

# Nifedipine and Diltiazem but Not Verapamil Up-Regulate Endothelial Nitric-Oxide Synthase Expression

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## ABSTRACT

We have recently shown that felodipine, a long-acting dihydropyridine L-type calcium channel blocker (CCB), up-regulates nitric oxide (NO) production and endothelial NO synthase (eNOS) expression and activity in cultured endothelial cells as well as in animals with chronic renal failure. This study was intended to compare the effects of prototypes of the three classes of L-type CCBs on the NO system in cultured human coronary artery endothelial cells. Thus, cultured endothelial cells were incubated either with nifedipine, diltiazem, or verapamil for 24 h at  $10^{-5}$  to  $10^{-7}$  M concentrations. Cells incubated with inactive vehicle served as controls. NO production, as discerned from total nitrate plus nitrite recovered in the medium, was significantly increased by nifedipine ( $P < .03$ ) and

by diltiazem ( $P < .05$ ). However, NO production remained unchanged with verapamil ( $P = \text{NS}$ ). Similarly, eNOS protein abundance was increased significantly by nifedipine ( $P < .05$ ) and diltiazem ( $P < .05$ ). In contrast, eNOS expression was not changed by verapamil ( $P = \text{NS}$ ). Likewise, NOS activity, as measured from [ $^3\text{H}$ ]L-arginine to [ $^3\text{H}$ ]L-citrulline conversion, significantly increased with nifedipine ( $P < .01$ ) and diltiazem ( $P < .01$ ). However, incubation with verapamil failed to alter NOS activity of the cultured endothelial cells ( $P = \text{NS}$ ). We concluded that prototypes of dihydropyridine and benzothiazepine classes, but not phenylalkylamine class of CCBs, up-regulate the NO system. This may, in part, account for the different biological properties of these agents.

We have recently shown that administration of felodipine, a dihydropyridine calcium channel blocker (CCB), enhances nitric oxide (NO) production and NO synthase (NOS) isotype expressions and activities in rats with chronic renal failure (CRF) (Vaziri et al., 1998) and in animals with erythropoietin-induced hypertension (Ni et al., 1998). In addition, we have found marked up-regulations of basal and acetylcholine-stimulated NO production and endothelial NOS (eNOS) expression and activity by felodipine in cultured endothelial cells (Ding and Vaziri, 1998; Wang and Vaziri, 1999). Together these in vivo and in vitro studies provided convincing evidence for the up-regulatory action of this dihydropyridine CCB on NOS expression. This study was designed to compare the effects of the three distinct classes of voltage-dependent L-type CCBs on eNOS expression in human coronary endothelial cells. To this end, cultured human coronary artery endothelial cells were incubated for 24 h with nifedipine, diltiazem, and verapamil, which are the prototypes of dihydropyridine, benzothiazepine, and phenylalkylamine classes of L-type CCBs, respectively. The results showed that both nifedipine and diltiazem significantly increased NO production and eNOS protein expression and activity, whereas ve-

rapamil had no discernible effect on NO system in human coronary artery endothelial cells in vitro.

## Materials and Methods

**Cell Culture.** Human coronary artery endothelial cells (BioWhittaker Inc., Walkersville, MD) were cultured in a manner similar to that described in our earlier studies (Ding and Vaziri, 1998). Cells obtained on passages three and four were used. After 90% confluence was reached, the cells were incubated in a medium that contained nifedipine, diltiazem, or verapamil (all purchased from Sigma Chemical Inc., St. Louis, MO) at  $10^{-5}$ ,  $10^{-6}$ , or  $10^{-7}$  M concentrations. For each condition, respective inactive vehicles were used as controls. The incubations were conducted for 24 h at 37°C and in the presence of 5%  $\text{CO}_2$ . At the end of the incubation period, cells were harvested and the culture media were collected and stored at  $-70^\circ\text{C}$  until processed. Cell viability was tested by trypan blue exclusion test, morphologic examination, and lactic dehydrogenase release test and was found to be greater than 95% in all experiments.

**Measurement of Total Nitrate and Nitrite (NO<sub>x</sub>).** NO production was assessed from NO<sub>x</sub> recovered in the extracellular medium. NO<sub>x</sub> was measured using the purge system of the Sievers NO Analyzer (model 270B NOA; Sievers Instruments Inc., Boulder, CO) as previously described (Wang and Vaziri, 1999). The amount of NO<sub>x</sub> produced was normalized against total cellular protein, which was measured with a Bio-Rad kit (Bio-Rad Inc., Hercules, CA).

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**ABBREVIATIONS:** CCB, calcium channel blocker; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; NO<sub>x</sub>, total nitrate and nitrite; CRF, chronic renal failure; CTL, inactive vehicle control.

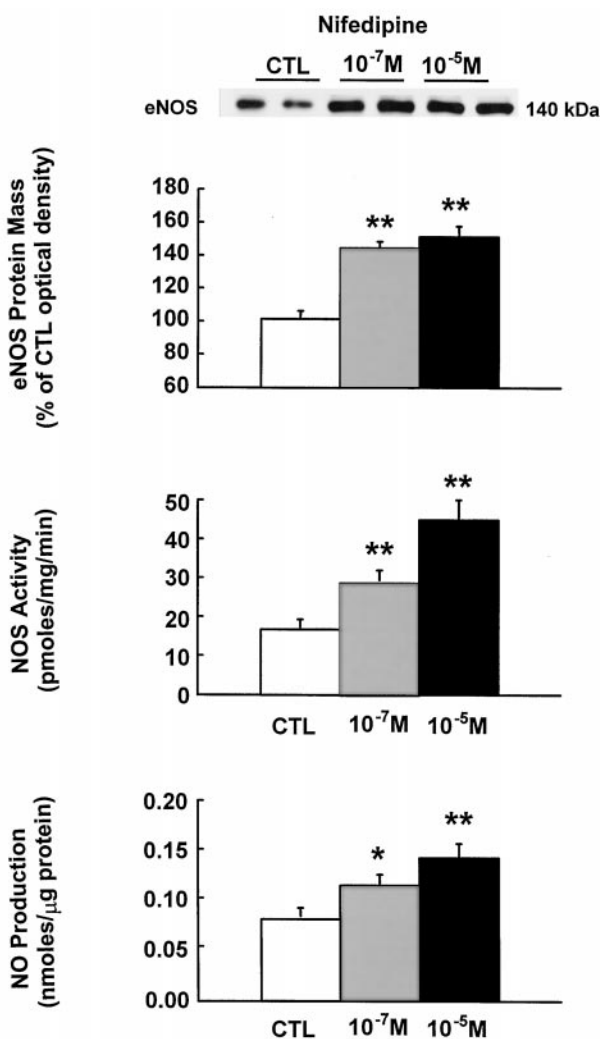
**Measurement of eNOS Protein.** Endothelial cells were processed, and eNOS protein abundance was determined by Western blot analysis using anti-eNOS antibody (Transduction Laboratories Inc., Lexington, KY) in a manner that was similar to that described in our earlier studies (Wang and Vaziri, 1999).

**Measurement of NOS Activity.** NOS enzymatic activity of the cell preparations was determined from the rate of [ $^3\text{H}$ ]L-arginine to [ $^3\text{H}$ ]L-citrulline conversion as described in our earlier studies (Wang and Vaziri, 1999). The results were expressed per microgram of cellular protein.

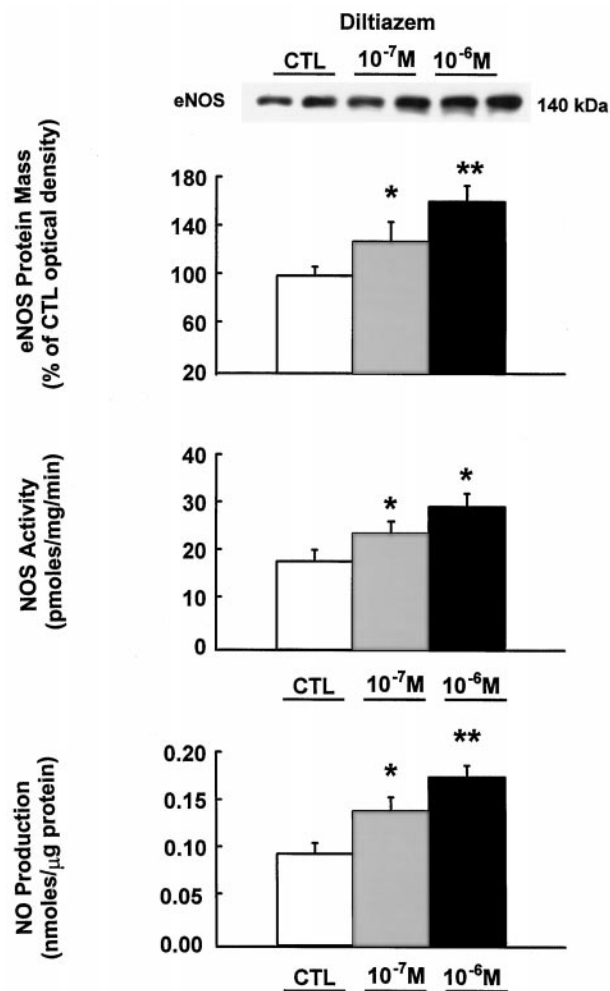
**Data Analysis.** ANOVA, Duncan's Multiple Range test, and regression analysis were used in statistical evaluation of the data. The results represent the mean  $\pm$  S.E. of at least four separate experiments.  $P$  values  $\leq .05$  were considered significant.

## Results

**NO Production.** Data are depicted in Figs. 1 to 3. NO production as discerned from NO $_x$  recovered in the extracellular medium was increased significantly by nifedipine ( $P < .01$ ) and diltiazem ( $P < .001$ ). A dose-dependent response was found with nifedipine within the  $10^{-7}$  and  $10^{-5}$  M concen-



**Fig. 1.** Representative Western blot and the corresponding group data depicting eNOS protein abundance (top), NOS enzymatic activity (middle), and NO production (measured as total nitrate + nitrite; bottom) in coronary artery endothelial cells incubated for 24 h in the presence of nifedipine at  $10^{-7}$  and  $10^{-5}$  M concentrations or inactive vehicle control (CTL).  $n = 4$  separate experiments. \* $P < .05$ . \*\* $P < .01$  versus CTL.

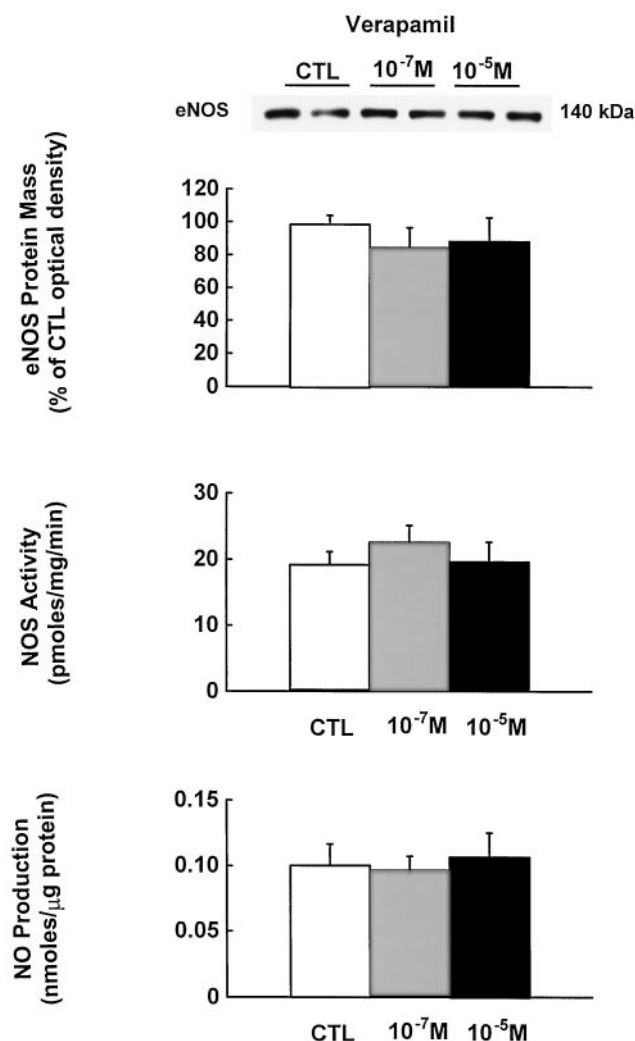


**Fig. 2.** Representative Western blot and the corresponding group data depicting eNOS protein abundance (top), NOS enzymatic activity (middle), and NO production (measured as total nitrate + nitrite; bottom) in coronary artery endothelial cells incubated for 24 h in the presence of diltiazem at  $10^{-7}$  and  $10^{-6}$  M concentrations or CTL.  $n = 4$  separate experiments. \* $P < .05$ . \*\* $P < .01$  versus CTL.

tration range. Similarly, a dose-dependent response was present with diltiazem within the  $10^{-7}$  and  $10^{-6}$  concentration range with no additional rise seen at  $10^{-5}$  M (data not shown). In contrast to nifedipine and diltiazem, verapamil had no effect on NO production by cultured endothelial cells.

**eNOS Protein Expression.** Data are illustrated in Figs. 1 to 3. At both  $10^{-7}$  and  $10^{-5}$  M concentrations, nifedipine caused a significant concentration-dependent increase in eNOS protein expression by cultured human coronary artery endothelial cells. Similarly, eNOS expression was enhanced dose dependently by diltiazem within  $10^{-7}$  and  $10^{-6}$  M concentrations. However, the effect of diltiazem was maximal at  $10^{-6}$  M with no additional increase noted at  $10^{-5}$  M concentration (data not shown). In contrast to nifedipine and diltiazem, verapamil had no effect on eNOS expression by cultured human endothelial cells.

**NOS Enzymatic Activity.** Data are shown in Figs. 1 to 3. The nifedipine-induced up-regulation of eNOS protein expression was accompanied by a parallel dose-dependent rise in NOS enzymatic activity of the cultured endothelial cells. Similarly, diltiazem dose dependently increased NOS enzy-



**Fig. 3.** Representative Western blot and the corresponding group data depicting eNOS protein abundance (top), NOS enzymatic activity (middle), and NO production (measured as total nitrate + nitrite; bottom) in coronary artery endothelial cells incubated for 24 h in the presence of verapamil at  $10^{-7}$  and  $10^{-5}$  M concentrations or CTL.  $n = 4$  separate experiments.

matic activity of the endothelial cells within  $10^{-7}$  and  $10^{-6}$  M concentrations. As with eNOS protein mass, the effect of diltiazem on NOS activity was maximal at  $10^{-6}$  M concentration with no additional rise in NOS activity observed at the higher drug concentration. As with eNOS protein mass, NOS enzymatic activity was unaffected by verapamil. A significant correlation was found between the eNOS protein mass and NOS enzymatic activity ( $r = 0.65$ ,  $P < .05$ ). Similarly, NO production was related significantly to NOS activity in this system ( $r = 0.72$ ,  $P < .05$ ).

**Cell Counts.** Cell counts, which were measured by cytometry at the conclusion of all incubation experiments, revealed comparable values (data not shown). This observation is in conformity with our earlier studies that showed that felodipine has no discernible effect on either DNA synthesis or cell proliferation in cultured endothelial cells (Ding and Vaziri, 1998).

## Discussion

Ionized calcium is the principal mediator of muscular contraction. Movement of  $Ca^{2+}$  across the cell membrane occurs

through calcium channels, of which several different types have been identified. The most extensively studied calcium channel is the L-type voltage-dependent channel that consists of  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -,  $\gamma$ -, and  $\zeta$ -subunits. Structurally, the L-type channels are highly diversified. The structural diversity of L-type calcium channels is responsible for their diverse functional activity, tissue specificity, and differential susceptibility to CCBs (Schwartz, 1992). For instance, unlike channels in the vascular smooth muscle, myocardium, sinoatrial node, atrioventricular node, and certain conductive tissues that are highly sensitive to the action of L-type CCBs, those in skeletal muscle are not. The L-type channels accommodate the flux of  $Ca^{2+}$  from extracellular to intracellular space. This calcium influx triggers the release of large intracellular stores of calcium into the cytoplasm, leading to cell contraction. Through their binding to specific sites on the  $\alpha_1$ -subunit of the L-type channels, the L-type CCBs inhibit this calcium current.

Nearly all CCBs that are used clinically act on the L-type calcium channels and belong to three distinct classes of compounds. They include dihydropyridines (e.g., nifedipine), benzothiazepines (e.g., diltiazem), and phenylalkylamines (e.g., verapamil). Although the action of all three classes of CCBs is mediated by their binding to the  $\alpha_1$ -subunit, the binding site for each class is distinctly different from the others (Luft and Haller, 1993).

In a recent study, we demonstrated that administration of felodipine, a dihydropyridine CCB, up-regulates NO production, renal and vascular eNOS and inducible NOS (iNOS) expressions, and NOS enzymatic activity in rats with CRF (Vaziri et al., 1998). Similarly, we found up-regulation of NO production and NOS isotype expression and enzymatic activity with felodipine administration in CRF rats with erythropoietin-induced hypertension (Ni et al., 1998). In a subsequent study, we examined the effect of felodipine on NO system in cultured rat aorta endothelial cells to discern whether the effects seen were due to a direct or indirect action of the drug on endothelial cells (Ding and Vaziri, 1998). The results showed a marked up-regulation of eNOS protein expression, eNOS enzymatic activity, and NO production after a 24-h incubation period with felodipine in cultured endothelial cells (Ding and Vaziri, 1998). More recently, we showed that erythropoietin-induced down-regulation of eNOS expression and NO production can be reversed by felodipine in cultured human coronary artery endothelial cells (Wang and Vaziri, 1999). These observations clearly demonstrated the up-regulatory action of this dihydropyridine CCB on endothelial NOS expression. This study was undertaken to determine whether the response seen with felodipine is unique to this particular compound or if it is a common property of the dihydropyridine class or all three classes of L-type CCBs. Thus, the effects of nifedipine, diltiazem, and verapamil, the prototypes of the three classes of L-type CCBs, were studied on human coronary artery endothelial cells.

The results of this study showed that both nifedipine and diltiazem significantly and dose dependently up-regulated immunodetectable eNOS expression in isolated human coronary endothelial cells. This was accompanied by parallel rises in NOS enzymatic activity and NO production in this system. Diltiazem produced maximum up-regulation of the

NO system at  $10^{-6}$  M concentration with no additional increase found at the higher concentration ( $10^{-5}$  M).

In contrast to nifedipine and diltiazem, incubation with verapamil at low ( $10^{-7}$  M) or high ( $10^{-5}$  M) drug concentration failed to alter either eNOS protein expression, NOS enzymatic activity, or NO production in human coronary endothelial cells. Thus, dihydropyridines and benzothiazepines directly enhance L-arginine-NO system in coronary artery endothelial cells. Accordingly, their effects on coronary and systemic arterial circulations must be, in part, due to up-regulation of the NO system. Based on the results of this in vitro study and our earlier in vivo studies (Ni et al., 1998; Vaziri et al. 1998) dihydropyridine and benzothiazepine CCBs enhance vasodilatory tone not only by limiting  $Ca^{2+}$  influx in the vascular smooth muscle but also via enhanced generation of endothelium-derived NO. This can account for enhanced acetylcholine-mediated vasorelaxation with chronic administration of dihydropyridine CCBs in spontaneously hypertensive rats (Gray et al., 1993). Additional support for the beneficial effect of these agents on endothelial function comes from our earlier studies demonstrating the reversal of CRF-induced down-regulation of renal and vascular NOS expressions with chronic administration of felodipine (Ni et al., 1998; Vaziri et al., 1998). In addition, use of another dihydropyridine CCB, nicardipine, has been shown to ameliorate age-related decline in brain NO production in old mice (Inada et al., 1997). Moreover, calcium channel blockade has been shown to ameliorate hypertension, prevent renal and vascular injury, and improve NO production in rats subjected to chronic NOS blockade (Tojo et al., 1996). The results of this in vitro study have uncovered the cellular mechanism to account for the reported effects of these CCBs in vivo (Gray et al., 1993; Tojo et al., 1996; Inada et al., 1997; Ni et al., 1998; Vaziri et al., 1998).

In contrast to dihydropyridine and benzothiazepine CCBs, verapamil had no effect on endothelial cell NOS expression or NO production. This may account, in part, for differences in biological and clinical properties of different classes of L-type CCBs. The mechanism by which dihydropyridine and benzothiazepine CCBs up-regulate eNOS expression in endothelial cells in vitro is presently unclear. To our knowledge, L-type calcium channels have not been demonstrated in endothelial cells. Accordingly, the effect of dihydropyridines and benzothiazepines on eNOS expression in endothelial cells is most likely due to a calcium channel-independent action of these drugs. The fact that verapamil, another L-type CCB, showed no such effect helps lend support for the latter possibility.

Additional studies are needed to elucidate the mechanism by which dihydropyridine and benzothiazepine CCBs up-regulate eNOS expression. It is of interest that perfusion with amlodipine, a long-acting dihydropyridine CCB, has been shown recently to increase NO release from the coronary microvasculature of failing human cardiac explants (Zhang et al., 1999). This phenomenon was blockable by concurrent use of a bradykinin-2 antagonist or a kallikrein inhibitor, suggesting a kinin-mediated mechanism. By raising cytosolic  $Ca^{2+}$ , kinins and acetylcholine enhance the enzymatic activity of eNOS, which is a  $Ca^{2+}$ -calmodulin-dependent NOS. Thus, this class of CCBs enhances endothelial NO system via a kinin-mediated activation of eNOS as shown by Zhang et al. (1999) as well as by the up-regulation of eNOS protein abundance shown in this study.

In conclusion, dihydropyridine and benzothiazepine CCBs up-regulated eNOS expression and activity and enhanced NO generation in cultured human coronary artery endothelial cells. However, no such effects were observed with verapamil, which belongs to the phenylalkylamine class of L-type CCBs. The mechanism by which the former CCBs enhance eNOS expression in endothelial cells is presently uncertain and awaits investigation.

#### References

- Ding Y and Vaziri ND (1998) Calcium channel blockade enhances nitric oxide synthase expression by cultured endothelial cells. *Hypertension* **32**:718–723.
- Gray GA, Clozel M, Clozel JP and Baumgartner HR (1993) Effects of calcium channel blockade on the aortic intima in spontaneously hypertensive rats. *Hypertension* **22**:569–576.
- Inada K, Yokoi I, Kabuto H, Namba Y and Ogawa N (1997) The effects of chronic administration of nimodipine on age-related changes in nitric oxide and its synthase in senescence-accelerated mouse brain. *Biochem Mol Biol Int* **41**:753–765.
- Luft FC and Haller H (1993) Calcium channel blockers in current medical practice: An update for 1993. *Clin Exp Hypertens* **15**:1263–1276.
- Ni Z, Wang XQ and Vaziri ND (1998) Nitric oxide metabolism in erythropoietin-induced hypertension: Effect of calcium channel blockade. *Hypertension* **32**:724–729.
- Schwartz A (1992) Molecular and cellular aspects of calcium channel antagonism. *Am J Cardiol* **70**:6F–8F.
- Tojo A, Kobayashi N, Kimura K, Hirata Y, Matsuoka H, Yagi S and Omata M (1996) Effects of antihypertensive drugs on nitric oxide synthase activity in rat kidney. *Kidney Int Suppl* **55**:S138–S140.
- Vaziri ND, Ni Z, Wang XQ, Oveisi F and Zhou XJ (1998) Downregulation of nitric oxide synthase in chronic renal insufficiency: Role of excess PTH. *Am J Physiol* **274**:F642–F649.
- Wang XQ and Vaziri ND (1999) Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. *Hypertension* **33**:894–899.
- Zhang X, Kichuk MR, Mital S, Michler R, Nasjletti A, Kaley G and Hintze TH (1999) Amlodipine promotes kinin-mediated nitric oxide production in coronary microvessels of failing human hearts. *Am J Cardiol* **84**:27L–33L.

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