Oxidative stress, sodium excretion and blood pressure in response to chronic stress in aged rats

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KEYWORDS
Hypertension; Aging; Oxidative status; Chronic stress; Endothelial dysfunction.

ABSTRACT
Hypertension is an important cause of cardiovascular morbidity and mortality and is age dependent. Moreover, the rate of aging and the onset of age-related diseases are modulated by the stress response. The aim was investigate the renal and oxidative response to chronic stress in aged rats, as predisposing factors to increase blood pressure with age. Male Wistar rats of three (young) or eight (old) months of age were divided into: controls or chronic immobilized rats. Blood pressure, natriemia, aldosterone, corticosterone, gamma-glutamyl-transferase, nitrites, uric acid and renal malonyldialdehyde, catalase and superoxide dismutase activity were measured. Corticosterone and blood pressure were higher in old than young stressed rats. Antinatriuresis in all stressed rats was found. Natriemia increased only in old stressed rats. No differences were found in superoxide dismutase and catalase activity in response to stress but a major increase in gamma-glutamyl-transferase and renal malonyldialdehyde and a decrease in nitrites were observed in old stressed rats. Aged rats have an increased oxidative state and a greater stress response. Increased natriemia, bigger oxidative stress and reduced NO bioavailability in stressed old than young rats could cause an attenuated vasorelaxant response and could explain, in part, the blood pressure increase with age.

INTRODUCTION
Hypertension (HT) remains the most important cause of cardiovascular morbidity and mortality worldwide. As HT is age dependent, with the prolongation of life expectancy it affects more and more older people. The prevalence of HT in this population is above 60% and continues to grow[1]. Moreover, the rate of aging and the onset of age-related diseases are modulated by the stress response[2]. Stress and other behavioral factors have been associated with a variety of cardiovascular diseases including hypertension[3]. Although the problem of stress-related hypertension has been addressed in several experimental studies, there are still conflicting data as to the nature of the cardiovascular changes induced by stressors.

Aged adults show heightened and more prolonged stress responses compared with younger adults[4]. It
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has been demonstrated that hypersecretion of gluco-corticoids in elderly involve prolonged activation of the stress axis, suggesting a difficulty to finish the response whit age due to an improper inhibitory feed-back\[5,6]\.

On the other hand, aging is also strongly correlated with endothelial dysfunction in animals and humans. It has been reported that during senescence are an increased production of endothelium-derived vaso-constrictor factors and superoxide anion. These changes may increase vascular resistance generating increases in blood pressure\[7\]. Another factor that may alter the vascular resistance is the increase in arterial stiffness which may be attributed to age-related loss of distensibility in the major central arteries\[8\] when elastic tissue is progressively replaced with collagen\[9\] or to endothelial dysfunction caused by free oxygen radicals in the arterial wall\[1,10\].

Age-related changes in renal structure and function have been described, not just in humans but in a wide range of other species, including rats, mice, hamsters, dogs, and cats. Modifications of the kidneys related to aging include changes to glomerular structure which can often be accompanied by tubular functional changes. These changes include a reduction in sodium homeostasis and blunted renin response\[11\]. In previous reports in our laboratory\[12-15\], we have demonstrated that daily exposure to acute or chronic immobilization stress (IMO) in normotensive young Wistar rats leads to a reduction in the renal sodium excretion. Antinatriuresis induced by chronic stress could be the result of sympathetic and the renin-angiotensin stimulation\[13,14\]. It is known that increased levels of angiotensine II and aldosterone stimulate oxidative NADPH activity and increased vascular superoxide anion with the consequent decrease of NO of bioavailability\[16,17\].

Taking into account that stress response is higher in aged animals, the purpose of this work was to investigate the renal and oxidative response to chronic stress in this age group, as predisposing factors to increase blood pressure with age.

**METHODS**

**Animals and general conditions**

Male Wistar rats bred in the animal house at the University of Rio Cuarto, Argentina, under standard conditions (kept with the light on from 07.00 to 19.00 h at 20 ± 2 °C) were used. Two groups of male rats of three (young) or eight (old) months of age, weighting 250-300 g or 450-500 g respectively were placed in individual cages, with wood shaving bedding. Food and water were freely available ad libitum. Before the beginning of the experiment 2, all rats were placed three times into metabolic cages to become accustomed to them. All experiments have been reviewed and approved by the UNRC Research Ethics Committee (Res CS 253/10 National University of Rio Cuarto, Argentina) who ensures that the animals were properly cared.

**Experiment 1**

Two groups of animals of each age were considered: controls rats and animals with 14 stress sessions (chronic stress). Stressed rats were immobilized 1h/day (from 11 AM to 12 PM) by taping their four limbs to metal mounts attached to wooden boards as it was previously described by Recepczeka and Mikula\[16\]. In the control rats a single record of blood pressure was performed, whereas in the stressed animals two subgroups were considered: stress (immediately post-stress record) and recovery (6 hours post-stress record). Systolic (SBP), diastolic (DBP) and media (MBP) blood pressure was recorded through a heparinized polyethylene catheter inserted into carotid artery and connected to a pressure transducer (Hewlett-Packard model 21080A) coupled to a polygraph Hewlett-Packard 78901 A in rats previously anesthetized with Ketamine hydrochloride, 5 mg/kg ip.

**Experiment 2**

Two groups of rats for each age were considered: control and chronic stress. Immediately after the last IMO, tail blood samples of control and stressed rats were collected into ice-cold heparinized capillary tubes. Immediately after, all the animals were placed into stainless steel metabolic cages for urine collection. The rats were maintained in the cages for 6 h since in previous studies, we found that stress-induced sodium excretion changes were observed only in the 6 h post IMO\[19\]. After this period, animals were decapitated and blood was collected. Kidneys and adrenal glands were removed within 2 minutes of decapitation.

**Determinations**

In the blood samples, corticosterone, aldosterone,
glucose, creatinine, osmolarity, gamma glutamyl transferase (GGT) and total nitrites (NOx) were determined. In the urine samples sodium, creatinine and volume were measured. Na⁺ levels were analyzed with an analyzer that determines ion concentrations through selective ion electrode. Total microequivalents of Na⁺ excreted were calculated taking into account the volume of urine excreted in the experimental period.

Plasma corticosterone levels were measured by radioimmunoassay (RIA) as described previously by Armario and Castellanos[20] with a modification: corticosterone-binding-globulin was denatured by heating the samples at 70 C for 30 min. Inter and intra-assay coefficients of variation were 12 and 8 per cent, respectively. Plasma aldosterone levels were determined by RIA. Inter- and intra-assay coefficients of variation were 5 and 13 per cent, respectively.

Plasma glucose, GGT (biomarker of oxidative stress), plasmatic and urinary creatinine were measured with commercial kits. Creatinine Clearance (CC) was calculated. Osmolarity was determined with osmometer that detects changes in the vapor pressure of a solution.

Nitric oxide (NO) was estimated through its stable metabolites NO₂, NO₃ and Total NO₂(NOx) by the Griess method[21].

Adrenal glands were immediately defatted, weighed and the adreno-somatic index (ASI), as adrenal weight (g)/body weight (g) x 1000, was calculated. Then, the glands were placed into 10% buffer formalin until cortex/medulla index (CMI) determination. For the cortex ratio determination, the adrenal were processed by routine histological techniques, sectioned at 5 um thickness and stained with haematoxilin-eosin. Sections were photographed in 5X magnification, and areas of each zone were determined in pixels, which were analyzed with the Image-J program (Wright Cell Imaging Facility, Toronto Western Research Institute, Toronto, Canada). The area of the entire gland and adrenal medulla was determined. The cortex area was calculated by subtracting the total area of the medullary area. With these areas, cortex/medulla ratio for each animal was calculated.

Renal thiobarbituric acid reactive substances (TBARs) concentrations, expressed as nmol MDA/g of tissues were measured spectrophotometrically at 532 nm in kidney homogenates by the method of Marcincak et al[22]. Renal SOD activity was determined by the method of Misra and Fridovich[23] based on the ability of SOD to inhibit the epinephrine auto-oxidation at alkaline pH. The absorbance was measured at 480 nm for 1 min, and the enzymatic activity was expressed as U/mg protein.

Catalase activity was determined according to the method described by Aebi[24] based on the consumption of H₂O₂ at room temperature. The absorbance was measured at 240 nm and catalase activity was expressed as pmol/mg protein.

The statistical significance was evaluated by the statistical software STATISTICA. Two-way analysis of variance (ANOVA) with the factors treatment (control; stress) and age (young; old) was used for renal sodium excretion, urinary volume, ASI, CMR, CC, GGT, MDA, SOD, CAT activity and NOx comparisons. A three-way ANOVA with the factors: Treatment (control; stress), Age (young; old) and Time (stress; Recovery) were used for plasma glucose, corticosterone, aldosterone, osmolarity, protein and sodium and systolic, diastolic and media blood pressure comparisons. In all experiment, Duncan test was applied as a post hoc test.

**RESULTS**

**Blood pressure**

Basal SBP, DBP and MBP were higher in old than young rats (p=0.00006, p=0.0003, p=0.00006, respectively). SBP, DBP and MBP increased in response to stress in old (p=0.0003, p=0.0007 and p=0.00004, respectively) and young animals (p=0.00003, p=0.00002 and p=0.00003, respectively), but this response was higher in old than young rats (SBP p=0.004, MBP p=0.03). SBP, DBP and MBP remain elevated in stress recovery period in old animals (p=0.0002, p=0.001 and p=0.04, respectively) while only SBP and MBP remain elevated en young rats (p=0.03 and p=0.02) (Figure 1).

**Plasma corticosterone**

Old animals showed higher basal corticosterone levels than young rats (p=0.00008). An increase in plasma corticosterone levels in response to stress in both age groups (young: p=0.00002, old: p=0.00002) were observed. This response was higher in old
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An increase in plasma glucose was observed in response to stress in both age groups (young: \( p=0.003 \); old: \( p=0.0001 \)). This response was higher in old (\( p=0.008 \)) than young animals and remained higher after stress in both age groups (young: \( p=0.03 \); old \( p=0.0002 \)) (TABLE 1).

**Plasma glucose**

* An increase in plasma glucose was observed in response to stress in both age groups (young: \( p=0.003 \); old: \( p=0.00001 \)). This response was higher in old (\( p=0.008 \)) than young animals and remained higher after stress in both age groups (young: \( p=0.03 \); old \( p=0.0002 \)) (TABLE 1).

**Adreno-somatic index and cortex/medulla ratio**

IAS was higher in stressed than controls animals (\( p=0.000002 \)) but cortex-medulla ratio increased in response to stress only in old rats (\( p=0.04 \)) (TABLE 1).

**Natriuresis**

Old control animals showed higher renal sodium excretion than young control (\( p=0.007 \)). Rats of the two age groups showed lower excretion in response to stress (young: \( p=0.03 \), old: \( p=0.0006 \)) (Figure 2).

**Urinary volume and creatinine clearance**

The urine output and creatinine clearance increased in old animals compared with young (\( p=0.000001 \), \( p=0.04 \), respectively). An increases in creatinine clearance in response to stress in both age groups was observed (\( p=0.00007 \)) (TABLE 2).

**Aldosterone levels**

Old control animals showed higher aldosterone levels than young control rats (\( p=0.0003 \)). Plasma aldosterone levels were higher in IMO than non stressed rats in both age groups (young: \( p=0.00006 \) and old: \( p=0.00006 \)).

In both stressed groups, aldosterone levels remained high in the recovery period (young: \( p=0.0001 \) and old

| TABLE 1 : Corticosterone, Glucose, Adreno Somatic Index and Cortex/Medulla Ratio |
|---------------------------------|---------|-----|-------|
|                                  | CORT (ug/dL) | Glucose (g/L) | ASI | CMR |
| Young Rats                       |           |               |     |     |
| C                                | 2.7±0.8  | 1.0±0.1       | 0.8±0.1 | 6.4±0.8 |
| S                                | 32.9±1.1 * | 1.2±0.1 *   | 1.2±0.2 | 6.4±0.1 |
| SR                               | 35.2±1.1 ** | 1.1±0.1 ** |       |     |
| Old Rats                         |           |               |     |     |
| C                                | 12.1±0.9 # | 0.9±0.1    | 0.9±0.1 | 6.2±0.3 |
| S                                | 49.9±2.6 * & | 1.4±0.1 * & | 1.6±0.1 * | 7.8±0.6 * |
| SR                               | 41.3±2.1 ** | 1.1±0.1 ** |       |     |

Means ± SEM are represented. CORT: corticosterone, ASI: adreno somatic index, CMR cortex/medulla ratio. C= Control, S= Stress, SR= Stress Recovery. * \( p<0.05 \) vs. C, ** \( p<0.05 \) vs. C; # \( p=0.000008 \) vs. Young C rats; & \( p<0.05 \) vs. Young S rats.
No significant differences were found in plasma osmolarity and proteins in both age groups (TABLE 2). Plasma sodium increased in response to stress only in old animals \((p=0.01)\) and did not return to control values at 6 hours post-stress \((p=0.001)\) (Figure 3).

### Oxidative stress markers

Plasma GGT activity increased in response to stress in both age \((young: p=0.001, old: p=0.00003)\) being greater in old animals \((p=0.00006)\). Old control animals showed greater MDA levels \((p=0.00006)\). An increase in renal MDA was observed in response to IMO in both age \((young: p=0.006, old: p=0.00003)\) but this response was higher in older than younger individuals \((p=0.0006)\) (TABLE 3).

### Renal superoxide dismutase and catalase activity

No differences in SOD and CAT activity in response to IMO in both age were found (TABLE 3).

### Total nitrites

Old control animals showed lower NOx levels than young control rats \((p=0.004)\). A decrease in NOx serum values was observed in stressed rats \((young: p=0.00003, old: p=0.04)\), but this response was greater in young than in old animals \((p=0.002)\) (Figure 4).

### Table 2: Urine volume, creatinine clearance and plasma aldosterone, osmolarity and proteins.

<table>
<thead>
<tr>
<th></th>
<th>Young Rats</th>
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<tbody>
<tr>
<td></td>
<td>U (mL/6h)</td>
<td>CC (mL/min)</td>
<td>ALDO (pg/mL)</td>
<td>Osmolarity (mmol/L)</td>
<td>Proteins (g/dL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>C</td>
<td>1.7±0.3</td>
<td>1.2±0.1</td>
<td>217.6±12.6</td>
<td>296±2.9</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td>S</td>
<td>1.4±0.1</td>
<td>1.7±0.1 *</td>
<td>1280.5±73.9 *</td>
<td>293.7±7.9</td>
<td>6.0±0.3</td>
</tr>
<tr>
<td>SR</td>
<td>867.7±34 **</td>
<td></td>
<td></td>
<td>293.5±4.8</td>
<td>5.8±0.2</td>
</tr>
</tbody>
</table>

**Means and SEM are represented. U: urine volume, CC creatinine clearance. ALDO: aldosterone. C= Control, S= Stress, SR= Stress Recovery. * p<0.05 vs. C, ** p<0.05 vs. Young C rats, ** p<0.05 vs. C.**

### Table 3: Plasma gamma glutamyl tranferase, renal malonyldialdehyde, renal superoxide dismutase and catalase.

<table>
<thead>
<tr>
<th></th>
<th>GGT (U/L)</th>
<th>MDA (nmoles/g Tissue)</th>
<th>SOD (U/mg Tissue)</th>
<th>CAT (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Young Rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>7.3±0.4</td>
<td>46.3±2.4</td>
<td>3.2±0.3</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>S</td>
<td>11.6±0.4 *</td>
<td>48±1.7 *</td>
<td>3.5±0.4</td>
<td>0.5±0.1</td>
</tr>
</tbody>
</table>

**Means ± SEM are represented. GGT= gamma glutamyl tranferase, MDA= malonyldialdehyde, SOD= superoxide dismutase, CAT= catalase. C= Control, S= Stress. * p<0.05 vs. C, & p<0.05 vs. Young S rats, # p=0.00006 vs. Young C rats.**
DISCUSSION

In the present work, higher corticosterone levels were found in response to stress in both groups, reflecting the activation of the hypothalamic-pituitary axis. Aged rats showed higher response than young rats which was consistent with the highest adrenosomatic index and cortex/medulla ratio found in this group. These results show a major stress response in aged rats and are coincident with those reported by Romeo et al. and Blake et al.\[4,25\] who indicate that corticosterone changes in response to stress appear to depend on alterations in HPA axis with age due to a decreased number of hippocampal neurons, essential in regulating structure of the termination of the response to stress\[6\]. Moreover, corticosterone values in old control rats were higher than those found in young control individuals and agree with those reported by Mc Ewen\[26\] who showed an increase HPA axis activity and glucocorticoids levels with age.

An increase in SBP, DBP and MBP values in response to stress were found in both age groups although these values were higher in old than young stressed rats. Moreover, blood pressure values in old control rats were higher than those found in young control animals. The greatest BP response to stress is coincident with a larger HPA axis activation in aged rats, indicating an association between these variables.

On the other hand, a decrease in sodium excretion was observed in all stressed rats which were coincident with a raise in the CC suggesting an increase in glomerular filtration rate. Therefore, lower sodium excretion would be independent of glomerular filtration and could be related to aldosterone increase observed in stressed rats. It is noteworthy that older control animals excreted more sodium than younger control animals despite having higher aldosterone levels.

It is known that blood pressure is regulated by sodium and water balance, the renin-angiotensin system, the sympathetic nervous system and vasoactive substances such as nitric oxide\[27,28\]. However, kidneys have a key role in long-term blood pressure regulation. Individuals without an adequate compensatory increase in the urinary sodium excretion in response to a stress-induced blood pressure increase show a delayed BP recovery after stress, which seemed to be at least partly, because of their increased blood volume\[29\]. In this work, no changes in plasma protein or osmolarity were observed suggesting that plasma volume remained unchanged despite antinatriuresis observed in stressed rats.

An increase in plasma sodium levels was found in aged stressed rats. Small rise in plasma sodium (1-3 mmol/L) are be related with hypertension but is not clear whether such a small increase in plasma sodium per se contribute to the development of hypertension\[29\]. In vitro experiments have shown that when extracellular sodium concentration is raised by about 5% endothelial cells stiffen within minutes by up to 25%. This strong response of the endothelium to small changes in sodium concentration is dependent upon aldosterone\[31\]. The increase in sodium and aldosterone plasma levels found in stressed aged rats could be related with higher blood pressure observed in this age group. In addition, natremia in old rats remains high still at 6 hours after finalization of the stress session. This alteration in sodium homeostasis in response to chronic stress may lead to changes in vascular elasticity and sustained increase in BP.

In the same way, several lines of evidence suggest that high salt intake is related to impair NO generation. Fujiwara et al.\[32\] reported that high salt intake is negatively correlated with total nitrite and nitrate concentrations in human plasma. In our work a decrease in serum NOx in all stressed rats was observed. Coincidentally about 2 mmol/L increase in plasma sodium in aged IMO rats with normal salt intake but diminished sodium excretion was found. This small increase in plasma sodium could be related with the minor NO bioavailability. However, in young animals, a decrease in plasma NOx levels without changes in natremia was
observed. It is known that increased ROS vascular production is another factor that may affect NO bioavailability\cite{33}. In the current study, an increment in renal MDA and plasma GGT without changes in CAT and SOD activity were observed in stressed rats. Aged animals showed higher increase in MDA and GGT response to stress. It has been reported that antioxidant system activity decrease with age leading to a progressive imbalance between prooxidants/antioxidants and generate oxidative damage\cite{34,35}. Stress would shift this imbalance toward oxidizing forms reducing vascular NO, increasing the vascular tone and blood pressure.

In summary, aged rats have an increased oxidative state due to the aging process and a greater stress response as evidences by higher activation of the HPA axis, increased natremia, oxidative stress and reduced NO bioavailability could cause an attenuated vasorelaxant response and could explain, in part, the blood pressure increase with age.

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