Oxidative Stress, Inflammation

Tumor Necrosis Factor-α
A Possible Priming Agent for the Polymorphonuclear Leukocyte–Reduced Nicotinamide-Adenine Dinucleotide Phosphate Oxidase in Hypertension

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Abstract—In the Sabra rat, oxidative stress (OS) and inflammation precede the development of hypertension. Inhibition of the phagocytic NADPH oxidase attenuates the rise in blood pressure. The present study was set to identify possible priming agents for this enzyme and to test the hypothesis that the phagocytic NADPH oxidase contributes to OS and inflammation. Sabra salt-sensitive and Sabra salt-resistant rats were salt loaded or provided regular chow for 60 days with or without apocynin to inhibit NADPH oxidase. Levels of interleukin 6, tumor necrosis factor-α, and interferon-γ served as indices of inflammation. Extracellular and intracellular levels of the polymorphonuclear leukocyte tumor necrosis factor-α receptors (p55 and p75) were assessed by flow cytometry in young and adult rats. NADPH oxidase activity and expression of p47phox were measured in polymorphonuclear leukocytes and aortic rings. Malondialdehyde and carbonylated fibrinogen served as indices of OS. Inflammatory and OS indices excluding interferon-γ were higher in the hypertensive state and reduced by apocynin. Levels of malondialdehyde and tumor necrosis factor-α were elevated already in the prehypertensive state. No differences were found in the levels of p75. The extracellular expression of p55 was higher in adult Sabra salt-resistant compared with Sabra salt-sensitive rats (7.46±2.2% versus 2.1±0.5%; P<0.05), whereas levels of the intracellular p55 were higher in adult Sabra salt-sensitive rats (3.2±2% versus 1.1±0.5%; P<0.05). In young normotensive rats, the extracellular levels of p55 were higher in Sabra salt-sensitive compared with Sabra salt-resistant rats (10.6±5.2% versus 2.9±1.5%; P<0.01). Tumor necrosis factor-α plays a role in activation of the polymorphonuclear leukocyte NADPH oxidase, thereby contributing to systemic OS, inflammation, and the development of hypertension in this model. (Hypertension. 2010;55:353-362.)

Key Words: hypertension ▪ TNF-α ▪ TNF-α receptor ▪ NADPH oxidase ▪ oxidative stress ▪ inflammation

Oxidative stress (OS) and inflammation are important components in the pathophysiology of hypertension.1–6 We have shown that the polymorphonuclear leukocyte (PMNL) is a significant contributor to OS and inflammation in hypertensive humans and in animal models of hypertension. In pathological disorders associated with atherothrombosis and cardiovascular diseases, such as hypertension, diabetes mellitus, and end-stage renal disease, PMNLs are primed.7–9 In the primed state, PMNLs are more sensitive to local or systemic stimuli because of a previous exposure to a priming agent. Hence, on encountering an additional stimulus, full cell activation occurs, resulting in a robust release of reactive oxygen species and tertiary granule contents into the bloodstream.10

The primary source for reactive oxygen species in PMNL is the NADPH oxidase, an enzymatic complex, composed of 3 cytosolic subunits (p40phox, p47phox, and p67phox) and 2 membrane subunits (p22phox and gp91). On cell activation, the cytosolic subunits translocate to the membrane, and the activated enzyme produces superoxide in a process known as the “respiratory burst.”11

We have reported previously that, in the Sabra rat model of hypertension, priming of PMNLs precedes the development of hypertension and is associated with an increased rate of superoxide release from PMNLs as result of PMNL priming and NADPH oxidase activation.12,13 We have also shown that inhibition of this enzyme with apocynin (APO) attenuates the development of hypertension, concomitantly with a decline in leukocyte count and cell priming.13 Nevertheless, the priming agent for the PMNL NADPH oxidase and the specific contribution of this enzyme to OS and inflammation in the Sabra rats are still poorly understood. In the current study, we set out to find possible priming agents for the NADPH oxidase that might explain its high priming state in the hypertensive rats and the effect of the enzyme inhibition by APO on OS and inflammation in these rats. We have studied inflammatory cytokines associated with hypertension, interleukin (IL) 6, tumor necrosis factor (TNF)-α, and interferon...
(IFN)-γ, which are also known as priming agents for PMNLs, probably via NADPH oxidase activation. We have also tested the hypothesis that the APO-related attenuation of the rise in blood pressure is mediated, at least in part, by inhibition of OS and inflammation resulting from activation of NADPH oxidase. Hence, we used APO to inhibit NADPH oxidase and investigated the effect of such inhibition on the levels of indices of systemic OS and inflammation in the Sabra rat model of hypertension.

Methods

Animals

Sabra hypertension-prone (SBH/y) and -resistant (SBN/y) rats from the colony at the Israeli Rat Genome Center (Ashkelon, Israel; www.irgc.co.il) were housed in compliance with the guidelines set forth by the American Physiological Society. Animal experiments were approved by the Institutional Ethics Committee for Animal Experimentation.

Feeding Protocol

Immediately after weaning, the animals were fed with either standard rat chow (RD) or chow supplemented with 8% NaCl ad libitum. Tap water was provided for drinking ad libitum.

Inhibition of NADPH Oxidase

We inhibited NADPH oxidase by administering APO in drinking water at a dose of 1.5 mmol/L. This dose has been shown in other models of hypertension to significantly inhibit the enzyme activity.14,15

Study Protocol

We administered APO or vehicle to SBH/y and SBN/y rats that had been either salt loaded with 8% NaCl in diet for 60 days or fed normal chow. Blood pressure was measured at baseline and at weekly intervals thereafter. On termination of the experiments, we drew blood from the bifurcation of the aorta in animals anesthetized with chloral hydrate/sodium pentobarbitone. Plasma was stored at −80°C until assayed. In separate experiments, we used young (4-week-old) SBH/y and SBN/y rats to study the distribution of TNF receptors.

Blood Pressure Measurement

We measured systolic blood pressure (SBP) at ambient temperature in awake animals by the tail-cuff method using the IITC-31 computerized blood pressure device (IITC Life Science), as described previously.12,13 Animals were placed in a restrainer and allowed 10 minutes of acclimatization. We derived SBP from ≥3 replicate measurements.

Plasma Proinflammatory Cytokines

Plasma levels of IL-6, TNF-α, and IFN-γ were measured using commercial ELISA kits (R&D Systems and DIACLONE), following the manufacturer’s instructions.

Extracellular Expression of TNF-α-R1 (p55) and TNF-α-R2 (p75) on PMNL

Surface levels of TNF-α-R1 and TNF-α-R2 were measured on PMNLs using flow cytometry (further details are available in the online Data Supplement at http://hyper.ahajournals.org).

Intracellular Expression of TNF-α-R1 in PMNL

The intracellular expression of TNF-α-R1 in PMNL in whole blood was carried out as above for the extracellular receptor with the addition of a permeabilization step after the lysis of red blood cells, using a ceperneablatisopm commercial permeabilization and staining kit (Invitrogen) following the manufacturers’ instructions.

Rate of Vascular Superoxide Release

The method describing measurements of vascular superoxide release is detailed in the online Data Supplement.

Immunohistochemistry for p47phox in the Rat Aorta

We determined p47phox expression in formaldehyde-fixed, paraffin-embedded specimens of the aorta. We prepared 4-μm-thick serial sections from each representative paraffin block and stained for p47phox with a specific anti-p47phox antibody (Santa Cruz Biotechnology) using the Histofine Simple Stain kit (Nichirei Corporation) following the manufacturer’s instructions.

Translocation of NADPH Oxidase p47 Cytosolic Component to the Plasma Membrane

We have previously described enhanced superoxide production in PMNLs from SBH/y rats.11 Because APO inhibits the NADPH oxidase by inhibiting p47phox translocation to the cell membrane, we measured its abundance in cytosol and membrane fractions of PMNLs obtained from all of the study groups. Cellular membranes and cytosolic fractions were prepared as described previously,16 and p47phox was detected in these fractions as detailed in the online Data Supplement.

Plasma Malondialdehyde Levels

Malondialdehyde (MDA) levels were measured using the assay for thiobarbituric acid-reacting substances, as described previously,12 and further detailed in the online Data Supplement.

Detection and Quantification of Carbonyls on Plasma Fibrinogen

Carbonylated fibrinogen was used as a marker of protein oxidation. We derivatized plasma with 2,4-dinitrophenylhydrazine and quantified for carbonyls, as described previously,12 and further detailed in the online Data Supplement.

Statistics

Data are presented as mean±SE. Statistical significance was tested by Student t test or 1-way ANOVA and applied Bonferroni multiple comparison test, as applicable. We set statistical significance at P<0.05.

Results

Blood Pressure

We have previously reported the effect of APO on attenuation of blood pressure in the SBH/y rats.13 In brief, at 6 weeks, basal SBP in SBH/y was 124±4 mm Hg, and in SBN/y rats it was 118±5 mm Hg (P value not significant [NS]). In salt-loaded SBH/y rats treated with vehicle, SBP increased over 60 days to 220±6 mm Hg. In animals treated with APO, the rise in SBP was significantly attenuated to 145±5 mm Hg (P<0.001, vehicle versus APO). In SBH/y rats provided a regular diet and treated with vehicle, SBP values slightly increased over 60 days to 134±7 mm Hg. In animals treated with APO, this mild increase in SBP was totally abolished, and SBP remained at 124±7 mm Hg. No changes in SBP over the duration of the experiment could be seen in either SBN/y salt-loaded rats or those provided regular diet, treated either with APO or vehicle.

Inflammatory Cytokines

Plasma IFN-γ Levels

In salt-loaded animals (Figure 1A), IFN-γ levels in hypertensive SBH/y animals treated with vehicle (SBH/yApo−) averaged
24.09 ± 3.97 pg/mL (n=9); treatment with APO (SBH/yApo+, n=9) did not affect these levels that remained unchanged at 22.05 ± 4.66 pg/mL (P=NS). In SBN/y animals treated with vehicle (SBN/yApo−) and those treated with APO (SBN/yApo+), IFN-γ levels were 14.53 ± 4.60 pg/mL (n=9) and 18.8 ± 3.81 pg/mL (n=9) respectively, levels that were also not significantly different from all of the groups of SBH/y rats.

In animals fed a regular diet (Figure 1B), IFN-γ levels in SBH/yApo− and SBH/yApo+ rats were similar, at 21.55 ± 5.9 and 17.94 ± 4.44 pg/mL (n=9), respectively (P=NS). In SBN/yApo− and SBN/yApo+ rats, the levels were 19.41 ± 4.32 and 18 ± 8.84 pg/mL (n=9), respectively (P=NS), levels that were also not significantly different from all of the groups of SBH/y rats.

Plasma IL-6 Levels
In salt-loaded animals (Figure 2A), plasma IL-6 levels were elevated in hypertensive SBH/yApo− rats, averaging 126 ± 13 pg/mL (n=9); treatment with APO (SBH/yApo+, n=9) reduced these levels to 82 ± 6.8 pg/mL (P<0.05). In SBH/y rats, IL-6 levels were not different in SBN/yApo− and SBN/yApo+ groups, at 67 ± 7.2 and 69 ± 8.5 pg/mL (n=9), respectively, and were not different from those measured in SBH/yApo+ rats.

In animals fed a regular diet (Figure 2B), IL-6 levels were low and similar in all of the groups, at 85 ± 12.1 pg/mL in SBH/yApo−, 73 ± 14.7 pg/mL in SBH/yApo+, 63 ± 8.6 pg/mL in SBN/yApo−, and 70 ± 10.2 pg/mL in SBN/yApo+ rats (n=9 in each group).

Plasma TNF-α Levels
In salt-loaded animals (Figure 3A), TNF-α levels were elevated in hypertensive SBH/yApo− rats, averaging 1123 ± 264 pg/mL (n=9); treatment with APO (SBH/yApo+) reduced these levels to 55 ± 17 pg/mL (n=9; P<0.01). In SBH/yApo− rats, TNF-α levels were 36 ± 7.4 pg/mL (n=9) and not different from those found in SBN/yApo− and SBN/yApo+ groups, averaging 71 ± 44 and 38 ± 26 pg/mL (n=9), respectively.

In animals fed a regular diet (Figure 3B), TNF-α levels in SBH/yApo− rats averaged 184 ± 23 pg/mL (n=9); treatment with APO (SBH/yApo+) significantly reduced these levels to 55 ± 17 pg/mL (n=9; P<0.01). In SBH/yApo− rats, TNF-α levels were 36 ± 7.4 pg/mL (n=9) and not different from those found in SBN/yApo− and SBN/yApo+ rats at 25 ± 9.7 pg/mL (n=9). These results indicate that inhibition of NADPH oxidase has a pronounced effect on levels of TNF-α in SBH/y rats.

Expression of TNF-α Receptors by PMNLs
The percentage of PMNLs expressing TNF-α-R1 (p55) on their cell surface was higher in SBH/y compared with SBH/y rats regardless of diet, at 2.1 ± 0.5% and 7.46 ± 2.2% for salt-loaded SBH/y versus SBH/y rats, respectively (P<0.05), and 2.94 ± 1.3% and 6.6 ± 1.1% for SBH/y versus SBN/y rats fed a regular diet, respectively (P<0.05; Figure 4A). Because internalization of the p55 receptor in the presence of high levels of TNF-α can explain its decreased expression on the cell membrane, we measured the intracellular expression of p55 in PMNLs (Figure 4B). Our results show that SBH/y rats, regardless of the type of diet, had higher levels of the

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**Figure 1.** Levels of IFN-γ in SBH/y and SBN/y rats fed a high-salt diet (A) or a regular diet (B) and treated with APO (black bar) or vehicle (gray bar). *P=NS among all of the groups.

**Figure 2.** Levels of IL-6 in SBH/y and SBN/y rats fed a high-salt diet (A) or a regular diet (B) and treated with APO (black) or vehicle (white). *P<0.05 SBH/yApo− vs SBH/yApo+, SBN/yApo−, and SBN/yApo+.
intracellular receptor compared with SBN/y (3.2±2% and 1.1±0.5% for salt-loaded SBH/y and SBN/y rats, respectively \([P<0.05]\), and 5.0±1.9% and 1.5±0.9% for SBH/y and SBN/y rats fed a regular diet, respectively \([P<0.005]\); \(n=5\) in each group).

Because we showed that elevated plasma TNF-\(\alpha\) precedes the rise in blood pressure in SBH/y rats (Figure 3B), we further tested the hypothesis that the lower expression of the extracellular p55 in SBH/y rats is an indication of a physiological response to the elevated levels of plasma TNF-\(\alpha\). We have, therefore, analyzed the expression of p55 in young (4-week–old rats) as opposed to 14- to 15-week–old SBH/y and SBN/y rats. Our results show that young SBH/y rats (4-week–old rats) as opposed to 14- to 15-week–old SBH/y rats, expressed compared with young SBN/y controls (\(0.05\); C).

Vascular Superoxide Release
In salt-loaded animals (Figure 5A), the rate of superoxide release from aortic rings in hypertensive SBH/yApo- rats \((n=9)\) was significantly reduced after treatment with APO (SBH/yApo+), at 5.8±0.7 versus 2.1±0.3 counts per minute per milligram of dry tissue, respectively \((n=9\); \(P<0.05\)). In vehicle-treated, SBN/yApo- rats, the rate of superoxide release was 2.2±0.3 counts per minute per milligram of dry tissue \((n=9)\), similar to SBH/yApo+ rats; in SBN/yApo+ rats, superoxide release remained unchanged at 2.0±0.2 counts per minute per milligram of dry tissue \((n=9)\).

In animals fed a regular diet (Figure 5B), the rate of superoxide release in normotensive SBH/y animals treated with vehicle was also elevated and not different from the salt-loaded animal; treatment with APO reduced the levels to control values \((4.9±1.6\) and \(2.3±0.2\) counts per minute per
Translocation of p47phox to the PMNL Membrane

Membrane p47phox

Translocation of p47phox to the PMNL membrane was measured as described in the Methods section, and a representative gel is shown in Figure 7A. In salt-loaded animals (Figure 7B), the levels of membrane p47phox in hypertensive SBH/y rats treated with vehicle were elevated but reduced after treatment with APO (98±20 and 61±22 relative density units [RDU], P<0.05). In normotensive SBN/y rats (Figure 7C), the levels of membrane p47phox remained unchanged at 58±10 (n=9).

Cytosolic PMNL p47phox

We have found no significant differences in the levels of cytosolic p47phox between all of the study groups (Figure 7D and 7E).

Markers of OS

MDA levels in SBH/yApo- rats (n=9) were elevated compared with SBH/yApo+ rats (n=9), at 12.85±1.09 versus 8.53±0.56 nmol/mL, respectively (P<0.005; Figure 8A). In SBN/yApo- rats (n=9), MDA levels were 8.83±0.60 nmol/mL, not different from the ones measured in SBH/yApo+ rats. Moreover, APO treatment in this group (SBN/yApo+; n=9) had no effect on MDA levels, which remained at 8.57±0.28 nmol/mL (P=NS; Figure 8A). In animals fed a regular diet (Figure 8B), MDA levels in the normotensive SBH/y rats treated with vehicle (SBH/yApo-; n=9) were also elevated, averaging 12.77±4.45 nmol/mL, levels similar to the levels of salt-loaded hypertensive SBH/yApo+ rats; however, treatment with APO of SBH/y rats on a regular diet (SBH/yApo+; n=9) reduced significantly the MDA levels to 9.47±1.97 nmol/mL (P<0.05). In normotensive SBN/y rats on a regular diet, treated with vehicle (SBN/yApo-; n=9) or with APO
Figure 7. Western Blot analysis of p47phox in PMNLs cytosol and membrane fractions. PMNLs were separated from all of the study groups, and p47phox was detected as described in the Methods section. A, Representative gel showing inhibition of p47phox translocation to the cell membrane in SBH/y rats after treatment with APO. B and C, Levels of membrane p47phox. In C, *P<0.001 salt-loaded SBH/y APO− vs SBH/y APO+. There were no significant differences in the levels of the cytosolic p47phox between groups fed a high-salt diet (D) or a regular diet (E).

Figure 8. Plasma MDA levels of SBH/y and SBN/y rats fed a high-salt diet (A) or regular diet (B) and treated with APO (black bar) or vehicle (gray bar). A, *P<0.005 SBH/y APO− vs SBH/y APO+, SBN/y APO−, and SBN/y APO+. B, *P<0.05 SBH/y APO− vs SBH/y APO+, SBN/y APO−, and SBN/y APO+. There was no significant difference between salt-loaded SBH/y APO− and SBH/y APO+ rats fed a regular diet. C, Levels of carbonylated fibrinogen in SBH/y and SBN/y rats fed a high-salt diet or a regular diet (D) and treated with APO (black bar) or vehicle (gray bar). *P<0.05 SBH/y APO− vs SBH/y APO+, SBN/y APO−, and SBN/y APO+.

(SBN/y APO+), MDA levels were 8.71±1.99 and 9.35±2.38 nmol/mL, respectively, not different from those measured in salt-loaded SBN/y rats treated with vehicle or APO. It should be noted that MDA levels were already elevated in the normotensive regular diet-fed SBH/y group.
Carbonyl levels on fibrinogen were elevated in SBH/y\textsuperscript{Apo−} rats (n = 9) compared with their APO-treated counterparts, averaging 3.46 ± 0.54 RDU (Figure 8C). In SBH/y\textsuperscript{Apo+} rats (n = 9), the carbonyls were significantly reduced to 1.33 ± 0.24 RDU (P < 0.05; Figure 8C). In SBN/y\textsuperscript{Apo−} rats (n = 9), carbonyl levels were 1.19 ± 0.36 RDU, not different from SBH/y\textsuperscript{Apo+} rats. In salt-loaded APO treated SBN/y\textsuperscript{Apo−} rats, APO did not change the carbonyl levels, remaining at 0.79 ± 0.23 RDU (n = 9). In animals fed a regular diet (Figure 8D), carbonyl levels were low and not different in all of the groups, at 1.33 ± 0.28 RDU in SBH/y\textsuperscript{Apo−} rats, 1.34 ± 0.34 RDU in SBN/y\textsuperscript{Apo−} rats, 1.09 ± 0.25 RDU in SBN/y\textsuperscript{Apo+} rats, and 0.88 ± 0.18 RDU in SBN/y\textsuperscript{Apo+} rats (n = 9 for each group).

**Discussion**

In the current study, we tested the hypothesis that, in the Sabra rat model of salt-sensitive hypertension, OS and inflammation are associated with hypertension and regulated in part by the NADPH oxidase. In addition, we propose a mechanism that could account for the high priming state of the PMNLs, which precedes elevation in blood pressure and, in particular, the activation of NADPH oxidase in this model. We suggest that high plasma levels of TNF-\(\alpha\) in SBH/y rats exist before the development of hypertension and possibly contribute to the high priming state of PMNLs. These high levels of TNF-\(\alpha\) were reduced by APO both in the normotensive and hypertensive SBH/y rats. The high levels of TNF-\(\alpha\) were associated with increased expression of the TNF-\(\alpha\) type 1 receptor in young normotensive SBH/y rats, which is involved in NADPH oxidase stimulation.\(^{17}\) We have also found that APO blunted both PMNL and vascular NADPH oxidase activity and reduced the levels of systemic OS and inflammation. In PMNLs, APO inhibited p47phox translocation from the cytosol to the cell membrane, limiting the production of superoxide, thus explaining our previous data showing the inhibition of PMNL superoxide release.\(^{13}\) In the vascular tissue, we show high expression of p47phox in SBH/y rats even before the development of hypertension, as well as inhibition of superoxide production in this tissue by APO.

The relationship between inflammation and hypertension is not novel, and a causal role for inflammation in the pathogenesis of hypertension has been suggested on multiple occasions.\(^{8,18,19}\) In the current study, we focused on inflammatory mediators that could explain the high priming state of PMNLs and the increased NADPH oxidase activity both in PMNLs and in the vascular tissue associated with hypertension and also found in our rat model.

Among the inflammatory cytokines we explored was IFN-\(\gamma\). We found that the levels of IFN-\(\gamma\) were not different between SBH/y and SBN/y rats, suggesting that IFN-\(\gamma\) does not actively participate in the pathophysiology of hypertension in our model. Interestingly, reports in the literature with regard to the role of IFN-\(\gamma\) on blood pressure are variable. Angiotensin II infusion in the rat, which causes hypertension, has been shown to increase the IFN-\(\gamma\) mRNA level.\(^{20}\) Administration of IFN-\(\gamma\) to New Zealand white mice has been reported to increase mean arterial pressure.\(^{21}\) On the other hand, administration of IFN-\(\gamma\) in Dahl salt-sensitive rats has been shown to reduce blood pressure,\(^{22}\) while having no effect in spontaneously hypertensive rats.\(^{23}\) Such variability in the data is consistent with the postulated pathophysiological heterogeneity of hypertension among different experimental models.

IL-6 is the second cytokine that we explored. We found elevated levels of IL-6 only in the hypertensive salt-loaded SBH/y group and not in normotensive SBH/y rats on a regular diet. We have also demonstrated that treatment with APO reduced IL-6 to levels that were similar to normotensive SBH/y and SBN/y rats. These data suggest that elevated IL-6 levels may be secondary to hypertension and that elevated IL-6 levels are not required to predispose the SBH/y animal to develop hypertension during salt loading. A review of the literature suggests a role for IL-6 in hypertension in other models. A role for IL-6 in hypertension has been proposed on the basis of the observation that high levels of plasma IL-6 are found in the spontaneously hypertensive rats\(^{24}\) and that angiotensin II–induced hypertension is attenuated in IL-6 knockout mice.\(^{25}\)

TNF-\(\alpha\) is a third cytokine that we explored. In the current study we found that TNF-\(\alpha\) levels are elevated in normotensive SBH/y rats before salt loading and the development of hypertension. These levels further increased in salt-loaded animals that developed hypertension. We further demonstrated that inhibition of the NADPH oxidase with APO significantly reduced TNF-\(\alpha\) plasma levels, both in normotensive and hypertensive SBH/y rats. Although the exact mechanism by which APO can reduce the levels of TNF-\(\alpha\) is unclear, we propose that this reduction is secondary to the inhibition of OS and inflammation triggered by the active enzyme. Our findings are consistent with elevated plasma TNF-\(\alpha\) found in another model of salt-sensitive hypertension, the Dahl rat.\(^{26}\) TNF-\(\alpha\) has been shown to stimulate the production of endothelin 1\(^{27}\) and angiotensinogen,\(^{28}\) both of which lead to vasoconstriction and hypertension. A role for TNF-\(\alpha\) in human hypertension is supported by the demonstration of increased secretion of this inflammatory cytokine in peripheral blood monocytes from hypertensive patients\(^{29}\) and by the presence of high TNF-\(\alpha\) levels in patients with essential hypertension.\(^{27–29}\)

What is the mechanism whereby TNF-\(\alpha\) could invoke hypertension? TNF-\(\alpha\) is an effective priming agent of the NADPH oxidase p47 subunit\(^{17}\) and has been shown to cause degranulation and release of oxygen metabolites.\(^{6,30–32}\) TNF-\(\alpha\) inhibition has been shown to reduce formation of PMNL-derived reactive oxygen species both in culture and in human blood.\(^{33,34}\) On the basis of these actions, we propose that elevated plasma TNF-\(\alpha\) in SBH/y rats could prime and directly activate NADPH oxidase, accounting, at least in part, for the high levels of superoxide release from primed PMNLs and vascular tissue. These reactive oxygen species could, in turn, contribute to the increased systemic OS and vascular tone and to the development of hypertension. Interestingly, Shahid et al\(^{15}\) demonstrated that TNF-\(\alpha\) infusion in mice exerts a vasoconstrictor action but also causes diuresis and natriuresis in the kidney, thereby resulting in no change of the mean arterial pressure. Although the vasoconstriction effect is mediated by TNF-\(\alpha\)-induced superoxide release, the exact cause of the natriuretic response to TNF is not yet clear. The
same group has also reported that chronic infusion of angiotensin II did not cause hypertension in TNF-α knockout mice. However, when a chronic replacement therapy was made with recombinant TNF-α, angiotensin II caused a similar hypertensive response to that observed in wild-type mice. These results suggest an active role for TNF-α in hypertension, although the exact mechanism by which TNF-α supports the development of hypertension needs to be further studied.

The biological activities of TNF-α are mediated by 2 structurally related, but functionally distinct, receptors, TNF-α-R1 (p55) and TNF-α-R2 (p75), belonging to the TNF receptor gene family. These receptors share 28% homology in their extracellular domain but are different in their intracellular sequence. p55, which also possesses a death domain, is a cell surface receptor, but large amounts of the receptor can be found localized at the perinuclear-Golgi complex. Stimulation of p55 leads to an array of responses depending on the specific cell type. Internalization of the p55 receptor was shown to be a mechanism used by cells to inhibit TNF signaling. The functions and characteristics of p75 are less understood than those of p55. Once activated, p75 is readily cleaved by metalloproteases into a soluble form that is still capable of TNF binding. Activation of p75 in T cells has been shown to be proliferative, but it is also known to have a function in regulating TNF-induced apoptosis. Although most studies have focused on TNF-induced apoptosis mediated by the TNF receptors, these receptors have also been found to be dominant in many other functions, including cell proliferation and the induction of inflammation.

On PMNLs, the p55 receptor is responsible for most of the TNF-induced signal transduction pathways and can activate the respiratory burst. Moreover, a neutralizing antibody against the p55 TNF-α receptor, rather than a neutralizing antibody against the p75 TNF-α receptor, successfully inhibited TNF-α–induced priming of the NADPH oxidase. We evaluated PMNL surface expression of p55 in our model, on the basis of our hypothesis that this receptor will be highly expressed in SBH/y rats. When measuring the levels of the p55 receptor in adult SBH/y and SBN/y rats, we unexpectedly found decreased surface expression of the p55 receptor in PMNLs of SBH/y rats compared with their SBN/y counterparts. This observation can be explained by the internalization of the receptor, a well-documented mechanism for the regulation of increased TNF-α activity. We subsequently found increased levels of the receptor inside PMNLs of adult SBH/y rats compared with their SBN/y controls. We suggest, therefore, that a decrease in p55 surface expression, along with the increase in p55 intracellular levels in SBH/y rats, reflects TNF-α–induced PMNL priming. Interestingly, in young normotensive SBH/y rats, we found the expected increased expression of the p55 receptor on the PMNL membrane, a result that should be further elucidated.

We have previously reported an antihypertensive effect of APO in the Sabra rat model of salt-sensitive hypertension. APO has been shown to prevent, inhibit, or reverse the development of hypertension in other experimental models of hypertension, including the spontaneously hypertensive rats, and in dexamethasone–, aldosterone–, and mineralocorticoid-induced hypertension. An open-ended issue has been the mechanism whereby APO affects blood pressure. APO is an NADPH oxidase inhibitor that has been shown recently to exert other effects, including an anti-inflammatory effect. In the current study, we also explored the hypothesis that the antihypertensive effect of APO is causally related to inhibition of NADPH oxidase–related OS and/or an active inflammatory state in SBH/y rats.

A causal relationship between OS and hypertension is not novel and has been demonstrated previously in the rat in several models of hypertension. The contribution of OS to the development of hypertension has been inferred by studies that have successfully attenuated the rise in blood pressure by administration of antioxidants or substances with antioxidative properties. In the current study, we demonstrate the presence of OS in the Sabra model of hypertension, the SBH/y rat, by the elevated level of protein oxidation (carbonyls) and lipids (MDA). We previously identified in SBH/y rats that the PMNLs are primed, generating high levels of superoxide through their NADPH oxidases. We currently demonstrated that the vascular NADPH oxidase is also activated, as suggested by the increased levels of superoxide release and increased expression of p47phox within vascular smooth muscle cells. Other studies have also reported enhanced activity of the vascular enzyme with increased expression of p47phox; however, in all of these studies, the PMNL enzyme was overlooked. Finally, we showed that the priming/activation of NADPH oxidase, which initiates a loop of OS and inflammation, could be disrupted by inhibition of the enzyme with APO.

Is the OS found in SBH/y rats mostly attributed to activation of the NADPH oxidase family? In support of this view is that, in SBH/y rats, APO (a specific NADPH oxidase inhibitor of both phagocytic and vascular enzymes) reduced the levels of MDA and carbonylated fibrinogen, both surrogates of systemic OS. APO prevented the development of hypertension. The temporal association between the reduction in OS and prevention of hypertension in SBH/y rats could be interpreted as suggesting that a high level of OS is required for hypertension to evolve in SBH/y rats. An alternative explanation is that the high levels of OS in SBH/y rats are not the cause of but are secondary to hypertension. This is unlikely, however, because high levels of OS are also apparent in normotensive SBH/y rats. In SBH/y rats fed a regular diet, as we demonstrated in the current and previous studies. Interestingly, it was reported that the Sabra rat model of salt-induced hypertension might involve a dysfunction in the production/availability of NO. This assumption is on the basis of studies demonstrating a greater pressor effect in the salt-resistant strain than in the salt-sensitive strain, after administration of an NO inhibitor and a vasoconstrictor, suggesting decreased NO generation. In light of the results presented herein, we propose that interactions of superoxide from primed NADPH oxidases present in PMNLs and the vascular wall with NO could contribute to the low levels of NO in these rats. Furthermore, these interactions could potentially elicit the generation of other active compounds, such as peroxynitrite, which, in turn, contribute to the elevated OS measured in the SBH/y rats.
Perspectives
The results presented herein enable future studies to specifically reduce PMNL priming as a new treatment modality in hypertension. The involvement of TNF-α and its interaction with its p55 receptor should be further investigated in other models of hypertension. Our results suggest that inhibition of this receptor would have an impact on PMNL priming and the development of hypertension. Finally, PMNL priming is associated with other chronic diseases associated with cardiovascular complications, such as hyperlipidemia, diabetes mellitus, and chronic renal disease. Our study enables the evaluation of TNF-α-p55 interactions as a possible priming mechanism in these conditions as well.

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Disclosures
None.

References


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TNF-α: a Possible Priming Agent for the PMNL NADPH Oxidase in Hypertension.

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Short title: TNF-α, phagocytic NADPH oxidase and hypertension
Extended Methods

**Extracellular expression of TNF-α-R1 (p55) and TNF-α-R2 (p75) on PMNL Surface**

Levels of TNF-α-R1 and TNF-α-R2 were measured on PMNLs using flow cytometry. Blood was drawn into sodium citrate containing tubes at 4°C. Erythrocytes were lysed by applying 1:9 volume of ACK buffer (NH₄Cl 0.15M, KHCO₃ 1nM, Na₄EDTA 1nM; PH=7.4) for 5 minutes, followed by centrifugation at 500xg for 5 min. After discarding the supernatant, cells were re-suspended in working buffer (2% heat-inactivated fetal calf serum (FCS), 0.09% sodium azide (NaN₃) in PBS; PH=7.4) and incubated with anti rat FcγIII receptor (CD32) (10µg/ml, Becton, Dickinson, USA) for 15 min at 4°C followed by incubation with rabbit anti rat TNF-α-R1 or mouse anti rat TNF-α-R2 (Abcam, USA) for 30 minutes. Cells were washed with working buffer and centrifuged at 500xg for 5min. The pellet was re-suspended in working buffer containing a fluorescent anti-IgG secondary antibody (Goat anti rabbit-PE, Abcam USA; and Chicken anti mouse-FITC, Santa Cruz,USA) for 30 min at 4°C in the dark. After incubation, cells were washed with working buffer, and re-suspended in working buffer before the analysis.

**Rate of vascular superoxide release** Rat aortas were rapidly removed from the anesthetized rat and placed in Krebs/HEPES buffer; the excessive fat was cleaned and the tissue was cut into 3-mm ring segments. We transferred the segments into a white 96-well microplate containing 50 µL/well of the same buffer at 37°C, added dark-adapted L-012 (50 µM, Wako, Japan) and after 5 minutes added NADPH (100 µM). We recorded the chemiluminescence every 2 minutes at 37°C in a microplate luminometer (Lucy 1, Rosys Anthos). After the assay was completed, the vessel was dried and weighed. The results were expressed as counts per minute per gram of dry tissue (counts/min/gram dry tissue).

**Translocation of NADPH Oxidase p47 Cytosolic Component to the Plasma Membrane** We have previously described enhanced superoxide production in PMNLs from SBH/y rats [13]. Since apocynin inhibits the NADPH oxidase by inhibiting p47phox translocation to the cell membrane, we measured its abundance in cytosol and membrane fractions of PMNLs obtained from all study groups. Cellular membranes and cytosolic fractions were prepared as previously described [16] and p47phox was detected
in these fractions. Proteins (40 μg) were electrophoretically transferred to nitrocellulose membranes and blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4). Blots were then incubated for 3 hours at room temperature in Tris-buffered saline, 5% milk containing antibody to p47phox, 1:500 (BD-Biosciences Pharmingen). Immunoblots were then incubated with peroxidase-conjugated secondary antibody and developed by the enzyme-linked chemiluminescence method.

**Plasma malondialdehyde (MDA) levels** MDA levels were measured using the assay for thiobarbituric acid-reacting substances (TBARS), as previously described [12]. In brief, plasma was diluted 1:1 with PBS and added to 0.12 mol/L TBARS solution at pH 7.0, containing 2.5% HCl, 0.12 mol/L thiobarbituric acid (TBA) and 15% trichloroacetic acid (TCA). We placed the samples in a 100°C water bath for 30 min, followed by centrifugation at 800g for 10 min. We measured absorbance of the supernatant at 532 nm and determined MDA levels according to a standard curve (0-7.5 nmol MDA).

**Detection and quantification of carbonyls on plasma fibrinogen** Carbonylated fibrinogen was used as a marker of protein oxidation. We derivatized plasma with DNPH and quantified for carbonyls, as previously described [12]. In brief, plasma was diluted 1:30 in 6% SDS and mixed with 1 volume of DNPH solution, incubated for 15 minutes at room temperature and neutralized by 2M Tris solution. The DNPH treated plasma samples were separated by SDS-PAGE containing 8% acrylamide and transferred to nitrocellulose filters in transfer buffer (25 mmol/L Tris, 192 mmol/L Glycine) for carbonyl detection by western blot analysis. Carbonyl signal on X-rays films was detected using the chemiluminescence reagents of the EZ-ECL kit (Biological Industries, Beit-Haemek, Israel). For identification of the fibrinogen band, we performed western analysis with rabbit polyclonal anti-fibrinogen serum (ICN Pharmaceuticals, Aurora, Ohio USA) and goat anti rabbit-HRP conjugate. We analyzed the density of the bands on the films in relation to a known oxidized fibrinogen marker using the BioCapt and Bio-Profil (Bio-1D) software and expressed carbonyl levels in relative density units (U).