Free Rad. Res. Comms., Vol. 17, No. 4, pp. 271–278
Reprints available directly from the publisher
Photocopying permitted by license only

GLOMERULAR INJURY INDUCED BY HYDROGEN PEROXIDE: MODIFYING INFLUENCE OF ACE INHIBITORS

LISA CLAYTON, CHRISTOPHER HILEY, SIMON DAVIES, RICHARD D'SOUZA, PETER JONES, RICHARD STRANGE and GEOFFREY ABER

Renal and Clinical Biochemical Research Laboratories, School of Postgraduate Medicine, Keele University, North Staffordshire Hospital Centre, Stoke-on-Trent, ST4 7QB, UK

(*Received June 22, 1992; in revised form July 6, 1992*)

The sensitivity of isolated glomeruli from normotensive (Wistar-Kyoto, WKY) and spontaneously hypertensive (SHR) strains to oxidant stress was studied by determining the incidence of pyknosis, karyohexis and karyolysis after incubation with different concentrations of hydrogen peroxide (H_2O_2) (4.7×10^{-9}–10^{-3} M). Even though the proportion of glomeruli containing nuclei that demonstrated these features increased progressively with increasing concentrations of H_2O_2, the number of severely damaged glomeruli was relatively small even at concentrations of 4.7×10^{-3} M.

Examination of the surface epithelial cells of glomeruli using scanning electron microscopy revealed no evidence of disturbance of the macroscopic or podocyte structure or, of increased blebbing after H_2O_2-treatment. These data suggest damage to nuclei is an early result of ROS stress on glomeruli.

Preincubation of WKY glomeruli with captopril or lisinopril resulted in a significant drop in the proportion of WKY glomeruli demonstrating structural damage after oxidant stress. In contrast, preincubation of SHR glomeruli with lisinopril had no effect on oxidant-induced changes in the morphology of SHR glomeruli, whereas captopril effected a significant increase in the proportion of glomeruli demonstrating damage at all concentrations of H_2O_2.

KEY WORDS: Glomerulus, oxidative stress, hydrogen peroxide, pyknosis, karyohexis, karyolysis.

INTRODUCTION

Recent evidence suggests that reactive oxygen species (ROS) play a major role in mediating the glomerular injury associated with many experimental forms of immunologically and non-immunologically determined glomerular disease.[1–3] Resident mesangial, epithelial and endothelial cells in the normal glomerulus produce low concentrations of ROS as a result of aerobic metabolism. Following insult, glomerular exposure to these species increases markedly primarily because of release by the macrophages and neutrophils that infiltrate the glomerulus as part of the inflammatory process.[3]

Many studies have shown that ROS have complex effects on both cell function and structure. These include acting as mitogens at low concentrations while high concentrations may cause cell damage and even death via mechanisms such as oxidation of lipid and DNA bases, scission of DNA and perturbation of intracellular calcium homeostasis.[4–6]

Because of the potentially harmful effects of ROS, cells modulate their concentration by mechanisms that include iron sequestration, scavenging and widespread expression of the antioxidant enzymes. CuZn- and Mn-superoxide dismutase, catalase and Se-glutathione peroxidase.[7] Previous studies from this and other laboratories have however shown only weak expression of antioxidant enzymes by glomerular cells suggesting either, the importance of scavengers (e.g. vitamins A, E and GSH) or that the glomerulus is particularly susceptible to ROS-induced damage.[8,9] Support for the latter suggestion comes from studies showing the protective influence of antioxidant enzymes, scavengers of the hydroxyl radical and the iron chelator, desferrioxamine, in experimental models of glomerular disease.[10] Angiotensin converting enzyme inhibitors (ACEI) have also been shown to limit the severity of many forms of experimentally determined glomerular injury although the mechanism(s) responsible for this effect is unknown.[11,12] It has been suggested in the case of one ACEI, captopril, that this results from scavenging of ROS by the sulphydryl group in its molecular structure. This view is not, however, supported by recent *in vitro* work.[13,14]

Most studies of the role of ROS in the pathogenesis of glomerular disease and the potentially protective effects of ACEI have been undertaken in whole animals. Observations on isolated glomeruli or cultured glomerular cells have been limited to the effects of ROS on metabolic activity and have failed to relate these to ROS-induced structural injury. The present study has several aims. First, to examine the sensitivity of the isolated glomerulus to oxidant stress by determining the effect of different concentrations of hydrogen peroxide (H_2O_2), on glomerular structure. Second, to assess whether this is modified by a sulphydryl and a non-sulphydryl-containing ACEI and third, to determine whether glomeruli from a spontaneously hypertensive strain of rats (SHR) respond similarly to H_2O_2 as those from the normotensive Wistar-Kyoto strain (WKY) since SHR are more vulnerable to injury than WKY.

MATERIALS AND METHODS

Preparation of Isolated Glomeruli

Glomeruli were isolated from kidneys from male WKY and SHR (16–18 weeks) using a modified sieving technique.[15,16] Kidneys were removed without perfusion, chilled in ice-cold Krebs-Henseleit buffer (pH 7.4) and decapsulated. Cortices were passed through 250, 100 and then 75 μm nylon sieves with glomeruli being collected from the 75 μm sieve. Purity of glomerular suspensions, assessed by light microscopy, was >95%.

Incubation of Isolated Glomeruli with H_2O_2

Suspensions (0.9 ml) of glomeruli (approx. protein 0.25 mg) in Krebs-Henseleit buffer were pipetted into pre-warmed (37°C) polypropylene tubes and after 10 min incubation, H_2O_2 was added to give final concentrations of 0 and 4.7×10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. Following further incubation (20 min, 37°C), with frequent gentle agitation, glomeruli were harvested by centrifugation (2200 g, 4°C, 2 min). In experiments to study the effects of ACEI, captopril and lisinopril (25 μM), were added to glomeruli 20 min before H_2O_2. The dose of ACEI was selected on the basis of previous studies.[15,16]

Examination of Glomerular Structure using Light Microscopy

Glomeruli were sedimented after incubation with H_2O_2 alone or H_2O_2 plus ACEI and fixed in 10% neutral buffered formalin for 24 h. The glomerular suspensions were washed three times in phosphate buffered saline, resuspended in 20% (w/v) human albumin (Blood Product Laboratory, Elstree, Herts) and recentrifuged (200 g, 10 min, 20°C). Absolute ethanol was gently layered on top of the pellet and left for 48 h. Glomeruli thus embedded in a block of denatured protein were processed to paraffin wax. Sections (4 μm) were cut, stained with haematoxylin and eosin and examined for pyknosis (shrinkage and condensation of nuclei), karyohexis (fragmentation of nuclei), karyolysis (lysis of nuclei) and for evidence of morphological damage to glomerular structure such as acellularity and gross enlargement. The finding of any or all of these parameters in any glomerulus resulted in it being recorded as damaged: the degree of damage to each glomerulus being graded 0, 1–30, 31–60 and >60% according to the percentage of nuclei demonstrating pyknosis, karyohexis and/or karyolysis. Each assessment involved examination of 100 glomeruli and was performed in quadruplicate at each concentration of H_2O_2. Glomeruli not exposed to H_2O_2 were examined under otherwise identical conditions. Examinations were carried out by C.H. who was unaware which experimental group the glomerular preparation had been obtained from.

Examination of Glomerular Structure using Scanning Electron Microscopy

Glomeruli were also examined by scanning electron microscopy after exposure to the following concentrations of H_2O_2; O, 4.7×10^{-7} M and 4.7×10^{-5} M. Glomeruli were sedimented after incubation with H_2O_2, fixed (20 h, 4°C) in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2 mM calcium chloride and washed three times in the cacodylate buffer. The glomerular pellet was incubated (1 h) with osmium tetroxide (1% w/v in the cacodylate buffer), washed three times with the cacodylate buffer and dehydrated through to 100% ethanol. Unsectioned specimens were mounted onto boats, coated with gold and examined using a Jeol 100CX instrument. Twenty glomeruli from the control group and each H_2O_2-treated group were examined at magnifications $\times 900$– $\times 25000$ for evidence of damage to macroscopic structure, blebbing and loss of podocyte structure.

Statistical Analysis

X^2 tests were used to test for differences in damage proportions.

RESULTS

Effects of H_2O_2 on Glomerular Structure

Figure 1 shows that both the proportion of glomeruli from WKY and SHR exhibiting pyknosis, karyohexis and karyolysis, and the proportion containing up to 30%, 31–60% or greater than 60% of cells demonstrating damaged nuclei increased progressively with increasing concentrations of H_2O_2. Although H_2O_2 in concentrations as low as 4.7×10^{-8} M, significantly increased ($p < 0.05$) the proportion of nuclei demonstrating damage, the number of glomeruli that were

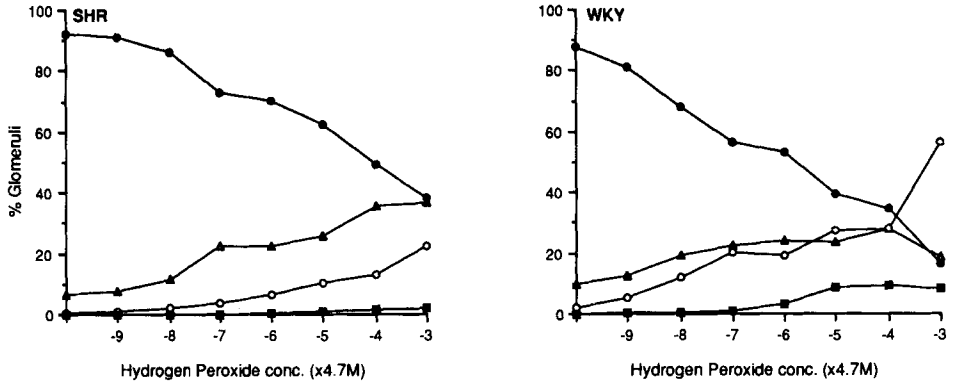


FIGURE 1 Extent of damage induced by H_2O_2 on isolated glomeruli from WKY and SHR. Undamaged glomeruli are shown by ●, those showing 1–30% damaged nuclei by ▲, and those showing 31–60% by ○, and >60% by ■.

severely damaged (i.e. >60% of cells) was relatively small even at H_2O_2 concentrations as high as 4.7×10^{-3} M.

After exposure to 0, 4.7×10^{-7} M and 4.7×10^{-5} M H_2O_2, glomeruli were further examined using scanning electron microscopy. Examination of WKY glomeruli at ×900 magnification revealed a surface view of the glomerulus comprising a network of capillary loops (Figure 3a). In a few glomeruli some localised separation of the capillaries was observed. Since the frequency of this phenomenon was similar in control and H_2O_2-treated samples, it is likely to be related to mechanical stress suffered during the preparation of glomeruli by sieving or during fixation. At magnifications of ×8800 and ×25000, epithelial cells and their inter-digitating foot processes were visible (Figure 3b, 3c). There was no evidence of membrane blebbing

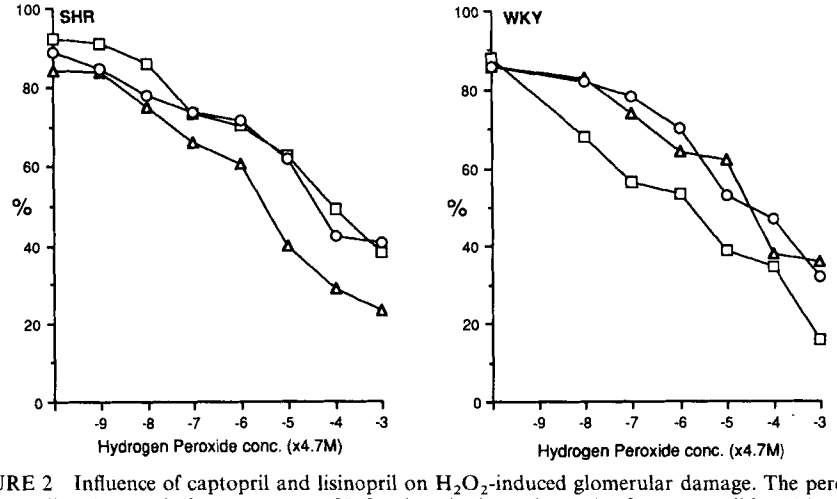


FIGURE 2 Influence of captopril and lisinopril on H_2O_2-induced glomerular damage. The percentage of glomeruli undamaged after exposure to H_2O_2 alone is shown by (□), after captopril by (Δ) and after lisinopril by (○). Since SEM values were small (0.8%–2.5%) they are not shown.

in any of the glomeruli examined or of increased disruption of podocyte structure in H_2O_2-treated preparations. Similar results were obtained from SHR glomeruli although preparations from this strain appeared more susceptible to damage during preparation.

Effects of ACEI on H_2O_2-Induced Changes in Glomerular Structure

The effects of captopril or lisinopril on the morphology of WKY and SHR glomeruli exposed to H_2O_2 concentrations between 10^{-8} M and 4.7×10^{-3} M are shown in Figure 2. Pretreatment with both ACEI resulted in similar, significant decreases ($p < 0.01$) in the proportion of WKY glomeruli demonstrating karyohexis, karyolysis and pyknosis at all concentrations of H_2O_2 compared with corresponding unstressed glomeruli.

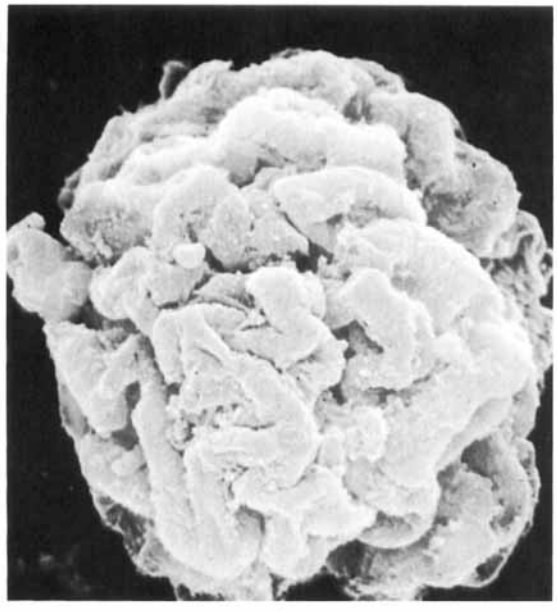
In contrast to its protective effect on glomeruli from WKY, preincubation of SHR glomeruli with captopril resulted in a significant increase ($p < 0.001$) in the proportion of glomeruli containing cells with damaged nuclei following exposure to all concentrations of H_2O_2. Lisinopril however, had no effect on H_2O_2-induced changes in the morphology of SHR glomeruli.

DISCUSSION

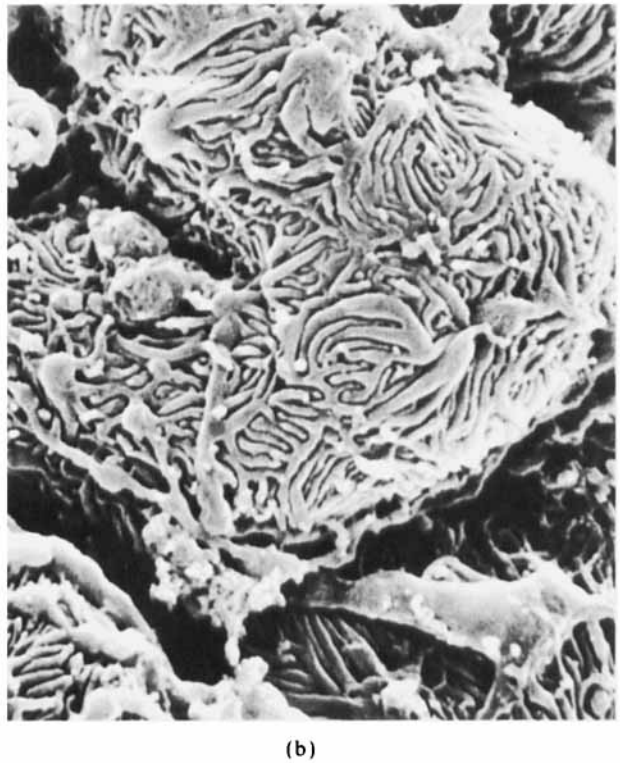
We have described concentration-dependent injury to isolated glomeruli from WKY and SHR by H_2O_2. H_2O_2 was selected as the most appropriate oxidant since the effects of superoxide anion may result from its disproportionation into H_2O_2. Preparations of isolated glomeruli were studied so that any systemic effects of ACEI were excluded.

The study shows that incubation of isolated glomeruli from both WKY and SHR with H_2O_2 resulted in a similar progressive increase in the proportion of cells demonstrating karyohexis, karyolysis or pyknosis. Although significant damage was evident at concentrations of H_2O_2 as low as 4.7×10^{-8} M, a substantial proportion of glomeruli showed no evidence of nuclear damage even after incubation with 4.7×10^{-3} M H_2O_2. Interestingly, our studies using scanning electron microscopal examination of the surface epithelial cells of control glomeruli, and preparations exposed to 4.7×10^{-7} M and 4.7×10^{-5} M H_2O_2 revealed no evidence of membrane blebbing, or of increased disruption of the macroscopic appearance or podocyte structure by H_2O_2. These initial observations support the view that ROS-mediated damage to DNA, presumably as a consequence of intranuclear formation of hydroxyl radicals,[6] is one of the earliest consequences of exposure of glomeruli to even apparently physiological levels of H_2O_2. They are, therefore, consistent with studies showing that damage to DNA is a rapid result of oxidant stress. For example, endothelial cells demonstrate strand breaks only 30 min after exposure to 50×10^{-6} M H_2O_2 while cell lysis and death were not evident until 3–6 hours post stress.[17]

The effects of H_2O_2 on WKY glomeruli were significantly attenuated by both lisinopril and captopril. Although there are no reports of the influence of ACEI on the structural integrity of glomeruli exposed to oxidant stress *in vitro*, the present findings complement those of *in vivo* studies in which the severity of glomerular injury, induced by both immunological and non-immunological challenges thought



(a)



(b)

(c)

FIGURE 3 Scanning electron microscopical examination of control WKY glomeruli. a shows an isolated glomerulus viewed at a magnification ×900. b shows surface epithelial cells and podocyte structure at a magnification of ×8800. c shows a higher power (×25000) view of podocyte structure.

to be partly mediated by ROS, has been attenuated by treatment with an ACEI.[11,12] By contrast, neither ACEI afforded protection to glomeruli from SHR; indeed the severity of H_2O_2-induced injury to glomeruli from SHR was significantly increased in the presence of captopril.

Although the mechanisms by which ACEI influence H_2O_2-induced glomerular damage are unclear it has been suggested that in the case of captopril, this involves scavenging of ROS by the sulphydryl group. This claim, however, is not supported by recent studies[14] and, since the extent of protection afforded during the present study by both ACEI to glomeruli from WKY was similar, yet different from that of SHR glomeruli it is unlikely that scavenging played a major role. Furthermore it appears unlikely that the relatively low concentrations of ACEI used would scavenge the amounts of H_2O_2 used.

The mechanism(s) of the strain differences in glomerular response to captopril is also unclear. There are currently no data on the relative levels of expression of antioxidant enzymes such as catalase and glutathione peroxidase in WKY and SHR glomeruli. However, since several ACEI cause strain-dependent changes in the ratio of glomerular $PGE_2 + PGI_2/TXA_2$ production,[15,16] it is possible this effect may

protect WKY but not SHR glomerular cells from oxidant stress since TXA_2 is cytotoxic to at least some cells (e.g. hepatocytes) and the extent of TXA_2 induced injury can be limited by both PGE_2 and PGI_2.[18]

Acknowledgments

We gratefully acknowledge the generous help of Dr. Carole Hackney and Dr. David Furness for providing electron microscopical facilities and interpreting data. We also thank ICI Pharmaceuticals, the North Staffordshire Medical Institute and the National Kidney Research Fund for support. R.D'S. is the holder of the Research Fellowship of the North Staffordshire Medical Institute.

References

1. S.V. Shah (1991) Oxidant mechanisms in glomerulosclerosis. *Seminars in Nephrology*, **11**, 320–326.
2. N.W. Boyce and S.R. Holdsworth (1986) Hydroxyl radical-mediation of immune injury by desferrioxamine. *Kidney International*, **30**, 813–817.
3. T. Yoshioka and I. Ichikawa (1989) Glomerular dysfunction induced by polymorphonuclear leucocyte derived reactive oxygen species. *American Journal of Physiology*, **257**, F53–F59.
4. B. Halliwell and S. Chirico (1992) Lipid peroxidation: Its mechanism, measurement and significance. *American Journal of Clinical Nutrition*, in press.
5. S. Orrenius, D. McConkey, G. Bellomo and P. Nicotera (1989) Role of Ca^{2+} in toxic cell killing. *Trends in Pharmaceutical Sciences*, **10**, 281–285.
6. B. Halliwell and O.I. Aruoma (1991) DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Letters*, **281**, 9–19.
7. D. Touati (1989) The molecular genetics of superoxide dismutase in *E. coli*. An approach to understanding the biological role and regulation of SODs in relation to other elements of the defence system against oxygen toxicity. *Free Radical Research Communications*, **8**, 1–9.
8. C. Hiley, J. Bell, R. Hume and R.C. Strange (1989) Differential expression of alpha and pi isoenzymes of glutathione S-transferase in developing human kidney. *Biochimica et Biophysica Acta*, **990**, 321–324.
9. R.C. Strange, C. Hiley, C. Roberts, P. Jones, J. Bell and R. Hume (1989) Studies on copper-zinc superoxide dismutase expression in developing human liver and kidney. *Free Radical Research Communications*, **7**, 105–112.
10. J.E. Bird, K. Milhoan, C.B. Wilson, S.G. Young, C.A. Mundy, S. Parthasarathy and R.C. Blantz (1988) Ischaemic acute renal failure and antioxidant therapy in the rat. The relation between glomerular and tubular dysfunction. *Journal of Clinical Investigation*, **81**, 1630–1638.
11. J.J.B. Beukers, A. Van Der Wall, P.J. Hoedemaeker and J.J. Weening (1987) Converting enzyme inhibition and progressive glomerulosclerosis in the rat. *Kidney International*, **36**, 272–279.
12. J.E. Heeg, P.E. Dejong, G.K. Van Der Hem and D. De Zeeuw (1989) Efficacy and variability of the antiproteinaemic effect of ACE inhibition by lisinopril. *Kidney International*, **36**, 272–279.
13. D. Bagchi, R. Prasad and D.K. Das (1989) Direct scavenging of free radicals by captopril, an angiotensin converting enzyme inhibitor. *Biochemical and Biophysical Research Communications*, **158**, 52–57.
14. O.I. Aruoma, D. Akanmu, R. Cecchini and B. Halliwell (1991) Evaluation of the ability of the angiotensin-converting enzyme inhibitor captopril to scavenge reactive oxygen species. *Chemico-Biological Interactions*, **77**, 303–314.
15. G.M. Aber, P. Harding, C. Stonier and A. Messenger (1989) Effect of angiotensin converting enzyme inhibition on isolated glomeruli. *Journal of Hypertension*, **7**, 515–520.
16. P. Harding, C. Stonier and G.M. Aber (1991) The effect of angiotensin converting enzyme inhibition on glomerular eicosanoid production in normotensive and hypertensive rats. *Clinical Science*, **81**, 491–499.
17. R.G. Spragg (1991) DNA strand break formation following exposure of bovine pulmonary artery and aortic endothelial cells to reactive oxygen products. *American Journal of Respiratory Cell and Molecular Biology*, **4**, 4–10.
18. A.A. Horton and J.M. Wood (1990) Prevention of thromboxane B_2-induced hepatocyte membrane bleb formation by certain prostaglandins and a proteinase inhibitor. *Biochimica et Biophysica Acta*, **1022**, 319–324.

Accepted by Professor B. Halliwell