Primed Polymorphonuclear Leukocytes, Oxidative Stress, and Inflammation Antecede Hypertension in the Sabra Rat

Shifra Sela, Rafi Mazor, Mazal Amsalam, Chana Yagil, Yoram Yagil, Batya Kristal

Abstract—Hypertension is accompanied by systemic oxidative stress, inflammation, and priming of peripheral polymorphonuclear leukocytes (PMNLs), yet the involvement of these factors in the pathophysiology of hypertension is incompletely understood. We investigated the relationship between oxidative stress, primed PMNLs, and inflammation and the development of hypertension in the Sabra rat model of salt-sensitive hypertension. Sabra hypertension-resistant rats (SBN/y) (salt-resistant) and Sabra hypertension-prone rats (SBH/y) (salt-sensitive) were studied under normal conditions or during salt loading. Systolic blood pressure (BP) was measured by the tail-cuff method. The extent of oxidative stress was evaluated by the rate of superoxide release from PMNLs, plasma-reduced glutathione (GSH) levels, malondialdehyde (MDA) levels (estimated by thiobarbituric acid–reacting substances), and plasma-carbonylated fibrinogen (Western blotting). Plasma fibrinogen levels and the peripheral PMNL count served as indices of inflammation. In SBH/y and SBN/y provided regular chow without salt loading, BP did not rise above baseline values, yet superoxide release, plasma MDA, carbonylated fibrinogen, and PMNL count were higher in SBH/y than in SBN/y, whereas GSH levels were lower in SBH/y. Four weeks of salt loading resulted in a gradual increase in systolic BP in SBH/y to 205±3 mm Hg, whereas BP remained in SBN/y at baseline normotensive levels. All the parameters reflecting oxidative stress and inflammation were further aggravated with the development of hypertension in salt-loaded SBH/y. We conclude that primed PMNLs, oxidative stress, and inflammation antecede the development of hypertension in this experimental model of hypertension. (Hypertension. 2004;44:764-769.)

Key Words: leukocytes ■ oxidative stress ■ rats, Sabra

Hypertension constitutes a major risk factor for cardiovascular, cerebrovascular, and renal disease. Among the known mechanisms associated with hypertension are salt sensitivity, insulin resistance, imbalance in the renin-angiotensin system, and perturbed endothelial function. In the last decade, oxidative stress (OS) has emerged as an additional major player in hypertension.1-7 Activation of vascular NAD(P)H oxidases and the production of reactive oxygen species (ROS) by these enzymes are common in hypertension and other cardiovascular diseases.8 An additional possible pathway for ROS production is through circulating leukocytes that may contribute to OS and thereby to the pathophysiology of hypertension.9-10

The polymorphonuclear leukocyte (PMNL) is an inflammatory cell type that circulates in 1 of 3 functional states: quiescent, primed, or activated. The quiescent PMNL that encounters a stimulus converts to a primed state; on encountering a second stimulus, the primed PMNL is fully activated, releasing ROS, proteases, and granule contents to the surrounding milieu and resulting in OS.11-13

There is evidence for a primed state of PMNLs in experimental models of hypertension as well as in humans. In the spontaneously hypertensive rat (SHR), circulating PMNLs have been reported to be in a primed state with increased ROS formation.14 In the Dahl model of hypertension, there has been a report of an increased number of activated neutrophils that undergo spontaneous degranulation in the circulation of the salt-sensitive compared with the salt-resistant strain.15 We have reported previously in patients with essential hypertension that PMNLs are in a primed state relative to healthy subjects and upon stimulation, release superoxide at a faster rate.5,6 We have also found in hypertensive patients that the PMNL count and the rate of superoxide release are significantly correlated with mean arterial pressure (S. Sela, B. Kristal, unpublished data, 2004). Other studies have shown that an elevated PMNL count per se constitutes a risk factor for cardiovascular and cerebrovascular disease and that increased superoxide release contributes to hypertension.2,15-19 Finally, in SHR in which superoxide release is increased,9 administration of superoxide dismutase normalizes blood pressure (BP).20,21
Although it is well established by now that in hypertension, there is a significant correlation between the rate of superoxide release from primed PMNLs and BP\textsuperscript{5,6} and that superoxide levels are increased in hypertension\textsuperscript{2,15,20} it is still uncertain whether these factors are causative of and contribute to the pathophysiology of hypertension or are merely secondary to hypertension. In the current study, we tested the hypothesis that primed PMNLs, OS, and inflammation precede the development of hypertension, using the Sabra rat model of experimental hypertension, the advantage of which is that it uniquely allows to study the hypertension-prone strain before and in the course of the development of hypertension\textsuperscript{22}.

Methods

Animals

Male Sabra hypertension-prone rats (SBH/y) and Sabra hypertension-resistant rats (SBN/y) were obtained from the colony bred at the Israeli Rat Genome Center (Ashkelon, Israel, www.irgc.co.il). Animals were housed in the animal facility on the campus of the Western Galilee Hospital (Nahariya, Israel) in strict compliance with the guidelines set forth by the American Physiological Society. The Technion institutional animal committee approved all animal experiments.

Feeding Protocol

Animals were allowed free access to standard rat chow (0.3% sodium chloride) and drinking water ad libitum, unless stated otherwise.

Salt Loading

To induce hypertension in this model, animals were salt loaded by implanting a 25-mg deoxycorticosterone acetate pellet (Innovative Technologies Co., Israel, www.intc.co.il). Animals were housed in the animal facility on the campus of the Western Galilee Hospital (Nahariya, Israel) in strict compliance with the guidelines set forth by the American Physiological Society. The Technion institutional animal committee approved all animal experiments.

Study Groups

Forty-eight animals were divided into 2 groups: an experimental and a control group, each consisting of 24 animals. The experimental group was salt loaded and consisted of 2 subgroups: SBH/y+ (n=12) and SBN/y+ (n=12). The control group was provided standard rat chow and tap water ad libitum and consisted of 2 additional subgroups: SBH/y− (n=12) and SBN/y− (n=12).

Study Protocol

Experiments were initiated shortly after weaning at age \(\sim6\) weeks. Systolic BP was measured before salt loading and at weekly intervals thereafter for a total of 4 weeks. Upon termination of the experiments, animals were anesthetized (10% chloralhydrate, 200 mg/kg with sodium pentobarbitone, 6 mg/kg IP), blood was drawn from the bifurcation of the aorta, and the animals were killed.

BP Measurements

Systolic BP measurements were performed at ambient temperature (26 to 28\(^\circ\)C) in awake animals by the tail-cuff method using the IITC-31 computerized BP device (IITC Life Science), as described previously\textsuperscript{22}. Each BP value was derived from \(\geq3\) replicate measurements.

Plasma and PMNL Isolation

Blood was drawn into heparinized plastic tubes and immediately centrifuged at 10\(^{\circ}\)C. The plasma was divided into aliquots and stored at \(-70\^{\circ}\)C. Immediately after plasma removal, the blood volume was substituted with saline, and PMNL separation was performed as described previously by Klebanoff and Clark\textsuperscript{7} with minor modifications, described in an online Methods supplement (available at http://www.hypertensionaha.org).

Rate of Superoxide Release Measurements

The rate of superoxide release was determined from 0.32\(\times10^{-7}\) mol/L phorbol 12-myristate 13-acetate (PMA)-stimulated 10\(^6\) PMNLs as described previously\textsuperscript{3,24}.

Determination of Plasma-Reduced Glutathione Levels

Determination of plasma glutathione (GSH) was performed according to Adams et al and Griffith\textsuperscript{25–27} using oxidized GSH reductase. Determination of GSH is described in the online Methods supplement.

Determination of Plasma Malondialdehyde Levels

Malondialdehyde (MDA) levels were determined using the assay for thiobarbituric acid–reacting substances (TBARS) as described previously\textsuperscript{28}. Details are described in the online Methods supplement.

Plasma Oxidability

Susceptibility of plasma lipids to oxidation (oxidability) was measured after oxidation with 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH; Wako Pure Chemical Industries) by determination of plasma MDA levels. Five plasma aliquots containing 0.5 mL plasma were diluted 1:1 in PBS and oxidized by the addition of 50 \(\mu\)L 1 mol/L AAPH. Samples were incubated for 0, 2, 4, 7, and 24 hours, followed by TBAR detection as described online.

PMNL Count

Two separate counts were performed: first, 20 \(\mu\)L of blood was added to 380 \(\mu\)L of 2% acetic acid colored pale violet solution, and incubated for 1 minute to lyse the erythrocytes. Leukocytes were counted in a counting chamber under light microscope. To determine the percentage of PMNLs, a second count was performed on a slide after cyto-spin of whole blood, using 2% acetic acid colored pale violet solution.

Preparation and Quantification of Carboxylated Fibrinogen Standard

Fibrinogen was oxidized in vitro by iron/ascorbate using the OxyBlot-Protein oxidation detection kit (INTERGENE) to yield a highly carboxylated protein. The quantification of carboxyl content was performed as described previously\textsuperscript{29}. The amount of carboxyls on fibrinogen was expressed as nanomoles carbonyl per milligram fibrinogen protein.

Preparation of Plasma Proteins for Carbonyl Detection

Because fibrinogen is highly susceptible to oxidation, carboxylated fibrinogen therefore served as a marker for protein oxidation\textsuperscript{30,31}. Plasma samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH), and carbonyls were quantified as described previously\textsuperscript{29} and briefly described in the online supplement.

Detection and Quantification of Carboxyls on Plasma Fibrinogen

The plasma–DNPH-treated samples and a fixed amount of the oxidized fibrinogen standard were separated by SDS-PAGE gels containing 8% acrylamide in parallel on 2 gels, as described in the online Methods supplement.

We estimated fibrinogen levels from the coomassie blue–stained gels as a measure of ongoing inflammation. To identify the fibrinogen band, Western analysis was performed with rabbit polyclonal anti-fibrinogen serum (ICN Pharmaceuticals) and goat anti-rabbit–horseradish peroxidase conjugate (OxyBlot Kit).
Statistical Analysis

Data are expressed as mean±SD, unless stated otherwise. In the boxes and whiskers presentations, the horizontal line in the middle shows the median (50th percentile). The top and bottom of the box show the 75th and 25th percentiles, respectively. The whiskers show the maximum and the minimum values. Statistical significance of differences in mean values was tested by 1-way ANOVA for repeated measures and the Bonferroni multiple comparison test or by Student unpaired t test, as applicable. The statistical significance was set at P<0.05.

Results

Blood Pressure

Tail-cuff systolic BP of the 4 study groups are shown in the table. Basal BP was ≈20 mm Hg higher in SBH/y than in SBN/y, as described previously in the Sabra strains.22 Four weeks of salt loading increased systolic BP in SBH/y, whereas BP remained unchanged in salt loaded SBN/y, as described previously.22

PMNL Count

The PMNL count in all groups is shown in Figure 1. The number of circulating PMNLs in salt-loaded SBH/y was 3-fold higher than in salt-loaded SBN/y (2990±880 cells/mm³ versus 1022±820 cells/mm³, respectively; P<0.001). The difference in the PMNL count between the strains could not be accounted for by salt loading, per se, because control SBH/y also had higher levels of circulating PMNL than control SBN/y (2340±900 cells/mm³ versus 776±230 cells/mm³, respectively; P<0.01). In addition, salt loading per se did not significantly affect the number of circulating PMNLs within strain, neither in SBH/y nor in SBN/y.

Rate of Superoxide Release

The rate of superoxide release from PMA-stimulated PMNLs, which reflects the priming state of these cells in vivo, is shown in Figure 2. Superoxide release was higher in salt-loaded SBH/y than in salt-loaded SBN/y (0.33±0.07 versus 0.23±0.07, respectively; P<0.05). Control SBH/y also released superoxide faster than control SBN/y (0.30±0.10 versus 0.22±0.05, respectively; P<0.05). Salt loading per se did not significantly affect the rate of superoxide release within strain, neither in SBH/y nor in SBN/y.

Plasma Levels of GSH

GSH is a ubiquitous intracellular and extracellular antioxidant and a neutralizer of oxidants such as hydrogen peroxide and superoxide. Plasma GSH levels are shown in Figure 3. GSH levels were significantly lower in salt-loaded SBH/y

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<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Blood Pressure (mm Hg)</th>
<th>Week 4 Blood Pressure (mm Hg)</th>
<th>Baseline vs Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBN/y</td>
<td>120±2</td>
<td>120±2</td>
<td>NS</td>
</tr>
<tr>
<td>SBN/y+ Salt</td>
<td>117±1</td>
<td>119±1</td>
<td>NS</td>
</tr>
<tr>
<td>SBH/y</td>
<td>140±1*</td>
<td>140±1</td>
<td>NS</td>
</tr>
<tr>
<td>SBH/y+ Salt</td>
<td>140±2</td>
<td>205±3</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Data are provided as mean±SE.*P<0.05 SBH/y vs SBN/y at baseline.
compared with salt-loaded SBN/y (2.52 ± 0.73 nmol/mL versus 3.52 ± 1.00 nmol/mL, respectively; P < 0.05). In the control groups, GSH levels tended to be lower in SBH/y compared with SBN/y, but the differences did not achieve statistical significance (3.07 ± 0.80 nmol/mL versus 3.53 ± 0.70 nmol/mL, respectively; P = NS).

Fibrinogen Oxidation
Fibrinogen levels and carbonylated fibrinogen, the latter being a marker of protein oxidation, are shown in Figure 4A and 4B, respectively. Fibrinogen and carbonylated fibrinogen levels were significantly higher in salt-loaded SBH/y than in salt-loaded SBN/y (3.86 ± 1.25 mg/mL versus 2.79 ± 1 mg/mL, respectively; P < 0.05 for fibrinogen; 11.14 ± 4.3 nmol/mg versus 6.97 ± 1.53 nmol/mg, respectively; P < 0.05 for carbonylated fibrinogen). There were more carbonyl groups per milligram of fibrinogen in SBH/y compared with SBN/y, indicating that the increase in carbonylated fibrinogen was of greater magnitude than the increase in fibrinogen. There was no difference in the fibrinogen level between control SBH/y and control SBN/y. The levels of carbonylated fibrinogen tended to be higher in control SBH/y than in control SBN/y, although not achieving statistical significance (8.21 ± 2.09 nmol/mg versus 5.47 ± 1.65 nmol/mg, respectively; P = NS).

Plasma Lipid Peroxidation and the In Vitro Susceptibility of Lipids to Oxidation
Plasma MDA levels, shown in Figure 5A, were significantly higher in SBH/y than in SBN/y, irrespective of salt loading.

As shown in Figure 5B, MDA levels were consistently higher in salt-loaded and control SBH/y compared with salt-loaded and control SBN/y at most time points of oxidation. In SBH/y but not in SBN/y, plasma lipid oxidability significantly increased during salt loading, suggesting a role of salt loading per se, which appears to be limited to the hypertension-prone group.

Discussion
In the current study, we addressed the role of primed PMNLs, OS, and inflammation in the pathophysiology of hypertension. We confirmed a high prevalence of primed PMNLs in hypertension. Our novel findings consist, among others, of a higher rate of superoxide release that is selective to the SBH/y strain, whereas the animal is, before salt loading and still, normotensive. OS was manifested in the SBH/y strain in its basal prehypertensive state by oxidative indices that included a low GSH, although significant only when hypertensive, increased MDA, and carbonylated fibrinogen levels. We also found evidence for markers of inflammation, including an elevated number of circulating PMNLs and an increased plasma fibrinogen levels. When hypertension eventually developed after salt loading in SBH/y strain, all of the parameters reflecting OS and inflammation were augmented. This study thus confirms previous reports by our group and by others showing a high prevalence of PMNL priming in hypertension in humans and in experimental models of hypertension and supports our hypothesis that primed PMNLs, OS, and inflammation

Figure 4. Plasma fibrinogen (A) and carbonylated fibrinogen (B) were measured in SBH/y and SBN/y with (+) or without (−) salt loading as described in Methods.*P < 0.05 SBH/y+ vs SBN/y+.

Figure 5. Plasma MDA levels before (A) and after (B) in vitro oxidation in SBH/y and SBN/y rats, with (+) or without (−) salt loading. A, *P < 0.05 SBH/y− vs SBN/y−; **P < 0.05 SBH/y+ vs SBN/y+. B, *P < 0.05 SBH/y− vs SBN/y− after 2 hours of oxidation; **P < 0.01 SBH/y+ vs SBN/y+ after 8 hours of oxidation; ***P < 0.05 SBH/y− vs SBN/y− after 24 hours of oxidation; ****P < 0.001 SBH/y+ vs SBN/y+ after 24 hours of oxidation.
The presence of OS has been demonstrated previously in several other rat models of hypertension. The contribution of OS to hypertension has been inferred indirectly by additional studies that have successfully attenuated the rise in BP by administration of antioxidants or substances with antioxidative properties. In the present study, OS was evident in SBH/y from oxidation of proteins (carboxylated fibrinogen), lipids (MDA), and a higher susceptibility for lipid peroxidation. It is noteworthy that OS was present already in the SBH/y strain under basal conditions when BP was normal. The relationship of OS to salt susceptibility per se (ie, the sensitivity to salt in terms of the development of hypertension) is also suggested by these findings. Our data also indicate that salt loading and the development of hypertension aggraves OS. This is observed exclusively in the SBH/y strain. In SBN/y, salt loading per se did not affect OS parameters. These results suggest that in the SBH/y strain, OS antecedes the elevation in BP.

The possible contribution of PMNL to hypertension is noteworthy because among the potential contributors to OS, the role of the primed PMNLs has largely been overlooked. A higher rate of superoxide release by primed PMNLs can in fact constitute a continuous and important source for ROS. In the current study, we demonstrated a higher rate of superoxide release in the SBH/y strain under basal conditions, even before salt loading or development of hypertension with only a small additional increase after salt loading. In the SBN/y strain, superoxide release by PMNLs was low and did not increase with salt loading. Because salt loading did not ensue in PMNL priming in SBN/y, we conclude that priming of PMNLs is part of the constitutional phenotype of the SBH/y. These findings are consistent with our previous reports in which we found that priming of PMNLs in humans and that an elevation in BP were associated with faster rates of superoxide release from PMNLs.

Priming of PMNLs and an increase in PMNL count can be viewed as markers of an ongoing inflammatory state. In clinical states such as hypertension, diabetes type 2, and uremia, which are often characterized by some as chronic noninfectious inflammatory states, a mild elevation in PMNL counts has been reported, albeit in the normal upper quintile only. In the current study, we found a significant increase in the circulating PMNL count in hypertensive salt-loaded SBH/y compared with normotensive salt-loaded SBN/y. These results are consistent with those reported in the Dahl model of salt-sensitive hypertension and in the SHR. However, it should be noted that an elevated circulating PMNL count was already apparent in the SBH/y strain under basal conditions and before salt loading or the development of hypertension. Salt loading per se did not affect the PMNL count nor in SBN/y. Development of hypertension in SBH/y did not significantly affect the PMNL count either. These findings relating to the PMNL count suggest indirectly that a chronic inflammatory state is ongoing in the salt-sensitive SBH/y strain before the development of hypertension. Combined with the elevated rate of superoxide release, the higher numbers of PMNLs within the circulation most likely impose a heavy burden on the antioxidative mechanisms resulting in OS, which further aggravates the inflammatory state. Such an inflammatory state may predispose the animal to the development of hypertension under the appropriate environmental conditions, accounting for the hypertension-prone phenotype of SBH/y. In addition, the high level of the acute phase protein fibrinogen in the hypertensive SBH/y group lends additional support to a chronic inflammatory state, once hypertension has developed, as reported previously in hypertension.

We conclude from this study that OS and an active inflammatory state anteced the development of hypertension, although no causal proof between OS and inflammation has been established. These conclusions are mostly based on the differences we found in markers of OS and inflammation between SBH/y and SBN/y under basal conditions, when both strains are still normotensive. However, because systolic BP is ~20 mm Hg higher in SBH/y than in SBN/y, we cannot entirely rule out that some of the differences we are reporting in this study are related to this BP difference. However, the likelihood is small because the major and significant rise in BP that occurred after salt loading did not alter significantly the priming expressed by the rate of superoxide release nor the PMNL count, whereas the parameters of OS and inflammation were significantly augmented by the development of hypertension. Finally, whether OS and inflammation, which precede hypertension, contribute to the susceptibility to develop hypertension or are epiphenomena linked but not causative of hypertension remains to be determined.

Perspectives

Our conclusions from this study extend well beyond hypertension because OS and inflammation are implicated in many other disease states. The development of novel treatment strategies such as prevention of PMNL priming and attenuation of OS and inflammation may delay or prevent development of the disease.

References

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