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We have extended our studies on the morphological characterization of C6 and astrocyte cells which we described in our last Progress Report, by exposing the cultured cells to oxidative stress and monitoring the time dependent morphological and biochemical changes. These include observing oxy-radical induced biochemical changes in these cells by following changes in structural lipids and characterizing lipase activities in these cells. During this past year we have also begun to study endothelial cells in the same manner and the C6 and astrocyte cells.

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PRINCIPAL INVESTIGATOR: W.B. Weglicki

CONTRACTOR: The George Washington University

CONTRACT TITLE: (U) Hyperbaric Pressure and Tissue Phospholipid Metabolism

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MORPHOLOGICAL STUDIES OF CELLULAR INJURY

In C6-astrocytes and endothelial cells we observed dramatic morphological changes upon exposure to an exogenous hydroxyl-radical generating system (1.67 mM dihydroxyfumarate, and iron(III)-ADP with 50 M $FeCl_3$, 500 M ADP). In both cases, after exposure to the free radical system, the cells developed blebs and eventually died. Although the final result of exposure to the oxy-radical generating system was the same for both cells, the time course of the development of blebs through to the death of the cell was quite different for these two cells.

A detailed morphological study of bleb formation has been performed on C6 astrocyte cells. A characteristic sequence of events developed after the exposure of these cells to the exogenous oxy-radical system. Within 2.5 minutes of exposure to the free radical generating system, 98% of the cells had lost all their villi. The time course of the loss of villi is described in Figure 1. Along with the loss of villi there is a concurrent appearance of blebs on the C6 cells. The bleb formation peaked at 7.5 minutes followed by a decrease in the number of blebbed cells. The decrease in blebbed cells followed a decrease in cell viability which was measured by trypan blue exclusion. There was a total loss of cells by 20 minutes. The decrease in the number of blebbed cells could not be explained by the decrease in total cells.

Bleb formation induced by an exogenous radical system also occurred in the endothelial cells. Endothelial cell blebbing required a longer time to form and the cells lived longer. In the presence of the exogenous radical system 80% of the endothelial cells developed blebs by 30 minutes (Figure 2), whereas the C6 cells reached this point within 7.5 minutes. As in C6 cells endothelial viability seemed to decrease with increasing bleb formation. At the same time there was an increase in the oxidation of the membrane lipids.

BIOCHEMICAL CORRELATES TO CELLULAR INJURY

Exposure of both endothelial and C6 cells to an exogenous free radical generating system resulted in significant cellular injury and death. One indicator of injury was the formation of lipid oxidation product such as malonyldialdehyde (MDA); the production of blebbed cells closely follow the formation of MDA, an indicator of phospholipid oxidative degradation. The MDA production reached a maximum within five to twenty minutes in C6 cells (Figure 3). In most cells, a lag-period was observed before MDA production. At the higher concentration of free radicals, the C6 cells appeared not to have such a lag-period and complete production of MDA occurred within the first ten minutes. However, significant lipid changes occur in C6 cells under these conditions at about 20 minutes (Table I). Phosphorylethanolamine concentration decreases most dramatically while lysophospholipid concentrations increased several times.

PHARMACOLOGICAL STUDIES WITH ANTIOXIDANT DRUGS

We have completed studies on the effects of exogenous free radicals on endothelial cell morphology, and have investigated the biochemical changes that occur with blebbing. Exposure to an exogenous free radical generating system initiated the formation of blebs on the endothelial cells, as was observed with the C6 cells, although the time course of bleb formation was more gradual for endothelial cells. Only 80% of the endothelial cells were blebbed after 30 minutes of exposure to free radicals, while on the same time scale the C6 cells were completely destroyed.

We have found that the number of blebbed endothelial cells could be reduced by 2 to 3 times when the cells were preincubated with the cardiovascular drugs propranolol and captopril (Figures 4, 5

and 6). The effects of propranolol and captopril on MDA formation and cell viability correlated with the observed blebbing.

WORK IN PROGRESS

To understand more clearly the events that lead up to blebbing and the subsequent measurable changes in the structural lipids we have begun the analysis of the lipid metabolizing enzymes which we hope will lead to an explanation of the observed changes in the cell membranes.

We are characterizing the lipid metabolizing enzymes, lipases and phospholipases, in C6, astrocytes, and endothelial cells. We probe the lipid metabolism of these cells by identifying phospholipase and esterase activities. We have analyzed pH profiles of specific enzymatic activities toward different radiolabelled substrates using the assay system describes in Appendix I. Under these conditions the substrate is held in a micelle which allows easy access of the enzyme to the substrate. In addition the detergent assists in further breaking down cell membranes releasing lipases into the assay mixture, any enzymes that may be sequestered in an organelle, such as a lysosome, will be exposed to the radioactive substrate.

The phospholipase activities in C6 cells (Figure 7) indicate that in the presence of calcium there is a wide spectrum of activities. While with EDTA the membrane fraction has a large activity at low pH and a broad high pH activity, and the cytosolic fraction has predominantly a neutral pH activity with lower, but
significant activities at low and high pH. In addition the lack of lysophospholipid production at neutral and high pH indicates that there are significant lysophospholipase activities in this region of the pH profile.

Assays using a lysophospholipase as the substrate gave a broad peak of activity, from pH 4 to pH 9. It is likely that there is only lysophospholipase activity at pH's above 7.0 since there is a large amount of lysophospholipid produced below this pH. These results suggest that there is also an acidic trans-acylase activity, that in conjunction with the acidic phospholipase A_1 produce a high activity with a lysophospholipase substrate. The astrocytes produces similar pH profiles to those of C6.

The only significant differences of the pH profiles of the endothelial cells (Figure 8) to those of the C6 cells are for cytosolic calcium independent activities. Comparing Figure 8D with 7B one sees that the C6 cells major activity is at about pH 6.5 whereas the endothelial activity is at pH 4.5. No other differences are significant.

PLANNED RESEARCH FOR 1990

In the next year we will concentrate our studies on the endothelial cell line. We will complete the analysis of the endothelial cell lipase activities, including calcium dependence, specific activities, time courses, and substrate form and specificity. In conjunction with these studies we will assay for changes in lipase activity after subjecting the cells to exogenous oxy-radical generating systems. Since we have yet to obtain access to the hyperbaric chamber at NMRI, we plan to purchase our own hyperbaric chamber in which we plan to repeat our experiments at 1 to 2 ATA. We also plan to do a chemical analysis of the structural phospholipids of the endothelial and astrocyte cells.

INVENTIONS: None

PUBLICATIONS AND REPORTS: 9

a) Papers Submitted: (2)

"Mechanisms of Cardiovascular Drugs As Antioxidants"
W.B. Weglicki, I.T. Mak and M.G. Simic

"Protective Effects of Sulfhydryl-Containing ACE Inhibitors Against Free Radical Injury In Endothelial Cells"
I.T. Mak, B.F. Dickens, B.R. Pflug, L.A. Kopyta and W.B. Weglicki

b) Meeting Abstracts (7)

"Spin-Trapping of Methyl Radicals from Sarcolemmal Lipids: Inhibition of Peroxidative Injury by Propranolol"
I.T. Mak, C.M. Arroyo B.F. Dickens, J.H. Liang and W.B. Weglicki

"Hydroxyl Radical Scavenging and Attenuation of Free Radical Injury in Endothelial Cells by SH-Containing ACE Inhibitors"
I.T. Mak, B.F. Dickens and W.B. Weglicki

"Potent Antiperoxidative Effect of Probucol on Isolated Membranes and Cultured Vascular Cells"
I.T. Mak, B.F. Dickens and W.B. Weglicki

"Endothelial Cells as A Source of Oxygen-Free Radicals: An ESR Study"
C.M. Arroyo, A.J. Carmichael, B. Bouscarel, J.H. Liang and W.B. Weglicki

"Synergistic Effect of Nitroprusside and FE(III) in Promoting Lipid Peroxidation in Hepatic, Cardiac and Aortic Membranes"
I.T. Mak, L.A. Kopyta and W.B. Weglicki

"Protective Effects of Sulfhydryl-Containing ACE Inhibitors Against Lipid Perioxidation in Endothelial and Smooth Muscle Cells"
W.B. Weglicki, B.F. Dickens, B.R. Pflug and I.T. Mak

"Effects of an Exogenous Free Radical Generating System on C6 Glioma Cells in Culture"
W.J. Goldberg, G. Tadvalkar, E. Laws Jr., B.F. Dickens, W.B. Weglicki and J.J. Berstein

TRAINING ACTIVITIES: Dr. Stafford is currently in his second year of his postdoc. Three high school students from the District of Columbia Public Schools, Denise Brown, Angela Bass and Gabriel Perez, are working on science projects under Dr. Stafford.

AWARDS AND FELLOWSHIPS: None

APPENDIX I

MATERIALS AND METHODS

All chemicals used were reagent grade unless otherwise stated. These chemicals were used without any further purification. The radiolabelled lipids were purchases from Amersham Ltd. the cold phospholipids were purchased from Avanti Polar Lipids, and the cold acyl glycerols were purchased from Sigma Chemical. For the HPLC the HPLC grade solvents were purchased from Fisher Chemical Co..

The method of exposing cultured cells to an exogenous oxy-radical generating system and the subsequent analysis of malonyldialdehyde (MDA) production has been described previously (1). The analysis of the structural phospholipids by HPLC has also been reported previously (2).

The lipase activities were measured using radiolabelled substrates in a lipid/Triton X-100 detergent mixed micelle assay system. The substrates include 1,2-di-[1-14C]palmitoyl-sn-glycerol phosphorylcholine, 1-[1-14C]palmitoyl-sn-glycerol phosphorylcholine, and 1,2,3-tri-[1-14C]palmitoyl glycerol. All the fatty acyl groups on the lipid are labelled. The radiolabelled substrates were mixed with cold lipids in toluene and chloroform, dried under nitrogen and then suspended in Milli-Q water (Millipore/Waters Asscociates). Triton X-100 was added to the suspension in a molar ratio of 2:1 detergent to lipid. The final substrate concentration was 100 M in 0.1 M buffer with 10 mM Calcium Chloride or 10 mM EDTA. The samples that were assayed were prepared by scraping cultured cells from plates, washing the cells three times in KSP buffer, suspending the cells in a measured volume of KSP, and then sonicating the cell suspension on ice at 100 watts for 30 seconds. The sonicate was then centrifuged at 20000 x g at 4 degrees C for 20 minutes. The final supernatant was designated the cytosolic fraction and the pellet, which was suspended in a measured volume of KSP, was designated the membrane fraction. pH profiles of each fraction were made as described previously (3) except MOPS buffer was replaced by citrate as seen in the results. The reaction products observed were lysophospholipids, mono and di-acyl glycerols and free fatty acids. The lipase activities are measured in nmoles of the above products produced per hour. C6, astrocytes and endothelial cells each produced characteristic profile of lipase activities.

APPENDIX II

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1. Mak, I.T., Misra, H.P., and Weglicki, W.B. (1983) J. Biol. Chem. 258, 13733-13737.

2. Mak, I.T., Kramer, J.H., and Weglicki, W.B. (1986) J. Biol. Chem. 261, 1153-1157.

3. Ross, M.I., Deems, R.A., Jesaitis, A.J., Dennis, E.A., and Ulevitch, R.J. (1985) Arch.Biochem. Biophys. 238, 247-258.

BLEBS AND VILLI ON C6 CELLS EXPOSED TO AN EXOGENOUS FREE RADICAL GENERATING SYSTEM

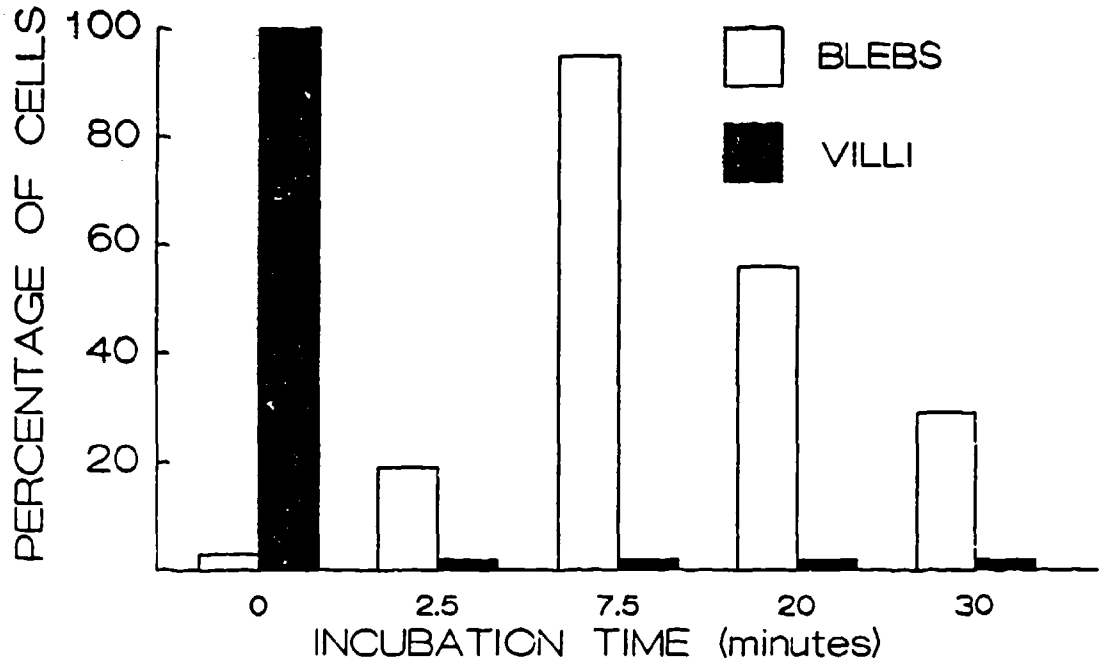


Figure 1: The percentage of cells that have list all their villi and the percentage of cells that have developed blebs over time after exposing the cells to the exogenous free radical generating system; DHF/Fe+3-ADP

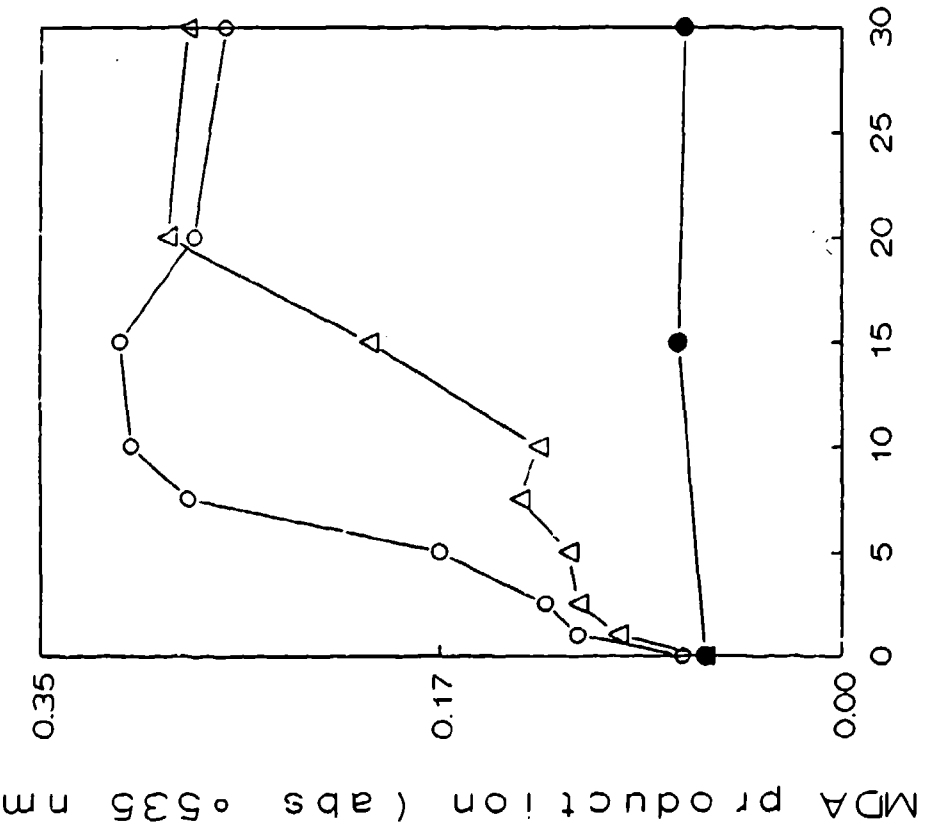
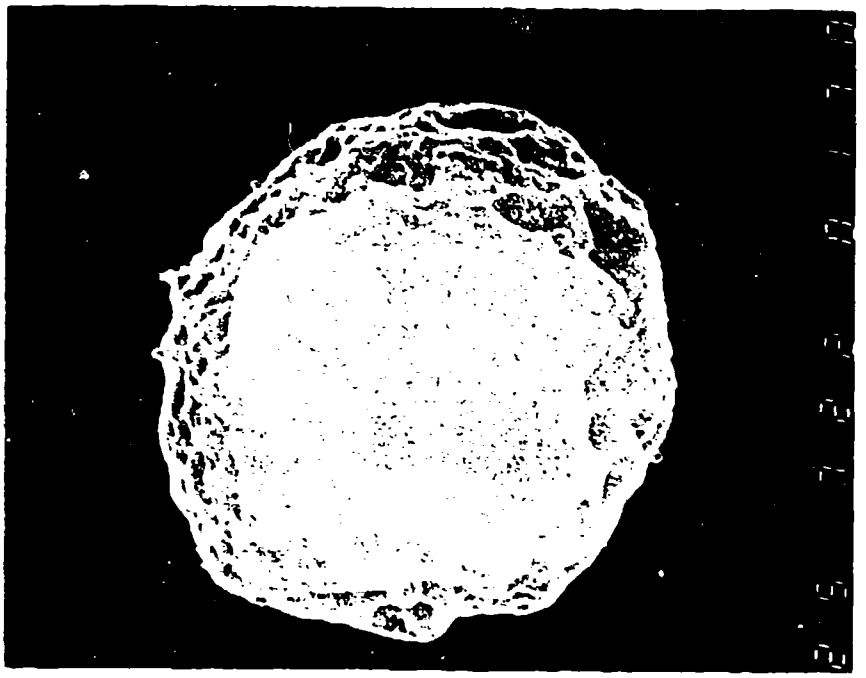


Figure 3: Effect of hydroxyl radical generating system on lipid peroxidation of C6 cells. Closed circles = control, open circle = 0.33 mM DHF, triangles = 0.17 mM DHF.

A



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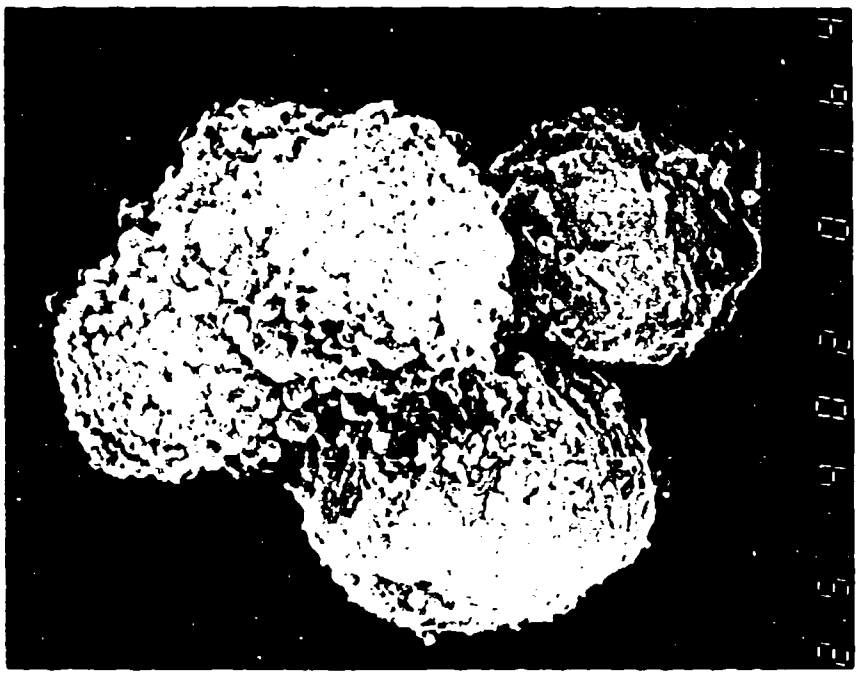

Figure 2: **A.** Scanning electron micrograph of an endothelial cell. The endothelial cells are grown to confluence in 150 cm2 flasks. The confluent cells are lifted from the culture dish with 0.05% trypsin. 0.53 mM EDTA. This procedure produces rounded-up smooth cells (7000x). **B.** Endthelial cells treated as in A and then exposed to the DHF/Fe+3-ADP radical system. At 30 minutes of exposure these cells are covered with blebs (4000x).

30 min Radical system

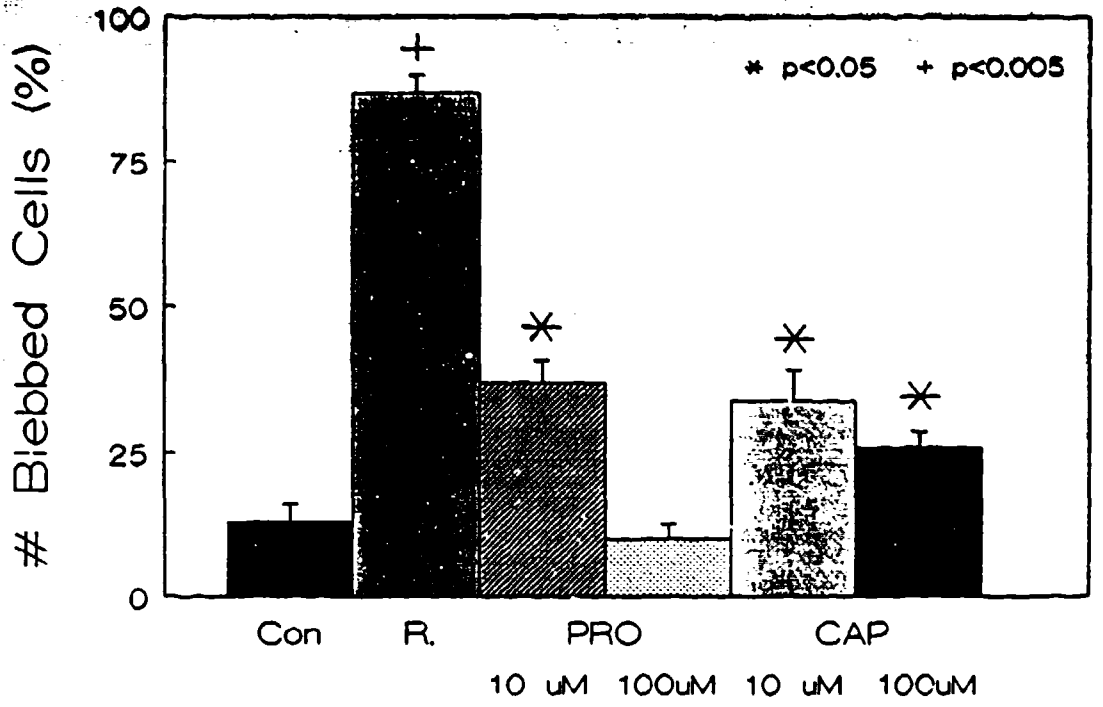

Figure 4: The effect of 10 uM and 100 uM propranalol and captopril on the number of endothelial cells which exhibit blebbing after 30 minutes of exposure to DHF/Fe+3-ADP.

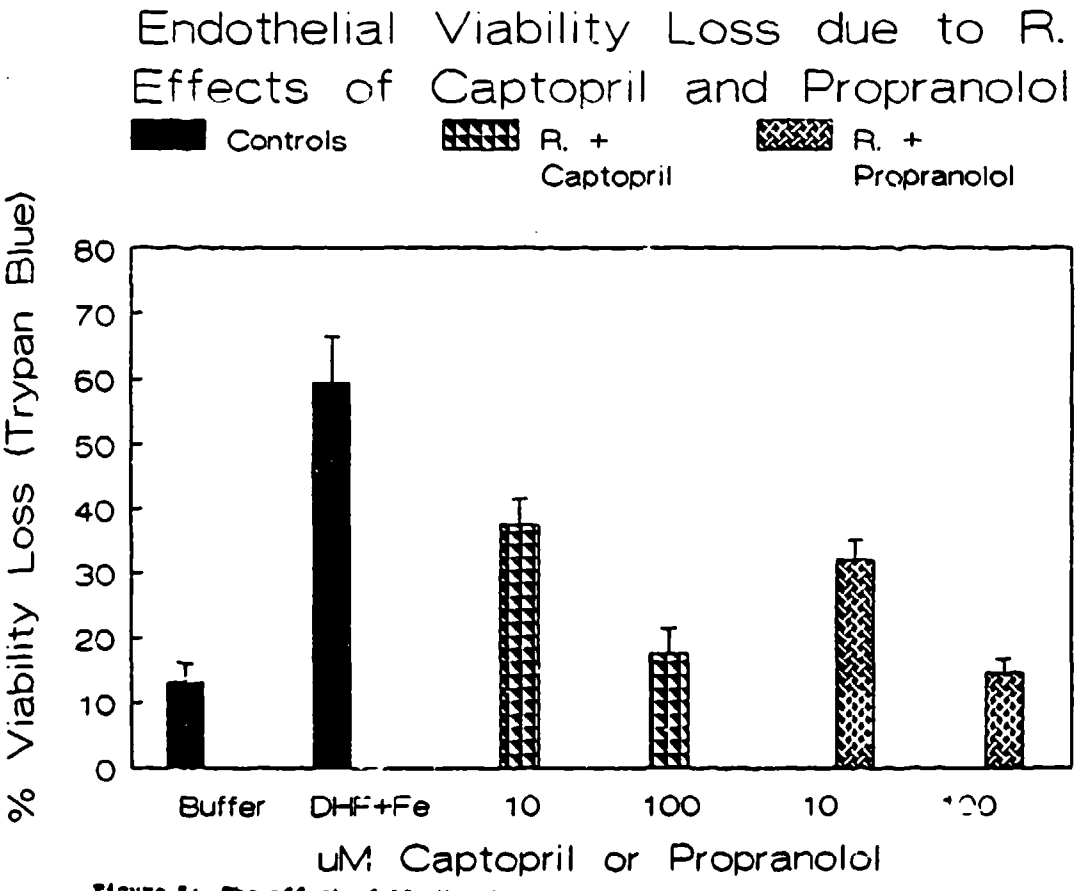

Figure 5: The effect of 10 uM and 100 uM propranalol and captopril on the viability of endothelial cells, measured by trypan blue uptake, after 30 minutes of exposure to DHF/Fe+3-ADP.

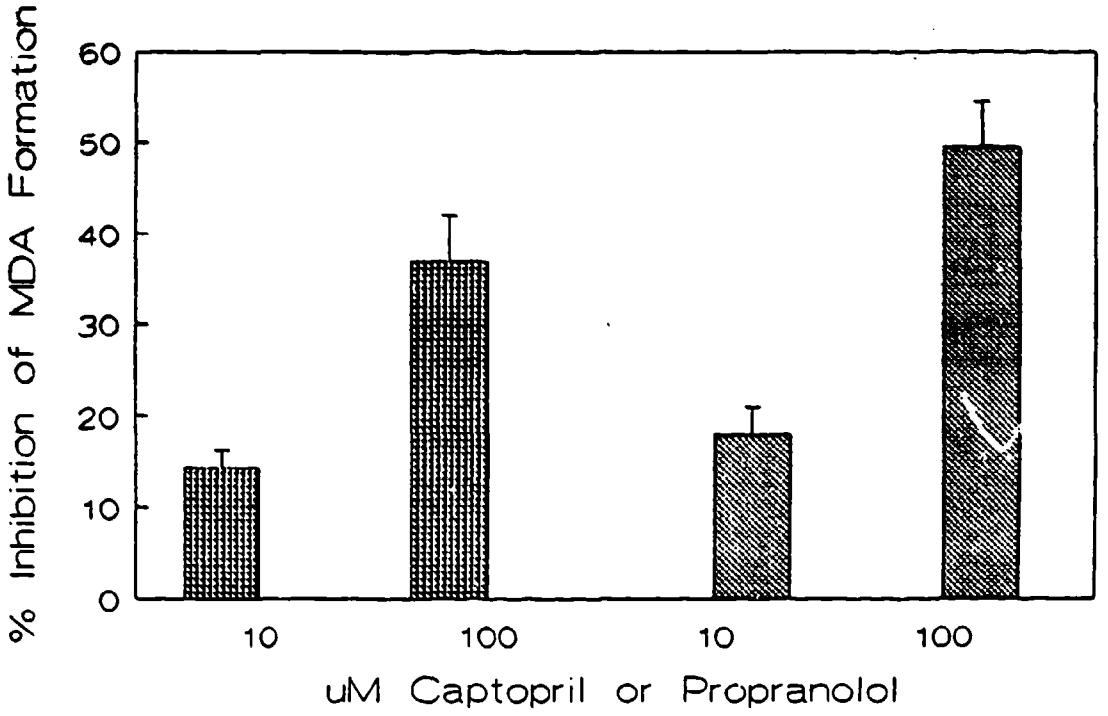

Figure 6: The precent inhibition of MDA production on endothelial cells by captopril and propranalol.

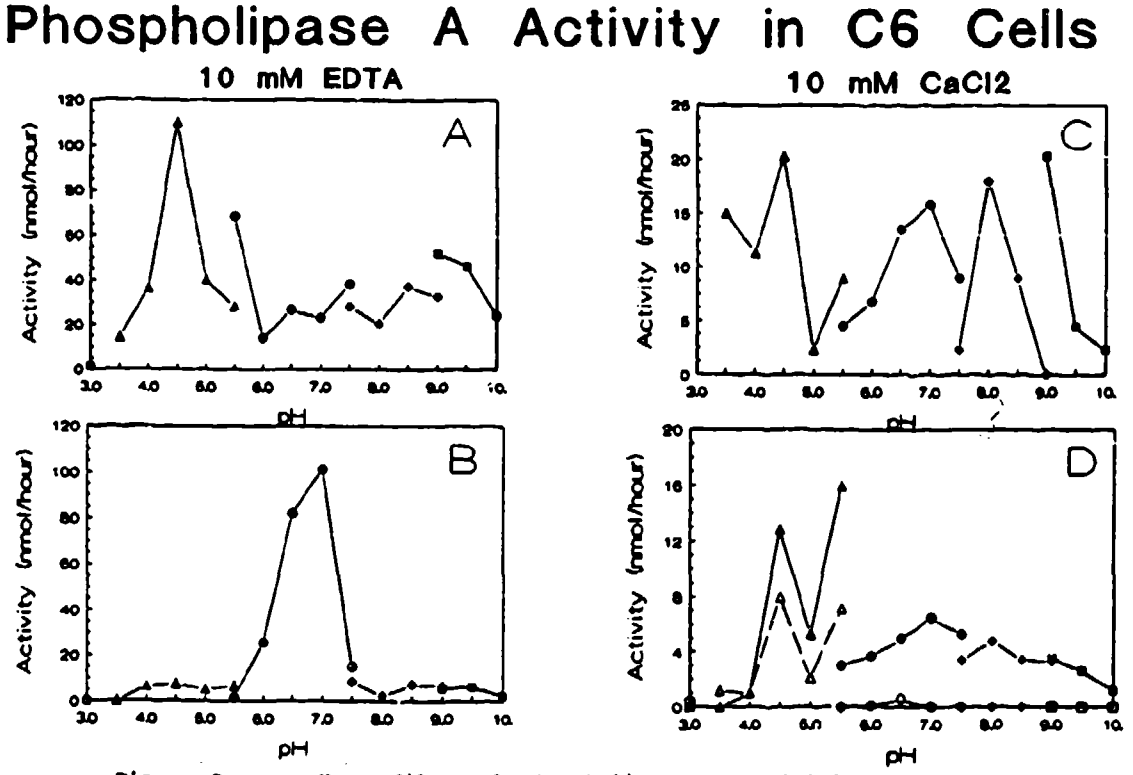

Figure 7: pH profiles of phospholipase A activities of endothelial cells in the presence of 10 mM EDTA or 10 mM CaCl2. The activity is measured as the production of free fatty acid (-) and lysophospholipid (---). The buffers used are: triangles = NaAcetate; circles = NaPhosphate; diamonds = Tris-Cl; squares = glycine.

Phospholipase A Activity of Endothelial Cells

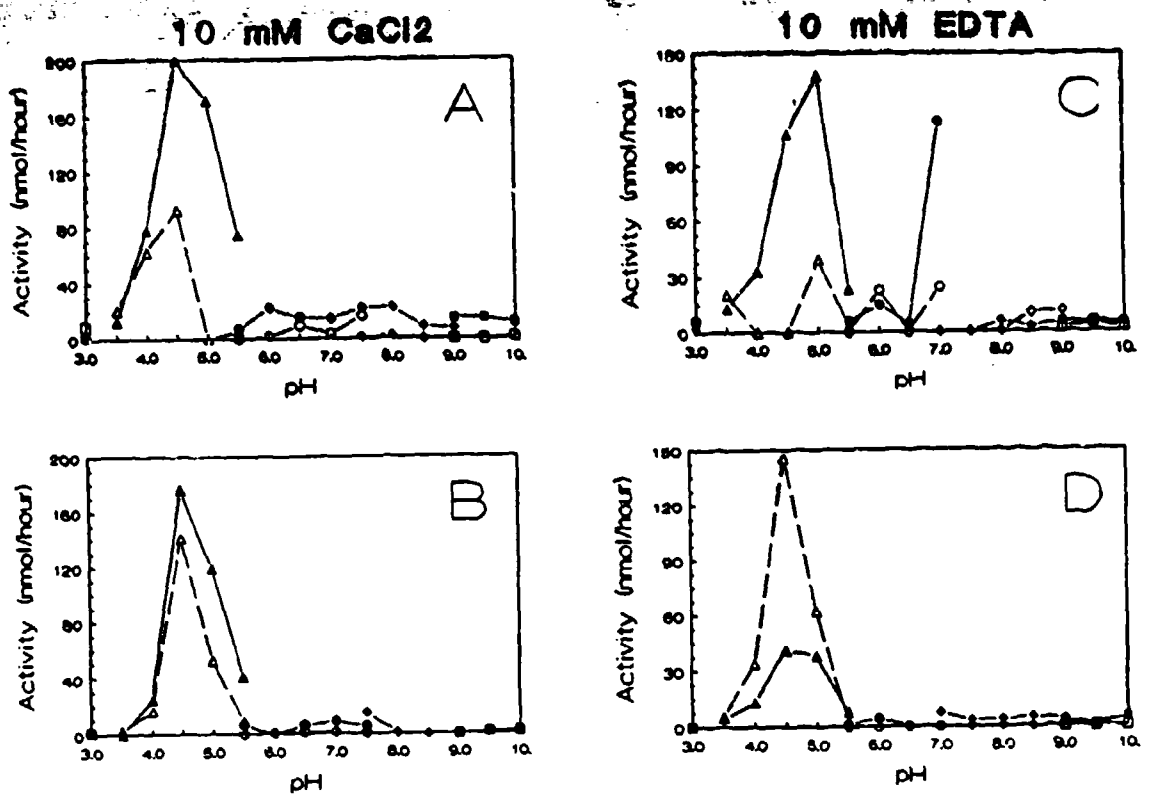

Figure 8: pH profiles of phospholipase A activities of endothelial cells in the presence of 10 mM EDTA or 10 mM CaCl2. The activity is measured as the production of free fatty acid (-) and lysophospholipid (---). The buffers used are: triangles = NaAcetate; circles = NaCitrate; diamonds = Tris-Cl; squares = glycine.

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