

Calcium Channel Blocker Prevents T Helper Type 2 Cell-mediated Airway Inflammation

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Rationale: Ca^{2+} signaling controls the production of T helper (Th) type 2 cytokines known to be deleterious in asthma. Recently, we showed that Ca^{2+} signaling was dihydropyridine (DHP)-sensitive in Th2 lymphocytes and that the DHP derivate, nifedipine, used in the treatment of cardiovascular pathologies, prevents Th2-dependent B cell polyclonal activation.

Objectives: We tested the effect of nifedipine in experimental allergic asthma.

Methods: BALB/c mice immunized with ovalbumin (OVA) in alum and challenged with intranasal OVA were treated with nifedipine once the Th2 response, or even airway inflammation, was induced. We also tested the effect of nifedipine in asthma induced by transferring OVA-specific Th2 cells in BALB/c mice exposed to intranasal OVA. We checked the impact of nifedipine on T-cell responses and airway inflammation.

Measurements and Main Results: Nifedipine inhibited *in vitro* Ca^{2+} response in Th2 cells. *In vivo*, it impeded the development of Th2-mediated airway inflammation and reduced the capacity of lymphocytes from lung-draining lymph nodes to secrete Th2, but not Th1, cytokines. Nifedipine did not affect antigen presentation to CD4^{+} T lymphocytes, nor the initial localization of Th2 cells into the lungs of mice exposed to intranasal OVA; however, it reduced the production of type 2 cytokines and the amplification of the Th2 response in mice with asthma. Conversely, nifedipine had no effect on Th1-mediated airway inflammation.

Conclusions: Nifedipine improves experimental asthma by impairing Th2-dependent inflammation. This study could provide a rationale for developing drugs selectively targeting DHP receptors of Th2 lymphocytes, potentially beneficial in the treatment of asthma.

Keywords: calcium signaling; IL-4; nifedipine

T helper type 1 (Th1) and T helper type 2 (Th2) cells have distinct functions, and deregulation of one or the other subset causes different types of pathology. Allergic asthma, a chronic inflammatory disease of the lungs, the prevalence and severity of which are increasing in developed countries (1), results from aberrant CD4^{+} Th2-mediated immune responses to inhaled allergens. The role of Th2 lymphocytes in the induction and maintenance of inflammatory responses in the lung has been supported by

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Nifedipine is a calcium channel blocker considered as potentially effective in asthma due to its expected effect on smooth muscle.

What This Study Adds to the Field

Nifedipine selectively impaired Ca^{2+} signaling in Th2 lymphocytes and prevented experimental asthma. The fact that nifedipine selectively inhibits Ca^{2+} -dependent production of cytokines IL-4, IL-5, and IL-13, all of which contribute to the development of asthma, offers a rationale for developing drugs targeting Ca^{2+} signaling in Th2 effectors.

the detection of Th2 cells in airways of patients with asthma (2, 3), and their importance has been confirmed in animal models (4, 5). Th2 cells produce IL-4, IL-5, and IL-13, which contribute to the multiple features of asthma: airway hyperresponsiveness (AHR), airway eosinophilia, and mucus hypersecretion. Ca^{2+} signaling is essential for Th2 cell functions (6–9), even if the mechanisms responsible for Ca^{2+} dynamics are poorly known in this subset. It was shown that Ca^{2+} was differentially regulated between Th1 and Th2 cells (8, 10–12). Indeed, in baseline conditions, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was higher in Th2 than in Th1 cells. Conversely, upon T-cell receptor (TCR)-driven stimulation, the increase in $[\text{Ca}^{2+}]_i$ was reduced in Th2 compared with Th1 cells (12). We have previously shown that Th2 cells, but not Th1 cells, expressed dihydropyridine (DHP) receptors (DHPRs) that can be blocked by DHPR antagonists, including nifedipine or nifedipine. Furthermore, nifedipine administration given at doses that control high blood pressure in hypertensive rats prevented the development of several models of Th2 cell-mediated autoimmune diseases without modifying the course of experimental autoimmune encephalomyelitis, a Th1-mediated autoimmune disease (13). This suggested that this compound could act on Th2 but not Th1 cells, and was not a global immunosuppressive drug. In the present study, we assessed whether nifedipine could impede the development of airway inflammation when administered after the Th2 cell response was already induced. We showed that nifedipine markedly reduced Th2- but not Th1-mediated airway inflammation. Nifedipine did not affect the capacity of antigen-presenting cells (APCs) to prime naive T cells or to induce Th2 cell proliferation. However, it altered Ca^{2+} signaling in T cells, the production of Th2 cytokines, and further Th2 cell differentiation, which suppressed asthma development. The demonstration that T lymphocytes from patients with asthma express DHP would be a

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good rationale for developing drugs targeting DHP on T lymphocytes. However, because we needed to use high doses of nicardipine to be effective in our model, it will be important to develop new drugs with a better affinity than nicardipine for DHP expressed by T cells. This work was partially presented as an abstract at the Sixth Annual Meeting of the Federation of Clinical Immunology Societies in 2006 (*see* Reference 14).

METHODS

DO11.10 TCR-transgenic BALB/c mice (7–10 wk old) (15), and female BALB/c mice (7–10 wk old) (Janvier Ets, Le Genest St. Isle, France), were cared for in our animal facility. Our institutional review board for animal experimentation (Ethical Committee of the “Institut Fédératif de Recherche” 30, Toulouse) approved all aspects of animal care.

Bone marrow-derived dendritic cells (prepared as in Reference 16) were incubated overnight with ovalbumin (OVA), without or with nicardipine (10 μ M). Cells were then washed, fixed, and used for stimulating Th2 cells.

Naive DO11.10 CD4⁺ cells stained with carboxyfluorescein succinimidyl ester (CFSE) (13) were intravenously injected into the footpad in BALB/c mice immunized with OVA in incomplete Freund’s adjuvant (IFA), and intraperitoneally injected, or not, with nicardipine (Loxen; Novartis, Rueil Malmaison, France) (13) 1 mg/day for 3 days. Draining lymph node cells were labeled with fluorescent anti-CD4 and the transgenic TCR-specific KJ1.26 antibodies (PharMingen BD, San Diego, CA). Doubly labeled CD4⁺KJ1.26⁺ cells were gated on and analyzed for CFSE staining.

OVA-specific transgenic Th1 or Th2 cells (differentiated as in Reference 13), were preincubated, or not, for 12 hours with 10 μ M of nicardipine and loaded with 5 μ M Fura-2AM (Sigma-Aldrich, St. Louis, MO), as previously described (17). They were stimulated with anti-TCR monoclonal antibody (mAb) plus anti-CD28 mAb or thapsigargin. $[Ca^{2+}]_i$ was determined as previously reported (17).

BALB/c mice referred to as OVA/OVA mice were primed intraperitoneally with OVA in alum (Sigma), and challenged 15 days later with intranasal OVA for 5 days. They were injected, or not, with nicardipine (Figure 1A). In transfer experiments, 5×10^6 DO11.10 Th2 or Th1 lymphocytes were injected intravenously into BALB/c recipients. One day later, mice were challenged with intranasal OVA and injected intraperitoneally, or not, with nicardipine (1 mg/d) for 7 days. At 24 hours after the last intranasal challenge, bronchoalveolar lavage (BAL) fluid was collected. Cytospin preparations of BAL cells were stained with May-Grunwald Giemsa to identify inflammatory cells. Lung tissue

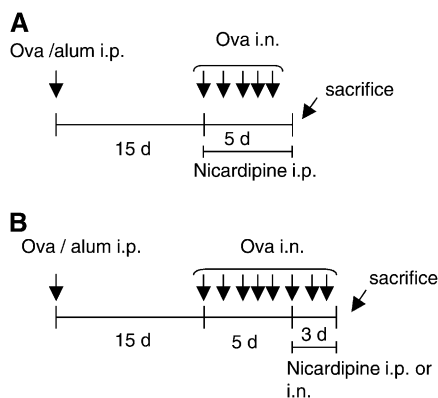


Figure 1. Experimental groups. All the mice were immunized intraperitoneally (i.p.) with 100 μ g of ovalbumin (OVA) mixed with 2 mg of alum. (A) After 15 days, mice were given intranasal (i.n.) OVA (50 μ g in 50 μ l of phosphate-buffered saline [PBS] per day) for 5 days and were simultaneously injected intraperitoneally, or not, with nicardipine (1 mg/d except when otherwise mentioned). (B) Other groups were immunized as in (A), except that intranasal OVA was given for 8 days and that mice were treated with nicardipine either intraperitoneally or intranasal during the last 3 days only. The control groups were given intranasal pH 7 or pH 5 PBS during the last 3 days.

was digested with collagenase type IV (150 U/ml; Sigma) and DNase I (10 U/ml; Sigma) for 30 minutes at 37°C, and passed through a wire mesh to dissociate cells. Mononuclear cells were recovered on Ficoll-Hypaque. In some experiments, CD4⁺ T lymphocytes (purified according to Reference 18) were labeled with fluorescein isothiocyanate-anti-CD4 mAb and ST-Bodipy-labeled DHP (Molecular Probes, Interchim, Montluçon, France), as previously described (19).

Peribronchial lymph nodes from OVA/OVA mice or from mice transferred with DO11.10 Th2 cells, injected, or not, with nicardipine, were collected at the time mice were killed. Purified CD4⁺ T cells were stimulated (10^5 /well), in the absence of nicardipine, with 1 mg/ml OVA and 10^6 irradiated BALB/c spleen cells, or with anti-TCR mAb, for 48 hours. Cytokine production was determined by ELISA.

Airway responsiveness to methacholine was assessed 24 hours after the final OVA inhalation by using a whole-body plethysmograph (EMKA Technologies, Paris, France). Unrestrained, spontaneously breathing mice were placed into a chamber of the plethysmograph and baseline enhanced pause values were recorded for 3 minutes before mice were exposed to intranasal methacholine. The enhanced pause values measured during 20-minute sequence were averaged and expressed as the percentage of baseline values.

Results are expressed as mean ($\pm$ SD). Differences between groups were analyzed by the Mann-Whitney U test.

RESULTS

Nicardipine Inhibits TCR-driven Ca^{2+} Response in Th2 Cells but Does Not Affect Store-operated Ca^{2+} Channel-mediated Intracellular Ca^{2+} Rise

Calcium influx from the external medium is essential for maintaining sustained increased $[Ca^{2+}]_i$ required for full-blown T-cell activation, and especially for IL-4 production by Th2 cells upon stimulation through the TCR. Nicardipine, a DHP derivate that we previously used to prevent Th2-mediated autoimmune B cell polyclonal activation (13), inhibited the increased $[Ca^{2+}]_i$ upon TCR activation (Figure 2A), confirming previous results (17).

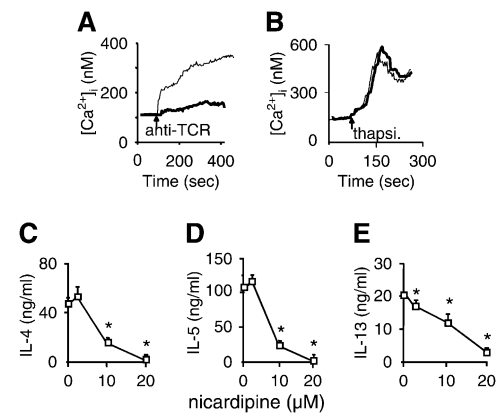


Figure 2. Nicardipine inhibits Ca^{2+} response and cytokine production by T helper (Th) type 2 cells. (A and B) Th2 cells were pretreated with nicardipine (10 μ M) (bold line), or not (control; thin line), after which basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was recorded at the single-cell level before adding 1 μ g/ml anti-T-cell receptor (TCR) plus anti-CD28 (2 μ g/ml) monoclonal antibodies (mAbs; arrow) (A) or 20 nM thapsigargin (thapsi.) (B). Each curve represents the mean of 35–45 cells and is representative of three experiments. (C–E) Nicardipine suppressed TCR-induced Th2 cytokines by differentiated DO11.10 Th2 cells in a dose-dependent manner. DO11.10 Th2 cells were stimulated with plate-bound anti-TCR mAb (10 μ g/ml) for 24 hours with or without increasing concentrations of nicardipine. The content in IL-4 (C), IL-5 (D), and IL-13 (E) was then determined in culture supernatants by ELISA. Values are expressed as mean ($\pm$ SD) of three experiments performed in triplicate (* $p < 0.01$ when compared with cells stimulated in the absence of drug).

Conversely, nicardipine had no effect on the rise in $[Ca^{2+}]_i$ elicited by thapsigargin, an inhibitor of the Ca^{2+} -ATPase expressed on the endoplasmic reticulum known to discharge intracellular Ca^{2+} stores, which is followed by an entry of Ca^{2+} through so-called store-operated Ca^{2+} (SOC) channels (Figure 2B). Nicardipine reduced IL-4 (Figure 2C), IL-5 (Figure 2D), and IL-13 (Figure 2E) release by Th2 cells in a dose-dependent manner. However, nicardipine did not modify the increase in $[Ca^{2+}]_i$ upon TCR stimulation in Th1 cells (data not shown), confirming previous results (17). In addition, nicardipine did not diminish IFN- γ production by Th1 cells at any dose tested (*see* Figure E1 in the online supplement). These results are consistent with a specific effect of nicardipine on Th2 cell signaling and suggest that it does not act on SOC channels. Considering the inhibitory effect of nicardipine on IL-4, -5, and -13 production by T cells, all these cytokines contributing to the pathogenesis of asthma, we assessed whether nicardipine may be beneficial in an experimental model of allergic asthma.

CD4⁺ T Cells That Infiltrate the Lungs Are Stained with Fluorescent DHP during the Course of Allergic Asthma

BALB/c mice were sensitized by intraperitoneal injection of OVA in alum and challenged 15 days later with inhaled OVA for 5 days (OVA/OVA group). Total cell number from BAL was increased in OVA/OVA mice when compared with control mice immunized with OVA and challenged with phosphate-buffered saline (PBS) (OVA/PBS) or mice exposed to inhaled OVA only (PBS/OVA) (data not shown). Around 40–60% of lung infiltrating CD4⁺ T cells were labeled with ST-Bodipy-DHP (Figure 3A). Analysis of fluorescence intensity on CD4⁺ T cells revealed that 35 ($\pm$ 15) % of cells showed no DHP staining (mean fluorescence intensity inferior to 3 arbitrary units, the value of which corresponds to background), 30 ($\pm$ 10) % displayed a fluorescence intensity comprised of between 3 and 10 units, and 25 ($\pm$ 15) % displayed a fluorescence intensity superior to 10 units, which corresponds to the staining of fully differentiated Th2 cells (data not shown). The staining was abolished when preincubating the probe with an excess of unlabeled DHP (Figure 3A). None of CD4⁺ T cells isolated from the lungs of OVA/PBS or PBS/OVA mice showed a staining with ST-Bodipy-DHP (100% of cells with a fluorescence intensity inferior to 3 units [data not shown]).

Nicardipine, a Ca^{2+} Channel Blocker, Abrogates Allergic Asthma

IL-4 and IL-5 production, and an increase in the number of inflammatory cells (mainly eosinophils), were demonstrated in the BAL fluid from OVA/OVA mice (Figures 3B and 3C). This was associated with the presence of severely inflammatory infiltrates around vessels and bronchioles in the lungs, with a marked hyperplasia of goblet cells and an increased production of mucus (Figure 3D). All the mice that were primed with OVA showed high-IgE titers at Day 15 (data not shown). The mice were then challenged with intranasal OVA instillation for 5 days and simultaneously injected with nicardipine, or not, as described in Figure 1A. At the time mice were killed, IgE titer was increased by two- to fourfold in OVA/OVA mice when compared with values before the beginning of intranasal OVA exposure. Conversely, IgE was not further up-regulated in mice treated with nicardipine (data not shown). The administration of nicardipine markedly reduced the concentration of IL-4 and IL-5 (Figure 3B), as well as the number of inflammatory cells in the BAL fluid, with a major effect on eosinophils (Figure 3C). We tested the effect of intraperitoneal daily injections of various doses of nicardipine (1.0, 0.5, and 0.1 mg/d), beginning at the

same time as the first intranasal OVA administration. Nicardipine at doses of 1.0 and 0.5, but not 0.1, mg/day reduced eosinophilic infiltration in the airways of OVA/OVA mice (Table 1). Nicardipine also completely prevented the appearance of inflammatory lesions, the thickening of the epithelial cells lining the bronchioles, and abnormal mucus production in the lungs in more than 85% of treated mice (12/14) (Figure 3D). The remaining 15% of nicardipine-treated mice (2/14) displayed a restrained infiltration, yet reduced compared with controls. Accordingly, AHR to methacholine was diminished in nicardipine-injected mice compared with control animals (Figure 4A). As shown in Table 2, nicardipine treatment decreased the number of lung infiltrating cells, including the number of CD4⁺ T cells. The number of CD4⁺ T cells in thoracic lymph nodes was also lower in mice treated with nicardipine relative to control mice, indicating that nicardipine prevented the recruitment and/or the proliferation of CD4⁺ T cells in lungs and in draining lymph nodes. In three independent experiments, nicardipine treatment diminished not only the number of CD4⁺ T cells in lung draining lymph nodes, but also their capacity to produce IL-4 (Figure 4B), IL-5 (Figure 4C), and IL-13 (Figure 4D) when stimulated by APCs plus OVA or by anti-TCR mAb *in vitro*. Substantial amounts of IFN- γ were produced only after stimulation by anti-TCR mAb, and were not affected by the *in vivo* treatment with nicardipine (Figure 4E). Therefore, nicardipine abolishes the development of allergic asthma in a previously sensitized individual by acting on the Th2-mediated secondary immune response.

Effect of Nicardipine Administered after Airway Inflammation Was Induced

We tested whether nicardipine administration could be effective once the airway inflammation was already induced. Mice were immunized with OVA in alum and, 15 days later, were given intranasal OVA for 5 days. At that time, OVA instillations were pursued for three additional days, and mice did or did not receive intraperitoneal injections of nicardipine (Figure 1B). We then analyzed the inflammation in the airways. Data in Table 3 show that nicardipine still strongly reduced lung inflammation. We also tested the effects of various doses of nicardipine given intranasally in this protocol (Figure 1B). Because nicardipine had to be dissolved in acidic PBS (pH 5), control mice received intranasal OVA dissolved in pH 5 PBS. These mice displayed the same inflammation as mice that received intranasal OVA in pH 7 PBS. At 1 mg/day, nicardipine induced the death of three out of four mice, and the remaining mouse was free of inflammatory reaction (data not shown). One out of four mice died after receiving 0.5 mg/day of nicardipine, which was innocuous at doses of 0.2 and 0.1 mg/day. Nicardipine at doses of 0.5 and 0.2 mg/day partially reduced the number of inflammatory cells and of eosinophils in the BAL fluid (Table 3). However, nicardipine at the dose of 0.1 mg/day was ineffective. Altogether, these data suggest that nicardipine can still be effective once the airway inflammation is already induced.

Effect of Nicardipine on Naive CD4⁺ T-Cell Proliferation, Antigen Presentation by Dendritic Cells, and Recruitment of Th2 Effectors in Lungs

CFSE-labeled OVA-specific transgenic DO11.10 CD4⁺ T cells were intravenously injected into normal BALB/c mice before immunization with OVA emulsified in IFA in the footpads. At the same time, intraperitoneal injections of 1 mg/day nicardipine were initiated. Three days later, popliteal draining lymph nodes were collected, and lymph node cells were labeled with anti-CD4 and anti-clonotypic KJ1.26 antibodies. CFSE staining was analyzed on KJ1.26⁺ CD4⁺ T cells. CD4⁺ T cells from

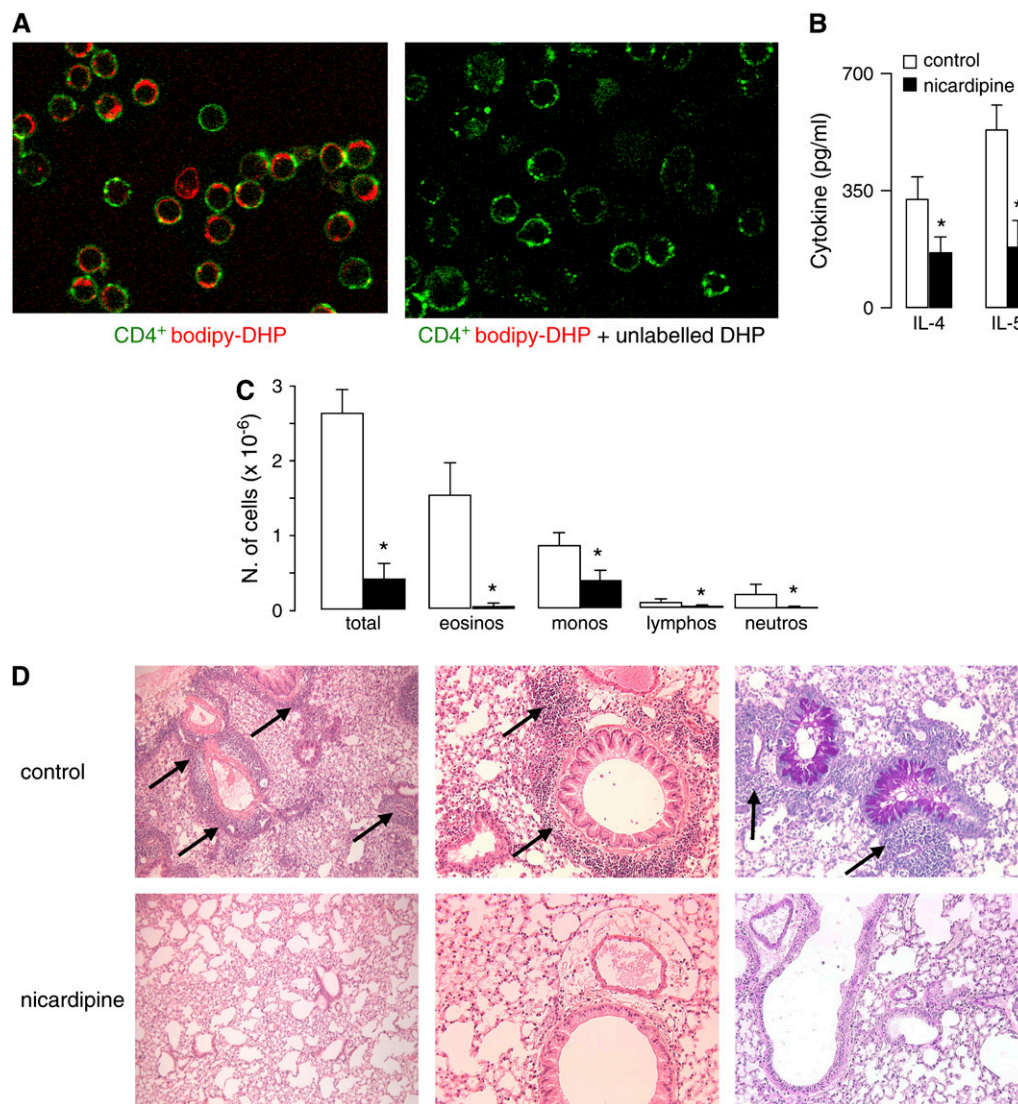


Figure 3. Nicardipine prevents asthma. BALB/c mice were primed and sensitized 15 days later with intranasal OVA for 5 days before analysis. (A) CD4⁺ T cells, purified from lungs, were stained with fluorescein isothiocyanate-anti-CD4 mAb and ST-Bodipy-dihydropyridine (DHP) (original magnification: $\times 40$). Preincubation of cells with unlabeled DHP abolished the staining. (B and C) At the time of intranasal instillation, mice were injected with nicardipine (filled bars) or not (control; open bars). (B) IL-4 and IL-5 secretion in the BAL fluid of four nicardipine-treated and untreated mice was analyzed by ELISA. One experiment out of two is shown. * $p < 0.05$ when compared with control mice with asthma. (C) Nicardipine reduced the number of inflammatory cells in the BAL fluid and especially the number of eosinophils (eosinophils). Results are represented by the mean ($\pm$ SD; $n = 5$). One experiment out of four is shown. * $p < 0.01$ compared with control animals. Lymphos = lymphocytes; monos = monocytes; neutros = neutrophils. (D) Diffuse leukocyte infiltration: hematoxylin and eosin staining, left and middle panels (original magnifications: $\times 12.5$ and $\times 50$, respectively), and mucus hypersecretion (right panel, periodic acid Schiff staining; original magnification: $\times 50$) were evidenced in lungs from control animals but not from nicardipine-treated mice. Arrows point to cellular infiltrates.

nicardipine-injected animals proliferated, as did those from control animals, with around 90% of cells undergoing division (Figure 5A). Thus, nicardipine does not prevent naive T-cell priming and proliferation *in vivo*. Because DHPs have been identified in dendritic cells, we next tested whether nicardipine

TABLE 1. DOSE-RESPONSE EFFECT OF NICARDIPINE ON THE PREVENTION OF EXPERIMENTAL ASTHMA

Nicardipine Dose (mg/d)	Total No. of Cells ($\times 10^{-6}$)	Total No. of Eosinophils ($\times 10^{-6}$)
1.0	$1.2 \pm 0.6^*$	$0.8 \pm 0.4^*$
0.5	$1.1 \pm 0.6^\dagger$	$0.9 \pm 0.5^\dagger$
0.1	2.6 ± 1.4	1.8 ± 1
0	3.9 ± 1.7	2.6 ± 1.2

Mice were immunized with ovalbumin in alum. After 15 days, daily intraperitoneal injections of various doses of nicardipine were initiated at the same time as the first intranasal ovalbumin exposure; 5 days later, the bronchoalveolar lavage fluid was recovered and the content of cells in the bronchoalveolar lavage fluid was analyzed (four to six mice per group).

* $p < 0.01$ when compared with control mice with asthma.

† $p < 0.02$ when compared with control mice with asthma.

could modulate the capacity of dendritic cells to internalize, process, and present antigenic complexes to Th2 cells. Bone marrow-derived dendritic cells from BALB/c mice were incubated overnight with OVA protein in the presence or absence of nicardipine. OVA-pulsed dendritic cells were then used to stimulate OVA-specific DO11.10 Th2 cells. As shown in Figure 5B, dendritic cells pulsed with OVA in the presence of various amounts of nicardipine were as efficient as control dendritic cells in their capacity to present antigen and to activate OVA323–339/H-2^d-specific DO11.10 Th2 cells. We next evaluated whether nicardipine could prevent *in vivo* the initial localization of OVA-specific transgenic Th2 cells in the lungs. We then analyzed the number of transgenic T cells recovered from the lungs of mice that were intravenously injected with 5×10^6 OVA-specific DO11.10 transgenic Th2 cells and sensitized with intranasal OVA for 3 days. Mice were or were not injected with nicardipine. At Day 3, transgenic KJ1.26⁺, CD4⁺ T cells were detected in equal numbers in the lungs of mice injected, or not, with nicardipine (Figure 5C). Altogether, these data show that nicardipine has no effect on naive CD4⁺ T-cell proliferation, and alters neither the capacity of antigen presentation by dendritic cells, nor the recruitment of Th2 effectors in lungs.

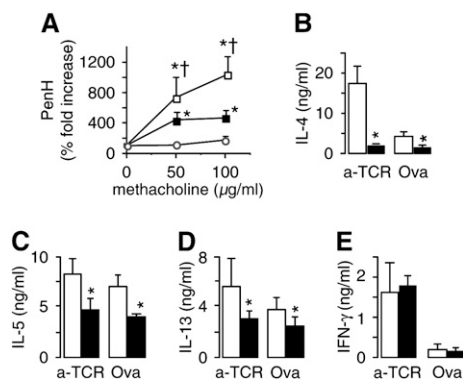


Figure 4. *In vivo*, nicardipine diminished airway hyperresponsiveness (AHR) and the ability of T cells to produce Th2 but not Th1 cytokines in an experimental model of asthma. (A) Hyperresponsiveness to methacholine was tested by whole-body plethysmography in mice primed and then sensitized to ovalbumin (OVA/OVA), treated (filled squares) or not (open squares) with nicardipine. Results from normal control mice (open circles) are included. Results represent the mean ($\pm$ SD) of four to five mice. Results of one experiment out of two are shown. * $p < 0.01$ comparing OVA/OVA mice injected with nicardipine or not; $^{\dagger}p < 0.005$ versus normal mice. (B–E) Th2 (B–D) but not Th1 (E) cytokine production by lung draining lymph node CD4⁺ T cells from nicardipine-treated mice (filled bars) was reduced (* $p < 0.01$) when compared with the response of CD4⁺ T cells from control animals (open bars), whether the cells were stimulated by antigen-presenting cells (APCs) plus OVA or anti-TCR mAb (mean $\pm$ SD from culture performed in triplicate; $n = 3$ experiments).

Nicardipine Abrogates Th2-mediated Airway Inflammation in an Adoptive Transfer Model of Asthma

Transfer of OVA-specific DO11.10 Th2 lymphocytes to syngeneic BALB/c mice, followed by intranasal challenge with OVA, induced airway inflammation (20). Nicardipine treatment reduced the total number of inflammatory cells in the BAL fluid, with a predominant effect on eosinophils (Figure 6A). This correlated with the absence (12/15 mice) or a lesser extent (3/15) of pulmonary infiltrates yet reduced compared with asthmatic mice (see Figure E2) and diminished mucus secretion in all nicardipine-treated mice. There was a two- to fivefold reduction in the number of CD4⁺ T cells recovered from lungs and draining lymph nodes of animals injected with nicardipine (Figure 6B and data not shown). Furthermore, thoracic lymph node CD4⁺ T cells from nicardipine-treated animals produced less IL-4, IL-5, and IL-13 after stimulation with APCs plus OVA than CD4⁺ T

cells from control mice (Figure 6C). To show that nicardipine selectively reduced airway inflammation induced by Th2 and not by Th1 cells, we evaluated the effect of nicardipine on the inflammatory reaction induced by the transfer of OVA-specific DO11.10 Th1 cells. Because Th1 cells express barely detectable levels of DHPs (13), nicardipine was not anticipated to modify airway inflammation in this adoptive transfer model. Indeed, Th1-mediated airway inflammation (in which neutrophils and monocytes predominate) was not affected by nicardipine treatment, as shown by the number of cells in the BAL fluid and the severity of pulmonary infiltrates (see Figure E3). Thus, nicardipine selectively prevented Th2- but not Th1-mediated airway inflammation.

DISCUSSION

In this article, we show that nicardipine, a calcium channel blocker used to treat cardiovascular diseases, altered TCR-driven calcium response in Th2 cells as well as the release of cytokines IL-4, IL-5, and IL-13, all of which are implied in the pathogenesis of asthma. Nicardipine hindered Th2-mediated airway inflammation and eosinophilia once the Th2 response and airway inflammation were already induced. Nicardipine did not prevent Th2 cells from localizing in the lungs after adoptive transfer of OVA-specific Th2 cells in mice sensitized with intranasal OVA, but it reduced type-2 cytokine production and subsequent Th2 cell priming and recruitment into the lungs. Nicardipine selectively inhibited airway inflammation induced by adoptive transfer of antigen-specific Th2, but not Th1 lymphocytes, strongly suggesting that this drug selectively affects Th2 cell effector functions *in vivo*.

These results indicate that nicardipine effectively inhibits differentiated Th2 cell functions and/or the priming of new Th2 cells after intranasal OVA sensitization. Indeed, it was previously shown that transferred transgenic Th2 cells permit the collateral priming and differentiation of newly recruited antigen-specific CD4⁺ T cells in the recipient (20). These Th2 cells may be specific for OVA or other antigens used for intranasal sensitization. Nicardipine was unlikely to act on antigen presentation to naive CD4⁺ T cells or to Th2 cells. Indeed naive OVA-specific DO11.10 T cells injected into BALB/c mice immunized with OVA proliferated as well, whether mice were injected with nicardipine or not. *In vitro*, the addition of nicardipine to dendritic cells did not alter their capacity to trigger IL-4 production by Th2 cells. Finally, DO11.10 Th2 cells injected *in vivo* localized equally well in the lungs, whether mice were injected with nicardipine or not. This shows that *in vivo* nicardipine did not interfere with the initial recruitment of transgenic Th2 cells into the lungs.

It has been suggested that the protective effect of nicardipine on AHR could be due to the beneficial action of DHPs on bronchorelaxation (21–23). Our data strongly argue that the protective effect of DHPs could also be explained by its inhibitory effect on Ca²⁺ signaling in Th2 cells, thereby preventing type-2 cytokine production and airway inflammation. Indeed, the number of cells, and especially eosinophils, is dramatically reduced in nicardipine-injected mice compared with control animals. This correlated with histologic examination of the lungs and with the reduced number of CD4⁺ T cells recovered from the lungs of nicardipine-injected mice in both models of asthma. The protective effect of nicardipine on pulmonary inflammation can be explained by its capacity to abolish IL-4 production by memory/effector Th2 cells at the time of secondary antigenic challenge. Indeed, CD4⁺ T cells from lung draining lymph nodes of mice injected with nicardipine produced less Th2 cytokines after stimulation with either the antigen or the anti-TCR mAb. IL-4 is a key cytokine because it directs Th2 cell differentiation

TABLE 2. NICARDIPINE REDUCES AIRWAY T-CELL INFLAMMATION IN AN EXPERIMENTAL MODEL OF ASTHMA

	Lung Infiltrating Cells		Total No. of CD4 ⁺ T Cells in LN*
	Total ($\times 10^{-6}$)	CD4 ⁺ ($\times 10^{-6}$)	
Nicardipine	2.3 $\pm$ 1.4 [†]	1 $\pm$ 0.5 [†]	2.5 $\pm$ 0.3 [‡]
Control animals	6 $\pm$ 2.3	3 $\pm$ 1.25	7.2 $\pm$ 2

Definition of abbreviation: LN = lymph node.

Each value represents the number of cells recovered from a pool of five mice. Results are expressed as the mean ($\pm$ SD) of five to six independent experiments.

* The number of CD4⁺ T cells was determined in lung draining LNs.

[†] $p < 0.01$ when compared with control mice with asthma.

[‡] $p < 0.02$ when compared with control mice with asthma.

TABLE 3. NICARDIPINE IS BENEFICIAL EVEN WHEN INITIATED AFTER THE INDUCTION OF AIRWAY INFLAMMATION

Nicardipine Dose (mg/d)	Total No. of Cells ($\times 10^{-6}$)	Eosinophils ($\times 10^{-6}$)	Lymphocytes ($\times 10^{-3}$)	Monocytes/Macrophages ($\times 10^{-3}$)
PBS	2.2 $\pm$ 0.6	1.7 $\pm$ 0.5	14 $\pm$ 10	29 $\pm$ 10
1, i.p.	0.6 $\pm$ 0.1*	0.4 $\pm$ 0.5*	5 $\pm$ 1*	19 $\pm$ 7
0.5, i.n.	1.3 $\pm$ 0.2*	1.1 $\pm$ 0.1*	6 $\pm$ 5	32 $\pm$ 5
0.2, i.n.	1.2 $\pm$ 0.6†	0.9 $\pm$ 0.4†	8 $\pm$ 2	21 $\pm$ 3
0.1, i.n.	2.4 $\pm$ 0.3	1.7 $\pm$ 0.3	8 $\pm$ 6	17 $\pm$ 7

Definition of abbreviations: i.n. = intranasal; i.p. = intraperitoneal; PBS = phosphate-buffered saline.

Mice were immunized with ovalbumin (OVA) in alum and challenged 15 days later with intranasal OVA for 5 days. Mice were then intraperitoneally OVA injected with 1 mg/day nicardipine and challenged with OVA for 3 additional days. Alternatively, mice were exposed to both intranasal OVA and nicardipine (0.5, 0.2, and 0.1 mg/d). After 3 days, the content of cells in the bronchoalveolar lavage fluid was analyzed (three to seven mice per group).

* $p < 0.01$ when compared with control mice with asthma.

† $p < 0.05$ when compared with control mice with asthma.

and induces the expression of other cytokines, chemokines, or adhesion molecules in asthma (4, 24, 25), thereby regulating cell recruitment to the lung and inflammation. This has been demonstrated in the Th2-adoptive transfer asthma model, where it has been shown that IL-4-deficient Th2 lymphocytes were unable to induce lung inflammation (4), as well as Th2 priming of endogenous CD4⁺ T cells to inhaled antigens (20). Accordingly,

nicardipine that reduced cytokine production by already committed transgenic Th2 cells would further impede differentiation of newly recruited naive CD4⁺ T cells toward a Th2 phenotype, resulting in disease inhibition.

Synthesis of IL-4, -5, and -13 by Th2 cells depends on Ca²⁺ signaling. Nicardipine suppressed TCR-driven increases in [Ca²⁺]_i in Th2 cells (Figure 1), confirming previous results (13), as well as the nuclear translocation of the Ca²⁺-regulated transcription factor, NFAT (14). However, nicardipine had no effect on the Ca²⁺ response induced by thapsigargin, an inhibitor of Ca²⁺-ATPase, which induces an entry of Ca²⁺ via SOC channels to replenish the intracellular Ca²⁺ stores. These results strongly suggest that nicardipine affects Ca²⁺ signaling without interfering with SOC channel-dependent Ca²⁺ entry. The molecular target of nicardipine in T cells is not yet completely solved. However, the fact that Th2 cells and lung-infiltrating CD4⁺ T cells were

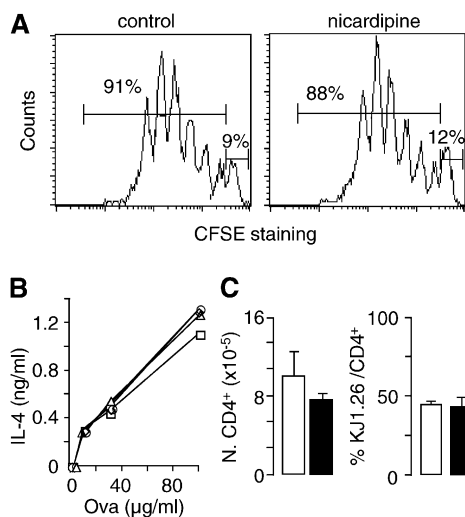


Figure 5. Nicardipine affects neither naive T-cell priming nor the capacity of APCs to induce IL-4 production by Th2 cells. (A) A total of 10^7 naive DO11.10 CD4⁺ cells, stained with carboxyfluorescein succinimidyl ester (CFSE), were intravenously injected into the hind footpads of BALB/c mice that were immunized with ovalbumin (OVA) (100 μg/mouse) emulsified in IFA. Three days later, draining lymph nodes were collected and the cells were labeled with anti-CD4 and KJ1.26 antibodies. The numbers indicate the percent of dividing and nondividing cells. Data are from one mouse out of three per group with similar results (left panel, control; right panel, nicardipine). (B) Dendritic cells were incubated overnight with various concentrations of OVA, with or without nicardipine (1, 5, or 10 μg/ml; squares, triangles, and circles, respectively), washed, and fixed before they were used for stimulating Th2 cells. IL-4 content was determined after 48 hours of culture. (C) BALB/c mice that were exposed to intranasal OVA and injected with nicardipine (filled bars) or not (open bars) were intravenously injected with DO11.10 Th2 cells. Three days later, lung infiltrating lymphocytes were prepared from three individual mice and stained with anti-CD4 and KJ1-26 antibodies. Data shown represent one of two experiments.

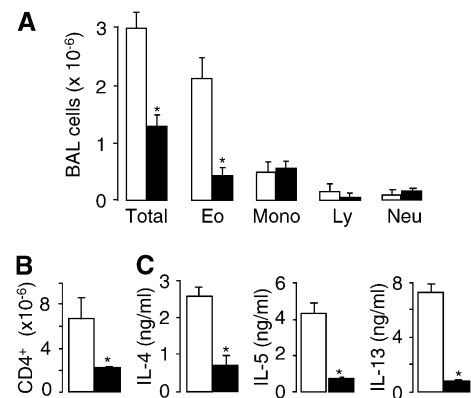


Figure 6. Nicardipine is effective in a model of asthma induced by adoptive Th2 cell transfer. BALB/c mice were transferred with 5×10^6 DO11.10 Th2 cells and further exposed to inhaled ovalbumin (OVA) for 7 days with or without daily nicardipine injection (filled bars, nicardipine-treated; open bars, control). (A) Mean cell counts ($\pm$ SD) from BAL from individual Th2 cell-transferred mouse. * $p < 0.01$; $n = 5$ mice per group. One representative experiment is shown of three. (B and C) Nicardipine reduced both the total cell number recovered from lung draining lymph nodes (B) and the capacity of the CD4⁺ T cells purified from these lymph nodes to produce Th2 cytokines after stimulation with APCs plus OVA (C). Mean cytokine production ($\pm$ SD) from culture performed in triplicate is shown. * $p < 0.01$. One experiment out of three is shown. Eo = eosinophils; Ly = lymphocytes; Mono = monocytes; Neu = neutrophils.

specifically labeled with DHP suggest that some T-cell populations, including Th2 cells, expressed DHPRs. SOC channels are considered to play a prominent role in Ca^{2+} entry upon stimulation through the TCR, and are indirectly activated by thapsigargin. The fact that nicardipine did not modify thapsigargin-induced increases in $[\text{Ca}^{2+}]_i$ suggests that these channels are not targeted by nicardipine. In addition, nicardipine interferes with Ca^{2+} signaling in Th2 cells, but not in Th1 cells, which is in keeping with the absence of effect of nicardipine on Th1-mediated airway inflammation. Differences in Ca^{2+} regulation observed between Th2 and Th1 cells have direct implications on the development of new drugs in the treatment of asthma. For example, Th2 cells failed to develop and to produce IL-4 in mice that did not express the tyrosine kinase, *itk*, whereas Th1 cell differentiation was spared. The defect in Th2 cells was related to impaired Ca^{2+} regulation, because it could be overcome by increasing the $[\text{Ca}^{2+}]_i$ (26). Moreover, *itk*^{-/-} mice are protected against asthma (27), and pharmacologic *itk* inhibitors (28) are beneficial in experimental models of asthma. Calcium channel blockers have previously been considered as potentially useful drugs in the treatment of asthma due to their ability to induce bronchorelaxation or to inhibit proinflammatory mediator release (29, 30). Our work suggests that this previously reported beneficial effect of DHP derivatives could also be due to their capacity to selectively inhibit Ca^{2+} signaling in Th2 cells.

Preliminary results show that fully differentiated human Th2 cell, but not Th1 clones, were labeled with fluorescent DHP (see Figure E4), suggesting that DHPR expression may also discriminate between human differentiated Th1 and Th2 lymphocytes. It will be important in the future to assess whether T lymphocytes from patients with asthma express DHPR. Before considering DHPR antagonists as a treatment for human asthma, the question of the dose and route of administration of the drug has to be solved. Indeed, the concentrations of nicardipine used to inhibit calcium signaling in Th2 cells, and to prevent or cure asthma, are higher than those used for inhibiting calcium signaling in excitable cells. For example, 3 mg/kg/day nicardipine protects spontaneously hypertensive rats against the cerebral damage associated with hypertension (31), whereas 0.2 to 1.0 mg/day nicardipine (doses ranging approximately from 6 to 30 mg/kg) given by injection, or even by intranasal instillation, are required to improve airway inflammation. Defining the molecular identity of DHPR in Th2 cells will help to design compounds with better affinity and selectivity than nicardipine. Such DHPR blockers might represent a promising approach in the treatment of asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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