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Short Communication

PROTECTIVE EFFECTS OF CALCIUM CHANNEL BLOCKERS AGAINST FREE RADICAL-IMPAIRED ENDOTHELIAL CELL PROLIFERATION

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Abstract—We have shown previously that the lipophilic calcium channel blockers exhibit membrane antioxidant activity. In the present study, when attached bovine aortic endothelial cells were exposed for 20 min to a low concentration of oxy-radicals generated from dihydroxyfumarate + Fe-ADP, no loss of glutathione or viability was detected; however, cell number, determined 48 hr later by the tetrazolium salt MTT assay, decreased to 45% of controls. Treatment of the cells for 1 hr with the calcium blockers (2–20 μM) prior to free radical exposure protected against the impaired cell growth in a concentration-dependent manner. The order of potency was nicardipine ≥ nifedipine ≥ verapamil > diltiazem, which appears to parallel their antioxidant potency. In addition, (+)-nicardipine, and its pharmacologically inactive isomer, (−)-nicardipine, were similarly effective. We conclude that it was the antioxidant activity of the calcium channel blockers that preserved the cell growth capacity against free-radical damage; such protective effects may contribute to their antiatherogenic effects.

Key words: L-type calcium blockers; antioxidant activity; endothelial cell growth; MTT assay

In previous studies [1, 2] using an isolated sarcolemmal membrane system, we demonstrated that certain calcium channel blockers (nicardipine, nifedipine, verapamil) exhibit varying degrees of membrane antioxidant activity. In agreement with our findings, several other laboratories also reported that certain calcium channel blockers can inhibit lipid peroxidation in hepatic microsomal and liposomal systems [3–6]. However, the clinical relevance of the antioxidant properties of these drugs is uncertain and remains to be explored. It has been suggested that the antiperoxidative actions of calcium antagonists may participate in mediating chronic antiatherogenic effects [1, 2, 7, 8]. In support of such a notion, we demonstrated that these calcium blockers provide endothelial cytoprotective effects against free radical-mediated acute loss of viability, as determined by retarding increased permeability to trypan blue [2]; however, the dye exclusion assay may underestimate membrane damage, which is not evident immediately and requires time for expression [9]. In the present study, we examined the ability of the calcium blockers to protect against delayed cell injury, which would only be manifested as impaired cell growth/survival after brief exposure to oxy-radicals. To separate the antioxidant effect of the drug from its calcium channel blocking activity, we assessed the difference between the (+) and (−) isomers of nicardipine; the (+) isomer is pharmacologically active, whereas the (−) isomer is not [10].

Materials and Methods

Chemicals and cell culture. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and most other chemicals were obtained from Sigma. The optical isomers (+)-nicardipine and (−)-nicardipine were provided by Syntex Research (Palo Alto, CA). Bovine aortic endothelial cells (GM 07372A) were obtained from the Coriell Institute for Medical Research, Camden, NJ, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. For sub-culturing, confluent plates were trypsinized by 0.05% trypsin in 0.02% EDTA solution.

Effect of calcium blockers on cell proliferation and survival. The index of endothelial cell survival-proliferation chosen was the colorimetric MTT (tetrazolium) assay according to the procedure of Mosmann [11]. Trypsinized endothelial cells were seeded at a density of $1 \times 10^4/cm^2$ in Corning 24-well flat-bottomed tissue culture-treated plates in Dulbecco's modified Eagle's medium supplemented with 0.01 M HEPES and 10% fetal bovine serum. After 24 hr of cell growth at 37° (cells in log phase), the medium was removed and replaced with 500 μL of the balanced salt buffer containing 10 mM glucose, 125 mM NaCl, 1.2 mM $MgCl_2$, and 10 mM potassium phosphate, pH 7.2. The attached cells were then incubated with or without the calcium blockers for 60 min before exposure (except controls) to the iron-catalyzed free radical system consisting of 0.83 mM dihydroxyfumarate and 0.025 mM $FeCl_3$ chelated by 0.25 mM ADP for 20 min. With the concentration of free radical reagents used in this study, the rate of superoxide anions generated was 2.9 nmol/min/mL, measured by superoxide dismutase-inhibitable cytochrome *c* reduction; this rate was about 56% of the magnitude of oxy-radical generation relative to the previous study [2]. At the end of the free radical incubation period, all wells were replaced with the normal growth medium. At 24, 48 and 72 hr post free radical exposure, all samples were quantitated for viable cells. Briefly, MTT, dissolved in PBS at 2 mg/mL, was added to all wells (125 μL/500 μL medium), and then incubated for an additional 4 hr at 37°. Finally, 800 μL of acidified isopropanol (0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly with a pipettor to dissolve the dark blue crystals. The supernatants were retrieved, briefly centrifuged, and read within 30 min using a test wavelength of 570 nm, and a reference one of 700 nm.

The trypan blue exclusion method for cell viability was assessed in 0.1% of the dye, and cell count was determined by using a hemocytometer. Immediately after the 20-min free radical exposure, cell samples pooled from 2–4 wells were processed for glutathione and protein-SH measurements. Total glutathione and protein-SH contents were determined as described previously [2, 12].

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All values are means of 3–8 separate determinations ± SD. Statistical analyses were performed using the Student's *t*-test.

Results

In this study, the effect of free radical-induced delayed injury was determined by cell growth/survival at 24, 48 and 72 hr. To assess cell growth, we used the MTT tetrazolium salt assay developed originally by Mosmann [11] with minimal modification. The assay relies on the conversion of the colorless tetrazolium salt (MTT) to the blue MTT formazan by the activity of mitochondrial dehydrogenase in living cells [11]. The MTT colorimetric assay has proven to be convenient and reliable to assess cell viability and proliferation [11, 13, 14]. Under our conditions, using a reference wavelength of 700 nm and a test wavelength of 570 nm, we observed that the color signal generated by the endothelial cells was linear for a wide range of cell densities (Fig. 1A). Figure 1B represents the viability/proliferation over the 72-hr time course following oxy-radical treatment for 20 min. With the seeding density employed, control samples were in log phase for the entire period of the experiments; at 72 hr, the control samples exhibited about 70% confluence. However, following free radical exposure, the cell number decreased significantly 36.3% at 24 hr ($P < 0.05$), 55.0% at 48 hr ($P < 0.01$), and 54.3% at 72 hr ($P < 0.01$) compared with controls. Separate experiments were performed confirming that decreases in the color products represented actual decreases in cell numbers. In results obtained immediately (within 30 min) after free radical exposure (data not shown), the free radical-treated cells exhibited no apparent damage, i.e. cell viability based on membrane exclusion of trypan blue was >90%. Also, both cellular glutathione and protein-thiol levels were maintained at >95% of controls, suggesting that most of the free radical-induced injury was subtle and beyond the routine biochemical detection assay. However, cell injury could eventually be elicited as a major inhibition of cell proliferation 48 hr later.

Of all the calcium channel blockers (nicardipine, nifedipine, verapamil and diltiazem) examined with our membrane model, nicardipine was the most potent. Figure 2 presents the effects of the four calcium channel blockers on the impaired cell growth. The data indicate that pretreatment of the attached endothelial cells for 1 hr with a 2, 5 or 20 μM concentration of each agent resulted in varying degrees of protection against the impaired cell proliferation; the effects were, in general, concentration dependent (except nifedipine at 20 μM) and the order of potency appeared to be nicardipine ≥ nifedipine ≥ verapamil > diltiazem. In separate controls (not shown), three of the four agents (excepting nifedipine) had no discernible inhibitory effects on the growth rate at 20 μM or less; nifedipine at 20 μM (but not at 2 or 5 μM), however, had a noticeable and significant inhibitory effect (15 ± 3%, $P < 0.05$ vs controls) on cell growth at 48 hr.

Of the four calcium blockers tested, nicardipine was the most effective in protecting against the impaired cell growth. To examine the possibility that the protective effect of the drug could be due to blockade of calcium overloading subsequent to free radical injury, we chose to examine the effects of the two isomers of nicardipine. Due to the presence of the asymmetric center at the 4-position, two isomers of nicardipine, (+)-nicardipine and (−)-nicardipine, can be derived. It has been determined that the (+) isomer is the active calcium channel blocking agent, whereas the (−) isomer is at least 5-fold less active [10]. In experiments using an isolated membrane system similar to that described earlier [1, 15, 16], we found that both the (+) and (−) isomers of nicardipine exhibited nearly identical concentration-dependent membrane antioxidant activity. In this study (Fig. 3), both the (+) and (−) forms provided similar concentration-dependent protection against the impaired endothelial cell growth, suggesting that this efficacy was mediated entirely by its antioxidant activity and little, if any, by calcium blockade.

Discussion

Results from the present study suggest that the calcium channel blockers can provide protection against free radical-impaired endothelial cell growth; these cytoprotective effects appear to be governed by antiperoxidative potency rather than calcium channel blocking activity. In our previous study [2], we subjected the endothelial cells to a higher concentration of free radicals for a longer time of incubation (30–60 min); cell injury was immediately apparent based on significant losses of cellular glutathione and increased membrane permeability to trypan blue dye. In the present study, a lower concentration of free radicals was applied to the attached endothelial cells; no significant increases in membrane permeability to trypan blue or depletion in cellular thiols were observed after the brief period of free radical exposure. However, significant inhibition of cell growth was evident 48 hr later. We consider the ability of endothelial cells to proliferate to be a more useful indicator of preservation of cellular function. Nevertheless, the exact loci of the subtle cell injuries remain to be determined; the possibilities include: (i) minor degree of lipid oxidation; and (ii) oxidation of the SH-moieties or redox susceptible amino acids of enzymes and proteins. The free radical stress was not severe enough to cause immediate cell death but critical enough to impair sub-

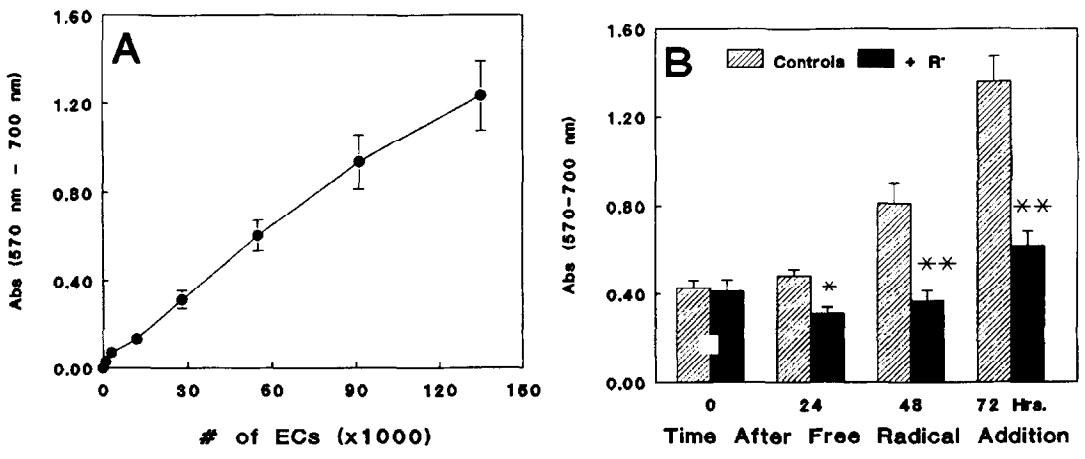

Fig. 1. (A) Relationship between absorbance of the MTT assay and cell number. (B) Time course of free radical-induced inhibition of endothelial cell proliferation. The attached endothelial cells in 24-well plates were exposed to free radicals generated from DHF + Fe-ADP for 20 min; 24, 48 and 72 hr later, cell proliferation was determined by the MTT assay as described under Materials and Methods. Values are means ± SD of 4–6 separate determinations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs the corresponding controls.

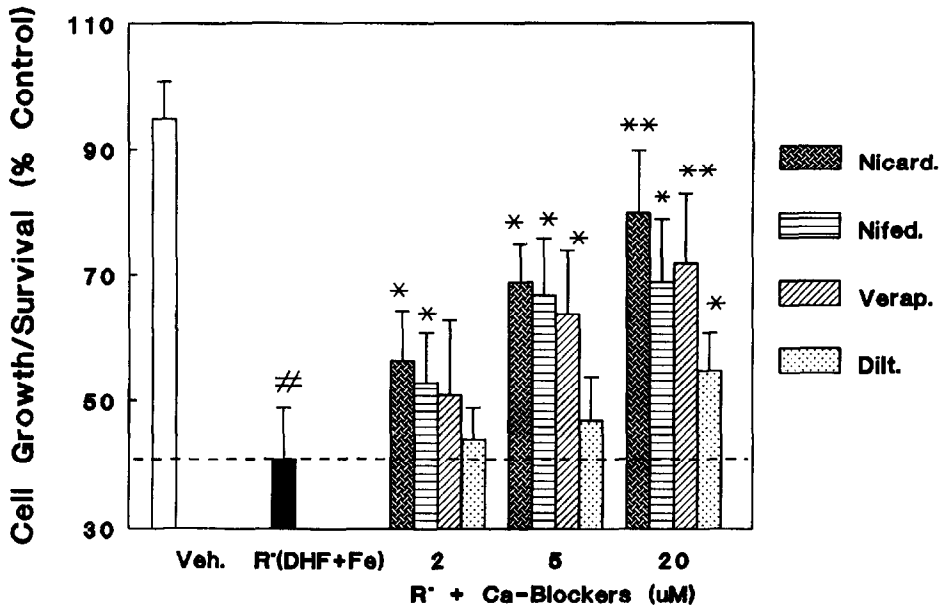

Fig. 2. Protective effects of calcium channel blockers against free radical-mediated impaired cell proliferation. The calcium blockers, initially dissolved in ethanol, were added to the drug-treated samples such that the final ethanol concentration was 0.25% (v/v). The attached cells, 24 hr after seeding at a density of $1 \times 10^4/cm^2$, were preincubated with each calcium blocker at different levels for 60 min before the incubation with the free radical components R*(DHF + Fe-ADP) for 20 min. Forty-eight hours later, cell proliferation was determined by the MTT absorbance assay as described under Materials and Methods and was expressed as percentage of controls without free radical treatment. Values are means ± SD of 3–8 separate determinations. Key: (#) $P < 0.001$ vs vehicle control (0.25% ethanol alone); (*) $P < 0.05$, and (**) $P < 0.01$ vs R* alone.

sequent cell growth. In either scenario, the presence of the calcium channel blockers may afford protection against such minor oxidative modifications and, subsequently, preserve the ability of the cells to grow. As has been emphasized by a number of reports, free radical-mediated endothelial cell injury could represent an important initial event leading to atherosclerosis [17–19]. Under this general premise, the ability of the endothelial cell to retain its capacity to proliferate after oxidative injury may be an important response that is essential to maintain endothelial integrity [20].

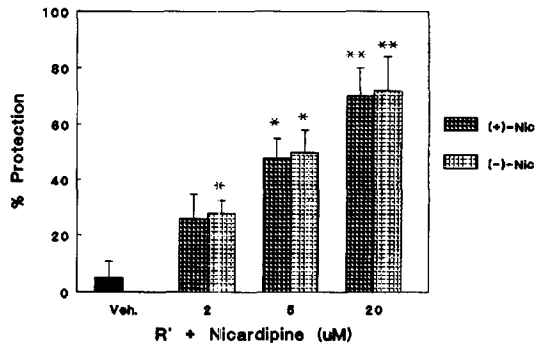

Fig. 3. Comparative protective effects of (+)-nicardipine and (−)-nicardipine against free radical-mediated impaired endothelial cell proliferation. The attached cells, 24 hr after seeding ($1 \times 10^4/cm^2$), were preincubated with (+)- or (−)-nicardipine for 60 min before the incubation with the free radical components for 20 min; 48 hr later, cell proliferation was determined by the MTT absorbance assay. Results are expressed as percent attenuation of the inhibited cell proliferation at 48 hr after free radical exposure; other conditions were as described for Fig. 2. Values are means ± SD of 4–6 separate determinations. Key (*) $P < 0.05$, and (**) $P < 0.01$ vs R* alone.

Although much has been written about the multiple effects of calcium antagonists, in the treatment of vascular disorders, the mechanisms responsible for their antiatherosclerotic effects are unclear [21–23]. During the past two decades, increasing evidence has accumulated which implicates the involvement of free radicals in the pathogenesis of atherosclerosis [16, 17]; presumably, oxygen free radicals might be produced endogenously within the arteries from auto-oxidizing catecholamines, redox active drugs, and activated neutrophils. The potential significance of the present findings lies in the possibility that the calcium channel blockers, especially the more lipophilic ones, could provide the described cytoprotective effects, which might be important during endothelium wound healing after oxidative injury. In reviewing the literature, Henry [8] has concluded that the antiatherogenic beneficial effects of calcium channel blockers may be related to their antiperoxidative activity. The present findings allow us to speculate that such drug intervention may facilitate healing of the vascular cells after oxidative injury, and thus contribute to their antiatherogenic effects.

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