Chronic blockade of angiotensin II AT₁-receptors increased cell-to-cell communication, reduced fibrosis and improved impulse propagation in the failing heart

Walmor C De Mello, Philip Specht

Abstract

Introduction. The influence of chronic administration of losartan on gap junction conductance (gj), conduction velocity and interstitial fibrosis was investigated in the failing heart of 4-month-old cardiomyopathic hamsters (TO-2). After two months of administration of losartan (25 mg/kg/day/po) the number of cell pairs showing very low values of gj (2–8 nS) was significantly reduced whereas the group of cell pairs with larger values of gj (18–45 nS) was significantly increased. The conduction velocity measured with intracellular microelectrodes in the wall of the left ventricle was enhanced from 38±2.3 cm/s (n=25) (control) to 49±2 cm/s (n=24) (p<0.05) after losartan administration. Moreover, the number of ventricular fibres showing non-propagated action potentials was significantly decreased (p<0.05) by losartan. The % area of interstitial fibrosis measured in the wall of the left ventricle was reduced from 22±1.4% (n=18) to 14±1.3% (n=18) (p<0.05) after losartan administration.

Conclusion. Chronic blockade of angiotensin II type 1 receptors increased gj in the failing heart. Moreover, the conduction velocity was enhanced in part by the increase of gj and in part by the decrease of interstitial fibrosis and structural remodelling.

Introduction

Evidence has been presented that the activation of the plasma and cardiac renin angiotensin systems during the process of heart failure is largely responsible for the ventricular remodelling, changes in intercellular communication, impulse propagation and the generation of cardiac arrhythmias.1,2

The activation of angiotensin II (Ang II) type 1 receptors (AT₁) is involved in the decline of gap junctional conductance (gj) in the failing heart with consequent impairment of impulse conduction.3,5 Previous studies performed at an advanced stage of the disease with overt heart failure (11 months of age) indicated a large group of cells with an extremely low value of gj (0.8–2.5 nS) and a second group with gj in the range of 7–40 nS.3 These findings indicate that the gj is gradually impaired during the development of heart failure.

A subject of seminal importance is the possible influence of chronic blockade of Ang II AT₁-receptors on gap junction remodelling. In the present work the influence of chronic administration of losartan on gj as well as on conduction velocity and interstitial fibrosis was investigated in the failing heart of cardiomyopathic hamsters.

Methods

Male Syrian cardiomyopathic hamsters (TO-2) at two and four months of age (Biobreeders, MA) were used. The animals were kept at the animal house at constant temperature (24°C) and humidity following the recommendations of NIH. The animals were maintained on a normal animal diet and tap water ad libitum. Cardiomyopathic animals were divided into two groups. The first group consisted of 2-month-old animals (prehypertrophic stage, n=14) that were treated with losartan (25 mg/kg/day) for an 8-week period. The drug was administered daily by gavage. The second group (2-month-old animals) was treated with vehicle for an 8-week period and was used as controls. All animals were housed in a temperature-controlled room, on a 12-hour light dark cycle.

Measurements of junctional conductance

Cell pairs were obtained by enzymatic dispersion of hamster ventricles following the method of Powell and Twist6 and Tanigushi et al.7 The heart was removed with the animals under deep anaesthesia and immediately perfused with a modified Krebs solution containing (mM): NaCl – 150; KCl – 4; CaCl₂ – 2; MgCl₂ – 1; CsCl – 2; BaCl₂ – 1; glucose 5; HEPES – 5, pH adjusted to 7.4. After 20 min, a calcium free solution containing 0.4% collagenase ( Worthington Biochemical Corp) was recirculated through the heart for 40–60 min. The collagenase solution was washed out with a recovery solution containing (mM): taurine-10; oxalic acid 10; glutamic acid 70; KC l25; KH₂PO₄ 10; glucose 11; HEPES –5, pH adjusted to 7.4. Two and four months of age (Biobreeders, MA) were used. The animals were kept at the animal house at constant temperature (24°C) and humidity following the recommendations of NIH. The animals were maintained on a normal animal diet and tap water ad libitum. Cardiomyopathic animals were divided into two groups. The first group consisted of 2-month-old animals (prehypertrophic stage, n=14) that were treated with losartan (25 mg/kg/day) for an 8-week period. The drug was administered daily by gavage. The second group (2-month-old animals) was treated with vehicle for an 8-week period and was used as controls. All animals were housed in a temperature-controlled room, on a 12-hour light dark cycle.
Pastel pipette. The suspension was filtered through a nylon gauze and the filtrate was centrifuged 4 minutes at 22g. The pellets were resuspended in normal Krebs solution. All experiments were made at 36°C.

Suction pipettes were pulled from micro-hematocrit tubing by means of a controlled puller (Narishige) and their tips polished with a microforge (Narishige). The pipettes, which were prepared immediately before the experiments, were filled with the following solution (mM): CsCl 110; NaCl 10; TEA 10; MgCl2 1; CaCl2 1; EGTA 10; HEPES 5, pH adjusted to 7.3.

**Experimental procedure**

All experiments were performed in a small chamber mounted on the stage of an inverted phase-contrast microscope (Diaphot, Nikon). The gj was measured using two separated voltage clamp circuits. Gigaohm sealing was achieved in each cell and then the surface membrane of both cells was broken by application of a stronger suction (-35 to -65 cm H2O) and a whole cell clamp configuration was produced. Each pipette was connected to a separated voltage clamp amplifier (Axon Instruments, CA) that made it possible to control the non-junctional membrane potential in each cell as well as the voltage gradient across the intercellular junction.

The experimental procedure consisted in applying the membrane potential of both cells at -40 mV. Cell 1 was then pulsed to 0 mV while the membrane of cell 2 was unchanged. Data acquisition and command potentials were controlled with a software program (PCLAMP 8, Axon Instruments).

Since the ratio of series resistance (Rs) to input resistance (Rm) is an important factor in the determination of the gj, the experimental protocol was designed to provide a maximised value of IR. This was accomplished by using Cs as a major action on the internal solution and Ba was added to the bath solution to block the potassium conductance. The resistance of the filled pipettes varied from 3–6 MΩ. These procedures resulted in an input resistant of about 2.5–3 GΩ. The pipettes Rs was carefully measured by applying an identical voltage step about simultaneously to both cells. The value of Rs was in the range of 6–10 MΩ. The membrane resistance (Rm) was also measured by applying an identical command voltage pulse to both cells at the same time and measuring the steady state currents in both cells. Values of Rm were found to be in the range of 1.5–1.6 MΩ and much higher than the values of Rs. Although Rs did not vary in the majority of the experiments, in two experiments there was a change in Rs and the value of gj was corrected using the following equation: $G_j = I_2/(V_1-I_2R_s + I_2R_s)$ where $V_1$, $I_1$ and $I_2$ are voltage command and currents recorded from cell 1 and 2, respectively while Rs is the series resistance.

**Measurements of membrane potential**

Intracellular microelectrodes connected to a high impedance DC amplifier (WP Instruments, FL, USA) were used to measure the transmembrane potentials. The left ventricular wall was dissected and transferred to a bath through which oxygenated Krebs solution flowed continuously (36°C). The composition of Krebs solution was as follows (mM): NaCl 150; KCl 5.1; CaCl2 1.8; MgCl2 1; HEPES 5; glucose 5, pH adjusted to 7.3. The solution was saturated with 100% O2. The muscle was stimulated with a fine pair of platinum electrodes (0.3 mm diameter) by using rectangular current pulses generated by an electronic stimulator and isolation unit (Grass Instruments, Boston). The intensity of current pulses was twice threshold and the duration of the pulses was 3 ms. Voltage calibration was determined by injecting known voltages between Krebs solution and ground.

**Determination of the % area of interstitial fibrosis**

To determine the presence of fibrosis coronal sections taken from the equator of the heart were obtained and immediately fixed in formalin (10%). Some sections were stained with haematoxylin-eosin and other with Sirius red or Masson for the study of interstitial fibrosis. Connective tissues and muscle areas were manually traced using a digitised pad connected to a computer. The area fraction of interstitial fibrosis was calculated as the sum of all connective tissue areas divided by the sum of all connective and muscle areas in the field. Tissues were analysed using a LSM 5 Pascal Confocal System, Carl Zeiss (Microimaging, Inc). Measurements of % of fibrosis were performed using a MCID (MicroComputer Imaging Device, Amersham Biosciences, Canada).

**Drugs**

Losartan was from Merck Sharp and Dohme.

**Statistical analysis**

Data are mean±SEM. Statistical significance for changes in conduction velocity, interstitial fibrosis and transmembrane potentials was determined with analysis of variance (ANOVA) and defined as a value of p<0.05. Since measurements of gj were clearly separated into two groups (high and low gj) the Fisher’s exact probability test was used.

**Results**

Measurements of gj performed on cell pairs isolated from the ventricle of control 4-month-old
CM hamsters (TO-2) showed a relatively large number of cells weakly coupled. Two major populations of cell pairs with respect to the values of gj were found; one in the range of 2 to 8 nS and the other with higher values of gj (18–45 nS) (figure 1). Since the value of Rs was in the range of 6–10 MΩ, it is reasonable to assume that the values of gj for these cell pairs are underestimated by about 5%.

Since Ang II reduces gj in the failing heart 3 the question whether chronic blockade of AT1-receptors reverses the gradual decline of gj during the process of heart failure merits serious consideration.

To investigate this possibility, 2-month-old cardiomyopathic hamsters were treated with losartan (25 mg/kg/day) for a period of 8 weeks. At the end of this period, the animals were anaesthetised and the heart was removed under deep anaesthesia. Measurements of gj were performed in isolated cell pairs as described above.

The results showed that the number of cells with very low values of gj (2–8 nS) was significantly reduced by losartan while the group with larger values of gj (18–45 nS) was significantly increased as shown in figure 1. Measurements of gj performed in control hamsters (F1B) showed no significant changes in gj (n=12) (p>0.05) after treatment with losartan (25 mg/kg/day) for a period of eight weeks.

An electrophysiological exploration performed in the isolated left ventricular wall with intracellular microelectrodes indicated a significant decrease of the number of fibres showing non-propagated action potentials when compared with age-matched controls (figure 2). Moreover, the conduction velocity measured in the longitudinal direction, was significantly increased in the animals treated with losartan as shown in figure 3.

**Losartan decreases the % area of interstitial fibrosis**

Confocal microscopy performed on the heart of cardiomyopathic hamsters at four months of age, showed interstitial fibrosis and severe separation of muscle fibres. Measurements of % area of fibrosis in the left ventricle was performed on the heart of animals treated with losartan for two months and the results were compared with age-matched controls. As shown in figure 4 the drug reduced the area of fibrosis from 22±1.4% (control) (n=18) to 14±1.3% (n=18) (p<0.05) after drug administration.

**Discussion**

It is known that overexpression of Ang II AT1-receptor transgene in the mouse myocardium causes myocyte hyperplasia and heart block.8 The present results provide evidence, by the first time, that chronic blockade of Ang II AT1-receptors reduced gap junction remodelling in the failing heart and increased the values of gj. Indeed, after
Evidence is available that Ang II causes cardiac longitudinal and transversal propagation. An increase of the spread of electrical activity in the ventricle of cardiomyopathic hamsters is the average from 14 animals. Vertical line at each bar SEM (p<0.05) b: Laser confocal microscopy showing interstitial fibrosis in the left ventricle of a cardiomyopathic hamster 4-month-old Calibration-100 µm.

In other studies it was found that losartan (25 mg/kg/day/po) plus hydrochlorothiazide (6.5 mg/kg/day/po) administered for a period of eight weeks, reduces the arterial blood pressure and the heart-to-body mass ratio. However, hydrochlorothiazide alone did not reduce cardiac remodelling despite the reduction in blood pressure.

The mechanism by which losartan increased the gap probably multifactorial. Ang II, for instance, is known to activate different intracellular pathways such as protein kinase C (PKC) and tyrosine kinases with consequent phosphorylation of junctional proteins and decrease of gj. Moreover, the activation of tyrosine kinases is involved in the effects of Ang II in different systems, which supports the notion that the suppression of AT1 receptor activation and consequent decrease of phosphorilation of junctional proteins elicited by losartan might represent an important factor in the effect of the drug on cell communication. Although it is known that the activation of AT1-receptors can activate tyrosine kinases the mechanism involved is not clear. In some cases, however, Ca2+ and PKC are involved. Since it is known that high enough intracellular calcium can uncouple gap junction channels and that Ang II increases Ca2+ an additional mechanism for the action of losartan might be a possible decrease of the intracellular calcium concentration.

In the failing heart, Northern and Western blot analysis demonstrated a marked decrease of connexin 43 transcript and protein. Therefore, an improvement of expression and distribution of the connexin 43 due to chronic losartan administration, might contribute to the improvement of cell coupling found with the drug. This view is supported by the finding that in hypertrophied ventricles of rats under pressure overload the decrease in number of Cx43 and its abnormal distribution was reversed by losartan. In vitro studies performed on cultured neonatal cardiomyocytes submitted to chronic administration of Ang II showed, however, increased expression of connexin 43.
Although the reason for this difference is not known, neonatal cardiac cells seem to be quite different from adult differentiated myocytes. Indeed, Saris et al.24 were unable to detect Ang II generation in neonatal cardiomyocytes after addition of prorenin to the medium.

Finally, considering that the activation of an intracrine RAS is involved in the regulation of cell communication in the failing heart1 and that intracellular Ang II generated inside the cell or receptors located at surface cell membrane. The generation of drugs with the ability to suppress the intracellular Ang II might represent an important step in the treatment of heart failure.

In conclusion, the chronic blockade of AngII AT1 receptors increased impulse propagation in the failing heart in part by increasing the gj and in part by reducing interstitial fibrosis and structural remodelling.

Acknowledgement
This work was in part supported by grants HL-34148, and G1280-0351 from NIH.

References
3. De Mello WC. Renin angiotensin system and cell communication in the failing heart. Hypertension 1996; 2 1267-72