angiotensin system in the pathogenesis of hypertension of different origins either essential (genetics) or diabetic, seems to be clear. Thus, in borderline hypertensive patients an elevated sympathetic nerve activity (Anderson et al., 1989) and augmented circulating levels of noradrenaline and adrenaline (Goldstein, 1983; de Champlain et al., 1999) have been reported. Also some studies have shown a parallel development of hypertension and diabetes associated to stress (Soucek and Kára, 2001). In another study, the involvements of the renin-angiotensin system and reactive oxygen species have also been shown to occur in type-2 diabetes (Leiter and Lewanczuk, 2005). Furthermore, in a latter study, vascular inflammation has been observed to be present in hypertension and diabetes (Savoia and Schiﬀrin, 2007).

As in hypertensive patients, spontaneously hypertensive rats (SHR) (Okamoto and Aoki, 1963; Pinto et al., 1998) also have elevated circulating levels of catecholamines (Iriuchijima, 1973; Grobecker et al., 1975), likely due to augmented noradrenaline release from sympathetic nerves and adrenaline release from adrenal medullary chromaﬃn cells (Arnaiz et al., 1978; Lim et al., 2002). A latter study demonstrated that in primary cultures of adrenal medullary chromaﬃn cells the release of catecholamine amperometrically monitored at the single-cell level, was considerably augmented in SHR with respect to normotensive rats (Segura-Chama et al., 2015). To our knowledge, whether an augmented quantal release of catecholamine from single chromaﬃn cells is present in diabetic rats, is unknown. Here, we present a comparative study performed in chromaﬃn cells from normotensive Wistar-Kyoto rats (WKY), rats exhibiting genetic hypertension (namely SHR), and rats that developed a diabetic state with hypertension after their treatment with streptozotocin (STZ). We found that chromaﬃn cells challenged with K⁺ depolarizing pulses, as well as those stimulated with angiotensin II responded with substantially higher catecholamine secretion responses in SHR and STZ, in comparison with WKY rats. Altered mitochondrial ultrastructure and function, with disturbed Ca²⁺ handling by chromaﬃn cells could explain those drastic differences.

2. Materials and methods

2.1. Animals

All experimental procedures with animals have been carried out following the rules approved by the Ethical Committee for the care and use of animals, of the Medical School, Autonomous University of Madrid, Spain, in accordance with the European Community Council Directive 2010/63/EEC and with the Spanish Real Decreto RD 53/2013. All eﬀorts were made to minimize animal suﬀering. Male 16-weeks-old WKYs (WKY/NHsd), SHRs (SHR/NHsd), and streptozotocin-induced diabetes group, weighing around 300 g, were housed at 24 ± 2 °C with 60 ± 20% relative humidity on a 12-h light/12-h dark cycle. The heart was weighed and normalized by the weight of the animals. Systolic blood pressure was monitored by non-invasive tail-cuff plethysmography (Cibertec®, Spain).

2.2. Induction of diabetes in WKY rats

Diabetes was induced by intraperitoneal administration to 3-month-old WKY of a single dose of streptozotocin (60 mg/kg). All animals were 4-month-old when taken to do the experiments, and all groups had the same genetic background (NHsd). Rats were considered diabetic when values of glycaemia were higher than 180 mg/dl after 48 h of diabetes induction; to measure glycaemia, we used a glucometer Accu-Chek Active® system (Roche, Barcelona). Streptozotocin, a glucosamine-nitrosourea, is taken up by the pancreatic cell glucose transporter GLUT-
2. promoting an alkylation of DNA and subsequent activation of poly-
(adenosine diphosphate (ADP)-ribose), which breaks the DNA molecule and depletes nicotinamide adenine dinucleotide, leading to destruction of β-cells in the pancreatic islets of Langerhans (Elsner et al., 2000). A timeline of the treatment of WKY rat with streptozotocin is depicted in Fig. 1A.

2.3. Culture of chromaffin cells

To prepare each chromaffin cell batch, we used one to two adult rats that were killed by cervical dislocation. An abdominal incision was performed, the adrenal glands were exposed, quickly removed and decapsulated, and both adrenal medullae were isolated under a stereoscope; they were placed in Ca²⁺- and Mg²⁺-free Locke solution composed in mM of 154 NaCl, 5.5 KCl, 3.5 NaHCO₃, 5.5 glucose and 9.98 of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), maintained at room temperature. Tissues were collected under sterile conditions and were prepared following the detailed procedure previously described (Padín et al., 2013). Chromaffin cells were cultivated on circular glass coverslips of 12 mm diameter for amperometry and patch clamp, and on 24 mm diameter coverslips for [Ca²⁺]c measurements. Before cell plating, coverslips were treated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich, USA) for 30 min, followed by a thorough washout with water. After 30 min, 2 ml of Dulbecco’s Modified Eagle Medium (DMEM) was added to each well. Cells were then incubated at 37 °C in a water-saturated, 5% CO₂ atmosphere; they were used within 1 day after plating.

2.4. Whole-cell calcium currents

For patch-clamp recording of Ca²⁺ currents (Iₛ), the perforated-patch mode of the patch-clamp technique was used, using amphotericin B (Sigma-Aldrich) as the permeating agent (Rosa et al., 2009). Tight seals (> 5 GΩ) were achieved in a bath solution for recording Iₛ (standard Tyrode solution) composed of the following (in mM): 137 NaCl, 1 MgCl₂, 2 CaCl₂, 5.3 KCl, 10 glucose, and 10 HEPES, pH 7.3 with NaOH. The intracellular solution for the recording of Iₛ under voltage clamp contained the following (in mM): 145 glutamic acid, 1 MgCl₂, 8 NaCl, and 10 HEPES, pH 7.2 with CsOH. Iₛ was recorded by means of an EPC-10 patch-clamp amplifier (HEKA, Germany) controlled by PULSE software (HEKA) running on a personal computer. The series resistance (Rₛ) was monitored until it decreased to < 20 MΩ. Rₛ averaged 8.6 ± 0.3 MΩ in chromaffin cells and was always compensated by 95%. In all recordings, the holding potential was −80 mV. Iₛ was activated in response to 20-ms depolarizing voltage steps from −60 to +60 mV in 10-mV increments.

2.5. Measurement of cytosolic calcium concentrations

The changes of [Ca²⁺]c were monitored as recently described (Padín et al. (2013)). Chromaffin cells were incubated for 1 h at 37 °C in DMEM containing the Ca²⁺ probe fura-2AM (Lifetechnologies, USA) (10 μM). After this incubation period, the coverslips were mounted in a chamber and cells were washed and covered with Tyrode solution. The setup for fluorescence recordings was composed of an inverted light microscope (DMI 4000 B; Leica Microsystems, Spain) equipped with an oil immersion objective (Leica × 40 Plan Apo; numerical aperture: 1.25). Cells placed on the microscope were continuously superfused by means of a five-way perfusion system at 1 ml/min with a common outlet 0.28-mm tube driven by electrically controlled valves with Tyrode solution at 22 °C. Fura-2AM was excited alternatively at 340 ± 10 and 387 ± 10 nm using a Küber CODIX xenon 8 lamp (Leica Microsystems). Emitted fluorescence was collected through a 540 ± 20 nm emission filter and measured with an intensified charge-coupled device camera (camera controller CI0600 orca R2, Hamamatsu, Japan). Fluorescence images were generated at 1-s intervals. Images were digitally stored and analyzed using LAS AF software (Leica Microsystems). A Tyrode solution was used to dissolve the drugs. Drug concentrations are indicated in the text and figure legends.

2.6. Monitoring of the quantal release of catecholamine

Carbon fiber microelectrodes were prepared by cannulating a 7 μm diameter carbon fiber in polyethylene tubing. The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp head stage and backfilled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The electrode was positioned at the middle right side of a spherical chromaffin cell, gently touching it. Amperometric currents were recorded using an EPC-10 amplifier and PULSE software running on an Apple Macintosh computer (Apple Computer, USA). Sampling was performed at 14.5 kHz, and samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments, using 50 μM noradrenaline as standard solution. Only fibers that rendered 200–300 pA of current increment after a 50 μM noradrenaline pulse were used for the experiments; cell secretion was stimulated by pulses of 30 mM K⁺ for 10 s or 1 μM angiotensin II for 30 s.

2.7. ATP measurement

Cells were seeded in 96-well plates with black wells at 7000 cells per well. We used the commercial kit (CellTiter-Glo®, Luminescent Cell Viability Assay Promega Biotech Ibérica, Spain). Overall ATP cellular levels were measured under basal conditions. The assays were performed according to the manufacturer’s instructions. The readings were made in a microplate reader (Labsystems, USA). We measured three wells of the same condition per plate.

2.8. Immunoblotting and image analysis

Adrenal glands were removed from rats, medullae were dissected out and proteins were extracted using the Mammalian Protein Extraction Reagent (MPEM®, Thermo Scientific, USA) following the manufacturer’s instructions. At all times, proteins were in the presence of protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Scientific). Proteins were quantified using the Bicinchoninic Acid Protein Assay (Bioscience, USA). Proteins (20 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred to Immobilon-P® Transfer Membrane (Millipore Corporation, USA). Membranes were blocked in Tris-buffered saline with 0.05% Tween 20 containing 4% bovine serum albumin, and incubated for 2 h at room temperature with primary antibodies anti-angiotein II receptor type 1 (anti-AT₁, 1:500; SAB3500209) or anti-angiotein II receptor type 2 (anti-AT₂, 1:1000; SAB3500098) and with anti-β-actin (ACTB, 1:10000; A3854) as loading control, all from Sigma-Aldrich; then, for 45 min with secondary antibodies conjugated with peroxidase (1:10000; Santa Cruz Biotechnology, USA) only for antibodies anti-angiotein AT₁ and AT₂ receptors. The membrane was developed using the ECL Select® Western Blotting Detection Reagent (GE Healthcare, UK). Different band intensities corresponding to immunoblot detection of protein samples were quantified using Scion Image® Alpha 4.0.3.2 program (Scion Corporation, USA).

2.9. Monitoring of the mitochondrial membrane potential

To calculate Ψₘ, Chromaffin cells were incubated in presence of 500 nM of the fluorescent mitochondrial probe tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Madrid, Spain) at 37 °C during 5 min. TMRE was excited at 545 ± 30 nm and emitted fluorescence was collected through a 610 ± 30 nm emission filter. Fluorescence images were generated at 10-s intervals. Cells were perfused with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 3 μM) during
30 s to induce mitochondria depolarization and after that, FCCP was washed out in order to measure the mitochondria repolarisation phase. \( \Psi_{m} \) was calculated as the lost from mitochondria of arbitrary units of fluorescence in the absence and presence of FCCP.

2.10. Electron microscopy

Adrenal glands dissected from at least three WKY, SHR or STZ were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The rat adrenal glands were removed and postfixed by immersion in the same fixative at 4 °C for 6 h. After adrenal glands of each animal were cryoprotected, coronal blocks tissue (1.2–2 mm, 250 µm) from each adrenal gland was fixed in 2% osmium tetroxide buffered with 0.1 M Sorensen phosphate buffer (0.133 M Na2HPO4, 0.133 M KH2PO4; pH 7.2, total osmolarity 320 mOsm) for 2 h at 4 °C. Then, the samples were dehydrated by passage through increasing concentrations of alcohol 30, 50, 70, 95% v/v (once, 10 min); absolute ethanol (twice, 10 min) and acetone (twice, 5 min) followed by propylene oxide, and embedded in Araldite resin (Durcupan ACM; Sigma, Spain). Sections were cut on a Leica Ultracut S with diamond knives on a Leica Ultracut S. Micrographs were made with JEOL transmission electron microscope 200-mesh grid, and stained with uranyl acetate and lead citrate.

2.11. Data analysis

The PULSE software and Igor Pro Software (Wavementrics, USA), were used to monitor the amperometric release of catecholamine either in terms of total spikes present in each stimulus trace or as the summation of spike areas (total secretion per stimulus). A threshold of 4.5 times the first derivative of the noise S.D. was calculated to clearly detect amperometric events. Whole-cell inward \( I_{\text{Ca}} \) amplitude was measured at the maximum peak current during the 50-ms depolarizing pulse. Regarding \( I_{\text{Ca}} \) data analysis was carried out on a personal computer, and data obtained by PULSE software and Igor Pro software were exported to Excel tables (Microsoft, USA). Only the cells that held up the entire protocol were included in the statistics. Differences between means of group data fitting a normal distribution were analyzed by repeated measures with one-way ANOVA followed by the Bonferroni test using the GraphPad Prism software, version 5.01 (GraphPad Software, USA). \( P < 0.05 \) was taken as the limit of significance. Data from measurement of changes in the \([\text{Ca}^{2+}]_{C}\) was obtained from LAS AF software. Graphs and the mathematical analyses were performed using the GraphPad Prism software, version 5.01. Areas and peak heights were calculated by integrating the calcium transient over time during the stimulus duration by means of Origin Pro 8 SR2 software, version 8.0891 (OriginLab Corporation, USA). Areas were worked out by the integration of the input data set by using the trapezoidal rule. Statistical analyses were carried out with one-way ANOVA followed by Tukey post hoc test. \( P < 0.05 \) was taken as the limit of significance. In the data from immunoblotting and image analysis the expression of proteins, comparisons between groups were performed by one-way ANOVA followed by the Newman-Keuls post hoc test using the GraphPad Prism software, version 5.01. \( P < 0.05 \) was taken as the limit of significance. Finally, statistical analyses from electron microscopy ultrastructure data were performed by one-way ANOVA followed by Dunnett’s post-hoc test comparing all columns with respect to WKY control rats. \( P < 0.05, P < 0.01 \) and \( P < 0.001 \) were taken as the limit of significance.

3. Results

We first studied the variations of some basal parameters of the animal models here studied. Basal blood levels of glucose (in mg/dl) were 110 ± 1 in WKY, 410 ± 40 in STZ, and 111 ± 10 in SHR (Fig. 1B). On the other hand, in WKY rats the systolic blood pressure was 122 ± 3.2 mmHg, in STZ it rose by 19.8%, and in SHR by 55% over WKY values (Fig. 1C). Furthermore, the dry weight of the heart normalized by body weight was 2.89 ± 0.3 g in WKY, 3.09 ± 0.5 g in STZ, and 3.87 ± 0.2 g in SHR (Fig. 1D). Finally, the bioenergetic steady-state in basal conditions was estimated as overall ATP levels in isolated chromaffin cells maintained in culture for 1 day. Overall ATP cellular content in WKY was 28 ± 3 pmol/cell, in STZ 12 ± 2 pmol/cell, and in SHR 18 ± 3 pmol/cell (Fig. 1E).

3.1. Whole-cell calcium currents

The whole-cell \( I_{\text{Ca}} \) were monitored with the perforated-patch configuration of the patch-clamp technique; the cell membrane potential was held at −80 mV and 20-ms depolarizing pulses to various test voltages (from −60 mV to +60 mV) were applied to chromaffin cells that were perfused with an extracellular solution containing 5 mM Ca\(^{2+}\). Fig. 2A shows the protocol used to elicit the family of \( I_{\text{Ca}} \) traces generated by the different test voltages applied in 10-mV steps to chromatim cells of WKY cells (Fig. 2B), STZ cells (Fig. 2C), and SHR cells (Fig. 2D) maintained in culture for 1 day. Of interest was the higher current inactivation occurring in the WKY chromaffin cell, compared with lesser or no inactivation of the currents generated in STZ and SHR cells (panels B, C, D of Fig. 2).

The Intensity-Voltage curves (I-V) made with pooled data of peak currents are graphed in Fig. 2E. In WKY cells the threshold current was at −30 mV, \( I_{\text{Ca}} \) peaked at −10 mV (243 ± 27 pA; Fig. 2E), and its reversal potential was at +50 mV. The I-V curve for STZ cells was shifted 10 mV to the right and had a threshold current at −20 mV, peaked at 0 mV (113 ± 11 pA), and its reversal potential was at +40 mV. Similarly, \( I_{\text{Ca}} \) of SHR cells had a threshold voltage at −20 mV, peaked at 0 mV (155 ± 13 pA), and reversed at +50 mV. The greatest difference in current amplitude between WKY cells, STZ cells, and SHR cells was found at −20 mV and −10 mV. Thus, at −20 mV (Fig. 2E) control cells had averaged \( I_{\text{Ca}} \) amplitude of 154 ± 24 pA. In STZ cells, \( I_{\text{Ca}} \) peak was considerably smaller, just 15% of control; on the other hand, in SHR cells \( I_{\text{Ca}} \) peak was 30% of control cells. At −10 mV, peak \( I_{\text{Ca}} \) of WKY cells was 243 ± 27 pA; peak \( I_{\text{Ca}} \) was reduced significantly to 25% and 40% of control, respectively, for STZ cells and SHR cells. At more depolarized voltages these differences gradually disappeared. Only STZ cells maintained significant differences at +10 and +20 mV.

3.2. Cytosolic calcium concentrations

These experiments were done in fura-2-loaded chromaffin cells challenged with angiotensin II (3 µM, 30 s), or with a K+ -enriched depolarizing solution (30 mM, 5 s). Superimposed records of the \([\text{Ca}^{2+}]_{C}\) transients elicited by the two stimuli in single WKY cells, STZ cells, and SHR cells are exemplified in Fig. 3A. Only 51% of WKY cells responded to angiotensin II; however, 74% of WKY cells responded to K+. The percentage of response to K+ was high in the three types of cells: 74%, 93% and 84%, respectively for WKY cells, STZ cells, and SHR cells (Fig. 3B).

Averaged amplitudes of peak \([\text{Ca}^{2+}]_{C}\) transients are graphed in Fig. 3C. The responses to angiotensin II were 67% and 80% higher in, respectively, STZ cells and SHR cells, with respect WKY cells (panel C). The K+ -elicited responses were 37% higher in STZ cells; however, in SHR cells the K+ response was 18% lower with respect WKY cells (panel D).
3.3. Quantal catecholamine release

The quantal release of catecholamine was amperometrically monitored with a carbon fiber microelectrode applied onto single WKY cells, STZ cells, and SHR cells, stimulated with high K⁺ (30 mM, 10 s) (Fig. 4) or with angiotensin II (1 µM, 30 s) (Fig. 5). Fig. 4A shows an original amperometric trace taken from an example WKY cell stimulated with K⁺; the single spike secretory events were scattered along the duration of the stimulus and ceased upon removal of the high K⁺ solution. The trace in Fig. 4B was taken from an example STZ cell; the spikes were more frequent with respect to the WKY cell and some of them were produced after K⁺ stimulation ceased. The third trace (Fig. 4C), which was taken from an example SHR cell, also exhibited a higher number of spikes compared with the WKY cell, although the activity ceased after 100 pA 10 ms.

Fig. 2. Characteristics of calcium currents (I_{Ca}) through voltage-activated calcium channels (VACCs) in chromaffin cells that were voltage-clamped at −80 mV and challenged with 10 ms depolarizing test pulses applied in 10 mV steps at 10 s intervals, using Ca²⁺ (5 mM) as charge carrier (protocol on panel A). Family of I_{Ca} traces obtained in WKY cells (panel B), STZ cells (panel C), or SHR cells (panel D). E, current (absissa) versus voltage (ordinate) relationship in WKY cells, STZ cells and SHR cells. Data are means ± S.E.M. of the number of cells and cultures shown in parentheses. The results were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison post-hoc test. **P < 0.05, ***P < 0.01 compared with WKY cells.

Fig. 3. Cytosolic Ca²⁺ ([Ca²⁺]c) elevations elicited by angiotensin II and K⁺ in WKY, STZ, and SHR chromaffin cells. A, original traces of representative cells during the experiments of [Ca²⁺]c measurements. B, percentage of WKY, STZ, and SHR cells that responded to their challenges with angiotensin II (3 µM, 30 s) or K⁺ (30 mM, 5 s) with a healthy transient elevation of the [Ca²⁺]c (at least 10% of the maximum response). The analysis of the peak of [Ca²⁺]c in all cells for the different stimuli are summarized in panels C (angiotensin II, 3 µM), D (K⁺, 30 mM). Data are means ± S.E.M. of the number of cells (first number) and cell cultures from different animals (second number) shown in parentheses. Significance was accepted at P < 0.05. *P < 0.05, **P < 0.01 with respect to WKY cells; #P < 0.01 with respect to STZ cells.
removal of the high K+ solution. The spike number per K+ stimulus was 20.1 ± 1.04 in 11 WKY cells (Fig. 4D); this number was increased by 70% in STZ cells, and by 115% in SHR cells. Cumulative total secretion was calculated by adding the areas of spikes in each secretory trace for each cell, and pooled data are presented in Fig. 4E; total secretion in WKY cells was 15 ± 2 picoCoulomb (pC); this value augmented 2.36-fold in STZ cells and 2.2-fold in SHR cells.

3.4. Immunoblot analysis of the expression of angiotensin AT1 and AT2 receptors

Western blot experiments were made to find out the relative expression of angiotensin AT1 and AT2 receptors in freshly isolated adrenal medulla of WKY, STZ, and SHR rats. Fig. 6 displays the relative densities of angiotensin type AT1 (A) and AT2 receptors in WKY, STZ, and SHR rat adrenal medulla of WKY, STZ, and SHR rats. Example immunoblots with the bands corresponding to angiotensin AT1, AT2 receptors, and control β-actin (ACTB) are displayed on top of Fig. 6. The results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison post-hoc test. *P < 0.05 compared with WKY cells.
3.5. Mitochondria ultrastructure and function

Alterations in \([\text{Ca}^{2+}]_e\) transients and catecholamine release could be secondary to impaired mitochondrial function. Therefore, ultrastructural and functional studies were carried out to define whether mitochondria were altered in chromaffin cells of STZ and SHR, with respect WKY rats.

Panels A, B and C of Fig. 7 are example electron microscopy pictures of mitochondria from adrenal medullae of WKY, STZ, and SHR rats, respectively. It is apparent that the three STZ mitochondria (panel B) have substantially lesser density of cristae, with respect the WKY large mitochondrion (panel A). Similarly, the SHR mitochondria of panel C have a notable decrease of cristae in comparison with control WKY mitochondria. On quantitative terms, the cristae of WKY mitochondria occupied 39.1 ± 1.6% of the averaged mitochondrial space (Fig. 7D), while in STZ this value was reduced to 26.5 ± 2.3%, and in SHR to 25.1 ± 2.4% (Fig. 7D). Reduction of cristae density was obviously accompanied of increased mitochondrial matrix space. Thus, in WKY the matrix amounted to 40.0 ± 2.9% of the total averaged space, that augmented to 59.3 ± 3.8% in STZ and to 50.7 ± 2.5% in SHR (Fig. 7E).

Concerning the mitochondrial membrane potential (\(\Psi_m\)), the greater TMRE fluorescence in control WKY cells also indicates more hyperpolarized mitochondria, with respect SHR and STZ chromaffin cells (Fig. 7F). Cell treatment with 3 μM of protonophore FCCP, caused a gradual loss of TMRE fluorescence indicating that mitochondria were being depolarized. Although the cells were exposed to FCCP for only 30 s, partial recovery of \(\Psi_m\) after its washout likely indicated some residual mitochondrial damage (Fig. 7F). Of interest was the observation that the relative depolarization change monitored as % of TMRE fluorescence loss, was 32.0 ± 3.7 Arbitrary Fluorescent Units (AFU) in WKY, 14.0 ± 2.5 AFU in STZ, and 22.0 ± 2.0 AFU in SHR (Fig. 7G). This suggests that before adding FCCP, STZ and SHR mitochondria were substantially more depolarized than mitochondria from control WKY chromaffin cells. This correlates reasonably well with the lower overall ATP levels found in STZ cells (12.6 ± 2.4 pmol/cell) and SHR cells (20.5 ± 3.4 pmol/cell), with respect WKY cells (28.6 ± 4.6 pmol/cell) (Fig. 7H).

3.6. Density of dense-core chromaffin granules

Differences observed in the catecholamine secretion among normotensive and hypertensive animals might be explained by an augmented number of vesicles near of cytoplasmic membrane. Therefore, images of chromaffin granules obtained from microphotographs of transmission electron microscopy were analyzed in the animals models studied.

Representative examples of microphotographs obtained by transmission electron microscopy of chromaffin cells containing dense-core vesicles are shown in Fig. 8. The pictures were taken from adrenal medullae of WKY (panel A), STZ (panel B), and SHR rats (panel C), respectively. Normotensive WKY rats (Fig. 8A) have lesser density of dense-core vesicles than STZ (Fig. 8B) and SHR rats (Fig. 8C). On quantitative terms, the number of chromaffin granules in the surface area (μm²) near the cytoplasmic membrane is significantly increased 1.45-fold in STZ (15.9 ± 1.2; \(P < 0.05\)) and 2-fold in SHR (21.5 ± 2.3; \(P < 0.001\); one-way ANOVA, Dunnett’s post hoc test), with respect to WKY rats (10.7 ± 0.6) (Fig. 8D).

4. Discussion

Two findings are central stage in this investigation: (1) angiotensin II triggered higher \([\text{Ca}^{2+}]_e\) elevations in chromaffin cells from STZ and SHR rats, in comparison with WKY rats (Fig. 3C); (2) the release of catecholamines in cells challenged with angiotensin II was also higher in STZ and SHR, with respect WKY rats (Fig. 5D, E). These drastic differences could have an explanation in one or more steps of the \(\text{Ca}^{2+}\) signaling pathway initiated at plasmaleminal angiotensin AT₁ and/or AT₂ receptors.

Concerning angiotensin II receptors it should be considered first that angiotensin AT₂ receptors are expressed more abundantly in rat chromaffin cells; however, the enhanced \([\text{Ca}^{2+}]_e\) and secretion triggered by angiotensin II is mediated by angiotensin AT₁ receptors (Wong et al., 1990; Cavadas et al., 2003). Although an altered regulation of angiotensin AT₁ receptors in the cortical glomerulosa layer of the adrenal gland, changes in angiotensin AT₁, or AT₂ receptor expression in the adrenal medulla was not observed (Song et al., 1995; Jöhref et al., 2003). This contrast with data from other studies on augmented angiotensin AT₁ expression in the adrenal medulla of SHR rats (Brown et al., 1997; Mansour et al., 2013). Here, we have corroborated that in the adrenal medulla of WKY, STZ and SHR rats the expression of angiotensin AT₂ receptors was twice higher than that of angiotensin AT₁ receptors, as previously reported (Wong et al., 1990; Cavadas et al., 2003). However, the relative expression of angiotensin AT₁ and AT₂ receptors in the adrenal medulla was similar in the three animal models here studied (Fig. 6). Thus, we may not attribute the differences in \(\text{Ca}^{2+}\) and exocytotic responses of cells stimulated with angiotensin II, to different expression densities of angiotensin AT₁ and AT₂ receptors.

We may invoke a kind of receptor “sensitization” associated to hypertension in STZ and SHR, with respect to WKY rats. This supposition is based in the local tissue presence of the renin-angiotensin system (Lavoie and Sigmund, 2003). This system mediates autocrine, paracrine or intracrine actions at the adrenal gland (Lavoie and Sigmund, 2003; Inagami, 2011). In this context, it has been suggested that locally synthesized angiotensin II could correlate with hypertension in a way closer than the systemic circulatory angiotensin II (Lavoie and Sigmund, 2003; Lee et al., 2012). Consistent with this postulate is the
observation that local tissue renin (Iwai et al., 1996) and angiotensin-converting enzyme-1 (Lee et al., 2012) are upregulated in the adrenal gland of SHR rats; this is consonant with low tissue levels of angiotensinogen found in hypertensive rats (Tamura et al., 1996). A similar outcome was found in STZ rats (Kalinyak et al., 1993; Nakayama et al., 1998). Additionally, lower local concentrations of angiotensinogen may be indicative of low local concentrations of angiotensin II, as happens to be the case in STZ rats (Campbell et al., 1999). If this were the case for chromaffin cells, we may speculate that lower local angiotensin II availability could lead to hyper-reactivity of angiotensin AT1 receptors, occurring as a kind of compensatory mechanism. This could partly explain the higher Ca2+ and exocytotic responses in STZ and SHR chromaffin cells exposed to angiotensin II in our present experiments.

However, a more plausible explanation could lie in a greater Ca2+ mobilization from the endoplasmic reticulum Ca2+ store. It is known since long that angiotensin II promotes the formation of inositol trisphosphate that causes endoplasmic reticulum Ca2+ release in chromaffin cells (Plevin and Boarder, 1988; Stauderman and Pruss, 1990). In line with this is the recent observation that a mixture of caffeine, ryanodine and thapsigargin causes higher [Ca2+]i transients in chromaffin cells from SHR rats, with respect to WKY rats (Segura-Chama et al., 2015). This indicates that the endoplasmic reticulum Ca2+ store may have a higher capacity in hypertensive, with respect normotensive rats. This possibility could also explain the enhanced Ca2+ and exocytotic responses in chromaffin cells from STZ and SHR rats compared with WKY rats, found in the present study.

The classical observation that high K+ triggers the Ca2+-dependent secretion of adrenal medullary catecholamine (Douglas and Rubin, 1963), it is today amply documented to be mediated by Ca2+ entry through subtypes of neuronal Ca2+ channels on the plasmalemma of chromaffin cells (García et al., 2006). In this context, the enhanced Ca2+ (Fig. 2A, D) and secretion responses (Fig. 4D, E) in chromaffin cells from STZ and SHR rats, in comparison with WKY rats challenged with high K+, could find an explanation if a greater Ca2+ entry occurs in the former, with respect to the latter animal model. The fact IcL was substantially lower in STZ and SHR rat chromaffin cells, with respect to WKY rats, against this explanation. However, we may invoke two plausible mechanisms underlying this augmented Ca2+ elevations: (1) lesser inactivation of IcL (Fig. 2C, D) indicates that voltage-activated calcium channels may open for a longer time in STZ and SHR cells; (2) as a consequence of a higher capacity endoplasmic reticulum Ca2+ store, a more effective calcium-induce calcium release mechanism

Fig. 7. Ultrastructural and functional aspects of mitochondria from WKY, STZ, and SHR chromaffin cells. A, B, and C are microphotographs of chromaffin cell cytoplasmic fields, obtained with transmission electron microscopy at 60.000x (scale bar = 0.5 µm) in sections of adrenal medulla of WKY, STZ, and SHR rats, respectively. Quantitative averaged data of mitochondrial density (20,000x magnification) and cristae density (30,000x magnification) in different cultures shown in parentheses. Data in panels B, C, and D are means ± S.E.M of 11 microphotograph analysis from 3 different rats of each type. *P < 0.05, **P < 0.01, ***P < 0.001, with respect WKY (ANOVA one-way test with Dunnett's post-hoc test). Panel G shows the ΔΨm changes (ordinate in arbitrary fluorescence units) elicited by FCCP. Data are means ± S.E.M. of the number of cells from 3 different cultures shown in parentheses (panel G). *, **, *** are P < 0.05, P < 0.01, P < 0.001, with respect to WKY cells (ANOVA one-way test with Dunnett's post-hoc test). Panel H is the overall ATP level of chromaffin cells in culture, prepared from WKY, STZ, and SHR rats, before and upon FCCP treatment (3 µM for 30 s). Data are means ± S.E.M. of the number of cells from 3 different cultures shown in parentheses. *P < 0.05, **P < 0.01, with respect to WKY cells (two way Student's t-test).
known to be present in chromaffin cells challenged with K⁺ (Alonso et al., 1999) could explain the greater Ca²⁺ elevations in STZ and SHR rats, compared with WKY rats. This together with a higher pool of dense-cored vesicles closer to the subplasmalemma (Fig. 8) could explain the augmented secretory responses in chromaffin cells of STZ and SHR rats challenged with angiotensin II or K⁺, with respect to WKY rats.

Many authors have related the renin-angiotensin system with mitochondrial damage. It is noteworthy that angiotensin II induces alterations in the mitochondrial structure and function, which in turn, promotes the generation of mitochondrial reactive oxygen species, consequently leading to the depression of energy metabolism (Dikalov and Nizarewicz, 2013; Manucha et al., 2015). These molecular mechanisms take place through at least two main pathways: i) the stimulation of NADPH oxidases throughout angiotensin AT₁ receptor; ii) direct mitochondrial angiotensin II interaction by means of an unknown mechanism (de Cavanagh et al., 2007). These mechanisms have been demonstrated to be common in the pathological base of diabetes and hypertension (Manucha et al., 2015). In relation to this, previous studies of SHR have described a global perturbation in the production of hypertension (Manucha et al., 2015). In relation to this, previous studies demonstrated to be common in the pathological base of diabetes and hypertension (Manucha et al., 2015). These mechanisms have been found to be present in chromaffin cells challenged with K⁺ (Alonso et al., 1999) could explain the greater Ca²⁺ elevations in STZ and SHR rats, compared with WKY rats. This together with a higher pool of dense-cored vesicles closer to the subplasmalemma (Fig. 8) could explain the augmented secretory responses in chromaffin cells of STZ and SHR rats challenged with angiotensin II or K⁺, with respect to WKY rats.

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unipporter plays an important function in $\text{Ca}^{2+}$ clearance (Duchen, 2000). This $\text{Ca}^{2+}$ uptake requires a $\text{H}^+$ gradient that is impaired in depolarized mitochondria. This will lead to higher $\text{Ca}^{2+}$ microdomains near subplasmalemmal exocytotic sites and enhanced secretory responses in chromaffin cells challenged with angiotensin II or high K+ form STZ and SHR rats, in comparison with WKY rats.

5. Conclusions

In conclusion, our data are compatible with the view that an altered morphofunctional mitochondrial function may be one of the starting points in the genesis of hypertension, whichever its origin. In chromaffin cells from hypertensive rats, an augmented $\text{Ca}^{2+}$, and catecholamine release responses elicited by angiotensin II or high K+ seem to have their origin in the following alterations: (1) augmented capacity of the endoplasmic reticulum $\text{Ca}^{2+}$ store likely due to (2) impaired mitochondrial $\text{Ca}^{2+}$ uptake; (3) augmented high-$\text{Ca}^{2+}$, microdomains at subplasmalemmal sites secondary to augmented calcium-induce calcium release (in the case of K+ stimulation) and to inositol tri-phosphate receptor mediated enhanced $\text{Ca}^{2+}$ mobilization from the endoplasmic reticulum (in the case of angiotensin II stimulation); and (4) enhanced secretory vesicle pool at subplasmalemmal exocytotic sites. These alterations are common to the models of human hypertension here explored, STZ diabetic rats and SHR hypertensive rats. As comorbidity of diabetes and hypertension are frequent in the clinic, our study may impinge on the one side, on a better understanding of the mechanism underlying such comorbidities and on the other, on the identification of new targets for better treatments of patients suffering them.

Declarations

Ethics approval and consent to participate

All experimental procedures with animals have been carried out following the rules approved by the Ethical Committee for the care and use of animals, of the Medical School, Autonomous University of Madrid, Spain, in accordance with the European Community Council Directive 2010/63/EEC and with the Spanish Real Decreto RD 53/2013. All efforts were made to minimize animal suffering.

Competing interests

The authors declare that they have no competing interests

Authors’ contributions

DCM, JFP, RMF, AJ NHJ and AGG designed the study. DCM, GBH, JAAT, IML, and JFP researched and analyzed the data. DCM, IML, JFP and AGG wrote the manuscript. DCM, GBH, IML, RMF, AJ, NHJ, and AGG contributed to the discussion and reviewed/edited the manuscript.

The guarantor for this work is JFP. All authors read and approved the final manuscript.

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