

The calcium channel blocker amlodipine exerts its anti-proliferative action via p21^(Waf1/Cip1) gene activation

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ABSTRACT Proliferation of vascular smooth muscle cells (VSMC) contributes to the progression of atherosclerotic plaques. Calcium channel blockers have been shown to reduce VSMC proliferation, but the underlying molecular mechanism remains unclear. p21^(Waf1/Cip1) is a potent inhibitor of cell cycle progression. Here, we demonstrate that amlodipine (10^{-6} to 10^{-8} M) activates de novo synthesis of p21^(Waf1/Cip1) in vitro. We show that amlodipine-dependent activation of p21^(Waf1/Cip1) involves the action of the glucocorticoid receptor (GR) and C/EBP- α . The underlying pathway apparently involves the action of mitogen-activated protein kinase or protein kinase C, but not of extracellular signal-related kinase or changes of intracellular calcium. Amlodipine-induced p21^(Waf1/Cip1) promoter activity and expression were abrogated by C/EBP- α antisense oligonucleotide or by the GR antagonist RU486. Amlodipine-dependent inhibition of cell proliferation was partially reversed by RU486 at 10^{-8} M ($58\% \pm 29\%$), antisense oligonucleotides targeting C/EBP- α ($91\% \pm 26\%$), or antisense mRNAs targeting p21^(Waf1/Cip1) ($96\% \pm 32\%$, $n=6$); scrambled antisense oligonucleotides or those directed against C/EBP- β were ineffective. The data suggest that the anti-proliferative action of amlodipine is achieved by induction of the p21^(Waf1/Cip1) gene, which may explain beneficial covert effects of this widely used cardiovascular therapeutic drug beyond a more limited role as a vascular relaxant.—Ziesche, R., Petkov, V., Lambers, C., Erne, P., Block, L.-H. The calcium channel blocker amlodipine exerts its anti-proliferative action via p21^(Waf1/Cip1) gene activation. *FASEB J.* 18, 1516–1523 (2004)

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ENHANCED PROLIFERATION of vascular smooth muscle cells (VSMC) is thought to contribute to the pathogenesis of vascular occlusive lesions including progression of atherosclerosis, restenosis following angioplasty, and graft atherosclerosis after coronary transplantation (1). Calcium channel blockers (CCB) are frequently used as standard therapeutics for patients suffering from atherosclerosis and for prevention of restenosis (1, 2).

Pharmacologically, CCB, such as amlodipine, are presumed to exert their biological effects by decreasing

intracellular calcium concentrations by inhibition of high voltage-gated L-type calcium channels that are expressed in VSMC (3–5). The latter are involved in the regulation of cellular activity, cellular energy supply, and the motility of VSMC (4–6). In addition to these calcium-dependent effects, α_1 subunits of the L-type calcium channel have been shown to interact with G-protein $\beta\gamma$ complexes and protein kinase C (PKC) (7, 8).

Amlodipine has recently been described to down-regulate cell proliferation by inhibiting the expression of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β_1 (TGF- β_1), and basic fibroblast growth factor (bFGF) (9, 10). These proteins are known to achieve their mitogenic effect via the mitogen-activated protein kinase (MAPK) signaling pathway, also involved in VSMC proliferation caused by oxidized low density lipoprotein or high blood pressure (11, 12). Activation of the MAPK signaling pathway resulted in VSMC proliferation and was linked to the activation of CCAAT/enhancer binding protein- β (C/EBP- β) and activating protein-1 (AP-1) (13).

C/EBPs represent a family of highly conserved transcription factors, of which six different isoforms have been characterized in humans (14). All C/EBPs bind to the same DNA consensus sequence, but exert different functions and interact with various other transcription factors (15). While C/EBP- β and C/EBP- δ activate cell cycle-stimulating factors (16), C/EBP- ϵ acts as a cell differentiation factor (17) and C/EBP- α inhibits cell proliferation by activating the expression of p21^(Waf1/Cip1) (9, 18). The function of C/EBP- ζ is uncharacterized, but has the potential to inhibit the action of C/EBP- α and C/EBP- β (18). Regarding cell cycle control by transcriptional mechanisms, it is noteworthy that transcription factors NF-IL6 and NF-IL6 β have been renamed C/EBP- β and C/EBP- δ , respectively (19, 20). We have previously shown that CCB activate NF-IL6 (or C/EBP- β) (21).

C/EBP- α induces p21^(Waf1/Cip1) leading to the inhibition of VSMC proliferation, macrophage infiltration, and restenosis in apolipoprotein knockout mice (22).

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Moreover, we have reported that maximal activation of p21^(Waf1/Cip1) depends on simultaneous activation of C/EBP- α and the glucocorticoid receptor (GR) (23). Thus, we have examined the possibility that this signaling pathway may underlie the anti-proliferative action of CCB.

MATERIALS AND METHODS

Vascular smooth muscle cell culture

Cultures of primary human lung VSMC were established from pulmonary arteries obtained during lung surgery. Cell cultures were established in minimal essential medium (MEM, Serotec, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Gibco/BRL, Basel, Switzerland), 5% colostrum (Chemie Brunschwig, Basel, Switzerland), 8 mmol/L L-glutamine (Serotec GmbH, Düsseldorf, Germany), and 20 mmol/L HEPES buffer (Serotec). VSMC were characterized as described earlier (24) by immunohistochemistry for positive smooth muscle cell actin and fibronectin, and negative staining for factor VIII. Subconfluent cultures (80% confluence) were used between passages 3 and 5. Before stimulation with amlodipine, VSMC were serum-deprived for 48 h in low serum medium (0.1% FCS). All experiments were done in MEM containing 5% FCS. Amlodipine was used at concentrations between 10^{-9} and 10^{-6} M. Signaling pathway inhibitors were obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach, Germany); L-type calcium channel agonist BayK8644, the p38 MAP kinase inhibitor SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), the Erk activation inhibitor peptide I (Ste-MP-KKKKPTPIQLNP-NH₂), or the PKC inhibitor D-erythro-sphingosine (trans-D-erythro-2-amino-4-octadecene-1,3-diol) were added to cell cultures 30 min before addition of CCB at concentrations recommended by the distributor.

Immunohistochemistry

Serum-deprived VSMC were grown on coverslips and treated with amlodipine (10^{-9} to 10^{-6} M) for various times (0, 0.5, 1, 3, 6, 9, and 12 h) before being washed (3 \times) with PBS, fixed (4% para-formaldehyde in PBS, 5 min), washed again with PBS, and finally fixed in 80% methanol containing 0.6% hydrogen peroxide. Nonspecific binding was blocked by 2.5% FCS in PBS with 0.05% Tween-20 (1 h). Cells were then incubated with a primary monoclonal antibody (rabbit anti-GR or rabbit anti-C/EBP- α , 1:500; 4°C, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer. Slides were washed (3 \times) and incubated with horseradish peroxidase (HRP) or Texas red-labeled second goat anti-rabbit antibody (1:5000, 1 h), then washed (5 \times) with blocking buffer. Bound antibodies were visualized by ACE enhancing stain (DAKO, Untermyli, Switzerland) or fluorescence microscopy as described previously (23).

Nuclear and cytosolic extracts

Transcription factors were assessed in nuclear and cytosolic extracts prepared from 80% confluent VSMC or from isolated circulating peripheral blood lymphocytes. Cells were resuspended in 80 μ L of low-salt buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM NaVO₄, 1 mM EDTA, 1 mM EGTA, 0.2% NP-40, 10% glycerol, Complete Protease InhibitorTM (Roche Diagnostics, Basel, Switzerland)] and incubated for 10 min on ice. After centrifugation (13,000 \times g, 1 min, 4°C), supernatant

was taken as the cytosolic fraction. The remaining pellet was dissolved in 50 μ L high-salt buffer [420 mM NaCl, 20 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM NaVO₄, 1 mM EDTA, 1 mM EGTA, 20% glycerol, Complete Protease Inhibitor] and kept on ice for 30 min, followed by centrifugation (13,000 \times g, 5 min, 4°C). The supernatant was taken as the nuclear fraction (21, 23). Protein concentration was determined by Bradford assay (Bio-Rad, Zürich, Switzerland).

Electrophoretic mobility shift assay (EMSA)

DNA binding proteins were characterized by EMSA using either a [³²P]-labeled glucocorticoid-responsive element (GRE; Santa Cruz Biotechnology), a C/EBP binding oligonucleotide (Santa Cruz), or a truncated human p21^(Waf1/Cip1) promoter that specifically binds the GR-C/EBP- α complex (23). Labeled DNA probe (60 fmol) was incubated with 1 μ g of nuclear protein extract (30 min, room temperature) using the following binding conditions: 10 mM Tris, 0.5 mM EDTA, 1 mM DTT, 50 mM NaCl, 1 mM MgCl₂, 8% glycerol, 1 μ g poly-[dI-dC]. The mixture was analyzed in a nondenaturing 4% polyacrylamide gel and subsequent exposure to X-ray films. Specificity of the observed DNA/protein complexes was confirmed by competitive binding of unlabeled GRE or p21^(Waf1/Cip1) or in the presence of antibodies against GR or C/EBP- α .

Western blot analysis

GR, C/EBP- α , or p21^(Waf1/Cip1) protein expression was assessed by fractionating 5 μ g of total protein on a gradient 4–15% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore Corp., Billerica, MA, USA) by standard protocols and protein transfer was confirmed with Ponceau red staining (22). Membranes were incubated (1 h) in blocking buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, and 5% skimmed milk), before addition of a protein specific monoclonal primary antibody (4°C, overnight, Santa Cruz). This was followed by three washes in blocking buffer and incubation with a secondary HRP-linked antibody (1 h, room temperature). Membranes were washed (3 \times) with blocking buffer and once with PBS before the protein bands were detected by enhanced chemiluminescence (ECL, Pierce, IL, USA).

Luciferase reporter gene assay

The p21^(Waf1/Cip1) promoter/luciferase construct (WWP-Luc) was a kind gift from B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA) and contained a 2.4 kb promoter fragment of the human p21^(Waf1/Cip1) gene promoter (23, 24). Luciferase assays were performed in serum-starved, 80% confluent cells that were transiently transfected for 1 h in the presence of serum-free medium containing 1.25 ng/ μ L of the WWP-Luc construct in TFX50 (Promega GmbH, Mannheim, Germany; TFX50: DNA ratio 3:1). Thereafter, cells were incubated for 24 h in growth medium (5% FCS) in either the presence or absence of drugs. Cells were then lysed and luciferase activity was determined for 10 s in an LUMAC Biocounter M1500P (Lumac, Landgraaf, The Netherlands) (21, 23).

Antisense oligonucleotides

Thioated antisense oligonucleotides (MWG, Biotech, Ebersberg, Germany) specific to C/EBP- α or p21^(Waf1/Cip1) mRNA were used in cell proliferation assays or in the WWP-Luc

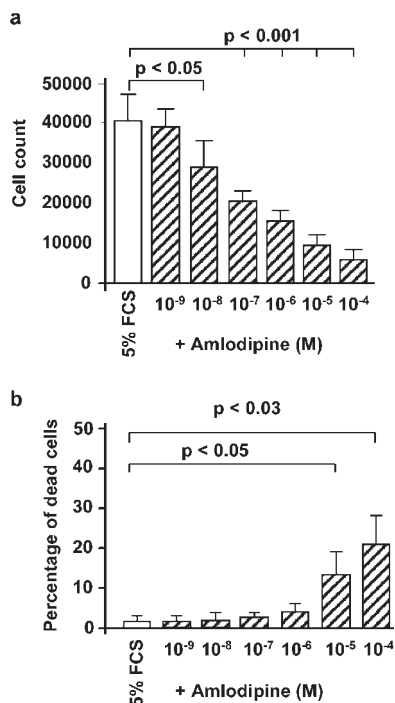


Figure 1. Growth inhibitory and cytotoxic effects of amlodipine in human VSMC. *a*) Serum-deprived VSMC (24 h) were stimulated with 5% FCS in the absence and presence of various concentrations of amlodipine and cell numbers were counted after 48 h. Each bar represents the mean \pm SD of data derived from 6 independent primary cell lines. *b*) Cell cytotoxicity of increasing concentrations of amlodipine was determined by standard Trypan blue staining (2 min) after 48 h. The % of positive stained cells was determined in all 6 VSMC lines in 100 cells in triplicate experiments. Each bar represents the mean \pm SD.

reporter gene assay. Nucleotide sequences of oligonucleotides used: antisense to C/EBP- α : 5'-GAA GGC GGC GCT GCT GGG CGC GT-3'; antisense to p21^(waf1/cip1): 5'-TGG GTT CTG ACG GAC ATC CCC A-3'; and a random sequence: 5'-AGC TCG GAT GCA TGG AGG AG-3' (negative control; all oligonucleotides from MWG Biotech AG, D-85560 Ebersberg, Germany). Cell cultures were transiently transfected by the addition of 0.5 or 1 μ mol of one of the oligonucleotides 24 h before each experiment. The oligonucleotides were re-added with the growth medium and present during the entire experiment. For cell counting experiments, the oligonucleotides were re-added at 1/4th the original concentration every day (23).

Cell counting

Cell proliferation experiments were performed in six independent cell lines and required a longer incubation of the cells (2 days) in the presence or absence of the drugs. Therefore, cells were seeded at a density of 10⁴ cells/cm², kept in low serum medium for 1 day, and exposed to growth medium in the absence or presence of CCB for 2 days. After two washes with PBS, cells were harvested with trypsin/EDTA for 1 min and resuspended in 400 μ L of low serum medium. Aliquots of 10 μ L were counted in a Neubauer improved hemocytometer. Data are representative for six independent experiments performed in triplicate \pm SD.

Statistical analysis

ANOVA and unpaired Student's *t* test was used to assess the data for cell count and WWP-Luc activity. Results were regarded as significant if $P \leq 0.05$.

RESULTS

The anti-proliferative and cytotoxic effect of amlodipine was assessed in six different primary human VSMC lines; each experiment was performed in triplicate. After an incubation of 48 h, amlodipine significantly inhibits serum-induced proliferation of VSMC at a concentration range of 1 \times 10⁻⁸ to 1 \times 10⁻⁶ M ($P \leq 0.05$) without any cytotoxic side effect (Fig. 1a). At concentrations \leq 1 \times 10⁻⁶ M, the cytotoxic effect of amlodipine was <5%; at concentrations above 1 \times 10⁻⁵ M amlodipine had a cytotoxic effect of >10%, as evaluated by Trypan blue staining (Fig. 1b).

Amlodipine induced the translocation/activation of two transcription factors, GR and C/EBP- α , as demonstrated by immunochemistry in human VSMC in Fig. 2a-d. In unstimulated cells, the GR was located in the cytosol (Fig. 2a) and was translocated into the nucleus 1 h after addition of amlodipine (10⁻⁶ M) (Fig. 2b). Staining for C/EBP- α was detectable in the cytosol of unstimulated cells (Fig. 2c), and a significant accumulation of nuclear C/EBP- α was observed 3 h after the

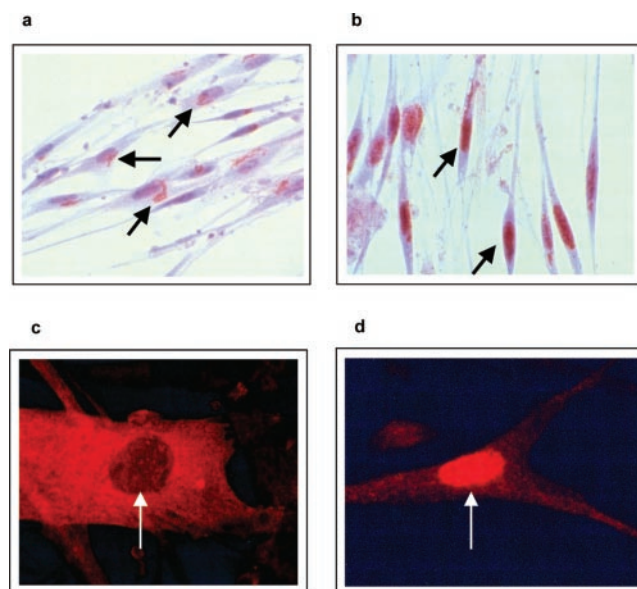


Figure 2. Immunofluorescence of the cellular distribution of the GR and C/EBP- α . *a*) Cytosolic localization of the GR in growing VSMC (5% FCS) and *b*) translocation into the nucleus after exposure to 10⁻⁶ M amlodipine (1 h). *c*) C/EBP- α staining by Texas red in growing VSMC and *d*) C/EBP- α translocation into the nucleus 3 h after addition of the drug. Arrows indicate compartmentalization before and after exposure to the drug. There was no translocation observed in unstimulated cell cultures without serum. Furthermore, amlodipine did not induce translocation in quiescent VSMC.

addition of the drug (10^{-6} M) while the cytosolic C/EBP- α pool was depleted (Fig. 2*d*).

GR translocation/activation by amlodipine (10^{-8} M) was confirmed by Western blot using 5 μ g of total protein per lane. Equal loading was controlled after transfer onto PVDF membranes by Ponceau staining. Amlodipine (10^{-8} M) and nifedipine (10^{-8} M) induced translocation of the GR at 30 min for up to 3 h. However, the translocation induced by amlodipine (10^{-8} M) was far more pronounced (Fig. 3*a*). In contrast, verapamil (10^{-8} M) failed to affect cellular compartmentalization of the GR (data not shown). The shift of GR by amlodipine was demonstrated by EMSA during a similar time course using a DNA consensus sequence containing the GRE (Fig. 3*b*). The time course of the amlodipine-induced functional activation of the GR was tested with a GRE oligonucleotide by EMSA and was similar to that observed by Western blot.

The activation of C/EBP- α by amlodipine (10^{-6} M) was demonstrated by EMSA using the same protein extracts used for GR activity, but in the presence of a CCAAT/enhancer consensus oligonucleotide (Fig. 3*c*). Binding of C/EBP could be detected 3 h after addition of amlodipine (10^{-6} M), persisting until 6 h.

Binding of C/EBP-isoforms and of the GR to the promoter of the negative cell cycle regulator p21^(Waf1/Cip1) (Fig. 3*d*) was demonstrated using the same p21^(Waf1/Cip1) promoter sequence later used to test the promoter activity with a luciferase reporter gene. The transcription factors' binding to the p21^(Waf1/Cip1) promoter sequence was characterized by supershift assay in the presence of the respective antibodies (Fig. 3*d*). Antibodies specific to the GR caused two supershifted bands and depleted the GR signal (GR Ab, lane 4, Fig. 3*d*). A similar double-band supershift was observed in the presence of anti-C/EBP- α antibodies (lane 5, Fig. 3*d*); anti-C/EBP- β antibodies did not produce any supershift (lane 6, Fig. 3*d*). Anti-C/EBP- ϵ antibodies showed a faint supershift band (lane 7, Fig. 3*d*). No supershift bands were observed in the presence of anti-C/EBP- γ or - δ antibodies or in the presence of anti-Oct-1 antibodies (Fig. 3*d*).

To achieve maximal activation of p21^(Waf1/Cip1), the GR has to form a complex with C/EBP- α that binds to the CCAAT/enhancer DNA sequence in the p21^(Waf1/Cip1) promoter (23). We therefore assessed the possible involvement of the GR and that of C/EBP- α on amlo-

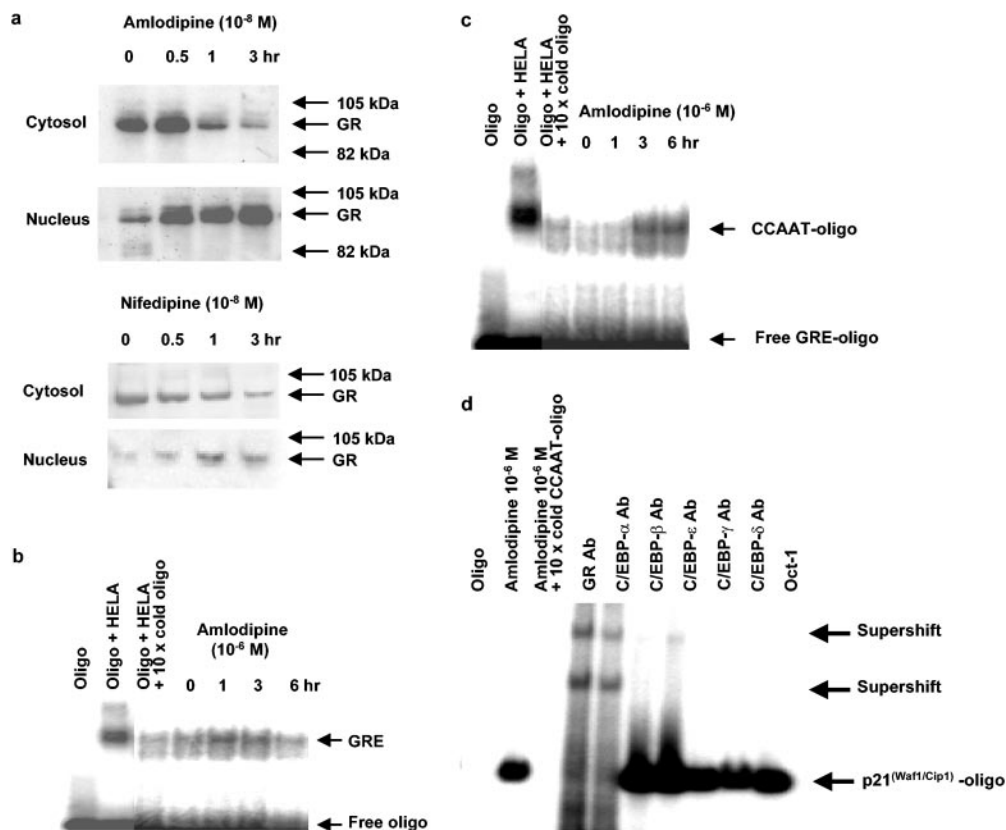


Figure 3. Dihydropyridines (10^{-6} M) activate the GR and C/EBP- α . *a*) A representative Western blot of amlodipine- and nifedipine-induced translocation of the GR. *b*) A representative EMSA using a GRE consensus oligonucleotide demonstrating the kinetic of GR activation by amlodipine (10^{-6} M). GR binding to GRE was abolished in the presence of 10 \times excess cold GRE oligonucleotide. *c*) Using the same extracts as in panel *b*, we demonstrate that amlodipine induced a C/EBP to bind to a CCAAT enhancer DNA consensus sequence. *d*) In a supershift assay using a human p21^(Waf1/Cip1) promoter fragment, we confirmed the binding of the GR and C/EBP- α to the p21^(Waf1/Cip1) promoter. Antibodies to C/EBP- β , - δ , or Oct-1 did not produce supershifts whereas an antibody to C/EBP- ϵ showed a faint supershift. To detect supershifts, the gels were run for an additional 3 h and exposed to a PhosphorImager screen overnight.

dipine-induced activation of the human p21^(Waf1/Cip1) promoter in growing VSMC. When cells were transiently transfected with a p21^(Waf1/Cip1)-luciferase reporter gene for 24 h, amlodipine induced luciferase activity in a dose-dependent manner (Fig. 4a). A similar dose-dependent effect on p21^(Waf1/Cip1) promoter luciferase activity was observed for nifedipine (10⁻⁸ M) but not for verapamil (10⁻⁸ M) (Fig. 4a).

In the presence of the GR inhibitor RU486 (10⁻⁶ M), amlodipine-dependent (10⁻⁶ M) activation of the p21^(Waf1/Cip1)-luciferase reporter gene was significantly decreased by 52% ± 12% (Fig. 4b). Similarly, amlodipine-induced activation of the p21^(Waf1/Cip1)-luciferase reporter gene was reduced by 56% ± 13%, when cells were pretreated with C/EBP-α antisense oligonucleotides (0.5 μM), whereas a scrambled oligonucleotide or an antisense oligonucleotide targeting C/EBP-β mRNA did not abrogate the stimulatory effect of amlodipine on luciferase activity (Fig. 4b).

Figure 4c reveals an up-regulation of p21^(Waf1/Cip1) protein expression starting after 6 h of treatment with amlodipine (10⁻⁶ M; lane 2) and increasing until 12 h

of treatment, with a slight decline thereafter at 24 h. In the presence of C/EBP-α antisense oligonucleotides (lane 5), the amlodipine-induced expression of p21^(Waf1/Cip1) protein was significantly down-regulated, whereas C/EBP-β antisense oligonucleotides (lane 7) had no such effect. RU486 (10⁻⁶ M, lane 8) at least partly inhibited the amlodipine-induced expression of p21^(Waf1/Cip1) protein in proliferating VSMC. p21^(Waf1/Cip1) protein is not expressed in growing VSMC in the presence of 5% FCS (lane 1, Fig. 4c).

As shown in Fig. 4d, amlodipine and nifedipine, but not verapamil (all at 10⁻⁸ M), activated the translocation of C/EBP-α within 6 h, which was followed by enhanced expression of p21^(Waf1/Cip1) protein 12 h after addition of the drugs. To delineate the signaling pathway involved in the CCB-mediated activation of p21^(Waf1/Cip1), we used SB203580, an inhibitor of p38 kinase, and observed that amlodipine-induced activation of C/EBP-α and the subsequent expression of p21^(Waf1/Cip1) were abrogated (lane 5, Fig. 4d). The extracellular signaling-related kinase (ERK) activation inhibitor peptide I (lane 6, Fig. 4d) had no significant

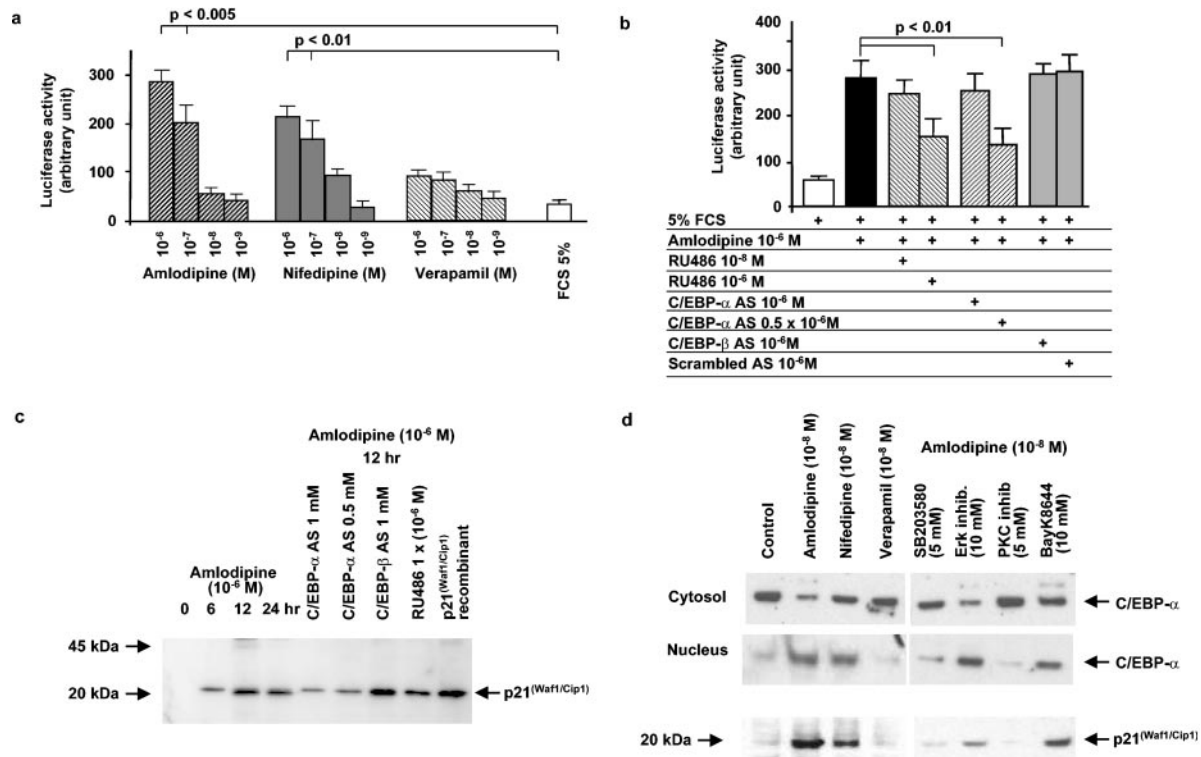


Figure 4. Dihydropyridines induce luciferase activity in a p21^(Waf1/Cip1) promoter-driven reporter gene. *a*) Dose-dependent induction of the p21^(Waf1/Cip1) promoter luciferase reporter gene construct by amlodipine, nifedipine, and verapamil. *b*) Inhibitory effect of the GR inhibitor RU486 and C/EBP-α antisense oligonucleotides on amlodipine-dependent activation of the p21^(Waf1/Cip1)-luciferase reporter gene. A scrambled oligonucleotide or an antisense oligonucleotide specific to C/EBP-β had no effect. Each bar represents the mean ± SD (n=6). *c*) A representative Western blot showing the amlodipine-induced kinetic of the expression of the p21^(Waf1/Cip1) protein (0–24 h) in human VSMC. After preincubation for 24 h of VSMC with C/EBP-α antisense oligonucleotides and subsequent addition of amlodipine for 12 h, the expression of p21^(Waf1/Cip1) protein was dose-dependently reduced, whereas antisense to C/EBP-β had no such effect. Preincubation of VSMC with RU486 (30 min) also reduced p21^(Waf1/Cip1) protein expression. Similar results were obtained in all other VSMC lines. *d*) Western blot analysis of CCB effects on C/EBP-α compartmentalization and on p21^(Waf1/Cip1) protein expression in the absence and presence of pharmacological modulators of intracellular signaling. C/EBP-α distribution was analyzed in cytosolic and nuclear protein fractions isolated 6 h after addition of the drugs. p21^(Waf1/Cip1) protein expression was determined 12 h after treatment. Various inhibitors of intracellular signaling were added 30 min before drugs in proliferating, subconfluent human VSMC.

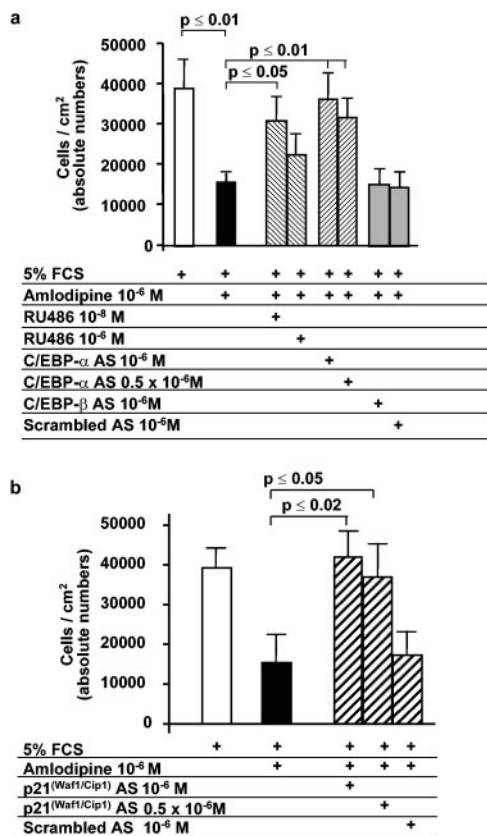


Figure 5. The anti-proliferative effect of amlodipine involves C/EBP- α , the GR, and p21^(Waf1/Cip1). *a*) Pretreatment of VSMC with RU486 (30 min) or a C/EBP- α antisense (AS) oligonucleotide (24 h) before addition of amlodipine (10⁻⁶ M) counteracted the inhibitory effect of the drug. Neither a C/EBP- β nor scrambled antisense oligonucleotides had such an effect. *b*) The effect of amlodipine was abolished when VSMC were pretreated with p21^(Waf1/Cip1) antisense oligonucleotides for 24 h. Each bar represents the mean \pm SD of triplicate experiments obtained in 6 primary VSMC lines.

inhibitory effect, nor on translocation of C/EBP- α or the expression of p21^(Waf1/Cip1). If activation of PKC was blocked by D-erythro-sphingosine (lane 7), both the amlodipine-induced activation of C/EBP- α and expression of p21^(Waf1/Cip1) were abrogated, whereas BayK8644, an L-type calcium channel agonist, had no effect (lane 8). When testing under the same experimental conditions, but without amlodipine, the addition of BayK8644 failed to result in a translocation of C/EBP- α (data not shown).

We confirmed the role of the GR, C/EBP- α , and p21^(Waf1/Cip1) in amlodipine-induced inhibition of VSMC proliferation (Fig. 5). As shown in Fig. 5*a*, amlodipine-dependent inhibition of cell proliferation was partially reversed by RU486 at 10⁻⁸ M (58% \pm 29%). This effect was even more pronounced if cells were pretreated with C/EBP- α antisense oligonucleotides (1 or 0.5 μ M), whereas scrambled or C/EBP- β antisense oligonucleotides failed to affect the amlodipine-inhibited VSMC proliferation. Finally, in the presence of a p21^(Waf1/Cip1) antisense oligonucleotide, the anti-proliferative effect of amlodipine was inhibited (Fig. 5*b*).

DISCUSSION

In this study, we show that the CCB amlodipine down-regulates VSMC proliferation by activating the p21^(Waf1/Cip1) gene, involving activation of GR and C/EBP- α .

Mason reported that amlodipine stabilizes atherosclerotic plaques in patients with coronary artery disease (2). The stabilization of plaques was explained as the result of a change in the differentiation status of several cell types in the plaques and may be based on the anti-proliferative potency of amlodipine. Amlodipine has been suggested to achieve its anti-proliferative action via the inhibition of growth factor synthesis (10) and by modulation of the interaction of LDL with proteoglycans (25).

Several studies indicated that the proliferation of VSMC is regulated by a signaling cascade that involves MAPK/ERK/PKC and subsequent activation of various transcription factors, including C/EBPs (26, 27). This signaling cascade is also activated by several risk factors for atherosclerosis and plaque development including LDL (11, 12), mechanical stress (13), and high blood pressure (27). It was reported that amlodipine (1–100 nM) dose-dependently inhibited p42/p44 bFGF-induced MAPKs (28). Several reports linked MAPKs with C/EBP and p21^(Waf1/Cip1) expression or activation: MAPKs were shown to 1) regulate binding of the FGF binding protein via p38 MAPK and C/EBPs (9), 2) affect the transcription and stability of p21^(Waf1/Cip1) mRNA, and 3) extend the stability of the p21^(Waf1/Cip1) protein (29). When delineating the underlying pathway for the anti-proliferative action of amlodipine, we found an apparent involvement of p38 MAPK and/or PKC, but not for ERK signaling or for changes of intracellular calcium, as evidenced from the use of millimolar BayK8644.

A potential role of the negative cell cycle regulator protein p21^(Waf1/Cip1) in atherosclerosis has been demonstrated by Nathe et al. (30) and by Condorelli et al. (31). The first study demonstrated that the cytokine IL-1 β in the presence of PDGF-BB down-regulates p21^(Waf1/Cip1), causing phosphorylation of p53, which results in decreased expression of inhibitors of cyclin-dependent kinases, enhanced expression of cyclins, and finally an increased proliferation of VSMC (30). The second study showed that transfection-dependent overexpression of a mutated p21^(Waf1/Cip1) inhibited VSMC proliferation in a mouse model of apolipoprotein E deficiency (31).

In this study, we provide data suggesting that the anti-proliferative action of amlodipine may be achieved, at least in part, by inhibition of the PKC and/or p38 MAP kinase signaling pathways (28) and that activation of p21^(Waf1/Cip1) is due to the activation of C/EBP- α and GR in human VSMC. Our observation is in agreement with a report showing that the natural inducer of p21^(Waf1/Cip1) gene expression, p53, is centrally involved in restenosis and progression of atherosclerotic plaques (32). A similar effect was observed after addition

of the dihydropyridine nifedipine; however, the translocation of GR by amlodipine was more efficacious, which could be explained by stereochemical differences between the two CCBs. It is noteworthy that the effect of the two dihydropyridines could not be mimicked by verapamil, which strengthens the argument for a specific antiproliferative efficacy of dihydropyridines independent of the role of intracellular calcium.

In conclusion, we suggest that the antiproliferative action of amlodipine and other dihydropyridines is mediated by the activation of p21^(Waf1/Cip1) via GR and C/EBP- α . **[F]**

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