Free Rad. Res., Vol. 28, pp. 393–402
Reprints available directly from the publisher
Photocopying permitted by license only

Hydralazine, but not Captopril, Decreases Free Radical Production and Apoptosis in Neurons and Thymocytes

PETER JOHNSON[a,b,*], YANZHANG WEI[c], MATTHEW J. HUENTELMAN[a], CRAIG M. PETERS[a] and ALEXANDER A. BOLDYREV[d]

[a] *Department of Chemistry,* [b] *College of Osteopathic Medicine,* [c] *Edison Biotechnology Center, Ohio University, Athens, OH 45701, USA;* [d] *Institute of Neurology, Medical Academy of Sciences of Russia, 123367 Moscow, Russia*

Accepted by Prof. M. B. Grisham

(Received 5 December 1997; In revised form 23 January 1998)

The effects of captopril and hydralazine, two commonly used antihypertensive drugs, on free radical generation and the onset of apoptosis in neuron and thymocyte preparations from 10–12 day old rats have been studied. Apoptosis was induced in neurons by kainate or N-methyl-D-aspartate and in thymocytes by heat shock. Intracellular free radical production was measured by 2′,7′-dichlorofluorescein fluorescence, and apoptotic cells were detected by cell staining with fluorescein-labelled annexin V. Captopril was found to have no effect on intracellular free radical generation and also had no significant effect on the early stages of apoptosis in neurons and thymocytes. In contrast, hydralazine was found to decrease free radical generation in both neurons and thymocytes, and it also significantly decreased the numbers of apoptotic cells when neurons and thymocytes were stimulated for apoptosis. Hydralazine had a greater effect on decreasing free radical generation in neurons than in thymocytes, but it had a more pronounced effect on decreasing apoptosis in thymocytes compared to neurons, suggesting that apoptosis, under our experimental conditions, may not solely be triggered by free radical generation. These results contrast with earlier reports that captopril is a free radical scavenger and can decrease apoptosis in T-lymphocytes and cardiomyocytes, and the results obtained with hydralazine are in apparent disagreement with earlier reports that this drug is a free radical generator and can cause intracellular damage suggestive of enhanced free radical formation.

Keywords: Captopril, hydralazine, DCF fluorescence, apoptosis, thymocyte, neuron

Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; NMDA, N-methyl-D-aspartate; PI, propidium iodide

INTRODUCTION

Captopril and hydralazine are two very commonly used antihypertensive drugs which are known to have different mechanisms of action, with captopril functioning as an angiotensin-converting enzyme inhibitor[1,2] and hydralazine

*Corresponding author. Department of Chemistry, Ohio University, Athens, OH 45701, USA. Tel.: (740) 593-1744. Fax: (740) 593-0148. E-mail: johnson@main.chem.ohiou.edu.

acting as a peripheral vasodilator primarily through its effect on calcium ion flux in smooth muscle cells.[3] In addition to their antihypertensive properties, both compounds have been reported to have effects on the free radical status of organisms or isolated tissues. Thus, captopril can protect isolated rat heart against free radical injury,[4–6] and attenuates lipid peroxidation in the erythrocytes of patients with type II diabetes,[7] whereas hydralazine has been shown to stimulate production of free radicals and reactive oxygen species in cultured fibroblasts[8] and can cause *in vitro* oxidative damage to DNA.[9] Further indications that these antihypertensive drugs can serve as free radical modulators in organisms come from recent reports that administration of these drugs alters the expression of a number of enzymes and proteins involved in antioxidant metabolism.[10–14]

The compound 2′,7′-dichlorofluorescein (DCF) has been used as an intracellular reporter molecule to measure the oxidative status of cells in terms of intracellular reactive oxygen species or free radicals,[15] and the conditions for this purpose in the presence of drugs which affect oxidative metabolism have been optimized.[16] In the present study, we have used the DCF fluorescence procedure to directly determine the effects of captopril and hydralazine on intracellular free radical species production in rat thymocytes and cerebellar granule cells (neurons). As free radical generation is a factor that can stimulate apoptotic transformation of cells,[17,18] we have compared the effects of captopril and hydralazine on reactive oxygen species formation and apoptosis under the same conditions, using fluorescein-labelled annexin V to detect the earliest step in apoptosis, namely the reorganization of the plasma membrane bilayer and the externalization of its phosphatidylserine.[19,20] In these experiments, a combination of annexin V as a label for early apoptotic cells and propidium iodide as a label for dead cells was used[21] in order to permit discrimination between apoptotic and necrotic processes of cell death.[22,23]

In contrast to expectations based on previous studies, we have found that, whereas captopril had no effect on intracellular free radical production, hydralazine decreased free radical production in both neurons and thymocytes. These results were found to be paralleled by data from measurements of stimulation of apoptosis in the cell preparations, with captopril having no effect on apoptosis but with hydralazine causing decreases in the percentages of apoptotic neurons and thymocytes.

MATERIALS AND METHODS

Materials

Sprague–Dawley inbred rat pups were bred in the Department of Laboratory Animal Resources at Ohio University. The Apoptosis Detection Kit (#KNX 50) was obtained from R & D Systems, Minneapolis, MN. and dispase (type II, lyophilized) was purchased from Boehringer Mannheim, Indianapolis, IN. Kainic acid and NMDA were purchased from RBI, Natick, MA, DCF was bought from ICN Biomedicals, Aurora, OH, and DCFH-DA was obtained from Molecular Probes, Eugene, Oregon. Fetal bovine serum (F 2442), captopril, hydralazine, PI and other laboratory reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Cell Preparations

Neurons (cerebellar granule cells) were prepared from cerebella of 10–12 day old rats (either sex) according to the general principles previously described.[22] Each cerebellum was gently macerated and the material was treated with 5 ml of a dispase solution (3.4 mg/ml in Tyrode's solution) for 1 h at 33–35°C, and the tissue was then washed twice with Tyrode's solution containing fetal bovine serum at a concentration of 0.01 ml/ml. After gentle disruption in a Pasteur pipet using 5 ml Tyrode's solution containing

fetal bovine serum (0.0025 ml/ml), cells were separated from debris by filtration of the suspension through a 44 μm nylon mesh. Cells were then incubated for restitution at 33–35°C for 1 h after dilution to an approximate concentration range of $2–5 \times 10^5$ cells/ml as determined by hemacytometry. Experimental procedures on these preparations were then performed immediately and the percentage of dead cells in the preparations was no larger than 20% as measured by flow cytometry using PI as a specific label for dead cells.[21]

Thymocytes were prepared from the same animals as neurons according to the exact procedure previously published[23] and were then diluted to 2×10^5 cells/ml for experimental purposes. Cells were used immediately following preparation and the percentage of dead cells measured by flow cytometry was less than 1%.

Treatment of Cells Prior to Flow Cytometry Measurements

Following cell suspension preparation, captopril or hydralazine was added at final concentrations of 100 μM (chosen to approximate the concentrations of these drugs previously used in animal feeding studies[14]) and the cells were incubated for restitution at 33°C for 15 min before apoptotic stimulation. Early apoptosis in thymocytes was induced by heat shock at 43°C for 15 min[24] followed by incubation at 37°C for a further 3 h. Neurons were stimulated for apoptosis by either 1 mM NMDA[25] or 100 μM kainic acid[26] at 37°C for periods of 30 min for DCF fluorescence measurement and 3 h for apoptosis detection. Appropriate control cell preparations in the absence of antihypertensive drugs and/or with no apoptotic stimulation conditions were used in each experiment.

Procedures for Flow Cytometry

All cytometry data were collected using 0.5 ml samples containing approximately 10^5 cells, with 10^4 cells counted per analysis. Triplicate or duplicate samples were counted, and each experiment was performed at least twice.

In order to characterize intracellular free radical formation and cell viability, two-color analysis by flow cytometry was performed simultaneously with DCF fluorescence as a specific indicator for intracellular free radical formation[15] and PI as a specific marker for necrotic cells.[21] In these experiments, the final concentration of DCFH-DA in the cellular suspensions was 100 μM for neurons and 50 μM for thymocytes (using a 10 mM stock solution of DCFH-DA in dimethylsulfoxide) and the cells were loaded with DCFH-DA for 1 h for neurons and 30 min for thymocytes, times which were found to be optimal for loading. PI was then added to DCF-loaded cell suspensions at a final concentration of 10 μg/ml approximately 1 min before measurement in the flow cytometer using a stock solution of 0.5 mg PI/ml H_2O. In the flow cytometer (Becton Dickinson FACSort), the excitation wavelength for DCF and PI was 488 nm and DCF emission was measured at 510–550 nm and PI emission at 555–580 nm.[15] Each peak was gated by the FACSort software program to permit calculation of the mean fluorescence intensity in arbitrary units, and mean fluorescence intensities from different experiments were compared after normalization by setting the fluorescence intensity of control samples to 100%.

For measurement of cell sub-populations which were viable, necrotic, pre-apoptotic and apoptotic, the Apoptosis Detection Kit was used in which fluorescein-labelled annexin V detects pre-apoptotic and apoptotic cells and PI detects dead cells. The flow cytometer was calibrated according to the instructions in the Detection Kit in order to discriminate between viable, necrotic, pre-apoptotic and apoptotic cell sub-populations.

Measurement of DCF Fluorescence Quenching

For measurement of the quenching of DCF fluorescence by hydralazine, the fluorescence of

20 nM DCF in Tyrode's solution in the presence of hydralazine within a concentration range of 10–500 μM was measured in a Perkin-Elmer LS-5 Fluorescence Spectrophotometer.

Statistical Methods

Data on DCF mean fluorescence intensities and the percentages of cell sub-types in comparison to control samples were analyzed by one-way ANOVA using the Minitab computer program. Results were calculated as mean ± SD and $P < 0.05$ was used to indicate a statistically significant difference between data sets.

RESULTS

Effects of Antihypertensive Drugs on DCF Fluorescence in Activated Cells

Curve 1 in Figure 1A shows a representative analysis of the DCF fluorescence of non-apoptotically stimulated neurons which reflects DCFH oxidation in these cells, curve 2 shows that the level of DCF fluorescence in kainate-stimulated neurons was elevated above the control value, and curve 3 shows that when hydralazine was present in the kainate-stimulated cell preparations, a dramatic left-shift of the DCF fluorescence curve occurred.

Table I shows the mean values of the mean fluorescence intensities of the DCF peaks from these experiments and these data show that, in comparison to the control value, kainate stimulation caused a statistically significant increase of 13% in the mean fluorescence intensity whereas the presence of hydralazine in the kainate-stimulated cells caused the mean fluorescence intensity to decrease to 25% of the control value.

This effect of hydralazine was observed both when hydralazine was added to the cells 1 h before the addition of DCFH-DA and when both compounds were added simultaneously (results not shown), demonstrating that the effect was

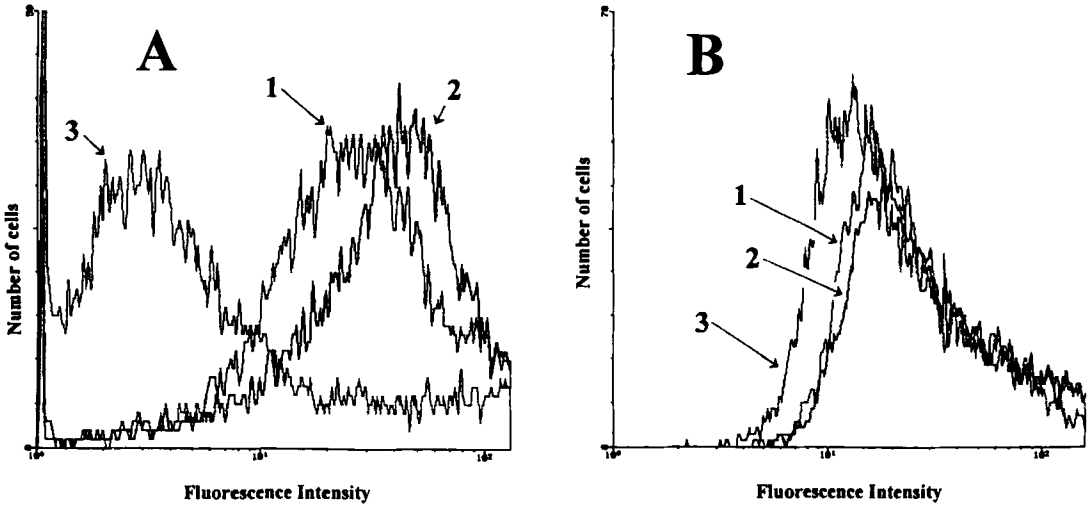

FIGURE 1 The effect of hydralazine on the DCF fluorescence of rat neurons and thymocytes. In graph A, curve 1 shows the DCF fluorescence peak of neurons not stimulated by kainate and curve 2 shows the DCF fluorescence of neurons stimulated by 100 μM kainate for 30 min at 37°C. Curve 3 shows the DCF fluorescence of kainate-stimulated neurons in the presence of 100 μM hydralazine. In graph B, curve 1 shows the DCF fluorescence peak of thymocytes not subjected to heat shock and curve 2 shows the DCF fluorescence of heat-shocked thymocytes. Curve 3 shows the DCF fluorescence of heat-shocked thymocytes in the presence of 100 μM hydralazine. Fluorescence intensity in arbitrary units (horizontal axis) is plotted on a logarithmic scale against cell numbers on a linear scale (vertical axis).

TABLE I The effects of apoptotic stimulation and hydralazine on intracellular DCF fluorescence intensity in neurons and thymocytes

Cell type	Treatment	Mean DCF fluorescence intensity (control = 100%)
Neurons	Control	100 ± 4.53
	Kainate	113 ± 4.80*
	Kainate plus hydralazine	24.7 ± 4.57*
Thymocytes	Control	100 ± 5.93
	Heat shock	106 ± 5.32
	Heat shock plus hydralazine	53.3 ± 1.03*

Mean fluorescence intensities obtained as arbitrary units in the experiments were normalized to percentage values based on the control value set to 100%. The data are the mean values from three experiments and the symbol * indicates that the experimental mean value is significantly different from the mean value of the respective control value.

not caused by a direct effect of hydralazine on DCFH-DA. Control experiments with DCF and hydralazine (varied between 10 and 500 μM) in Tyrode's solution also demonstrated that very little quenching of DCF fluorescence by hydralazine occurred with only a 4% quenching effect even at 500 μM hydralazine. The results obtained by flow cytometry therefore indicate that the decrease in cellular DCF fluorescence was primarily the result of an inhibition of free radical production by hydralazine in the cells.

In contrast to the left-shift caused by hydralazine, the same concentration of captopril (100 μM) had no effect on the position of the DCF fluorescence curve (results not shown), indicating that free radical formation induced by activation/overloading of the glutamate receptors under our conditions was unaffected by captopril. Similar results (not shown) for the effects of hydralazine and captopril on DCF fluorescence in neurons were also obtained when apoptosis was stimulated by NMDA.

Figure 1B shows representative analyses of the DCF fluorescence of thymocytes not subjected to heat shock (curve 1), of heat shocked thymocytes (curve 2), and of thymocytes subjected to heat shock in the presence of hydralazine (curve 3). These curves and the statistical data in Table I of the mean fluorescence intensities of the DCF peaks show that there was no significant increase in DCF fluorescence caused by heat shock, but that hydralazine treatment caused a large and statistically significant decrease in DCF fluorescence. In agreement with the results obtained with neurons, captopril was found to have no effect on DCF fluorescence intensity (results not shown).

Effects of Antihypertensive Drugs on Apoptotic Cell Percentages

The percentages of apoptotic and pre-apoptotic cells in rat neuron and thymocyte preparations were determined using the Apoptosis Detection Kit and flow cytometry. Figure 2 shows representative dot plot data obtained for this procedure, with plot A showing an unstimulated neuron preparation and plot B showing a thymocyte preparation which had not been subjected to heat shock. In these plots, the types of cells found in each quadrant are based on their staining with annexin V and/or propidium iodide and are as follows: lower left, viable cells; lower right, pre-apoptotic cells; upper right, early apoptotic cells; upper left, necrotic cells.

As shown in Table II, statistically significant increases in the percentages of early apoptotic cells were found as a result of kainate or NMDA (results not shown) stimulation of neurons and

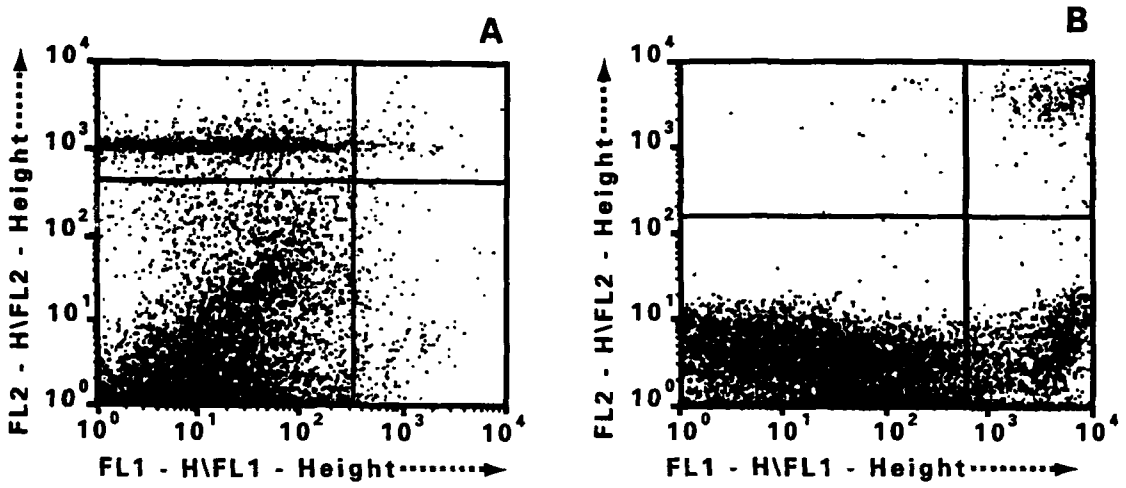

FIGURE 2 Measurement of apoptosis of rat neurons and thymocytes using the Apoptosis Detection Kit. The data are presented as dot plots in which the vertical FL2-H\FL2 axis detects PI-positive cells, and the horizontal FL1-H\FL1 axis detects annexin V-positive cells. The upper left quadrant region contains necrotic (PI-stained) cells, the upper right region contains apoptotic (PI- and annexin-V-stained) cells, the lower left region contains viable (PI- and annexin-V-unstained) cells, and the lower right region contains pre-apoptotic (PI-unstained and annexin-V-stained) cells. Plot A is for neurons not stimulated by kainate: and plot B is for thymocytes without heat shock treatment.

TABLE II The effects of apoptotic stimulation on the percentages of apoptotic, pre-apoptotic, viable and necrotic cells in neuron and thymocyte preparations

Cell type	Treatment	Relative percentage of cells counted			
		Apoptotic	Pre-apoptotic	Viable	Necrotic
Neurons	Control	0.68 ± 0.05	1.96 ± 0.30	82.3 ± 2.96	15.07 ± 2.60
	Kainate	1.32 ± 0.01*	1.93 ± 0.86	81.9 ± 4.16	14.83 ± 3.76
Thymocytes	Control	3.14 ± 0.08	17.11 ± 0.66	79.43 ± 0.86	0.19 ± 0.05
	Heat shock	4.03 ± 0.22*	14.61 ± 2.18	81.36 ● 2.28	0.22 ± 0.05

The cell percentages in the preparations were determined by counting cells in each of the four quadrants of the flow cytometer dot plot obtained by use of the Apoptosis Detection Kit. The data are the mean values from two experiments with neurons and three experiments with thymocytes. The symbol * indicates that the experimental mean value is significantly different from the mean value of the respective control value.

by heat shock treatment of thymocytes. The early apoptotic population was increased about two-fold for neurons after kainate stimulation and slightly less than this (1.4-fold) for heat shock treatment of thymocytes, and in both cases, the increases appeared to be generated by a transformation of pre-apoptotic cells (lower right quadrant) to the early apoptotic state (upper right quadrant). In our experiments, the percentages of necrotic cells (upper left quadrant) were much higher in the neuron than in the thymocyte preparations, in agreement with previous results.[22]

When this procedure was used to analyze the effects of treatment of rat neurons with antihypertensive drugs (Figure 3), it was found that captopril treatment had no significant effect on the percentage increase in apoptotic cells caused by kainate stimulation. In contrast, hydralazine treatment significantly reduced the percentage of apoptotic cells in comparison to the kainate-treated control value, although the

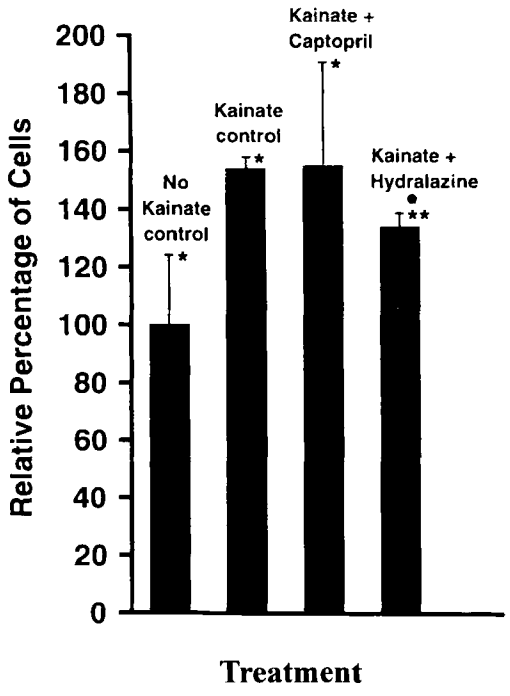

FIGURE 3 The effects of captopril and hydralazine on apoptosis in rat neurons subjected to stimulation by 100 μM kainate. The bar chart shows data for the amounts of apoptotic cells expressed as mean percentages of total cells (± SD) normalized to the no drug, no kainate control percentage which was 0.86%. Each bar represents the mean of three experiments. The symbol * indicates a significant difference compared to the no kainate control and the symbol ** indicates a significant difference compared to the no drug, kainate-treated control.

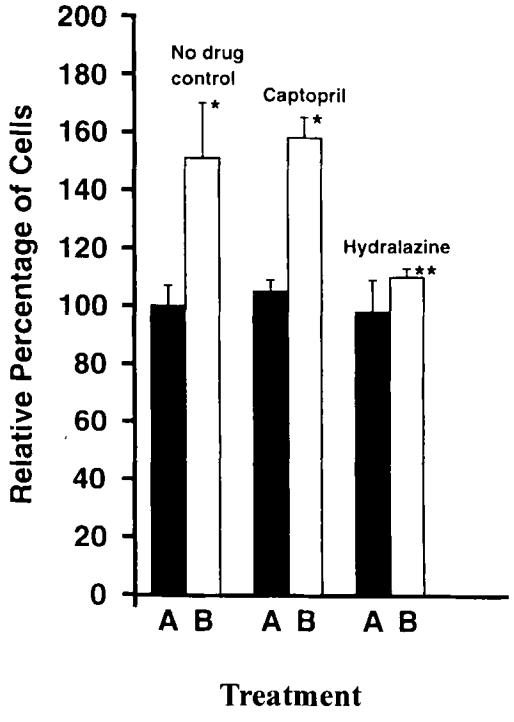

FIGURE 4 The effects of captopril and hydralazine on apoptosis in rat thymocytes subjected to heat shock. The bar chart shows data for the amounts of apoptotic cells expressed as mean percentages of total cells (± SD) normalized to the no drug, no heat shock control percentage which was 2.82%. The dark-colored bars (A) correspond to no heat shock controls in the absence or presence of antihypertensive drugs (100 μM), and the light bars (B) show data for heat shock-treated cells. Each bar represents the mean of three experiments. The symbol * indicates a significant difference from the no heat shock control and the symbol ** indicates a significant difference compared to the no drug, heat shock control value.

value for the hydralazine-treated cells was still significantly above the no kainate, no drug control value.

For rat thymocytes, similar measurements were performed and in this case comparisons were made between heat shock-treated cells and cells not subjected to heat shock (Figure 4). It was found that neither captopril nor hydralazine had a significant effect on the percentage of apoptotic cells in preparations which were not subjected to heat shock. With heat-shocked thymocytes, captopril had no significant effect on the percentage increase in apoptotic cells, but the presence of hydralazine decreased this percentage to the no heat shock, no drug control value.

DISCUSSION

Captopril has previously been shown to have *in vivo* antioxidant properties[4–7,27] which may be a consequence of its direct antioxidant activity,[27,28] and it can act as a scavenger of free radicals in various tissues and cells at micromolar concentrations.[4,7] Our studies, which show that captopril at a pharmacological concentration of 100 μM in the medium does not affect intracellular hydrogen peroxide production, therefore contrast with these earlier results

despite the use of similar concentrations in the studies. A possible reason for this difference is that in our studies, the exposure time of the cells to captopril was relatively short (no more than a few hours), whereas in the *in vivo* studies, the animals were treated chronically with captopril for periods up to three months. This difference in exposure time could affect the modulation of the expression of antioxidant enzymes by captopril,[10,14] and therefore the antioxidant status and free radical content of the cell.

In previously published work on the effect of captopril on apoptosis,[29,30] it was found that captopril decreased apoptosis in T-lymphocytes and cardiomyocytes, which contrasts with our results which show that captopril does not significantly decrease early apoptosis in neurons and thymocytes. The results obtained in the lymphocyte and cardiomyocyte studies might be explained by the longer periods of exposure to captopril and the fact that early apoptosis was not being measured in these studies. Our results also suggest that captopril does not have a direct antioxidant effect as it does not decrease DCF fluorescence and that its protection of cells and tissues over longer periods of time may be explained by its indirect effects on antioxidant systems such as modulation of antioxidant enzyme levels.[10,14]

In contrast to captopril, hydralazine has previously been shown to elevate free radical levels in cultured cells[8,31] which could be related to its modulation of antioxidant enzymes[13,14] or to direct radical effects.[8,32] These results, and the reports that much higher concentrations of hydralazine can promote oxidative damage to DNA,[9] contrast with the data obtained from the present studies which show that a pharmacological concentration of 100 μM hydralazine decreases free radical production in neurons and thymocytes. A possible explanation for this difference is that, in the previous studies, a much longer period of exposure to hydralazine was used, such as daily feeding of hydralazine to rats for three weeks[33] and incubation of fibroblasts *in vitro* for up to 120 h in the presence of hydralazine.[31] The latter studies found that lipid peroxidation in the presence of hydralazine was time-dependent, and further experiments will be required to determine if this time-dependent effect is related to the metabolism of hydralazine over longer periods of time to other compounds with pro-oxidant properties which can cause oxidative damage to cellular components.

Our results have shown that decreases in both free radical levels and apoptosis are caused by hydralazine treatment of stimulated neurons and thymocytes, but also show that the decrease in apoptosis is less affected by a decrease in intracellular free radicals in neurons than in thymocytes. This lack of a correlation between the extent of the decrease in DCF fluorescence and the decrease in apoptosis suggests that processes stimulating apoptosis in neurons may be much less dependent on free radical formation than in thymocytes or even that there is no direct connection between these two effects. Further evidence that, at least in thymocytes, apoptosis may not be caused by an increase in free radicals is also seen from the studies performed on these cells in the absence of hydralazine, which show that heat shock does not increase intracellular free radical levels but does induce apoptosis. These results therefore support previous conclusions from different laboratories that, in addition to free radical stimulation of apoptosis, other processes which do not involve free radical formation may also stimulate apoptosis.[29,34]

An earlier study[35] concluded that hydralazine had no effect on apoptosis in aortic smooth muscle cells from SHR rats as measured by DNA content and fragmentation analysis, whereas our studies with thymocytes and cerebellar neurons have shown that hydralazine significantly reduced the proportion of cells undergoing early apoptosis. Although both of these studies indicate that hydralazine did not promote apoptosis in the cell types studied, hydralazine has been reported to elevate the expression of the c-fos proto-oncogene in non-cerebellar regions of

the brain,[11] and as c-fos induction has been associated with the initiation of apoptosis in some cells,[36,37] it may therefore be possible that, under certain circumstances, hydralazine could favor apoptosis. In this context, a study of the effects of hydralazine on mature cardiomyocytes would be of considerable interest in view of its widespread use as an antihypertensive drug and recent observations that, although cardiomyocyte apoptosis is associated with heart failure in hypertensive animals,[38] apoptosis (as opposed to necrosis) may play a beneficial role in myocardial infarction and viral infection.[39]

Acknowledgements

We thank Ms. Stephanie M. Polutnik for excellent technical assistance. This research was supported by a Ohio University Putnam Visiting Professorship to A.A.B. and by grant #96-04-49078 (1997) from the Russian Foundation for Fundamental Research.

References

[1] P. A. van Zwieten (1993) New avenues in antihypertensive drug treatment. *Clinical and Experimental Hypertension*, **15**, 1205–1220.

[2] M. Chopra, H. Beswick, M. Clapperton, H. J. Dargie, W. E. Smith and J. McMurray (1992) Antioxidant effects of angiotensin-converting enzyme (ACE) inhibitors: free radical and oxidant scavenging are sulfhydryl dependent, but lipid peroxidation is inhibited by both sulfhydryl- and nonsulfhydryl-containing ACE inhibitors. *Journal of Cardiovascular Pharmacology*, **19**, 330–340.

[3] A. M. Gurney and M. Allam (1995) Inhibition of calcium release from the sarcoplasmic reticulum of rabbit aorta by hydralazine. *British Journal of Pharmacology*, **114**, 238–244.

[4] X. J. Pi and X. Chen (1989) Captopril and ramiprilat protect against free radical injury in isolated working rat hearts. *Journal of Molecular and Cellular Cardiology*, **21**, 1261–1271.

[5] W. Westlin and K. Mullane (1988) Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation*, **77**, I30–I39.

[6] P. Sogaard, I. C. Klausen, J. Rungby, O. Faergeman and K. Thygesen (1996) Lipoprotein (a) and oxygen free radicals in survivors of acute myocardial infarction: effects of captopril. *Cardiology*, **87**, 18–22.

[7] Y. Altuntas, M. Guven, E. Ince, O. Acbay, M. Caner and G. Kanigur-Sultuybek (1995) The *in vitro* effects of captopril on the levels of lipid peroxidation and glutathione of erythrocytes in type II diabetes. *Journal of Basic Clinical Physiology and Pharmacology*, **6**, 281–288.

[8] L. Weglarz and G. Bartosz (1991) Hydralazine stimulates production of oxygen free radicals in Eagle's medium and cultured fibroblasts. *Free Radical Biology and Medicine*, **11**, 149–155.

[9] M. Runge-Morris, N. Wu and R. F. Novak (1994) Hydrazine-mediated DNA damage: role of hemoprotein, electron transport, and organic free radicals. *Toxicology and Applied Pharmacology*, **125**, 123–132.

[10] E. M. V. de Cavanagh, F. Inserra, L. Ferder, L. Romano, L. Ercole and C. G. Fraga (1995) Superoxide dismutase and glutathione peroxidase activities are increased by enalapril and captopril in mouse liver. *FEBS Letters*, **361**, 22–24.

[11] J. C. Graham, G. E. Hoffman and A. F. Sved (1995) c-Fos expression in brain in response to hypotension and hypertension in conscious rats. *Journal of the Autonomic Nervous System*, **55**, 92–104.

[12] N. Iwai, K. Hanai, I. Tooyama, Y. Kitamura and M. Kinoshita (1995) Regulation of neuronal nitric oxide synthase in rat adrenal medulla. *Hypertension*, **25**, 431–436.

[13] M. Runge-Morris, Y. Feng, R. C. Zangar and R. F. Novak (1996) Effects of hydrazine, phenelzine, and hydralazine treatment on rat hepatic and renal drug-metabolizing enzyme expression. *Drug Metabolism and Disposition*, **24**, 734–737.

[14] K. S. Cabell, L. Ma and P. Johnson (1997) Effects of antihypertensive drugs on rat tissue antioxidant enzyme activities and lipid peroxidation levels. *Biochemical Pharmacology*, **54**, 133–141.

[15] A. Maftah, O. Huet, P. -F. Gallet and M. -H. Ratinaud (1993) Flow cytometry's contribution to the measurement of cell functions. *Biology of the Cell*, **78**, 85–93.

[16] Y. Oyama, A. Hayashi, T. Ueha and K. Maekawa (1994) Characterization of 2′,7′-dichlorofluorescein fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide. *Brain Research*, **635**, 113–117.

[17] T. A. Sarafian and D. E. Bredesen (1994) Is apoptosis mediated by reactive oxygen species? *Free Radical Research*, **21**, 1–8.

[18] J. Bustamente, A. Tovar-B, G. Montero and A. Boveris (1997) Early redox changes during rat thymocyte apoptosis. *Archives of Biochemistry and Biophysics*, **337**, 121–128.

[19] G. Koopman, C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals and M. H. van Oers (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*, **84**, 1415–1420.

[20] J. P. Fabisiak, V. E. Kagan, V. B. Ritov, D. E. Johnson and J. S. Lazo (1997) Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. *American Journal of Physiology*, **272**, C675–C684.

[21] R. P. Haugland (1989) *Handbook of Fluorescent Probes and Research Chemicals*. Molecular Probes, Eugene, Oregon.

[22] Y. Oyama, D. Carpenter, L. Chikahisa and E. Okazaki (1996) Flow-cytometric estimation of glutamate- or kainate-induced increases in intracellular Ca^{2+} of brain neurons: a technical aspect. *Brain Research*, **728**, 121–124.

[23] L. Chikahisa, Y. Oyama, E. Okazaki and K. Noda (1996) Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol levels of dissociated rat thymocytes. *Japanese Journal of Pharmacology*, **71**, 299–305.

[24] G. Migliorati, I. Nicoletti, F. Crocicchio, C. Pagliacci, F. D'Adamio and C. Riccardi (1992) Heat shock induces apoptosis in mouse thymocytes and protects them from glucocorticoid-induced cell death. *Cellular Immunonology*, **143**, 348–356.

[25] I. J. Reynolds and T. G. Hastings (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *Journal of Neuroscience*, **15**, 3318–3327.

[26] N. A. Simonian, R. L. Getz, J. C. Leveque, C. Konradi and J. T. Coyle (1996) Kainate induces apoptosis in neurons. *Neuroscience*, **74**, 675–683.

[27] D. Jay, A. Cuella and E. Jay (1995) Superoxide dismutase activity of the captopril–iron complex. *Molecular and Cellular Biochemistry*, **146**, 45–47.

[28] D. Lapenna and F. Cuccurullo (1995) Captopril: Does it act as a direct antioxidant by free radical scavenging in humans? *Free Radical Biology and Medicine*, **18**, 377–378.

[29] O. Deas, C. Dumont, B. Mollereau, D. Metivier, C. Pasquier, G. Bernard-Pomier, F. Hirsch, B. Charpentier and A. Senik (1997) Thiol-mediated inhibition of FAS and CD2 apoptotic signaling in activated human peripheral T cells. *International Immunology*, **9**, 117–125.

[30] Z. Li, O. H. L. Bing, X. Long, K. G. Robinson and E. G. Lakatta (1997) Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat. *American Journal of Physiology*, **272**, H2313–H2319.

[31] L. Weglarz, M. Drodz, M. Wardas and M. Goss (1989) Activities of antioxidant enzymes in fibroblasts cultured in vitro in the presence of hydralazine. *Biomedica Biochimica Acta*, **48**, 489–493.

[32] D. C. Goodwin, S. D. Aust and T. A. Grover (1996) Free radicals produced during the oxidation of hydrazines by hypochlorous acid. *Chemical Research in Toxicology*, **9**, 1333–1339.

[33] A. Jendryczko and M. Drozdz (1988) Lipid peroxidation in the nuclear fraction of rat lungs induced by hydralazine. *Neoplasma*, **35**, 37–40.

[34] H. Hug, M. Enari and S. Nagata (1994) No requirement of reactive oxygen intermediates in Fas-mediated apoptosis. *FEBS Letters*, **351**, 311–313.

[35] D. deBlois, B. S. Tea, V. D. Than, J. Tremblay and P. Hamet (1997) Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension*, **29**, 340–349.

[36] S. Pandey and E. Wang (1995) Cells en route to apoptosis are characterized by the upregulation of c-fos, c-myc, c-jun, cdc2, and RB phosphorylation, resembling events of early cell-cycle traverse. *Journal of Cellular Biochemistry*, **58**, 135–150.

[37] D. G. Herrera and H. A. Robertson (1996) Activation of c-fos in the brain. *Progress in Neurobiology*, **50**, 83–107.

[38] M. O. Bolyut, O. H. L. Bing and E. G. Lakatta (1995) The ageing spontaneously hypertensive rat as a model of the transition from stable compensated hypertrophy to heart failure. *European Heart Journal*, **16** (suppl. N), 19–30.

[39] K. A. Krown, M. T. Page, C. Nguyen, D. Zechner, V. Gutierrez, K. L. Comstock, C. G. Glembotski, P. J. E. Quintana and R. A. Sabbadini (1996) Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. *Journal of Clinical Investigation*, **98**, 2854–2865.