Felodipine Inhibits Free-Radical Production by Cytokines and Glucose in Human Smooth Muscle Cells

Keiichi Hishikawa, Thomas F. Lüscher

Abstract—An imbalance between nitric oxide (NO) and superoxide is importantly involved in the pathogenesis of vascular disease. Inflammatory stimuli and risk factors contribute to these alterations. Calcium antagonists and angiotensin-converting enzyme inhibitors are commonly used cardiovascular drugs. To clarify the effect of felodipine and ramiprilat on the balance of these free radicals, we stimulated human aortic smooth muscle cells (HASCs) with cytokines (human interleukin-1β, tumor necrosis factor-α, lipopolysaccharide, and/or interferon-γ) or high glucose in the presence and absence of these compounds. Felodipine, but not ramiprilat, concentration-dependently inhibited cytokine-induced NO production and NO synthase (NOS) mRNA induction. The antioxidant N-acetylcysteine also inhibited cytokine-induced NO production and induction of inducible NOS mRNA. Moreover, felodipine inhibited cytokine-induced superoxide production both in the presence and absence of an NOS inhibitor, suggesting that it acted as a superoxide scavenger and not as an inhibitor of inducible NOS induction. High glucose treatment (22 mmol/L for 48 hours) also significantly increased superoxide production in HASCs, and this increase was inhibited in a concentration-dependent manner by felodipine but not by ramiprilat. These results suggest that felodipine may exert vascular protective effects by suppressing free radical generation in human smooth muscle cells during activation of inflammatory mechanisms and diabetic conditions. (Hypertension. 1998;32:1011-1015.)

Key Words: nitric oxide ■ free radicals ■ superoxides ■ cytokines ■ glucose

Free radicals such as nitric oxide (NO) and superoxide are produced by cells of the blood vessel wall, and their imbalance may play an important role in the development of vascular disease. NO is synthesized from the amino acid L-arginine by a family of 3 related isoenzymes, the NO synthases (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are expressed constitutively, whereas inducible NOS (iNOS), which normally is not expressed in resting cells, requires inflammatory cytokines to induce its expression. The constitutive NO production by eNOS exerts a continuous vasodilator tone in the circulation and exerts a protective effect in the vessel wall. In contrast, NO production from iNOS, which produces a large amount of NO, has been implicated in atherosclerosis1 and heart failure.2

Superoxide anion formation has been described mainly as a specific function of phagocytes. Recent studies, however, have shown that the ability to generate superoxide is not limited to phagocytes. Indeed, superoxide production has been demonstrated in B-lymphocytes, fibroblasts, glomerular mesangial cells, epithelial cells, endothelial cells, and smooth muscle cells. Moreover, recent studies have shown that vascular superoxide production is stimulated by angiotensin II, and superoxide may act as a mediator of Ras-induced cell cycle progression.3

Only recently has it been recognized that the interaction between NO and superoxide may be equally important. The reaction rate between NO and superoxide occurs at the near diffusion limited rate of 6.7 × 1010 mol/L−1 s−1, which is 6 times faster than the scavenging of superoxide by copper, zinc superoxide dismutase (SOD) at physiological ionic strength. Hence, nearly every collision between NO and superoxide results in the irreversible formation of peroxynitrite (ONOO−).4 Thus, excess production of NO or superoxide yields the powerful oxidant ONOO− and may damage vascular tissue function.5

Calcium channel blockers retard the progression of atherosclerosis.6–8 The mechanisms involved have not been fully clarified but may relate to endothelial protection and inhibition of smooth muscle proliferation, as well as antiplatelet effects. On the other hand, inflammatory cytokines in many patients and high plasma glucose levels9 in diabetics have been implicated in the pathogenesis of atherosclerosis and coronary artery disease. In this article, we investigated the effect of felodipine on free radical production (especially NO and superoxide) stimulated by cytokines and high glucose in human aortic smooth muscle cells (HASCs).

Methods

Materials

Cytochrome c, SOD, lipopolysaccharide (LPS), and insulin-transferrin-selenite (ITS) were purchased from Sigma Chemical Co.
Smooth muscle cell growth medium (SmBM) was purchased from Clonetics. Recombinant human tumor necrosis factor-α (TNF-α), human interleukin-1β (IL-1β), and human interferon-γ (IFN-γ) were purchased from Boehringer Mannheim. DMEM without phenol red was purchased from Gibco. Griess reagent was purchased from Alexis. Felodipine and ramiprilat were kindly provided by Astra (Sweden).

**Cell Culture**

HASCs were purchased from Clonetech. HASCs were cultured in SmBM medium (Clonetech), and passages 3 through 6 were used for experiments.

**Induction of NO and Measurement of Nitrite**

When HASCs reached confluency with SmBM, they were cultured in DMEM without phenol red containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (ITS) for 48 hours. The cells were then incubated with DMEM without phenol red containing ITS, IL-1β (1 ng/mL), TNF-α (5000 U/mL), LPS (10 ng/mL), and human IFN-γ (500 U/mL) for 8 to 48 hours in the presence or absence of various drugs. N-Acetylcysteine was added 2 hours before treatment with cytokines. At the indicated times, a 100-μL sample of the culture medium was collected, and the nitrite level was measured by Griess reaction.10,11 The nitrite accumulation observed in wells without cells, which were run in parallel with those containing HASCs, were then subtracted from each cumulative value.

**Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was extracted using Trizol Gibco (Basel, Switzerland) and was reverse transcribed using random hexamers by superscript (II) Gibco (Basel, Switzerland). The human NOS (II) primers 12 were 5′-ATTCAAGTACGCTGTGTTTGG-3′ and 5′-CATGGTGAACACCGTTCTTGG-3′. The human GAPDH primers were 5′-CAGGAAATTCGCTTGAGTGAAAGCTCGGAGTCACCGG-3′ and 5′-AGTGAGTCCCGCTATGAGCTCCACCGAT-3′. PCR reactions were performed in a Biometra Trioblock thermocycler for 30 cycles at an annealing temperature of 60°C for 30 seconds, denaturation at 94°C for 30 seconds, and primer extension at 72°C for 1 minute. PCR products were then separated by 1% agarose gel electrophoresis and visualized and photographed by use of the visionary gel documentation system (Fotodyne).

**Measurement of Superoxide Production (O$_2^-$)**

Superoxide production was measured as the SOD-inhibitable reduction of cytochrome c. After each treatment, HASCs were washed with PBS and preincubated in DMEM without phenol red for 30 minutes at 37°C. Then cytochrome c (final concentration, 1 mg/mL) with or without SOD (final concentration, 500 U/mL) was added. After 60 minutes of incubation, the medium was removed from the cells, and the absorbance was read at 550 nm against a distilled water blank. Reduction of cytochrome c in the presence of SOD was subtracted from the values without SOD. The portion of superoxide-specific reduction of cytochrome c was between 20% to 35% according to the experiments. The OD difference between comparable wells with or without SOD was converted to equivalent O$_2^-$ production by use of molar extinction coefficient for cytochrome c (21.0 × 10$^{5}$ mol/L$^{-1}$ · cm$^{-1}$).

**Statistical Analysis**

Data are presented as mean±SEM. Multiple comparisons were evaluated by ANOVA and then by Fisher’s protected least-significant difference test. Student’s paired or unpaired t tests were used for comparisons between 2 experiments. $P<0.05$ was considered significant.

**Results**

**Effects on Cytokine-Induced NO Production**

To induce NO production in HASCs, we used human IL-1β, human TNF-α, LPS, and human IFN-γ, alone and in combination. The combination of IL-1β (1 ng/mL), TNF-α (5000 U/mL), LPS (10 ng/mL), and IFN-γ (500 U/mL) was found to be the most potent combination to induce NO production in HASCs at the passages used, whereas other combinations were ineffective (Figure 1). We therefore used this combination (ITL-γ) in the following experiments. Treatment with ITL-γ significantly increased NO production from HASCs compared with the control condition (no treatment). Felodipine, but not ramiprilat, inhibited NO production in a concentration-dependent manner (Figure 2).

**Effects on Induction of iNOS mRNA**

The effect of N-acetylcysteine on ITL-γ-induced NO production was also examined to further characterize the inhibitory effects of felodipine. As in the case of felodipine,
N-acetylcysteine (20 mmol/L) significantly inhibited ITL-γ induced NO production (Figure 3A).

Furthermore, to clarify the effect of these compounds on mRNA level of human iNOS induction, we performed RT-PCR. Treatment of HASCs with ITL-γ caused a clear single band of the expected size (Figure 3B). Felodipine (10⁻⁴ mol/L) and N-acetylcysteine (20 mmol/L) completely inhibited induction of mRNA by ITL-γ. No iNOS band was detected in these HASCs by RT-PCR up to 40 cycles. On the other hand, all compounds used had no effect on mRNA of the housekeeping gene GAPDH (Figure 3B).

**Effects on Cytokine-Induced Superoxide Production**

To clarify the effects of felodipine and ramiprilat on cytokine-induced superoxide production, we measured superoxide production in both the presence and absence of N⁵-monomethyl-L-arginine (L-NMMA). ITL-γ treatment significantly enhanced superoxide production, and this effect was further enhanced after treatment with L-NMMA. Under both conditions, felodipine, but not ramiprilat, inhibited superoxide production in a concentration-dependent manner (Figure 4).

**Effects on High Glucose–Induced Superoxide Production**

Although high glucose treatment (22 mmol/L) for 48 hours showed no effect on iNOS induction (data not shown), it significantly increased superoxide production compared with the osmotic control (mannitol, 22 mmol/L). Moreover, this increase was significantly inhibited by felodipine, but not by ramiprilat, in a concentration-dependent manner (Figure 5).

**Discussion**

Our results demonstrate for the first time that the calcium antagonist felodipine, but not the angiotensin-converting enzyme (ACE) inhibitor ramiprilat, inhibits (1) cytokine-induced NO production at the mRNA level and (2) cytokine as well as high glucose–induced superoxide production in HASCs. These results suggest that felodipine can diminish formation of the toxic product ONOO⁻, which is formed after reaction of NO and superoxide.¹⁴,¹⁵ This may explain at least in part the antiatherogenic effects of calcium antagonists.⁶⁻⁸

In human cells,¹⁴ induction of iNOS requires multiple cytokines, whereas the induction of this enzyme can be very effectively induced by even a single cytokine in rat smooth muscle cells,¹¹ mesangial cells,¹⁶ and macrophages.¹⁵ These results strongly suggest that the stimuli and mechanism required for iNOS induction differ not only among different cell types but also across species. Nonetheless, dihydropyridine calcium antagonists also are able to inhibit iNOS induction in J774 cells,¹ rat vascular smooth muscle cells,¹⁵,¹⁶ and human aortic smooth muscle cells.¹⁴

![Figure 3. Effect of felodipine and N-acetylcysteine on cytokine-induced NO production and mRNA induction. A, HASCs were treated with ITL-γ in the absence or presence of felodipine (10⁻⁴ mol/L) or N-acetylcysteine (NAC; 2×10⁻² mol/L). Values are mean±SEM (n=6). *P<0.05 vs no cytokines treatment; **P<0.05 vs ITL-γ alone. B, Representative result of RT-PCR assays for detection of iNOS and GAPDH expression.](image)

![Figure 4. Effect of felodipine and ramiprilat on cytokine-induced superoxide production. HASCs were treated with ITL-γ in the absence or presence of felodipine or ramiprilat for 48 hours. After treatment, superoxide production was measured by the cytochrome c method with or without an NOS inhibitor (L-NMMA, 10⁻⁵ mol/L). □ indicates basal production (without any treatment); ◻, felodipine without L-NMMA; ●, felodipine with L-NMMA; △, ramiprilat without NMMA; and ▲, ramiprilat with L-NMMA. Values are mean±SEM (n=6). *P<0.05 vs basal; **P<0.05 vs ITL-γ alone without L-NMMA; ***P<0.05 vs ITL-γ alone with L-NMMA.](image)

![Figure 5. Effect of felodipine and ramiprilat on high-glucose-induced superoxide production. HASCs were treated with high glucose (22 mmol/L) in the absence or presence of felodipine or ramiprilat for 48 hours. After treatment, superoxide production was measured by the cytochrome c method. □ indicates no treatment; ◻, treatment with mannitol (22 mmol/L); ●, high glucose treatment with felodipine, and ▲, high glucose treatment with ramiprilat. Values are mean±SEM (n=6). *P<0.05 vs mannitol; *P<0.05 vs high glucose treatment alone.](image)
and rat mesangial cells, as felodipine does in HASCs. Hence, these results suggest that dihydropyridine calcium antagonist and in particular felodipine are able to inhibit iNOS induction in different cell types and species, including humans.

Impaired NO production by eNOS is thought to play a key role in the pathophysiology of vascular disease. Upregulation or downregulation of eNOS, however, may also contribute to this process. Upregulation of iNOS by cytokines leads to the production of large amounts of NO by HASCs. This may at first be considered beneficial. However, particularly in the presence of increased superoxide production, upregulation of iNOS may produce large amounts of the toxic oxidant ONOO⁻ unless superoxide production is concomitantly suppressed.

In contrast with calcium antagonists, ACE inhibitors increase NO production by endothelial cells by inhibiting the breakdown of bradykinin, an effect that was not investigated in this study. Our results with ramiprilat in HASCs, however, demonstrate that ACE inhibitors are not able to reduce iNOS induction by cytokines or to suppress superoxide production. Thus, our results suggest different roles of calcium antagonists and ACE inhibitors in controlling free radical formation in the vessel wall: whereas the former compounds reduce superoxide and suppress excessive NO production by iNOS in smooth muscle cells, ACE inhibitors normalize NO from eNOS in endothelial cells. Compared with the therapeutic plasma levels of these compounds (1 to 10 nmol/L) required to lower blood pressure in patients, these compounds may be needed in much higher levels to reduce free radical formation in vitro. Tissue levels of these compounds during chronic treatment are still unknown but may be considerably higher, particularly with compounds with high tissue affinity. Thus, our in vitro results suggest that combination therapy with calcium antagonists and ACE inhibitors may represent a good rationale not only for lower blood pressure but also for long-term cardiovascular protection.

Dihydropyridine derivatives such as felodipine act as lipophilic chain-breaking antioxidants. In this study, however, we provide evidence for an additional mechanism by which these drugs may exert antioxidant effects. First, we measured superoxide production by the cytochrome c method in the absence and presence of L-NMMA. By use of this method, SOD-inhibitable extracellular superoxide production can be detected. To evaluate intracellular superoxide, one could consider the lucigenin method. However, as lucigenin itself stimulates NADPH-dependent superoxide production, we chose the cytochrome c method in this study. Because felodipine inhibited iNOS induction, it is possible that the calcium antagonist led to a depression of superoxide production by this mechanism; indeed, NO may be a source of superoxide. However, felodipine inhibited superoxide production in both the absence and presence of the NOS inhibitor L-NMMA. Hence, felodipine is more likely to quench active superoxide similar to N-acetylcysteine, and therefore both substances most likely act via the same mechanisms. Calcium antagonists have also been shown to prevent glutathione loss, and this might be a possible mechanism as well. Intracellular thiols regulate nuclear factor-kB (NF-kB) activation at one or more levels in the signal transduction cascade. High intracellular thiol levels could influence protein folding or enzyme activation and thus block the activation of protein kinases (eg, protein kinase C) that phosphorylate the IkB/NF-kB complex and liberate activated NF-kB. Alternatively, high intracellular thiol levels could interfere directly with IkB phosphorylation or with the transport of activated NF-kB into the nucleus. These results suggest the possibility that calcium antagonists inhibit NF-kB activation. NF-kB activation also plays a key role in iNOS mRNA induction, and antioxidants such as N-acetylcysteine are also potent NF-kB inhibitors. The fact that felodipine inhibited induction of iNOS at the mRNA level further supports the notion that felodipine acts as an antioxidant and NF-kB inhibitor very much like N-acetylcysteine. Indeed, lacidipine, another dihydropyridine calcium antagonist, inhibits the activation of NF-kB antipression of adhesion molecules induced by pro-oxidant signals in endothelial cells. As felodipine inhibits calcium entry, we should consider whether attenuation of intracellular calcium by felodipine can modulate NO and superoxide production. It is well established that cytokine-induced NOS is a calcium-independent enzyme; however, the role of calcium for superoxide-generating systems in human smooth muscle cells has not been well clarified. Hence, further studies are needed to clarify the precise mechanism. Taken together, this study suggests that felodipine acts not only as a direct antioxidant but also as an indirect antioxidant like N-acetylcysteine, which also increases intracellular free thiol levels in HASCs.

In conclusion, this study demonstrates that felodipine inhibits NO and superoxide production induced by cytokines and high glucose in HASCs. Our findings support the concept of a combination therapy of calcium antagonists with ACE inhibitors for cardiovascular protection.

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References


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