

## COMMENT

# Angiotensin II Receptor Blocker Valsartan Suppresses Reactive Oxygen Species Generation in Leukocytes, Nuclear Factor- $\kappa$ B, in Mononuclear Cells of Normal Subjects: Evidence of an Antiinflammatory Action

PARESH DANDONA, VIKRAMJEET KUMAR, AHMAD ALJADA, HUSAM GHANIM, TUFAIL SYED, DEBBORAH HOFMAYER, PRIYA MOHANTY, DEVJIT TRIPATHY, AND RAJESH GARG

*Division of Endocrinology, Diabetes and Metabolism, State University of New York at Buffalo, and Kaleida Health, Buffalo, New York 14209*

In view of the pro-oxidant and proinflammatory effects of angiotensin II, we have tested the hypothesis that valsartan, an angiotensin receptor blocker, may exert a suppressive action on reactive oxygen species (ROS) generation, nuclear factor  $\kappa$ B (NF- $\kappa$ B) in mononuclear cells. Four groups of eight normal subjects were given 1) 160 mg daily of valsartan, 2) 80 mg daily of simvastatin, 3) 40 mg quinapril, or 4) no treatment. Fasting blood samples were obtained before treatment and at d 1, 8, and 14 (7 d after the cessation of the drug). After valsartan, ROS generation by polymorphonuclear cells and mononuclear cells fell significantly by more than 40% ( $P < 0.01$ ). NF- $\kappa$ B binding activity and the expression of total cellular p65, a protein component of NF- $\kappa$ B, fell significantly ( $P < 0.01$ ). The expression of inhibitor  $\kappa$ B (I $\kappa$ B) increased significantly ( $P < 0.05$ ). Plasma C-reactive protein (CRP) concentration fell significantly ( $P < 0.01$ ). All indices, except I $\kappa$ B, re-

verted toward baseline, 7 d after the cessation of the drug. I $\kappa$ B persisted in an elevated state. Neither quinapril nor simvastatin given for 7 d produced a suppression of ROS generation, intranuclear NF- $\kappa$ B, p65, or CRP, and these two agents did not alter cellular I $\kappa$ B either. The untreated controls also did not demonstrate a change in their ROS generation or NF- $\kappa$ B binding activity or plasma CRP concentration. We conclude that valsartan at a modest dose exerts a profound and rapid ROS and inflammation-suppressive effect that may be relevant to its potential beneficial effects in atherosclerosis, diabetes, and congestive cardiac failure. In contrast, quinapril and simvastatin produced no similar effect over the period of 1 wk. Our observations may also have implications to clinical situations in which a rapid antiinflammatory effect is required. (*J Clin Endocrinol Metab* 88: 4496–4501, 2003)

ANGIOTENSIN II IS now known to be a proinflammatory mediator that causes an increase in the release of reactive oxygen species (ROS), including H<sub>2</sub>O<sub>2</sub>, which in turn cause phosphorylation of inhibitor  $\kappa$ B (I $\kappa$ B) and its ubiquitination *in vitro* (1–3). This allows cytosolic nuclear factor  $\kappa$ B (NF- $\kappa$ B) to translocate to the nucleus to induce an increase in the transcription of proinflammatory cytokines, adhesion molecules, and enzymes generating ROS (4–6). Thus, apart from being a major vasopressor effector of the renin-angiotensin system, angiotensin II is probably also a modulator of ROS generation and of inflammation. Its proinflammatory action is probably of relevance to atherosclerosis (7). It is noteworthy that angiotensin converting enzyme (ACE) inhibitors (8, 9) and angiotensin receptor blockers (ARBs) improve cardiovascular outcomes in patients with coronary heart disease and diabetes mellitus (8, 9).

Thus far, there is no information regarding the effect of either ARB or ACE inhibitors on either ROS generation or other mediators of inflammation *in vivo*. It is, therefore, im-

portant to investigate whether such agents are antiinflammatory and/or ROS suppressive. Recent work has shown that the key proinflammatory transcription factor, NF- $\kappa$ B, is activated in proinflammatory states including atherosclerosis. NF- $\kappa$ B regulates the transcription of genes for proinflammatory cytokines (*e.g.* TNF $\alpha$  and IL-6), adhesion molecules (*e.g.* intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), chemokines (*e.g.* monocyte chemoattractant protein-1), and protein subunits of ROS-generating enzymes [*e.g.* reduced form of nicotinamide adenine dinucleotide phosphate oxidase (p47<sup>phox</sup>)] (4, 6, 10, 11). I $\kappa$ B is a protein that binds to NF- $\kappa$ B and prevents its translocation into the nucleus and, thus, prevents the transcription of proinflammatory genes. We have recently used mononuclear cells (MNCs) prepared from blood samples to investigate the pro- or antiinflammatory actions of various agents and drugs including hydrocortisone (12), troglitazone (13, 14), and insulin (15). Monocytes and T cells are known to initiate the endothelial inflammation leading to atherosclerosis (16). The rapid antiinflammatory and ROS-suppressive effect of hydrocortisone is observed in 4–6 h. Using the same model, we have shown that carvedilol and nadolol suppress ROS generation by leukocytes and oxidative stress, as observed in

Abbreviations: ACE, Angiotensin converting enzyme; ARB, angiotensin receptor blocker; CRP, C-reactive protein; I $\kappa$ B, inhibitor  $\kappa$ B; MNC, mononuclear cell; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PMN, polymorphonuclear cell; ROS, reactive oxygen species.

lipid peroxidation or amino acid oxidation after 1 wk of administration of modest doses of these drugs. HMG-CoA reductase inhibitors (statins) and ACE inhibitors improve cardiovascular outcomes, and both have been shown to lower plasma C-reactive protein (CRP) concentrations (17–19). This indicates that they have an antiinflammatory effect.

In view of the above, we have now investigated the effect of valsartan, an ARB, on ROS generation by leukocytes [polymorphonuclear cell (PMN) and MNC], NF- $\kappa$ B, I $\kappa$ B, and plasma CRP concentrations, key indices of inflammation, after a short period of therapy in normal subjects. We also compared the action of quinapril (an ACE inhibitor) and simvastatin with that of valsartan over the same period of time.

## Subjects and Methods

### Subjects

Four sets of eight subjects [mean age,  $32.4 \pm 5.6$  yr (range, 26–42); mean body mass index,  $24.6 \pm 3.0$  kg/m<sup>2</sup> (range, 19.5–28)] were included in the study. All subjects came to the Research Center at the Diabetes-Endocrinology Center of Western New York at 0800–0900 h in the fasting state. A blood sample was obtained, and they were started on either valsartan (160 mg daily) or quinapril (40 mg daily) or simvastatin (80 mg daily) for 7 d. A second fasting blood sample was obtained on d 8, 24 h after the last dose of the drug. A third blood sample was obtained 7 d after the cessation of the drug. The fourth group was not given any drug but had fasting samples obtained on d 1, 8, and 14, as in the three drug-taking groups. All subjects participating in the study signed informed consent. The study protocol was approved by the Human Research Committee of the State University of New York at Buffalo based at Millard Fillmore Hospital. Compliance regarding drug intake was assured by administering eight tablets of the drugs to each subject and by determining that each subject had one remaining tablet at the end.

### ROS generation assay

Respiratory burst activity of PMN or MNC was measured by detection of superoxide radical via chemiluminescence, as described previously (20, 21). Briefly, blood samples were collected in Na-EDTA as an

anticoagulant. Three and a half milliliters of anticoagulated blood sample were carefully layered over 3.5 ml PMN medium (Robbins Scientific Corp., Sunnyvale, CA). Samples were centrifuged, and at the end of the centrifugation, two bands separated at the top of the red blood cell pellet. The top band consisted of MNC, whereas the bottom band consisted of PMN. The bands were harvested and repeatedly washed with Hanks' balanced salt solution. This method provides yields greater than 95% pure PMN and MNC suspensions. Five hundred microliters of MNC or PMN ( $2 \times 10^5$  cells) were delivered into a Chronolog Lumi-Aggregometer cuvette. Fifteen microliters of 10 mM Luminol were then added, followed by 1.0  $\mu$ l of 10 mM fMLP. Chemiluminescence was recorded for 15 min.

### NF- $\kappa$ B EMSA

DNA-binding protein extracts were prepared from MNC by the method described by Andrews and Faller (22). Total protein concentrations were determined using bicinchoninic acid protein assay (Pierce, Rockland, IL). EMSA was performed using a NF- $\kappa$ B activity and binding protein detection kit (Life Technologies, Long Island, NY). The double-stranded oligonucleotide containing the consensus sequence for the NF- $\kappa$ B binding site was radiolabeled with  $\gamma$ -P<sup>32</sup> by T<sub>4</sub> kinase. Then, 5  $\mu$ g of the nuclear extract were mixed with the 5 $\times$  incubation buffer [50 mM Tris (pH 7.5), 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol, and 0.4 mg/ml sonicated salmon sperm], and the mixture was preincubated at 4 C for 15 min. Labeled oligonucleotide (60,000 cpm) was added, and the mixture was incubated at room temperature for 20 min. Samples were then applied to wells of 6% nondenaturing polyacrylamide gel. The gel was dried under vacuum and exposed to x-ray film. Densitometry was performed using molecular analyst software (Bio-Rad, Hercules, CA).

### Total I $\kappa$ B and p65 (Rel A) Western blotting

I $\kappa$ B and p65 Western blotting were performed as described previously (12, 13).

### Plasma CRP measurement

Plasma CRP was assayed with an ELISA kit from Diagnostic Systems Laboratories Inc. (Webster, TX).

### Statistical analysis

Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). All data on ROS generation and CRP were

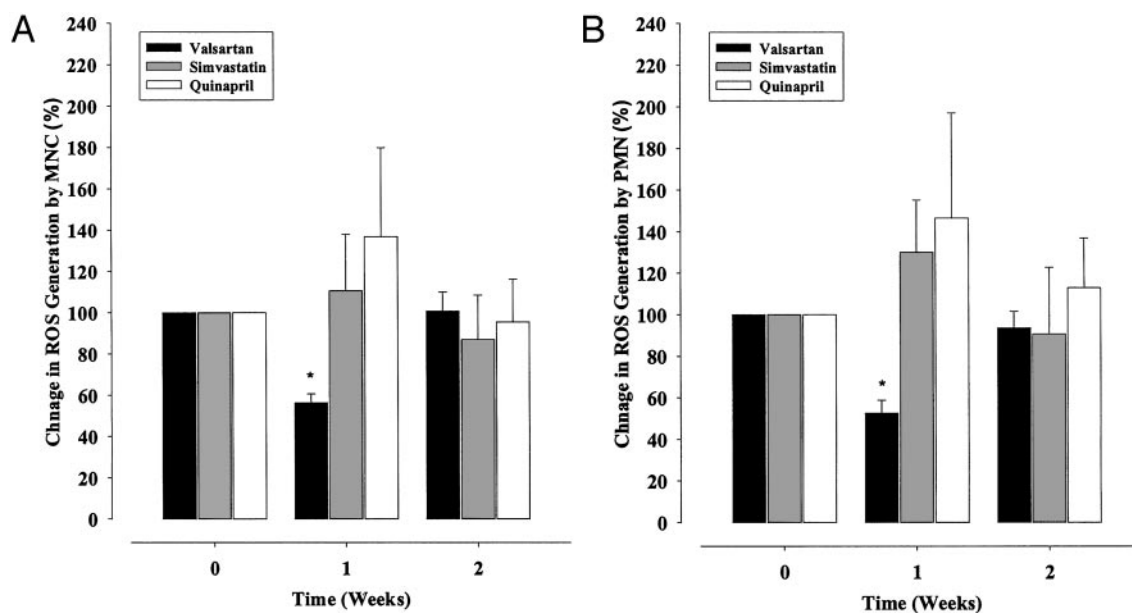


FIG. 1. ROS generation by MNC (A) and PMN (B) after valsartan or simvastatin or quinapril intake (\*,  $P < 0.05$ ).

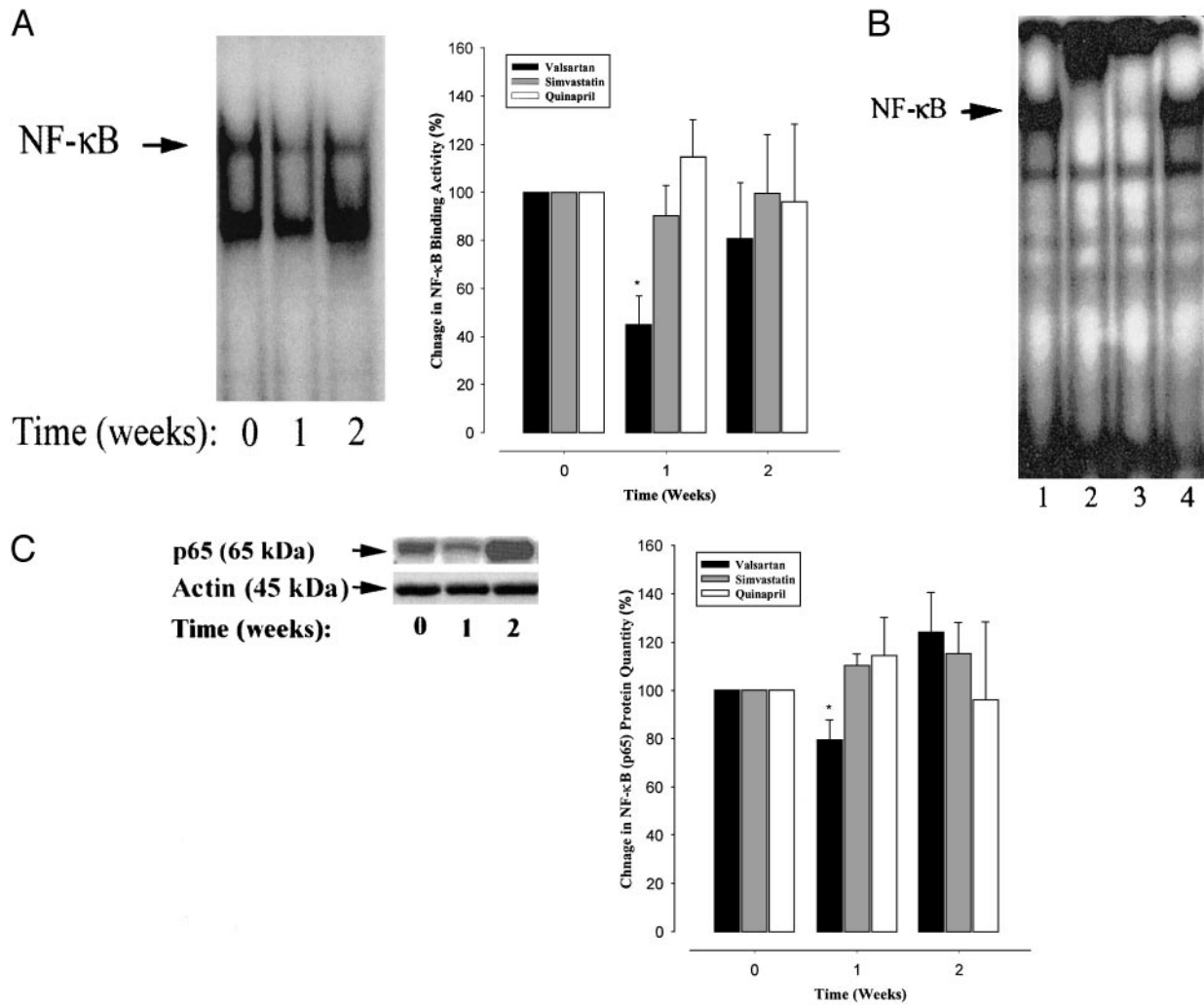


FIG. 2. A, A representative gel shift assay showing the decreased NF- $\kappa$ B binding activity to the double-stranded oligonucleotide containing NF- $\kappa$ B binding site after valsartan intake for 1 wk and the densitometry results of intranuclear NF- $\kappa$ B binding activity after valsartan or simvastatin or quinapril intake. \*,  $P < 0.05$ . Band-shift assays were performed using 5  $\mu$ g MNC nuclear extract for each time point. B, Lane 1, NF- $\kappa$ B binding activity in the baseline sample nuclear extract for one of the subjects; lane 2, super shift using an antibody against p50 subunit of NF- $\kappa$ B; lane 3, super shift using an antibody against p65 subunit of NF- $\kappa$ B; lane 4, p75 (c-Rel) of NF- $\kappa$ B. The super shift EMSA shows that NF- $\kappa$ B band at a p50 and a p65 heterodimer and does not contain p75 (c-Rel). C, A representative Western blot showing the decrease in NF- $\kappa$ B (Rel A) subunit quantity in MNC homogenates and no significant change in actin quantity after valsartan intake and the densitometry results of total NF- $\kappa$ B in MNC homogenates after valsartan or simvastatin or quinapril intake (\*,  $P < 0.05$ ).

normalized to a baseline of 100% in view of the interindividual variability and are expressed accordingly as percentage of the basal. A paired  $t$  test was used to compare all the indices measured in this study. Results are expressed as mean  $\pm$  sn.

## Results

### ROS generation by PMN and MNC

ROS generation by PMN fell from  $175 \pm 96$  mV (100%) to  $53 \pm 18.6\%$  of the basal on the eighth day, 24 h after the last dose of valsartan ( $P < 0.001$ ). It returned to the baseline 7 d after cessation of the drug ( $95 \pm 23.4\%$ ). ROS generation by MNC fell from  $404 \pm 242$  mV (100%) to  $56.3 \pm 11.5\%$  of the basal on the eighth day, 24 h after the last dose of valsartan ( $P < 0.001$ ). It returned to the baseline 7 d after cessation of the drug ( $99 \pm 25.2\%$ ). In contrast, quinapril and simvastatin were not able to suppress ROS generation by PMN and MNC

(Fig. 1). ROS generation did not alter in the control group either.

### NF- $\kappa$ B binding activity and total NF- $\kappa$ B (Rel A: p65) in MNC homogenates

Valsartan intake caused a significant decrease in intranuclear NF- $\kappa$ B binding activity levels ( $P < 0.01$ ) (Fig. 2A). This decrease was statistically significant on the eighth day. NF- $\kappa$ B binding activity returned to the basal level after the cessation of valsartan intake. NF- $\kappa$ B gel retardation assay confirmed that it was a p50 and p65 heterodimer (Fig. 2B). p65 (Rel A) protein expression fell significantly as well after valsartan intake ( $P < 0.05$ ) (Fig. 2C). Thus, both the total NF- $\kappa$ B as protein as well as its activity in terms of DNA binding fell significantly. Neither

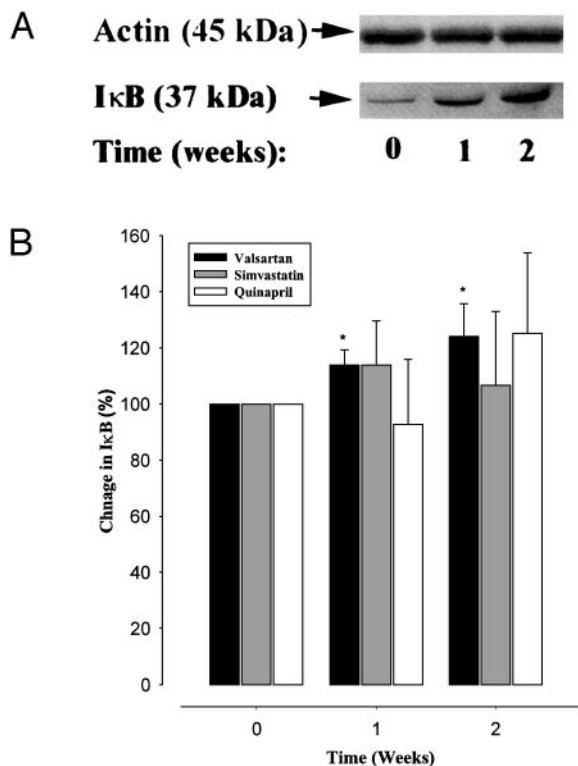


FIG. 3. A, A representative Western blot showing the relative increase in IκBα quantity in MNC homogenate after valsartan intake for 1 wk. Note that valsartan intake did not change actin concentrations. B, Densitometry results of IκB in MNC homogenates after valsartan or simvastatin or quinapril intake (\*,  $P < 0.05$ ).

quinapril nor simvastatin or the control group altered either p65 expression or NFκB binding activity.

#### IκB expression in MNC homogenates

IκB expression in MNC homogenates increased significantly after valsartan intake ( $P < 0.05$ ) (Fig. 3), at wk 1; it remained elevated at wk 2. IκB expression did not alter after simvastatin or quinapril or in control subjects.

#### Plasma CRP concentration

Plasma CRP concentration fell significantly from a basal level of  $127.9 \pm 154.6$  ng/ml (100%) to  $67.4 \pm 22.2\%$  of the basal at wk 1 and to  $122.3 \pm 122.0\%$  of the basal level at wk 2 ( $P < 0.05$ ). Plasma CRP concentration did not alter in the quinapril or simvastatin or the control group (Fig. 4).

### Discussion

Our data show clearly that the angiotensin II receptor blocker valsartan inhibited ROS generation by both PMN and MNC. The magnitude of inhibition was more than 40% for both. There was a concomitant suppression of NF-κB, as reflected in its binding to the consensus sequence polynucleotide and the diminished levels of p65 (Rel A), the protein that is a part of the NF-κB heterodimer. IκB expression increased whereas plasma CRP concentration fell. Therefore, valsartan has a comprehensive antiinflammatory effect at the cellular and molecular level, as well as in the plasma. It is of

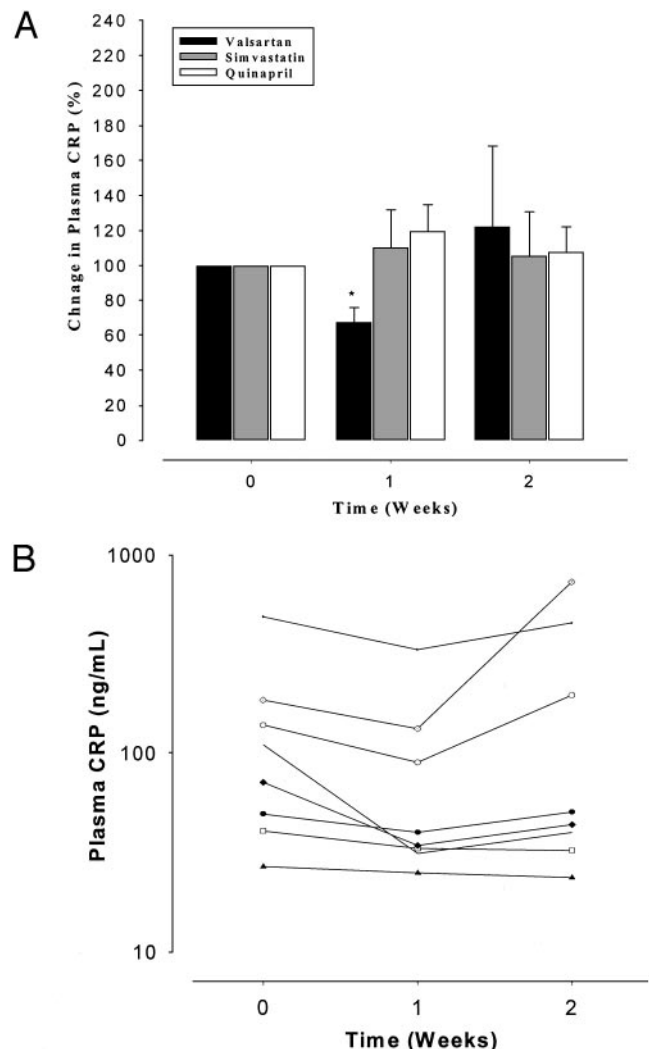


FIG. 4. A, Percentage change in plasma CRP after valsartan or simvastatin or quinapril intake (\*,  $P < 0.05$ ). B, Scatter diagram of CRP concentration (ng/ml) at 0, 1, and 2 wk after valsartan intake.

interest that quinapril, an ACE inhibitor, did not suppress any of the mediators of inflammation investigated in this study. Because angiotensin II is proinflammatory, it is surprising that this should be so. However, it is possible that the reduction in plasma angiotensin II concentrations after the administration of quinapril takes longer than the blockade of the angiotensin II receptor after valsartan. Whether valsartan shares this property with other ARBs is worthy of additional investigation. Similarly, an investigation of quinapril after longer administration would be of interest. It should be noted that ramipril (10 mg) caused a reduction in CRP in the Heart Outcomes Prevention Evaluation (HOPE) study quite apart from reducing the frequency of cardiovascular events. Simvastatin at a high dose (80 mg daily) also did not produce a significant effect during this period.

This antiinflammatory effect of valsartan would potentially inhibit the transcription of NF-κB-modulated proinflammatory cytokines and adhesion molecules and enzymes responsible for ROS generation, like the reduced form of nicotinamide adenine dinucleotide phosphate oxidase. It is

of interest that I $\kappa$ B $\alpha$  expression remained elevated 1 wk after the withdrawal of the drug, whereas NF- $\kappa$ B reverted to levels greater than the baseline. The mechanism underlying this discrepancy is not clear. It is possible that the persistent increase in I $\kappa$ B $\alpha$  at wk 2 is a reflection of the increase in NF- $\kappa$ B, because the latter is one of the regulators of the expression of I $\kappa$ B $\alpha$  gene. It is noteworthy that CRP fell significantly by over 30% in 1 wk; the magnitude of this fall is greater than that reported recently by following pravastatin therapy (14%) for 6 months (23). It is also of interest that simvastatin and quinapril (at high doses) did not induce a reduction in CRP, although both reduce CRP after a long duration of administration.

The reduction in ROS generation would potentially reduce oxidative damage of lipids, amino acids, and proteins. Oxidative damage of lipids leads to lipid peroxidation, which is crucial in the formation of foam cells from the monocyte-macrophage, which takes up oxidized low-density lipoprotein by the scavenger receptor (7, 24).

These data provide a mechanism that may explain the beneficial effects of valsartan and other ARBs in long-term outcome studies in patients with atherosclerosis, those with congestive cardiac failure, and those with renal complications of diabetes. These drugs block the action of angiotensin II at the AT<sub>1</sub> receptor level. Angiotensin II is known to promote oxidative stress and to be proinflammatory. Leukocytes are known to express angiotensin II receptors (25). Thus, valsartan, which blocks angiotensin II, may be expected to inhibit inflammation and oxidative stress and, thus, the progression of atherosclerosis. The blockade of the AT<sub>1</sub> receptor by valsartan may allow the activation of the AT<sub>2</sub> receptor by angiotensin II (26). This may, in turn, facilitate nitric oxide generation (27) that may contribute to an antiinflammatory effect. These observations also have implications for the action of ACE inhibitors because they reduce the concentration and bioavailability of angiotensin II in plasma. However, ACE inhibitors probably take longer to exert this effect.

The recent Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study demonstrating a significant fall in cardiovascular mortality and morbidity in both diabetic and nondiabetic populations after intake of another angiotensin receptor blocker, losartan, is consistent with the mechanistic data presented here (28). Furthermore, it is also of interest that our short-term studies with  $\beta$ -blockers (nadolol) and a combined  $\alpha$ - and  $\beta$ -blocker (carvedilol) (21, 29) demonstrated a significant suppression of ROS generation by leukocytes but did not alter plasma CRP concentrations.

The comprehensive antiinflammatory effect of valsartan is similar to that we have described for insulin and the insulin sensitizers of the thiazolidinedione class (13–15, 30). The antiinflammatory effects of these drugs are of relevance to potential prevention of atherosclerosis as well as the restoration of insulin sensitivity in insulin resistant states. Thus, losartan has been shown to prevent both cardiovascular complications and type 2 diabetes (28).

The fact that these rapid and marked antiinflammatory changes have been observed in normal subjects within the period of 1 wk of the administration of a modest dose of valsartan suggest 1) a potent antiinflammatory effect of this drug and 2) a tonic proinflammatory effect of angiotensin II

in normal subjects. It is likely that these effects would be more impressive in patients with inflammatory changes given higher doses of the drug for longer periods. The ability of valsartan to induce a rapid antiinflammatory effect like that of thiazolidinediones may also allow it to be used in clinical situations in which we require a rapid antiinflammatory effect. The absence of an effect by simvastatin or quinapril at relatively high doses in our study also suggests that the antiinflammatory effect of these drugs is relatively slow to develop.

In conclusion, valsartan inhibits ROS generation by PMN and MNC and, thus, reduces the oxidative load. It also suppresses NF- $\kappa$ B while inducing I $\kappa$ B $\alpha$  and suppressing plasma CRP concentration. These actions are consistent with an antiinflammatory and potential anti-atherogenic effect of valsartan and possibly other ARBs in the long term. Our data are also the first to distinguish between ARBs, on the one hand, and ACE inhibitors and statins in terms of the rapidity of onset.

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Address all correspondence and requests for reprints to: Paresh Dandona, M.D., Ph.D., F.R.C.P., F.A.C.P., F.A.C.C., Diabetes-Endocrinology Center of Western New York, 3 Gates Circle, Buffalo, New York 14209. E-mail: pdandona@kaleidahealth.org.

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