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Modulation by dihydropyridine-type calcium channel antagonists of cytokine-inducible gene expression in vascular smooth muscle cells

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1 The 1,4-dihydropyridine nifedipine is frequently used in the therapy of hypertension and heart failure. In addition, nifedipine has been shown to exert distinct anti-arteriosclerotic effects both in experimental animal models and in patients. In the present study we have investigated the hypothesis that the latter effect of this class of drugs is mediated by an interference with the expression of pro-arteriosclerotic gene products in the vessel wall. Moreover, to elucidate as to whether nifedipine acts *via* L-type calcium channel blockade, its effects were compared to those of another dihydropyridine, Bay w 9798, which has no calcium-antagonistic properties in concentrations up to 10 μM, as verified by superfusion bioassay.

2 Both, nifedipine and Bay w 9798, in concentrations ranging from 0.01 to 1 μM, augmented the interleukin-1β/tumour necrosis factor-α (IL-1β/TNF-α)-induced expression of the inducible isoform of nitric oxide synthase (iNOS) in rat aortic cultured smooth muscle cells (raSMC) 2–3 fold, as judged by RT–PCR and Western blot analyses.

3 In contrast, cytokine-induced mRNA expression of monocyte chemoattractant protein 1 (MCP-1) in these cells was down-regulated by more than 60% in the presence of both dihydropyridines, as judged by RT–PCR and Northern blot analyses.

4 Nuclear run-on assays and incubation with the transcription-terminating drug actinomycin D revealed that both drugs acted at the level of mRNA synthesis rather than stability.

5 These findings suggest that 1,4-dihydropyridines such as nifedipine affect the expression of both potentially pro-arteriosclerotic (MCP-1) and anti-arteriosclerotic (iNOS) gene products in the vessel wall at the level of transcription, and that these effects are unrelated to their calcium channel-blocking properties.

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Abbreviations: AP-1, activator protein 1; Bay w 9798, 1,2,6-trimethyl-4-(4-trifluoromethylphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid trimethylester; C/EBP, CCAAT-enhancer binding protein; EMSA, electrophoretic mobility shift assay; IL-1β, interleukin-1β; iNOS, inducible isoform of NO synthase; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor κB; nifedipine, 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid dimethylester; O_2^-, superoxide anion; TNF-α, tumour necrosis factor α; VCAM-1, vascular cell adhesion molecule-1

Introduction

Nifedipine was the first calcium antagonist introduced in antihypertensive therapy (for review see Fleckenstein, 1983). Its beneficial effects in the development of arteriosclerosis were soon recognized. Thus, nifedipine was shown in clinical trials (Lichtlen *et al*., 1990; Waters *et al*., 1990; Weinstein & Heider, 1989; Keogh & Schröder, 1990) as well as in animal models (Habib *et al*., 1986; Henry & Bentley, 1981; Willis *et al*., 1985; for review see Rafflenbeul, 1997) to inhibit the formation of new arteriosclerotic plaques, whereas pre-existing plaques were not affected. In contrast to its antihypertensive and cardioprotective action, which are thought to be understood at the molecular level (for review see Spedding & Paoletti, 1992), up to now the mechanism underlying its anti-arteriosclerotic effect has not been elucidated, but is believed to be due to antioxidative (Mak *et al*., 1992), or calcium-antagonistic (Hof & Ruegg, 1991) properties of the dihydropyridines.

Arteriosclerosis is a chronic vascular inflammation resulting in various severe diseases of the circulatory system that are responsible for the majority of death cases in industrial countries (for review see Ross, 1995). Especially in the early phase of arteriosclerosis, a variety of cytokines such as interleukin-1β (IL-1β) are involved in the development and maintenance of the inflammation (Libby *et al*., 1995) by up-regulating monocyte chemoattractant protein-1 (MCP-1; Takeya *et al*., 1993) or vascular cell adhesion molecule-1 expression (VCAM-1, Li *et al*., 1993). These proteins in turn promote arteriosclerotic plaque formation through an increased recruitment of monocytes and other leukocytes to the vessel wall, hence augmenting cytokine production in the inflammatory focus.

On the other hand, vessels undergoing remodelling in arteriosclerosis contain only small amounts of nitric oxide (NO; Darley-Usmar *et al*., 1995), an autacoid not only important in the regulation of vascular tone but also in the negative modulation of smooth muscle cell growth (Cornwell *et al*., 1994). In addition, expression of the inducible isoform of NO synthase (iNOS) in endothelial or smooth muscle cells in such circumstances may have an anti-arteriosclerotic effect, as NO also effectively suppresses the expression of pro-arteriosclerotic gene products such as MCP-1 or

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VCAM-1 in the endothelium (Cooke & Tsao, 1994; Peng *et al*., 1998).

Here we have investigated whether nifedipine modulates the expression of iNOS and MCP-1 in rat aortic smooth muscle cells and how this effect is brought about.

Methods

Materials

All laboratory chemicals were from Roth, Darmstadt or Sigma-Aldrich, Deisenhofen, Germany. Oligonucleotides, molecular biology reagents and cell culture materials were from Gibco Life Technologies BRL, Paisley. Cytokines were from R&D Systems, Wiesbaden, Germany. The antibody against iNOS was from Biomol, Hamburg, Germany; the anti-MCP-1 antibody was from Research Diagnostics, Flanders, NJ, U.S.A. The HRP-conjugated secondary antibodies were from Sigma-Aldrich, Deisenhofen, Germany. Nitrocellulose membranes and the Super Signal Blaze™ chemiluminescent reagent were from Pierce, Rockford, IL, U.S.A.; the nylon membranes from Qiagen, Hilden, Germany. Sodium pentobarbitone was from Sigma-Aldrich, Deisenhofen, Germany; actinomycin D from Roche, Mannheim, Germany, and nifedipine and 1,2,6-trimethyl-4-(4-trifluoromethylphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid trimethylester (Bay w 9798) were from Bayer, Wuppertal, Germany.

Cell culture

Rat aortic smooth muscle cells (raSMC) were isolated by the explant technique. Briefly, aortae (1.5 mM i.d.) were isolated from pentobarbitone-anaesthetized male Wistar rats (250–300 g bodyweight), cleaned under sterile conditions of adherent fat and connective tissue, and cut open longitudinally. The endothelium was scraped off, the adventitia removed with the aid of small forceps and the remainder of the blood vessel cut into segments of approximately 3 mm width. These were incubated in non-coated 6-well plates in 1 ml of Waymouth medium, supplemented with 10% FBS, 50 μ ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 10 μ ml^{-1} nystatin, HEPES 5 mM and TES 5 mM until there was a visible outgrowth of cells (usually within less than 1 week). Thereafter the segments were removed and the culture medium changed every 2 days for approximately 1 week until the cells reached confluence. They were harvested by using 0.05% (w v^{-1}) trypsin and 0.02% (w v^{-1}) EDTA, pooled and seeded at a ratio of 1 : 5 into 100 mm i.d. Petri dishes.

Cells for the experiments described were derived from passages 2–4 of individual preparations. They were identified after fixation with *p*-formaldehyde by positive immunostaining for smooth muscle α-actin with a monoclonal anti-α-smooth muscle actin antibody (dilution 1 : 1000, Sigma-Aldrich, Deisenhofen, Germany) and a secondary anti-mouse IgG-FITC conjugate from goat (dilution 1 : 80; Sigma-Aldrich). Great care was taken to differentiate the smooth muscle cells from contaminating fibroblasts by visualizing the actin fibres with confocal laser scanning microscopy (Leica CLSM; Leica Microsystems, Heidelberg, Germany). According to this procedure, the cultured smooth muscle cells appeared to be essentially homogenous.

On the day of the experiment, cells were pre-incubated with nifedipine or Bay w 9798 for 3 h, after which time the cells were exposed to 60 u ml^{-1} IL-1β plus 1000 u ml^{-1} TNF-α or vehicle for 6 h in an incubator. Experiments were terminated by washing the cells and scraping them off with the aid of a cell scraper (Western blot and nuclear run-on analyses) or lysing them with 700 μl of GTC buffer (4.0 M guanidiniumisothiocyanate, 25 mM sodium citrate, 0.5% (w v^{-1}) sodiumlaurylsarcosine, 1% β-mercaptoethanol, pH 7.4) for RNA isolation (RT–PCR).

Superfusion bioassay

Rings of 5–7 mm width from freshly prepared rat aortae were mechanically denuded with the tips of a forceps, mounted between force transducers and a rigid support for measurement of isometric force (TSE, Bad Homburg, Germany) and superfused with Krebs-Henseleit solution ((mM) NaCl 119, KCl 4.7, $CaCl_2$ 2.1, $MgSO_4$ 2.5, $NaHCO_3$ 25.2, KH_2PO_4 1.2, glucose 12.2, 26 μM EDTA and 1 μM diclofenac, pH 7.4, $pO_2 \geqslant 400$ mmHg) at 1 ml min^{-1}. Passive tension was adjusted to approximately 3 g over a 30 min equilibration period. Thereafter, the segments were superfused with a potassium-rich modified Krebs-Henseleit solution ((mM) NaCl 53, KCl 66, $CaCl_2$ 2.1, $MgSO_4$ 2.5, $NaHCO_3$ 25.2, KH_2PO_4 1.2, glucose 12.2, 26 μM EDTA and 1 μM diclofenac, pH 7.4, $pO_2 \geqslant 400$ mmHg) for depolarization until a new plateau, representing a total force of approximately 6 *g* was reached. Bolus injections of nifedipine and Bay w 9798 in the range of 0.1 pmol to 1000 nmol resulting in final drug-concentrations of 0.1 nM to 1 mM were injected into the perfusate and changes in tension were recorded with the aid of a digital PC-operated analysis system (Biosys, TSE).

RT–PCR analysis

Total RNA was isolated according to the method described by Chomczynski & Sacchi (1987). First strand cDNA synthesis from 3 μg of total RNA was performed with Superscript™ reverse transcriptase (Gibco Life Technologies) according to the manufacturer's instructions. To normalize the amount of cDNA in the samples from one experiment, 2.5% of the resulting cDNA was used for performing PCR reactions for the house keeping genes, elongation factor 2 (EF-2) or glycerinealdehydephosphate dehydrogenase (GAPDH). PCR was performed with as few cycles as possible to detect the PCR products on an ethidium bromide-stained agarose gel. According to densitometric analysis of the PCR products (One-Dscan Gel analysis software from Scanalytics, Billerica, MA, U.S.A.), cDNA volumes were adjusted for consecutive analyses. Programmes and primers for the measurement of steady state levels of mRNA of the other gene products were as follows: iNOSfor: 5′-ATG GCT TGC CCC TGG AAG TTT CTC-3′; iNOSrev: 5′-CCT CTG ATG GTG CCA TCG GGC ATC TG-3′, (product spans two introns in the human gene; cDNA length is 826 bp); MCP-1 for: 5′-ACC TGC TGC TAC TCA TTC ACT-3′; MCP-1rev: 5′-CAT CTT GCA TTT AAG GAT TTC T-3′, (product spans one intron in the rat MCP-1 gene, cDNA length is 454 bp), GAPDH for: 5′-TCA CCA TCT TCC AGG AGC G-3′; GAPDH rev: 5′-CTG CTT CAC CAC CTT CTT GA-3′ (product spans one intron in the rat GAPDH gene; cDNA-length is 553 bp); EF-2 for: 5′-GAC ATC ACC AAG GGT GTG CAG-3′; EF-2 rev: 5′-GCG GTC AGC ACA CTG GCA TA-3′ (no intron spanning product; cDNA length is 220 bp).

All PCR reactions were performed in OmnE cyclers from Hybaid, Heidelberg, Germany. The primers for iNOS and EF-2 were kindly provided by Dr E. Schütz, Department of

Clinical Chemistry, University of Goettingen. For all primers, 58°C was established to be the optimal annealing temperature. The programme performed for PCR amplification was as follows: An initial period of 2 min at 94°C, followed by a variable number of cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C and finally 60 s of extension at 72°C. The programme was terminated with a period of 5 min at 72°C. To be within the exponential phase of the semi-quantitative PCR reaction (Wang *et al*., 1989), the appropriate number of cycles was newly established for every set of samples.

Northern blot analysis

Electrophoresis of total RNA and Northern blot analysis were performed with standard techniques as described by Ausubel (1997). The probes for GAPDH and MCP-1 were generated with the same primers as described for the RT–PCR analysis and sequenced for identity. As hybridization probes, 30 ng of DNA were labelled by random priming (Amersham, Freiburg, Germany). Blots were hybridized overnight at 42°C in 50% formamide as described in Ausubel (1997). High stringency washing and exposure to Kodak X-OMAT film (Sigma, Deisenhofen) for 1–7 days at −80°C was performed after the hybridization.

Nuclear run-on assays

All steps were performed on ice. Approximately 5×10^6 cells in 100 mm Petri dishes were used for the incubation. They were harvested with a cell scraper and incubated for 10 min in 2 volumes of lysis buffer ((mM) HEPES 5, $MgCl_2$ 1, DTT 1, 20% glycerol, 0.05% Triton X-100 pH 7.4). After that period, the volume was adjusted to 2 ml with cold wash buffer ((mM) Tris 20, 20% (w v^{-1}) glycerol, KCl 140, $MgCl_2$ 10, DTT 1, pH 7.4) to which protease inhibitors had been added at the following concentrations: 1 mg ml^{-1} Pefabloc^{TM}, 0.5 μg ml^{-1} pepstatin A, 0.5 μg ml^{-1} leupeptin (protease mix). The cells were lysed with 15 strokes in an EMBL-cell cracker device as described by Balch *et al*. (1984). The lysate was centrifuged for 10 s at $300 \times g$ and 0°C in a microcentrifuge, and the supernatant was used for further isolation of the nuclei. After centrifugation for 5 min at $1650 \times g$, the cytosolic supernatant was quantitatively discarded or used for the isolation of steady state RNA and consecutive RT–PCR analysis as described above. The raw nuclear pellet was washed three times with 1 ml of ice-cold cold wash buffer and centrifuged at $1650 \times g$ for 5 min. The nuclear pellet from the last centrifugation step was suspended in approximately four volumes of reaction buffer (2 mM each CTP, GTP and UTP, 3 mM ATP, 20 μ ml^{-1} RNAsin (Fermentas, Vilnius, Lithuania), 8.5 mM creatine-phosphate and 0.1 mg ml^{-1} creatin-kinase (Roche, Mannheim, Germany) in cold wash buffer). Half of the nuclei were immediately lysed in four volumes of GTC as the negative control and the other half was incubated at 30°C for 30 min and then lysed in GTC. RNA was isolated and RT–PCR analysis was performed as described before, except for the use of random primers instead of oligo-dT primers in the reverse transcription step. When RT–PCR analysis with RNA from control nuclei was performed, the amount of DNA synthesized typically was not detectable or reached an amount of maximally 20% compared to cDNA amounts from the incubated nuclei, demonstrating that the PCR products obtained from this procedure reflected *de novo* mRNA synthesis in the isolated nuclei.

Western blot analysis

After 6–12 h of incubation, conditioned media were collected and the cells were washed with 1 ml of PBS without calcium, harvested with a cell scraper, cooled to 4°C and sedimented for 5 min at $800 \times g$ in a microcentrifuge. The supernatant was quantitatively discarded, the cells were lysed by five cycles of freeze thawing and the resulting lysate was resuspended in two volumes of lysis buffer ((mM) Tris × HCl 25, KCl 70, EDTA 1, glucose 2.5, DTT 1, protease mix at pH 7.4). After mixing thoroughly, the lysate was centrifuged consecutively for 5 min each at 1300 and $8000 \times g$ and the resulting supernatant used for electrophoresis. The protein content was estimated with the modified Bradford protein assay from BioRad (Munich, Germany) according to the manufacturer's instructions. The protein quantification was confirmed by staining of the gels after blotting with a coloidal Coomassie blue solution (Pierce) and densitometry of prominent protein bands. For detection of iNOS protein, aliquots of 50 μg protein were subjected to a 8% SDS–PAGE according to Lämmli (1970). The fully developed gel was blotted onto nitrocellulose filters at 600 V for 2 h in a semi-dry electroblotter. Transfer and development of the blot was done with standard buffers and protocols (Ausubel, 1997) by using a primary rabbit polyclonal anti-iNOS antibody (Transduction Laboratories, Heidelberg, Germany) together with a secondary HRP-conjugated anti-rabbit antibody (Sigma-Aldrich) and the Super Signal Blaze^{TM} chemiluminescent reagent (Pierce).

For the detection of MCP-1 protein, a Schägger SDS–PAGE of 12% acrylamide (Schägger & von Jagow, 1987) was performed in a minigel device. Aliquots of 120 μl cell culture supernatant were subjected to electrophoresis. The gel was blotted onto a nitrocellulose filter at 90 mA for 45 min in a semi-dry blotter and immunostaining was performed with a primary goat anti-MCP-1 antibody (R&D Research Diagnostics, Wiesbaden, Germany) and a secondary HRP-conjugated anti-goat/sheep antibody (Sigma-Aldrich).

Electrophoretic mobility shift analysis (EMSA)

Nuclear extracts from raSMC were prepared as described previously (Schreiber *et al*., 1989) except that Igepal CA-630 (Sigma-Aldrich) was used instead of Nonidet-P40 as a detergent. Protein concentrations were determined by the method of Bradford (1976). The double-stranded gel shift oligonucleotides (Santa Cruz Biotechnology, Heidelberg, Germany) AP-1 (5′-CGC TTG ATG ACT CAG CCG GAA-3′), C/EBP (5′-TGC AGA TTG CGC AAT CTG CA-3′), C/EBPmut (5′-TGC AGA GAC TAG TCT CTG CA-3′) and NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were end-labelled with γ-^{32}P-ATP by using the 5′-end labelling kit from Amersham Pharmacia Biotech (Freiburg, Germany). Typically the binding mixture contained 3–10 μg of nuclear extract, 10–20,000 c.p.m. of the ^{32}P-labelled oligonucleotide probe (0.5 ng), 1 μg poly[d(I-C)] and 1.33 mM DL-dithiothreitol in a total volume of 15 μl binding buffer (Hecker *et al*., 1997). After 30 min at room temperature, the DNA-protein complexes were resolved by non-denaturing electrophoresis through a 4% polyacrylamide gel. The gel was dried and

the ^{32}P-labelled protein-DNA-complexes visualized by autoradiography. The specificity of the binding reaction was monitored by performing the assay in parallel with the same samples in the presence of a 100–1000 fold excess of the non-labelled oligonucleotide. For supershift analyses, 1.0–2.0 μl of the appropriate gel supershift antibody (2 mg ml^{-1}, Santa Cruz Biotechnology) per 6.0–7.0 μl of nuclear extract (3–10 μg protein) were pre-incubated overnight at 4°C at room temperature for 60 min, before the EMSA was performed.

Determination of superoxide generation

Superoxide (O_2^-) formation was determined essentially as described previously (Hecker *et al*., 1996). Segments of the rat aorta were incubated in the presence or absence of 0.1 to 10 μM (final concentration) of nifedipine or Bay w 9798 in a HEPES-modified Krebs-Henseleit solution ((mM) NaCl 135.3, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 10, EDTA 26 μM and diclofenac 1 μM, pH 7.4) containing 250 μM lucigenin. Both basal and phorbol-12,13-dibutyrate (5 μM)-stimulated O_2^- generation was measured as relative light units per second (RLU s^{-1}) in a Lumat LB 9501 chemiluminescence reader (Berthold, Bad Wildbad, Germany).

Statistics

Unless indicated otherwise, results are expressed as mean ± s.e.mean of n observations. Student's unpaired t-test was used to determine the statistical significance of differences between the means. A P value of <0.05 was considered to be statistically significant.

Results

Effects of nifedipine and Bay w 9798 on calcium channel activity

To compare the effects of nifedipine and Bay w 9798 on L-type calcium channel activity, endothelium-denuded rat aortic segments were actively constricted with a depolarizing concentration of potassium chloride (66 mM) to a tension of 2.7 $g \pm 0.35$ g ($n=14$). Under these conditions, constriction is mediated almost exclusively by the opening of L-type calcium channels. In three independent experiments, nifedipine elicited a dose-dependent relaxation with an ED_{50} value of 100 pmoles (corresponding to a final concentration of approximately 0.1 μM). Bay w 9798 was more than four orders of magnitude less active with an $ED_{50} > 1$ μmol (corresponding to a final concentration >1 mM), suggesting that it is only a very weak calcium channel blocker (Figure 1).

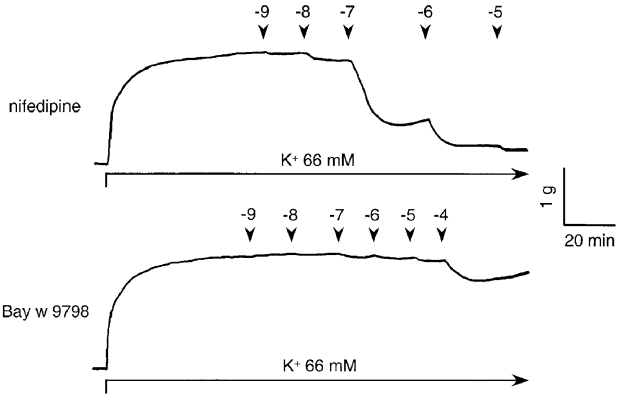

Figure 1 Dose-dependent relaxation by nifedipine and Bay w 9798 of potassium-constricted endothelium-dependent segments of the rat aorta. Arrows indicate bolus injections of nifedipine and Bay w 9798 with the resulting approximate concentrations in the superfusate (log M) displayed above. The figure shows two typical original traces. Virtually the same results were obtained in two other independent experiments.

Effects on O_2^- formation

In concentrations up to 1 μM neither nifedipine nor Bay w 9798 had a significant inhibitory effect on basal or phorbol ester-stimulated O_2^- formation in isolated segments of the rat aorta (not shown). Direct antioxidative effects of nifedipine as well as of Bay w 9798 were detectable only at a concentration $\geqslant 10$ μM when xanthine oxidase/xanthine was used to generate O_2^- (not shown).

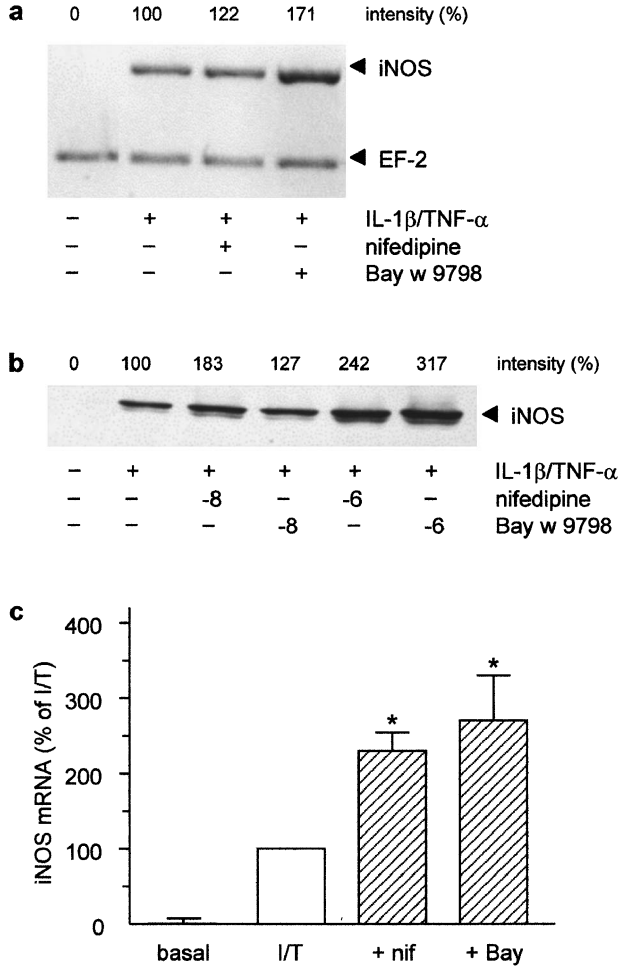

Figure 2 Effect of nifedipine and Bay w 9798 on (a, c) the steady state mRNA level of iNOS and (b) iNOS protein abundance in cultured raSMC. The cells were pre-incubated for 3 h with 1 μM nifedipine or Bay w 9798 and then stimulated for 6 h with 60 u ml^{-1} IL-1β and 1000 u ml^{-1} TNF-α (I/T). The mRNA of the house-keeping gene, elongation factor 2 (EF-2) was used as a standard for total cDNA load. (a) Typical RT–PCR analysis and (b) Western blot analysis typical of four individual experiments with the concentrations of nifedipine and Bay w 9798 indicated in log M. Corrected densitometric intensity values are indicated at the top of each panel. (c) Statistical summary of the RT–PCR data (expressed as percentage of the mRNA level in IL-1β and TNF-α stimulated raSMC) derived from six independent experiments (*$P<0.05$ vs I/T).

Effects on MCP-1 and iNOS expression in cultured raSMC

According to RT–PCR analysis, there was no basal expression of iNOS mRNA detectable in these cells, whereas MCP-1 was detectable also under control conditions in most but not all experiments (cf. Figure 4a). Exposure to IL-1β plus TNF-α resulted in a marked increase in mRNA abundance of both gene products (Figures 2, 3). Nifedipine as well as Bay w 9798 at concentrations of up to 1 μM had no effect on basal iNOS expression, but significantly augmented the stimulating effect of IL-1β plus TNF-α 2–3 fold at a concentration of 1 μM (Figure 2a,c). This effect was, albeit weaker, also detectable at a concentration of 10 nM. The increase in iNOS mRNA abundance in the presence of both dihydropyridines could also be verified at the protein level after incubation for 18 h by Western blot analysis (Figure 2b).

In contrast to iNOS mRNA abundance, cytokine-inducible MCP-1 expression was reduced in the presence of both dihydropyridines (1 μM final concentration) to approximately 50% of the level attained by stimulation with IL-1β and TNF-α alone, as judged both by RT–PCR (Figure 3a,c) and Northern blot analysis (Figure 3b). Whereas it was possible to detect MCP-1 protein in the supernatant of human umbilical vein and porcine aortic cultured endothelial cells by Western blot analysis, this was not possible in the supernatant of raSMC (not shown).

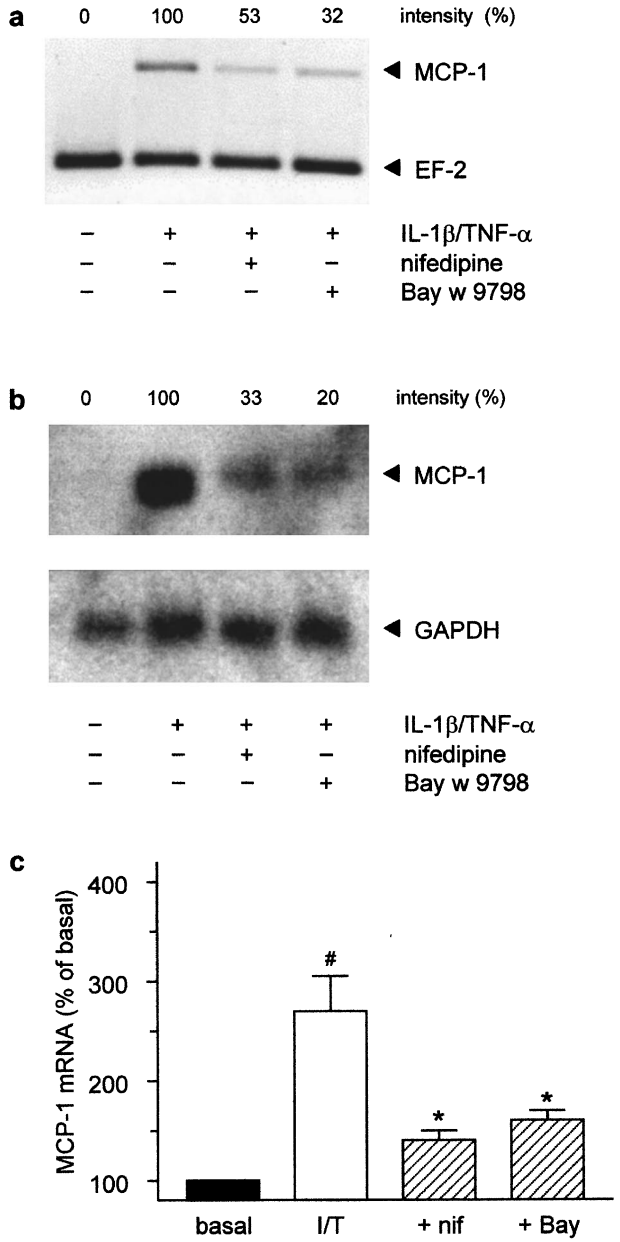

Figure 3 Effect of nifedipine or Bay w 9798 (1 μM, 1 h preincubation) on MCP-1 mRNA expression in cultured raSMC exposed to IL-1β (60 u ml^{-1}) and TNF-α (1000 u ml^{-1}) for 6 h. (a) Typical RT–PCR analysis with elongation factor 2 (EF-2) as house-keeping gene, and (b) Northern blot analysis with GAPDH as house-keeping gene (typical Northern blot representative of two additional experiments with different batches of raSMC). (c) Statistical summary of the RT–PCR-based mRNA data (expressed as percentage of the basal MCP-1 mRNA level) from six individual experiments ($P>0.05$ vs basal, $^*P<0.05$ vs I/T).

Nifedipine and Bay w 9798 act at the transcriptional level

To elucidate as to whether the dihydropyridines affected iNOS and MCP-1 mRNA synthesis or stability, nuclear run-on experiments were performed. Cells were first incubated for 3 h with 1 μM nifedipine, Bay w 9798 or vehicle and then exposed to IL-1β plus TNF-α for 3 h. After this time nuclei were isolated and mRNA synthesis determined as described in the Methods section. As shown in Figure 4a,b, both dihydropyridines clearly acted at the transcriptional level. Similar results were obtained by RT–PCR analysis for iNOS, when mRNA stability was assessed in the presence of the RNA synthesis inhibitor actinomycin D (Figure 4c,d). After exposure to 1 μM actinomycin D, 4 h after the beginning of the incubation, iNOS mRNA was barely detectable after a total period of 8 h in samples from IL-1β plus TNF-α treated cells as well as in samples additionally treated with nifedipine or Bay w 9798, indicating that stability of iNOS mRNA is not elevated by these compounds. For the analysis of MCP-1 mRNA this kind of experiment is not feasible, as it would be impossible to differentiate whether the actinomycin D-induced decrease in MCP-1 mRNA is due to low transcriptional activity or decreased stability.

Analysis of the activity of potentially involved transcription factors

Electrophoretic mobility shift analyses (Schreiber *et al*., 1989) with nuclear extracts of raSMC were performed to analyse transcription factors possibly involved in dihydropyridine-induced modulation of iNOS- and MCP-1 gene expression. Nuclear extracts were incubated with ^{32}P-labelled consensus oligonucleotides for AP-1, C/EBP (β and δ) and NF-κB, transcription factors known to be activated upon IL-1β/TNF-α induction and being involved in iNOS as well as MCP-1 gene expression (Eberhardt *et al*., 1998; Martin *et al*., 1997; Hu *et al*., 1998). However, no modulation of the IL-1β/TNF-α-induced activation of these transcription factors by nifedipine or Bay w 9798 could be detected (cf. Figure 5 for NF-κB).

Discussion

The present findings demonstrate that 1,4-dihydropyridines such as nifedipine affect the cytokine-inducible expression of arteriosclerosis-related gene products in cultured raSMC at the level of *de novo* mRNA synthesis, presumably by interfering with the cytokine-mediated activation of one or several crucial transcription factors. This effect of nifedipine

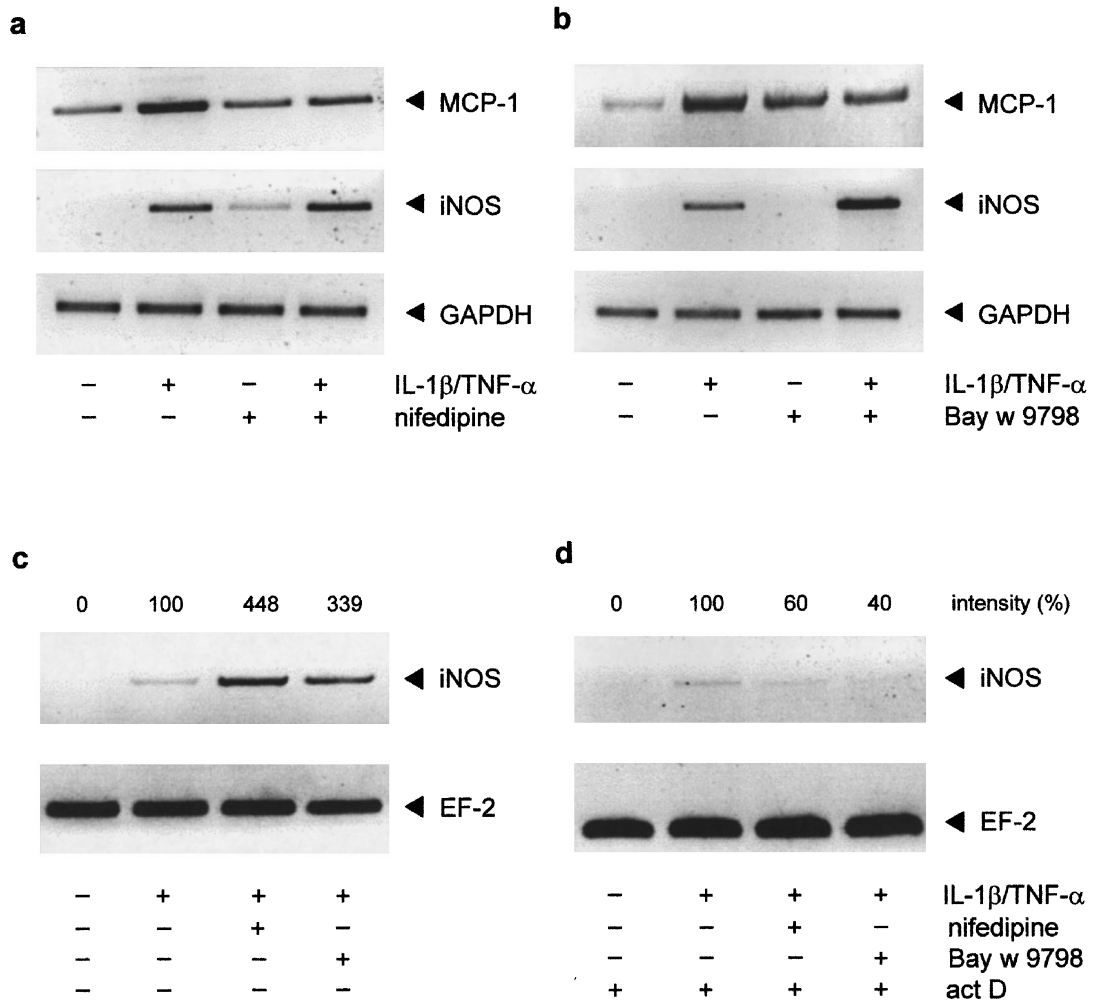

Figure 4 (a,b) Up- and down-regulation by (a) nifedipine and (b) Bay w 9798 of cytokine-inducible *de novo* synthesis of iNOS and MCP-1 mRNA, respectively in nuclei prepared from cultured raSMC, as determined by the RT–PCR-based nuclear run-on technique described in the Methods section. The figure shows two typical RT–PCR analyses; very similar results were obtained in four independent experiments. The cDNA for GAPDH was used as a standard for total cDNA load. (c,d) Determination of iNOS mRNA stability. Experiments were performed as described in the legend to Figure 2 except that incubation times were 8 h and actinomycin D was added at a final concentration of 1 μM to the cells 4 h after beginning of the incubation. The figure shows two RT–PCR analyses (c, control, d, actinomycin D), typical of three additional experiments with different batches of raSMC. Densitometric intensity values are indicated at the top of each panel.

is neither related to its putative antioxidative properties nor to its calcium channel-blocking activity, as verified by the virtually identical effects of Bay w 9798, a 1,4-dihydropyridine that appears to be only a very weak L-type calcium channel antagonist.

In this study we used IL-1β and TNF-α, classical pro-inflammatory cytokines, as a model for the situation in the early phase of arteriosclerosis. In recent studies, it was shown that these two cytokines indeed play an important role in the development of arteriosclerosis. For example, aortic smooth muscle cells undergo a dedifferentiation towards a proliferative neointima-like phenotype *in vivo* when exposed to exogenous IL-1β as well as TNF-α in a porcine model of arteriosclerosis (Fukumoto *et al*., 1997). Moreover, acute neointima formation and coronary stenosis can be prevented or strongly ameliorated by blocking the action of TNF-α in cholesterol-fed rabbits after heart transplantation (Clausell *et al*., 1994).

Interestingly, the effects on gene expression by both dihydropyridines clearly differed with respect to the potentiation and inhibition of cytokine-inducible iNOS and MCP-1 expression, respectively. Whereas iNOS was up-regulated both at the mRNA and protein level, MCP-1 was down-regulated. This holds true at least for mRNA expression, as was shown independently by RT–PCR and Northern blot analysis. The failure to detect MCP-1 protein by Western blot analysis may have resulted from a rather low rate of constitutive exocytosis in the cultured raSMC or a cell-specific blockade of the binding site for the antibody used, as MCP-1 protein was readily detectable in samples from both human and porcine endothelial cells. Especially O-glycosylations are likely to occur at the C-terminus of MCP-1, as confirmed by motif searches of its amino acid sequence. These glycosylations might mask the antigenic domain of MCP-1 in a tissue- or cell specific way. Unfortunately, only antibodies against this MCP-1 epitope (JE-protein, a C-terminal peptide common to all known species homologues of MCP-1) are available thus far.

Notwithstanding this technical problem, the effects of the dihydropyridines described in this study, occur at concentrations that can still be viewed as therapeutically relevant, especially if one considers that these drugs may accumulate in the vessel wall. As mentioned before, Bay w 9798, although structurally related to nifedipine, displayed no calcium channel-blocking activity in concentrations relevant for this study, but its effects on MCP-1 and iNOS gene expression were

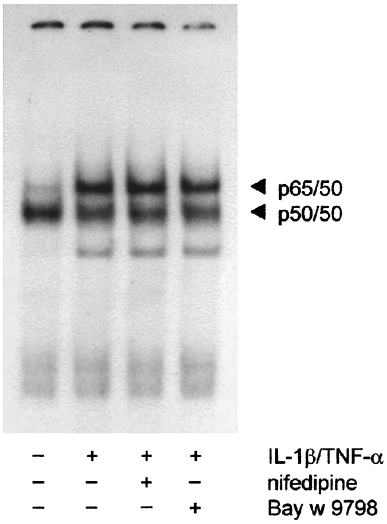

Figure 5 Effects of nifedipine or Bay w 9798 (1 μM, 1 h pre-incubation) on nuclear translocation of NF-κB in cultured raSMC after 30 min exposure to IL-1β (60 u ml^{-1}) plus TNF-α (1000 u ml^{-1}). The figure shows a representative EMSA. Similar results were obtained in two further experiments with different batches of raSMC. The localization of the p50/p50 homodimer as well as that of p65/p50 heterodimer of NF-κB was confirmed in a different series of experiments by supershift analysis with the corresponding antibodies.

indistinguishable from those of nifedipine. This clearly rules out L-type calcium channel blockade as the underlying mechanism, and suggests that the dihydropyridines have another common target, putatively the protein kinase-dependent activation of (a) transcription factor(s) that are essential for the cytokine-induced up-regulation of iNOS and MCP-1 expression.

The hypothesis, that the antioxidative properties of nifedipine are responsible for the observed effects on gene expression (e.g., by altering the activity of redox-sensitive transcription factors such as NF-κB) could also be decisively ruled out in our experiments (cf. Sugawara *et al*., 1993). Reasons for this apparent discrepancy with previous studies by other authors might be that they either used a much higher concentration of nifedipine (Aruoma *et al*., 1991; Szabo *et al*., 1993) or may have had problems with the sensitivity of nifedipine to light, as the product of light-induced decomposition of nifedipine, nitrosopine, is a very good antioxidant (Jakobsen *et al*., 1979; Bauer *et al*., 1995).

The transcription factor(s) that is (are) responsible for cytokine-induced iNOS expression affected by nifedipine and Bay w 9798 remain(s) to be characterised. The EMSA data clearly indicate that the nuclear translocation of none of the three transcription factors analysed (AP-1, C/EBP family, NF-κB) was affected by the dihydropyridines. Moreover, it remains unclear for example, as to how C/EBPβ or δ, transcription factors that are responsible for the up-regulation of iNOS as well as MCP-1 gene expression (Hu *et al*., 1998; Eberhardt *et al*., 1998), affect the expression of these gene products differentially upon dihydropyridine treatment. So there must be other transcription factors involved or the activity of the transcription factors studied was influenced by the dihydropyridines through a mechanism that is not detectable by EMSA.

Interestingly, such a divergent effect on gene expression has previously been reported for another dihydropyridine, manidipine, which inhibited the platelet-derived growth factor BB-induced expression of IL-1β and granulocyte/monocyte-colony-stimulating factor, but not that of IL-6 in human mesangial cells (Roth *et al*., 1992).

Since plaque formation requires the recruitment and extravasation of monocytes, a reduction in MCP-1 synthesis in the vessel wall is likely to attenuate the initiation and progression of new arteriosclerotic lesions (Gosling *et al*., 1999). Moreover, the local concentration of biologically active NO seems to be reduced in arteriosclerosis due to an increased formation of reactive oxygen species such as O_2^- which chemically neutralises NO at a rate that is only limited by diffusion (White *et al*., 1994). NO attenuates both the expression of pro-arteriosclerotic gene products (e.g. chemokines and cell adhesion molecules) in the vessel wall (by blocking the activity of NF-κB; Zeiher *et al*., 1995; Tsao *et al*., 1997; Spiecker *et al*., 1998) and the proliferation of smooth muscle cells (by elevating intracellular cyclic GMP), two hallmarks of the early phase of arteriosclerosis. Therefore, the inhibitory effect of nifedipine on cytokine-inducible MCP-1 synthesis and its stimulatory effect on iNOS expression may well contribute to its anti-arteriosclerotic action *in vivo*. However, additional studies are necessary to demonstrate that the present findings obtained with raSMC can indeed be extrapolated to the situation in human patients.

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