

# The Expression of RAC1 and Mineralocorticoid Pathway-Dependent Genes are Associated With Different Responses to Salt Intake

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## BACKGROUND

Rac1 upregulation has been implicated in salt-sensitive hypertension as a modulator of mineralocorticoid receptor (MR) activity. Rac1 could affect the expression of oxidative stress markers, such as hemoxygenase-1 (HO-1) or nuclear factor- $\kappa$ B (NF- $\kappa$ B), and the expression of neutrophil gelatinase-associated lipocalin (NGAL), a cytokine upregulated upon MR activation.

## AIM

We evaluated RAC1 expression in relation of high salt intake and association with MR, NGAL, HO-1, and NF- $\kappa$ B expression, mineralo- and glucocorticoids levels, and inflammatory parameters.

## SUBJECTS AND METHODS

We studied 147 adult subjects. A food survey identified the dietary sodium (Na) intake. RAC1 expression was considered high or low according to the value found in normotensive subjects with low salt intake. We determined the gene expression of RAC1, MR, NGAL, HO-1, NF- $\kappa$ B, and 18S, isolated from peripheral leukocytes. We measured aldosterone, cortisol, sodium, potassium excretion, metalloproteinase (MMP9 y MMP2), and C-reactive protein.

## RESULTS

We identified 126 subjects with high Na-intake, 18 subjects had high, and 108 low-RAC1 expression. The subjects with high-RAC1 expression showed a significant increase in MR ( $P = 0.0002$ ), NGAL ( $P < 0.0001$ ) HO-1 ( $P = 0.0004$ ), and NF- $\kappa$ B ( $P < 0.0001$ ) gene expression. We demonstrated an association between RAC1 expression and MR ( $R_{sp}$  0.64;  $P < 0.0001$ ), NGAL ( $R_{sp}$  0.48;  $P < 0.0001$ ), HO-1 ( $R_{sp}$  0.53;  $P < 0.0001$ ), and NF- $\kappa$ B ( $R_{sp}$  0.52;  $P < 0.0001$ ). We did not identify any association between RAC1 and clinical or biochemical variables.

## CONCLUSIONS

RAC1 expression was associated with an increase in MR, NGAL, NF- $\kappa$ B, and HO-1 expression, suggesting that RAC1 could be a mediator of cardiovascular damage induced by sodium, and may also useful to identify subjects with different responses to salt intake.

**Keywords:** blood pressure; essential hypertension; hypertension; PBMC; RAC1; gene expression; salt intake.

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Arterial hypertension (AH) is one of the most frequent pathologies in the general population and is estimated to affect approximately 26% of the world's population.<sup>1</sup> According to the latest Health National Survey, the current prevalence of AH in Chile is 27% (Ministry of Health Chile, ENS 2009). As a result of current changes in epidemiological patterns, the global prevalence of hypertension

could increase to 30% by 2025 (1.5 billion individuals).<sup>1</sup> Essential AH is a polygenic disorder that results from the complex interplay between genetic predispositions and environmental influences. In addition to the well-known effects of high-dietary salt intake and its subsequent increase in blood pressure (BP), salt loading also causes cardiovascular (CV) and kidney damage, induces hypertrophy of vascular

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smooth muscle cells, increases NADPH oxidase activity and oxidative stress, and reduces the availability and production of nitric oxide in animals and humans.<sup>2-4</sup>

The recent National Health Survey (Ministry of Health Chile, ENS 2009) showed the average Chilean salt intake is approximately 9 g, higher to the 5 g intake recommended by the World Health Organization (WHO). In United States, several studies from 1957 to 2003 also showed a growing daily sodium intake, a phenomenon that is independent of age, gender, and ethnicity.<sup>5</sup> It is also important to highlight that the BP response to sodium intake differs among individuals; thus, individuals can be classified as salt-sensitive (SS) and salt-resistant (SR) hypertensives.<sup>6,7</sup> Several research groups have attempted to identify individuals that belong to each group of subjects, and salt sensitivity has been defined as a change in BP of at least 10 mm Hg following a salt-loading test (2 l of NaCl 0.9% in 4 hours).<sup>3,8</sup> The increase in BP in response to dietary sodium depends on the amount of sodium intake (NaCl, halide salts, ionic salt (Na<sup>+</sup>))<sup>9</sup> and the ethnicity of the individual.<sup>5</sup> However, there is no direct and useful tool or biochemical marker to identify SS and SR subjects, independent of interventional studies.

Recently, it was shown that excessive salt intake can cause MR activation,<sup>10</sup> which leads to the synergistic action of aldosterone and high salt intake, although salt decreases the circulating aldosterone levels.<sup>11</sup> The mechanism mediating the paradoxically activation of the mineralocorticoid receptor (MR) cascade due to high salt had long been elusive. Although several research groups have proposed that MR biological activity is influenced by other factors than aldosterone, recent studies have revealed a cross-talk between Rac1, a small GTP-binding-protein, and MR activation independent of aldosterone, which has been associated with the development of SS hypertension and CV damage.<sup>12,13</sup> Rac1 is a member of the Rho family of small GTPases involved in signal transduction pathways, and it has been proposed as a potential MR activator.<sup>13</sup> This mechanism of MR activation would be associated with the development of SS hypertension and CV damage.<sup>12,13</sup>

Rac1 is part of the NADPH oxidase complex, which induces the generation of reactive oxygen species (ROS), such as superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), leading to increased alterations in the cell membrane and endothelial damage. Oxidative stress has been shown to play a key role in cardiac pathologies associated with MR,<sup>14</sup> potentially through the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), an important transcription factor of many proinflammatory cytokines.<sup>15</sup> Another stress-response protein is hemeoxygenase-1 (HO-1), which appears to have a protective role in the vascular wall against atherogenesis through several pathways. However, few reports have examined the regulation of Rac1 expression through a MR-dependent mechanism in renal disease. The consequences of MR activation are able to induce CV damage independent of the increase in arterial blood.<sup>16</sup> Neutrophil gelatinase-associated lipocalin (NGAL) is a mineralocorticoid target gene in the CV system and is modulated by aldosterone/sodium and MR antagonism.<sup>17</sup>

In this study, we evaluated the effects of high salt intake on the RNA expression of RAC1 (MIM 602048) to identify

RAC1-sensitive (RAC1-S) and RAC1-resistant (RAC1-R) subjects. Clinical and biochemical parameters were assayed to determine any potential association with RAC1, MR (MIM 600983), NGAL (MIM 600181), HO-1 (MIM 141250), and NF- $\kappa$ B (MIM 164014) gene expression.

## SUBJECTS AND METHODS

### Subjects

We designed a cross-sectional study and recruited 147 subjects with an age from 16 to 60 years. Patients with renal disease, diabetes mellitus, hepatic failure, cardiac failure, primary aldosteronism, clinical Cushing's disease, and patients treated with glucocorticoids were excluded. Patients who were using antihypertensive drugs that affect the renin-angiotensin system, such as  $\beta$ -blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, diuretics, and spironolactone, were also excluded.

The protocol followed in this study was written according to the guidelines of the Declaration of Helsinki and was approved by the Ethical Committee of the Faculty of Medicine, Pontificia Universidad Catolica de Chile. The study and protocol were explained to all participants and written informed consent was obtained.

### Clinical characteristics and study protocol

All subjects underwent a complete physical exam. Height was measured using a wall-mounted Harpenden stadiometer Holtain (Crymych, Pembrokeshire, UK), and weight and total fat mass percentage were assessed by bioelectrical impedance (Tanita; Corporation of America, Arlington Heights, IL). Trained nurses measured the BP and heart rate in all subjects. Three measurements were obtained from the right arm at consecutive 5-minute intervals using an oscillometric method (Dinamap CARESCAPE V100, GE Healthcare, Medical Systems Information Technologies, Milwaukee, WI) with the subjects in a seated position. A food survey was conducted to determine the intake of sodium/day with a threshold of 5 g salt/day (2000 mg sodium/day), according to the recommendation of the World Health Organization (WHO) 2013. Following these criteria, we determined an upper cutoff for RAC1 expression based on the percentile 95 (p95) observed in normotensive subjects with normal salt intake (<2000 mg sodium/day), whose value is 88.6 relative units (RU) to 18S expression in RNA isolated from peripheral blood monocyte cells (PBMC).

### Biochemical and hormonal analyses

Following an overnight fast in all subjects, basal blood samples were obtained between 8:00 and 10:00 AM. The subjects were in a sitting position for at least a 15-minute rest period before blood sampling.

Serum aldosterone (SA) was assayed using a commercial radioimmunoassay kit (Coat-A-Count kit, Siemens, CA). Plasma renin activity (PRA) was measured by radioimmunoassay using a commercial kit (DiaSorin, Stillwater, MN).<sup>18</sup>

Cortisol and cortisone were quantified using HPLC-MS/MS (Agilent 1200, ABI Sciex API4500-Qtrap).

Serum MMP-9 and MMP-2 activities were estimated by zymography as previously described.<sup>19</sup> The results are expressed as arbitrary units of the number of changes with respect to the reference plasma used as an internal control. hsCRP was measured with a nephelometric assay (BN ProSpec Systems, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Total 24-hour urine was also collected and measured; 50 ml was used to measure creatinine, potassium, and sodium and to store aliquots for further urinary biochemical assays. Urinary creatinine was measured by the Jaffe method with automated equipment (Modular Analytics, Roche, Germany). We also calculated the fractional excretion of sodium in 24 hours (FENa, %) and the fractional excretion of potassium (FEK, %). The serum, plasma, and urine samples were stored at -80 °C until analysis. Patients without a full sample at the 24-hour urine collection were not included in this analysis.

### Quantification of mRNA

Total RNA was isolated from PBMCs with TRIZOL reagent (Invitrogen, San Diego, CA). Real-time polymerase chain reaction (qRT-PCR) amplification of the RAC1, MR, NGAL, HO-1, NF-κB, and 18S genes was performed using the RotorGene-6000 instrument (Corbett Research, Sydney, Australia). The sequence of primers used in this study are described in Table 1.

RNA expression was quantified by RT-PCR using fluorescent SYBR-Green technology and was expressed in RU with respect to the 18S housekeeping gene. The PCR analysis was performed in a 12.5 μl of MAXIMA (K0222, Fermentas), 2.5 pmol of both forward and reverse primers, 1 μl of cDNA (from 2 μg RNA), and nuclease-free water up to 25 μl of the final volume. The amplification reactions were performed as follows: initial denaturation at 95 °C for 5 minutes, 40 cycles of 95 °C for 15 seconds, annealing temperature (Table 1) for 20 seconds and 72 °C for 20 seconds, and final extension at 72 °C for 7 minutes. The amplified gene products were visualized using SYBR Safe DNA Gel Stain (Life Technologies) on a 2% agarose gel.

### Data analysis

The results are expressed as the median (inter-quartile range, (Q1–Q3)). Statistical comparisons were performed

using the Mann–Whitney or unpaired t-tests. Associations were analyzed by Spearman ( $R_{sp}$ ) correlation with GraphPad Prism v5.0 software. Differences and associations were considered significant at  $P < 0.05$ .

## RESULTS

From the nutritional survey, we identified 21 subjects with normal Na-intake and 126 subjects with high Na-intake. In the high Na-intake group, 18 subjects had high RAC1-expression and 108 subjects had low-RAC1 expression; the subjects were defined as RAC1-S and RAC1-R subjects, respectively. The baseline characteristics of both groups are shown in Table 2. Both groups were comparable in terms of age, gender, body mass index, sodium intake, and urinary sodium excretion (Table 2). Diastolic (DBP) and systolic blood pressure (SBP) showed a trend to higher values in RAC1-S than RAC1-R. DBP was higher in RAC1-S than RAC1-R by parametric comparison ( $t$ -test,  $P = 0.046$ ).

### RAC1 expression and mineralocorticoid pathway associated genes

We analyzed the effects of high salt intake on the gene expression in both groups of subjects, including RAC1-S and RAC1-R. We determined that the expression of MR, NGAL, HO-1, and NF-κB was increased in the RAC1-S subjects compared with the RAC1-R subjects: MR (13.9 (6.7–40.0) vs. 1.1 (0.3–5.4) RU;  $P = 0.0002$ ), NGAL (22.3 (11.6–43.9) vs. 2.8 (0.8–9.7) RU;  $P < 0.0001$ ), HO-1 (21.7 (6.9–26.0) vs. 1.3 (0.3–5.8) RU;  $P = 0.0004$ ) and NF-κB (17.6 (12.1–23.4) vs. 3.1 (0.7–6.2) RU;  $P < 0.0001$ ) (Table 3).

We also performed association studies that assayed RAC1 expression with MR, NGAL, HO-1, and NF-κB. In the group with high salt intake ( $n = 126$ ), we observed a positive association between RAC1 and MR ( $R_{sp} 0.64$ ;  $P < 0.0001$ ), NGAL ( $R_{sp} 0.48$ ;  $P < 0.0001$ ), HO-1 ( $R_{sp} 0.53$ ;  $P < 0.0001$ ), and NF-κB ( $R_{sp} 0.52$ ;  $P < 0.0001$ ) (Figure 1).

### RAC1 expression and biochemical parameters

In both groups of subjects (RAC1-S and RAC1-R), the blood pressure and levels of PRA, aldosterone and cortisol were similar. The inflammatory variable hsCRP had a tendency for greater values in RAC1-S compared with RAC1-R (1.85 vs. 1.44 mg/l,  $P = 0.09$ ). The biochemical variable urinary

**Table 1.** Primers used for RT-PCR amplification of RAC1 and MR, NGAL, HO-1, NF-κB in human samples

GENE	FW	RV	pb	Tm
RAC1	ACGCCCTATCCTATCCGCAAAC	CGCTGTGTGAGCGCCGAGCA	345/288	58 °C
MR	TGCACCAAATCAGCCTTCAGTTTCG	TGAGGCCATCCTTTGGAATTGTGC	105	58 °C
NGAL	CGAGTTCACGCTGGGCAAC	CGATGTGGTTTTTCAGGGAGG	230	56 °C
HO-1	GACAGTTGCTGTAGGGCTTTA	CATAGGCTCCTTCCTCCTTTC	198	54 °C
NF-κB	CTGTCCTTTCTCATCCCATCTT	CCGTGAAATACACCTCAATGTC	159	54 °C
18S	ACTGGCTCAGCGTGTGCCTAC	TAGTAGCGACGGCGGTGTGTAC	179	58 °C

Abbreviations: HO-1, hemoxigenase-1; MR, mineralocorticoid receptor; NGAL, neutrophil gelatinase-associated lipocalin.

**Table 2.** Clinical characteristics of individuals with high intake of sodium stratified as RAC1-sensitive or RAC1-resistant subjects

Parameters	RAC1-sensitive	RAC1-resistant
N	18	108
Gender, % female	77.7	59.3
Age, years	36.3 (26.3–47.9)	41.7 (29.2–50.5)
Height, cm	160.2 (155.0–167.5)	163.0 (157.0–171.8)
BMI	27.0 (22.5–32.5)	27.6 (24.9–29.9)
SBP, mm Hg	127.5 (112.8–153.2)	122.3 (112.1–138.7)
DBP, mm Hg	81.8 (74.2–98.6)	80.2 (73.7–88.2)
Sodium intake, mg/24 h <sup>a</sup>	2,685 (2,394–4,571)	2,978 (2,672–3,790)
Urinary sodium excretion, mEq/24 h	128.0 (90.3–204.3)	141.0 (112.5–183.0)

Values correspond to median Q1–Q3. Statistical analyses were performed by Mann–Whitney test (\* =  $P < 0.05$ ). Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

<sup>a</sup>A food survey was conducted to determine the intake of sodium/day (higher salt intake >5 g salt/day or equivalent >2000 mg sodium/day).

**Table 3.** Gene expression of MR, NGAL, NF-κB, and HO-1 genes in RAC1-resistant and RAC1-sensitive subjects

Gene expression	RAC1-sensitive	RAC1-resistant	P value
MR	13.98 (6.75–40.02)	1.13 (0.30–5.42)	0.0001
NGAL	22.34 (11.64–43.92)	2.80 (0.78–9.69)	<0.0001
NF-κB	17.59 (12.11–23.41)	3.14 (0.74–6.19)	<0.0001
HO-1	21.73 (6.96–26.02)	1.29 (0.34–5.64)	0.0003

Statistical analyses were performed by Mann–Whitney test (significant  $P < 0.05$ ).

Abbreviations: HO-1, hemoxygenase-1; MR, mineralocorticoid receptor; NGAL, neutrophil gelatinase-associated lipocalin.

potassium was increased in RAC1-S subjects compared with the RAC1-R subjects (46.8 (38.5–63.8) vs. 40.8 (32.3–53.2) mEq/g creatinine;  $P = 0.03$ ). Other variables in the serum and urine were not significantly different (Table 4).

When all subjects were analyzed together, we did not identify a significant association between RAC1 and SBP, DBP, PRA, aldosterone, cortisol, or the other biochemical variables evaluated (data not shown). We only observed a weak significant association with urinary potassium ( $R_{sp}$  0.188;  $P = 0.035$ ).

**DISCUSSION**

In this study, we examined the effects of high-salt intake on the RNA expression of RAC1 in Chilean adult subjects. We identified two groups of subjects, i.e., individuals with high RAC1 expression, named RAC1-S, and individuals with low RAC1 expression, named RAC1-R. The RAC1-S subjects exhibited increased expression of specific genes related to the mineralocorticoid pathway, such as MR and NGAL, and genes related to oxidative stress, such as HO-1 and NF-κB.

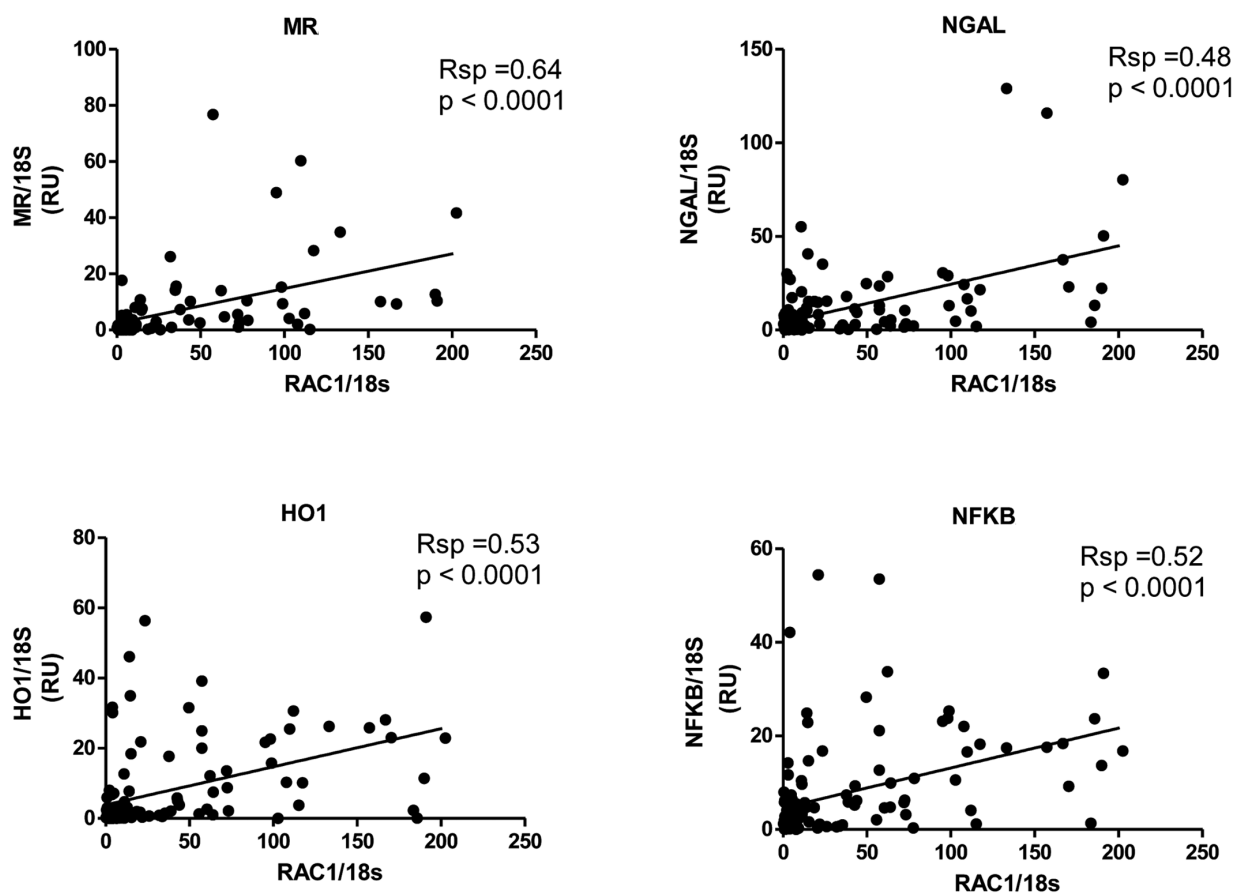
The RAC1-S subjects exhibited increased MR expression compared with the RAC1-R subjects in a high-salt intake condition.<sup>20–23</sup> In the distal nephron of the kidney, the mineralocorticoid pathway has a pivotal role in the homeostatic regulation of electrolytes, fluid volume, and blood pressure.<sup>24</sup> In the present study, the evidence suggests that RAC1 affected sodium reabsorption or potassium excretion, where urinary potassium excretion was increased in RAC1-S compared with RAC1-R, and in the total group urinary

potassium excretion was also associated with RAC1 expression.<sup>25–27</sup> In a high-salt diet, the Rac1-MR pathway could contribute to the development of hypertension, proteinuria, glomerular sclerosis, and tubule-interstitial injury, which have been observed in Dahl-S rats.<sup>20,21</sup>

We observed that NGAL expression was higher in the RAC1-S subjects compared with the RAC1-R subjects. In addition to previous evidence that NGAL functions as a biomarker in ischemic and nephrotoxic renal injury,<sup>28</sup> it has also been observed that aldosterone induced NGAL RNA expression in a dose-dependent manner.<sup>17</sup> NGAL is a MR-dependent cytokine, which may increase in expression in patients with renal failure<sup>29</sup>; furthermore, in agreement with the current results, NGAL might also act as a potential early marker of RAC1 activation in a high-salt diet.

Rac1-MR could also induce oxidative stress through ROS formation.<sup>22,23</sup> We observed that the gene expression of two known markers of oxidative stress, including NF-κB and HO-1, increased in RAC1-S but not in RAC1-R subjects. The increment of ROS might be triggered by different mechanisms, including Rac1-dependent activation of NADPH oxidase.<sup>30</sup> In hypertensive rats, both the expression and activity of NADPH oxidase subunits are increased, which supports a role for ROS production in the pathogenesis of hypertension.<sup>4</sup> In this respect, Sulciner *et al.* showed in HeLa cells that Rac1 protein activates NF-κB and regulates ROS production.<sup>31–33</sup> Highlighting the ROS formation, we detected higher levels HO-1, which has been shown to have a physiological role in the protection of cells from oxidative stress and pathological functions when ROS are





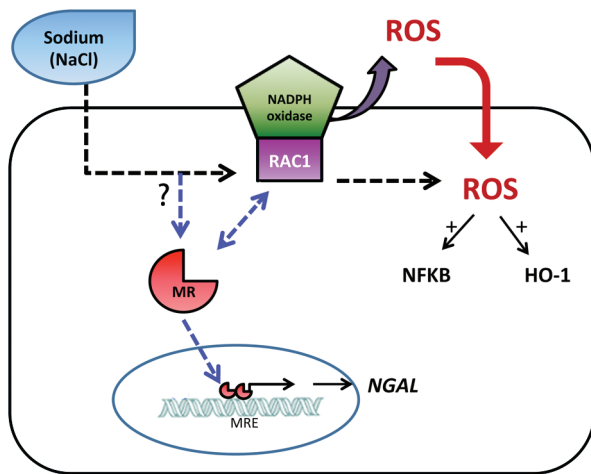
**Figure 1.** Spearman correlation (Rsp) of RAC1 expression with MR, NGAL, HO-1 and NF-κB in 126 subjects with high-salt intake. Abbreviations: HO-1, hemoxygenase-1; MR, mineralocortic receptor.

**Table 4.** Biochemical characteristics of individuals with high intake of sodium stratified as RAC1-sensitive or RAC1-resistant subjects

Characteristics	RAC1-sensitive	RAC1-resistant	P value
<b>Serum</b>			
Aldosterone, ng/dl	8.55 (5.27–17.73)	7.25 (5.12–11.40)	0.28
PRA, ng × ml/h	1.70 (0.72–2.15)	1.15 (0.61–1.68)	0.08
hsCRP, mg/l	1.85 (1.16–6.18)	1.44 (0.68–3.13)	0.09
MMP9 activity	1.04 (0.95–1.54)	1.29 (1.05–1.59)	0.40
MMP2 activity	1.07 (1.03–1.24)	1.11 (1.00–1.23)	0.87
<b>Urinary</b>			
Sodium excretion, mEq/g creatinine	129.2 (84.97–179.3)	122.4 (96.61–157.0)	0.66
FENa (24h), %	0.70 (0.46–0.88)	0.65 (0.53–0.81)	0.70
Potassium excretion, mEq/g creatinine	46.86 (38.45–63.79)	40.81 (32.26–53.20)	0.03
FeK (24h), %	8.51 (6.46–9.85)	7.40 (6.04–8.84)	0.13
MAC, mg/24 h	8.25 (2.65–21.58)	4.70 (2.60–8.62)	0.15
F/E urinary	0.87 (0.28–0.43)	0.38 (0.30–0.47)	0.29
Cortisol urinary, ng/ml	11.52 (5.69–15.03)	11.73 (8.22–18.40)	0.16
Cortisone urinary, ng/ml	28.40 (18.40–37.70)	31.80 (25.70–39.20)	0.19

Values correspond to median (Q1–Q3). Statistical analyses were performed by Mann–Whitney test (significant P value < 0.05).

Abbreviations: hsCRP, high sensitive c-reactive protein, PRA, plasma renin activity; MMP, metalloproteinase; FENa, fractional excretion of sodium; FEK, fractional excretion of potassium; F/E, cortisol to cortisone ratio.



**Figure 2.** Hypothetical model showing the effect of sodium inducing a higher expression of RAC1 and MR, which could induce NGAL expression and also stimulate the production of ROS in NADPH oxidase complex. Secondary, ROS can also induce the oxidative stress markers NF-κB and HO-1. Abbreviations: HO-1, hemoxigenase-1; MR, mineralocorticoid receptor; ROS, reactive oxygen species.

present.<sup>34</sup> The higher NF-κB and HO-1 RNA levels suggests that RAC1-induced ROS generation, which has a critical role in salt-induced vascular and renal damage.<sup>13</sup>

We determined that C-reactive protein (hsCRP) had a tendency to increase in RAC1-S compared with RAC1-R. Increased hsCRP is indicative of an inflammatory response, and it is now widely accepted as a marker of atherosclerosis and CV disease.<sup>35,36</sup> Rac1 has been shown to play a role in the regulation of gene expression involved in vascular cell function and inflammation,<sup>37,38</sup> which have been associated with hypertensive disease<sup>39</sup>; therefore, it is an attractive possibility that high-salt intake promoted injury through a Rac1-mediated mechanism. We did not identify a correlation with other biochemical markers of inflammation or endothelial damage, which suggests that more sensitive and specific markers are required to detect this early damage.

This study has some limitations, because we did not examine salt-sensitivity of blood pressure and we only examined Rac1 mRNA expression not Rac1 activity. Future studies are needed to elucidate both RAC1 expression and activity in PBMC and other local tissues (i.e., adipose, kidney, blood vessels) of subjects with and without a diet-interventional study.

In summary, we identified higher expressions of RAC1 and genes related to the mineralocorticoid pathway, renal damage (NGAL), inflammation (NF-κB), and ROS formation (HO-1) in Chilean subjects with a high-salt intake. These findings suggest that RAC1 could represent a mediator of CV damage induced by sodium and a potential marker of salt responsiveness.

**SUPPLEMENTARY MATERIAL**

Supplementary materials are available at *American Journal of Hypertension* (<http://ajh.oxfordjournals.org>).

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**DISCLOSURE**

The authors declared no conflict of interest.

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