1,4-Dihydropyridine Calcium Channel Blockers Inhibit Plasma and LDL Oxidation and Formation of Oxidation-Specific Epitopes in the Arterial Wall and Prolong Survival in Stroke-Prone Spontaneously Hypertensive Rats

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Background and Purpose—Calcium-channel blockers (CCBs) reduce systolic blood pressure and stroke-related mortality in stroke-prone spontaneously hypertensive rats (SPSHR). Brain ischemia is associated with loss of intracellular antioxidants. Increased formation of oxygen radicals and oxidation of LDL may enhance arterial vasoconstriction by various mechanisms. CCBs that also exert antioxidative properties in vitro may therefore be particularly useful. To investigate such antioxidant effects in vivo, we determined several parameters of LDL oxidation in SPSHR treated with two 1,4-dihydropyridine–type (1,4-DHP) CCBs of different lipophilic properties and compared them with antioxidant-treated and untreated controls. We also tested whether these drugs decrease the formation of oxidation-specific epitopes in arteries.

Methods—Five groups of 9 to 14 SPSHR each (aged 8 weeks) were treated with 80 mg/kg body wt per day nifedipine, 1 mg or 0.3 mg/kg body wt per day lacidipine, vitamin E (100 IU/d), or carrier for 5 weeks. A group of Wistar-Kyoto rats was used as normotensive control. Plasma samples were taken, and LDL was isolated by ultracentrifugation. Then LDL was exposed to oxygen radicals generated by xanthine/xanthine oxidase reaction (2 mmol/L xanthine +100 mU/mL xanthine oxidase), and several parameters of oxidation were determined. The presence of native apolipoprotein B and oxidation-specific epitopes in the carotid and middle cerebral arteries was determined immunocytochemically.

Results—1,4-DHP CCBs completely prevented mortality. Normotensive Wistar-Kyoto rats showed less oxidation than control SPSHR. Plasma lipoperoxide levels were 0.87±0.27 μmol/L in control SPSHR, 0.69±0.19 and 0.63±0.20 μmol/L in the groups treated with 0.3 and 1 mg lacidipine, respectively, and 0.68±0.23 μmol/L in nifedipine-treated animals (P<0.05 versus control SPSHR for all values). Both CCBs significantly decreased formation of conjugated dienes and prolonged the lag time in LDL exposed to oxygen radicals. Similarly, liperoxides and malondialdehyde were significantly reduced (P<0.05). Reduced relative electrophoretic mobility and increased trinitrobenzenesulfonic acid reactivity of LDL from treated rats (P<0.01) also indicated that fewer lysine residues of apolipoprotein B were oxidatively modified in the presence of 1,4-DHP CCBs. Finally, these drugs reduced the intimal presence of apolipoprotein B and oxidized LDL (oxidation-specific epitopes) in carotid and middle cerebral arteries.

Conclusions—In the SPSHR model, 1,4-DHP CCBs reduce plasma and LDL oxidation and formation of oxidation-specific epitopes and prolong survival independently of blood pressure modifications. Our results support the concept that the in vivo protective effect of these drugs on cerebral ischemia and stroke may in part result from inhibition of oxidative processes. (Stroke. 1999;30:1907-1915.)

Key Words: atherosclerosis ■ calcium channel blockers ■ lipoproteins, LDL ■ oxygen radical ■ stroke
Much effort has been spent to identify medical treatments that contribute to reduce the incidence of cerebral ischemia and stroke. Reactive oxygen species have long been implicated in the development of brain lesions in reperfusion after cerebral ischemia (reviewed in Reference 1). Superoxide radicals are generated in vivo in cerebral inflammation and ischemia, whereas endogenous antioxidants are depleted as a consequence of an excessive production of oxygen radicals very early after the ischemic insult. Furthermore, the activity of the radical scavenger superoxide dismutase in serum is reduced in stroke patients and inversely correlated with the size of the brain infarction and the severity of neurological deficits. Radicals are generated by brain endothelial cells through the xanthine/xanthine oxidase (X/XO) pathway and via mitochondrial electron transport escaping cellular antioxidant defenses after transient brain ischemia. Taken together, these studies support the view that brain ischemia and ischemic stroke are associated with loss of intracellular antioxidants and increased formation of oxygen radicals.

Oxidation of LDL begins very early in human development. Much evidence now exists that it promotes atherogenesis by a number of mechanisms, including rapid uptake of oxidized LDL (ox-LDL) by macrophage scavenger receptors (reviewed in Reference 7). Ox-LDL also enhances arterial vasoconstriction by various mechanisms. Furthermore, it is increasingly recognized that oxygen radicals act as intracellular second messengers and modulate expression of many genes hypothesized to influence plaque formation, vasotonia, and hemostasy. In this pathophysiological scenario, the reduction of oxidation-related processes could provide additional benefit in the treatment of cerebral ischemia–related syndromes.

A meta-analysis of studies on patients with hypertensive stroke and transient ischemic attack indicates that antihypertensive therapy reduces stroke recurrence by 38%. Calcium channel blockers (CCBs) not only reduce systolic blood pressure and the incidence of stroke-related mortality in humans but have been demonstrated to exert several additional protective effects against stroke in stroke-prone spontaneously hypertensive rats (SPSHR). This is generally considered a suitable model for mechanistic and intervention studies because the cerebral lesions in these animals are similar to those in humans.

1,4-Dihydropyridine (1,4-DHP) CCBs vary markedly in their chemical structure and antihypertensive effect but contain aromatic rings capable of stabilizing oxygen radicals, and a hydrogen-donating reaction may also account for their antioxidant activity. In particular, in vitro studies have shown that they protect sarcolemmal and brain microsomal membranes and reperfused rabbit hearts against lipid peroxidation. They also protect endothelial cells against radical-mediated injury and exert antioxidant effects in liposomes and hepatic microsomal systems. 1,4-DHP CCBs reduce LDL oxidation in vitro and in vivo. When used clinically, 1,4-DHP CCBs do not reach the very high concentrations required for antioxidant activity in vitro. It therefore remains to be determined whether antihypertensive doses of 1,4-DHP exert any significant antioxidant effects in vivo and whether such effects provide clinical benefits. Beneficial effects of 1,4-DHP CCBs have been previously reported, but it is unclear whether they are associated with antioxidant effects.

The present study had 2 main objectives. The first was to determine whether treatment of SPSHR with antihypertensive dosages of two 1,4-DHP CCBs with different lipophilic properties reduces total plasma oxidation, LDL oxidation in circulating LDL, and/or oxidation-specific epitopes into arterial wall. For this purpose, we used lacidipine and nifedipine at dosages equally active on systolic blood pressure (1 mg/kg per day lacidipine, 80 mg/kg per day nifedipine). The second goal was to ascertain whether a lower dose of lacidipine (0.3 mg/kg per day) that does not reduce blood pressure provides significant antioxidant protection and prolongs survival.

Materials and Methods

Experimental Animals

At age of 8 weeks, male SPSHR (Iffa Credo, L’Arbresle, France) were divided at random into 5 groups maintained on 1% NaCl drinking water and regular diet. One group served as control (n = 12). The ordinary food and water of the other 4 groups was supplemented with vitamin E (n = 9) as (±)-α-tocopheroyl acetate 100 IU/d (this group was used to compare 1,4-DHP CCBs with a classic antioxidant), 0.3 or 1 mg/kg body wt lacidipine (n = 9 and n = 14, respectively), or 80 mg/kg body wt nifedipine (n = 14), as previously described in detail. In addition, untreated age-matched male Wistar-Kyoto rats (WKY) (Iffa Credo, n = 9) were included in the study as normotensive controls. At the beginning and end of the study (ie, at 5 and 13 weeks of age), the systolic blood pressure was 120.8 ± 3.5 and 127.8 ± 3.7 mm Hg, respectively. After 5 weeks of intervention, SPSHR were killed by decapitation, and plasma samples were taken. In parallel experiments, 5 additional groups of SPSHR (control, 0.3 and 1 mg/kg lacidipine, 80 mg/kg nifedipine, and 100 IU/d vitamin E; n = 8 for each group) were observed for 12 weeks of long-term treatment (ie, up to the 20th week of age) to evaluate the survival rate of animals. All groups were kept in the same environment, the average daily intake of the diet was measured every day, and the systolic blood pressure was measured every week by the tail-cuff method in conscious animals in thermostatic cages prewarmed to 35°C (Physiograph Narco). LDL was isolated by rapid ultracentrifugation (see below). Hearts and aorta were immediately removed and immersed in physiological solution (mmol/L: NaCl 122, KCl 5.9, NaHCO3, 15, MgCl2, 1.25, CaCl2, 1.25, glucose 11) maintained at 37°C and aerated with a gas mixture of 95% O2/5% CO2. Hearts and aorta were dissected free of atria, dried on a filter paper, and weighed to determine ratio of ventricle to body weight. The protocol of the study was approved by the Institutional Animal Investigation Committee of the Catholic University of Louvain, Brussels, Belgium. The animals were managed in accordance with the Guidelines of the American Physiological Society.

Plasma and LDL Oxidation

Plasma was obtained from venous blood samples, and lipoperoxide levels in EDTA-containing plasma (2 mmol/L EDTA and 10 IU/mL aprotinin) were evaluated spectrophotometrically with a lipid peroxide kit (Kamiya Biomedical Company). LDL was isolated by 2 consecutive steps of discontinuous density ultracentrifugation in a KBr gradient, as previously described in detail. A Sephaeryl S-300
column (5 × 0.9 cm, equilibrated with 150 mmol/L NaCl-PBS, 1 mmol/L EDTA) was used both to desalt and to remove molecular-weight-lowering components from the samples, and LDL was used within a few hours to prevent spontaneous oxidation.32,33 LDL purity was checked by both agarose, under nondenaturing conditions, and SDS-PAGE, performed on a 5% to 16% linear gradient slab gel. Samples were dissolved in Laemmli buffer and subjected to electrophoresis at a constant current of 7 mA for 14 hours. Protein bands were detected by Coomassie brilliant blue R250 staining, and molecular weight was calculated by comparison with protein standards. Relative LDL mobility was evaluated by electrophoresis on agarose gel (0.8% agarose in 0.08 mol/L Tris-HCl buffer at pH 8.3) stained by a saturated solution of Sudan black. This assay allows detection of changes in electric charge induced by oxidation. Protein content was measured as described by Lowry et al.54 with bovine serum albumin used as a standard.

To determine the susceptibility of LDL to ex vivo oxidation, the X/XO system was chosen. Oxygen radicals generated by the X/XO reaction can modify LDL.8,31,35 In vitro, X/XO generates a peak of oxygen radicals within the range that can be encountered in vivo and similar to that released by arterial wall cells, fibroblasts, and mesangial cells.35 The fact that XO activity is present in human atherosclerotic plaques8,36 further supports the role of X/XO-induced radical generation in humans. LDL (100 μg/mL) was incubated for 18 hours at 37°C, in the presence of xanthine (2 mmol/L, final concentration) and xanthine oxidase (100 μg/mL, salicylate free, from bovine milk; specific activity, 1 U/mg of protein) in 0.150 mol/L NaCl/0.01 mol/L sodium phosphate, pH 7.4, as previously described.8,31,35 In parallel experiments, superoxide radical production by X/XO reaction was monitored after the reduction of cytochrome-c (1.2 mmol/L) at 550 nm in a double-beam spectrophotometer (Uvikon 810, Kontron). The X/XO reaction yields both superoxide radicals and hydrogen peroxide,35 which in turn may produce hydroxyl radicals in the presence of trace amounts of iron or other transition metals. This system generates ~20 nmol/min per milliliter of superoxide radicals and ~40 nmol/min per milliliter of hydrogen peroxide at peak activity (ie, 90 seconds), which in turn progressively declines within 6 minutes.35

To measure the oxidation resistance of LDL samples, we determined the length of the lag phase preceding the onset of rapid oxidation in LDL, as previously described.33 In this method the 234-nm absorption develops through the conversion of polyunsaturated fatty acid with isolated double bonds into lipid hydroperoxides with conjugated double bonds. The lag time, obtained from the diene versus time curve, is considered an index of the resistance of LDL against oxidation. Peroxidation was also evaluated from the amount of both lipoperoxides (lipid peroxide kit, Kamiya) and malondialdehyde (MDA) produced. This latter compound is an end product of peroxidation of unsaturated fatty acids and is a widely used marker of lipid oxidation. MDA content was assayed by the thiobarbituric acid method, modified as previously described.31,35 Amplification of oxidation during this assay was prevented by adding the chain-breaking butylated hydroxytoluene (100 μmol/L final concentration) to the sample before the thiobarbituric reagents were added. This reduces artifacts due to variations in sample lipid content and/or antioxidation concentration and possible iron contamination of reagents. Oxidative fatty acids were analyzed by mass spectrometry, as previously described in detail.31 Free amino groups present on LDL was checked by both agarose, under nondenaturing conditions, and SDS-PAGE, performed on a 5% to 16% linear gradient slab gel. The primary antibody were devoid of specific staining.

Immunocytochemical staining was performed as previously described.6 Briefly, duplicate serial sections of the fixed and paraffin-embedded arterial segments were stained with the following antibodies: MDA2, a murine monoclonal antibodies against MDA-lysine epitopes, and NP153388, a mouse monoclonal antibody (IgG1) to rat apolipoprotein B (Boehringer Mannheim Italia). MDA2 antibody was previously generated by Dr Palinski and colleagues (University of California–San Diego, La Jolla) by immunizing mice with homologous MD-A-LDL; epitope specificity and binding in atherosclerotic lesions has been extensively characterized in the literature (see References 6, 37, and 38 for more details). Both antibodies were used at a dilution of 1:500. Epitopes recognized by the primary antibody were detected by an avidin-biotin-peroxidase method, as previously described in detail.6,37,38 Control sections stained without the primary antibody were devoid of specific staining.

**Chemicals**

Agarose, acrylamide, SDS, and other electrophoresis grade reagents were purchased from Bio-Rad. Chemicals were purchased from Sigma Chemical Co. Cholesterol content of LDL was measured by enzymatic assay method with the use of a commercial kit (Cholesterol 50, Sigma Chemical Co) according to the manufacturer’s instructions. Nefilpine and lacinidipine were purchased from Bayer and Glaxo-Wellcome, respectively. Vitamin E was purchased from Walgreen Co.

**Statistical Analysis**

Data are presented as mean±SD. Differences between treated groups and controls were primarily tested by 1-way ANOVA followed by Bonferroni’s corrected t test. A value of P<0.05 was considered significant.

**Results**

**Characteristics of Experimental Animals**

Biometric parameters of experimental groups are shown in Table 1. As expected, WKY were normotensive and had a normal ratio of ventricle to body weight (Table 1). The mean body weight was not significantly different among groups. The estimated drug intake, based on the amount of food consumed by each animal, was 0.28±0.02 mg/kg per day for SPSHR receiving the lower dose of lacinidipine (0.3 mg) and 1.02±0.01 mg/kg per day for SPSHR receiving the higher dose of lacinidipine (1 mg). In nifedipine-treated SPSHR, the estimated drug intake was 81.3±2.4 mg/kg per day. In rats receiving 1 mg/kg per day lacinidipine, the plasma concentration of the drug was 2.78±0.99 ng/mL, and the estimated free plasma concentration, assuming 90% bound to plasma proteins, was 0.278 ng/mL. As previously reported,27,30 lacinidipine 0.3 mg/kg per day did not significantly affect systolic blood pressure but reduced cardiac hypertrophy. Lacinidipine 1 mg/kg per day and nifedipine 80 mg/kg per day reduced systolic blood pressure as well as cardiac and aortic hypertrophy by the same extent we previously reported26–28 (Table 1). The SPSHR group treated with vitamin E had biometric characteristics similar to those of control SPSHR (Table 1).

**Effect of 1,4-DHP CCB and Vitamin E Treatment on SPSHR Survival**

1,4-DHP CCBs completely prevented stroke-related mortality in our experimental conditions (Figure 1). In fact, during the 12 weeks of treatment, all control SPSHR (8/8) died. In contrast, all SPSHR treated with 0.3 or 1 mg/kg lacinidipine or 80 mg/kg nifedipine survived during the trial until the 20th week. Interestingly, after 12 weeks of treatment with vitamin
E, 3 rats died; 5 survived the 12 weeks of intervention (Figure 1) but died within the 18th week. These latter results suggest that vitamin E may contribute to reduce stroke-related mortality in the SPSHR model.

### Plasma and LDL Peroxidation

Lipid oxidation may be initiated by any primary free radical that has sufficient reactivity to extract a hydrogen atom from a reactive methylene group of a polyunsaturated fatty acid. Formation of the initiating species is accompanied by bond rearrangement into diene conjugates. The radical then takes up oxygen to form the peroxyl radical. These radicals are also capable of abstracting hydrogen from fatty acid side chains and thus perpetuating the peroxidative chain reactions. Hence, a single initiation event can result in the conversion of hundreds of fatty acid side chains into lipid monohydroperoxides or cycloperoxides. These are fairly stable molecules under physiological conditions, and the cleavage of the carbon bonds during peroxidation results in formation of alkanals such as MDA.

Under our experimental conditions, total plasma lipoperoxide levels were $0.87 \pm 0.27 \mu$mol/L in SPSHR controls but decreased to $0.65 \pm 0.20 \mu$mol/L in the group treated with 1 mg lacidipine, $0.69 \pm 0.19 \mu$mol/L in the group treated with 0.3 mg lacidipine, and $0.68 \pm 0.23 \mu$mol/L in nifedipine-treated animals ($P<0.05$ versus control SPSHR for all values). The dose of 1 mg lacidipine had been selected on the basis of preliminary experiments with doses ranging from $300 \mu$g to $3 \text{mg/kg}$ per day, which demonstrated progressive reduction of plasma and LDL oxidation from 0.1 mg to 1 mg/kg per day (data not shown). Vitamin E significantly reduced plasma peroxidation in SPSHR ($0.58 \pm 0.25 \mu$mol/L; $P<0.05$ versus control SPSHR). Normotensive WKY had lower levels of plasma peroxidation than control SPSHR ($0.73 \pm 0.15 \mu$mol/L; $P<0.05$). These results demonstrate that both 1,4-DHP CCBs and vitamin E reduce the total amount of lipid oxidative compounds in the bloodstream.

In general, all measures of lipid oxidation were lower in normotensive WKY than in control SPSHR (Table 2 and Figure 2). LDL from SPSHR treated with CCBs or vitamin E yielded significantly longer lag times (ie, was better protected) when exposed to X/XO-induced oxidation than control LDL (Table 2). Figure 2 shows the effects of CCBs on the sequential steps of LDL peroxidation, ie, formation of conjugated dienes (A), lipoperoxides (B), and MDA (C). Although both doses of lacidipine were more effective than nifedipine ($P<0.05$), both CCBs significantly decreased formation of conjugated dienes ($P<0.01$ versus control SPSHR). Similarly, the time courses of lipoperoxide and MDA formation showed that these end compounds were significantly reduced by both CCBs ($P<0.05$). As expected, vitamin E had a potent antioxidant effect (Table 2 and Figure 2). The major fatty acids of LDL subject to oxidative modification were analyzed by mass spectrometry. The concentration of C18:1(12-OH) was $9.5 \pm 4.5$ before oxidation and $14.9 \pm 4.2 \mu$g/mg of protein after oxidation ($P<0.05$). Similarly, the concentration of C18:1(10-OH) was $4.9 \pm 1.8$ before oxidation and $10.7 \pm 2.0 \mu$g/mg of protein after oxida-

### Table 1. Characteristics of Treated and Control SPSHR and Normotensive WKY

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body Weight, g</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Ratio of Ventricle to Body Weight, mg/g</th>
<th>Aorta Weight/Length, mg/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY controls</td>
<td>9</td>
<td>252.0 ± 7.2</td>
<td>127.8 ± 3.7†</td>
<td>2.56 ± 0.04†</td>
<td>Not determined</td>
</tr>
<tr>
<td>SPSHR Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>12</td>
<td>262.4 ± 8.4</td>
<td>243.8 ± 6.9</td>
<td>3.67 ± 0.07</td>
<td>1.09 ± 0.07</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>14</td>
<td>254.6 ± 5.9</td>
<td>204.1 ± 6.4*</td>
<td>3.18 ± 0.05*</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>Lacidipine, 1 mg/(kg · d)</td>
<td>14</td>
<td>258.4 ± 5.5</td>
<td>195.8 ± 3.9*</td>
<td>3.24 ± 0.05*</td>
<td>0.90 ± 0.02*</td>
</tr>
<tr>
<td>Lacidipine, 0.3 mg/(kg · d)</td>
<td>9</td>
<td>260.9 ± 4.2</td>
<td>232.8 ± 8.8</td>
<td>3.36 ± 0.04</td>
<td>Not determined</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>9</td>
<td>258.7 ± 8.8</td>
<td>243.6 ± 7.4</td>
<td>3.68 ± 0.08</td>
<td>1.09 ± 0.07</td>
</tr>
</tbody>
</table>

*P<0.05 vs SPSHR controls, SPSHR lacidipine 0.3 mg/(kg · d), and SPSHR vitamin E groups by ANOVA followed by t test and Bonferroni’s correction.†P<0.01 vs SPSHR controls.

![Figure 1](https://stroke.ahajournals.org/). Prevention of mortality in SPSHR by CCBs or vitamin E (n=8 for each group). See the text for more details.
tion; we also observed a significant increase of C18:0(10–0H) from 6.9 ± 2.4 to 13.7 ± 3.9 mg/mg of protein after oxidation (*P < 0.05 versus before oxidation). These peroxidative compounds are derived from linoleic and oleic fatty acids, respectively.

Apolipoprotein B Oxidation Analysis
Proteins are particularly susceptible to direct attack from oxygen radicals and peroxidative intermediates, such as alkoxyl (LO°) and peroxyl (LOO°) radicals. To investigate whether the presence of CCBs could also protect apolipoprotein B from oxygen radical–induced damage, in additional experiments LDL was oxidized and its relative electrophoretic mobility in agarose gel was evaluated. Agarose gel mobility of LDL from WKY subjected to X/XO oxidation was reduced compared with LDL from control SPSHR (Table 3). In contrast, mobility of LDL from SPSHR treated with 1,4-DHP CCBs or vitamin E was significantly reduced (Table 3). Figure 3 shows a typical experiment of LDL agarose gel mobility in SPSHR groups.

We also tested the degree of modification of protein amino groups inducible by X/XO, using TNBS. When control LDL of SPSHR were incubated with oxygen radicals, >20% of TNBS reactivity was lost, whereas >16% was lost in ox-LDL from WKY (Table 3). The loss of TNBS reactivity was significantly smaller in animals treated with 1,4-DHP CCBs or vitamin E compared with both control groups (Table 3), indicating that fewer lysine residues of apolipoprotein B were oxidatively modified. Moreover, lacidipine and vitamin E were more effective than nifedipine in the prevention of apolipoprotein B modifications (*P < 0.05).

Immunocytochemistry
As another measure of oxidation in tissue, paraffin-embedded serial sections of arteries were immunostained and assessed for the intimal presence of oxidation-specific epitopes (ox-LDL) and native apolipoprotein B. Oxidation-specific epitopes do not occur in normal arteries, but small intimal lesions and vascular dysfunction are frequently found in the SPSHR arteries.39,40 Results are expressed in Figure 4. The carotid artery and the middle cerebral artery of the control group contained significantly more intimal apolipoprotein B and ox-LDL epitopes than those of the groups treated with CCBs or vitamin E (P < 0.05). Moreover, the percentage of sections showing immunostaining for each of these epitopes was significantly lower in middle cerebral artery than in the carotid artery, but no significant differences in relative lesion composition were seen. The fact that not only ox-LDL but also native LDL seemed decreased in treated animals was in agreement with the qualitative observation of fewer small intimal lesions in these groups (data not shown). Finally, Figure 5 shows typical staining pattern in control SPSHR and SPSHR treated with 1 mg lacidipine.

Discussion

Our results demonstrate that prolonged treatment of SPSHR with 1,4-DHP CCBs conveys significant protection against
plasma and LDL oxidation (both lipid and protein components) and formation of oxidation-specific epitopes in the arterial wall. Normotensive WKY had lower levels of oxidative compounds than control SPSHR. Treatment with 1,4-DHP CCBs also prevented mortality of SPSHR compared with untreated control. The latter is consistent with the data of Shinya et al.,17 who reported that the CCB AE0047 reduced mortality and caused a significant improvement of neurological scores immediately preceding and during stroke. Similarly, Cristofori et al.16 showed that lacidipine increased survival in SPSHR. The severe hypertension seen in the SPSHR impairs multiple protective functions of the endothelium, such as the release of endothelium-derived vasodilators, and leads to increased penetration of plasma proteins into the vascular wall and the perivascular parenchyma.12–17 The marked reduction of stroke-related mortality could therefore be explained by the antihypertensive effects of CCBs alone. However, previous reports have reported a decreased mortality even at doses that no longer significantly affect blood pressure. For example, nonantihypertensive doses of lacidipine,16,41 the 1,4-DHP CCB nilvadipine,42 and the CCB AE004717 prevent stroke in rats, suggesting that pathophysiological mechanisms other than decrease of high blood pressure play a role. The present study confirms this for low-dose lacidipine (Figure 1) and demonstrates that at this dose, lacidipine still exerts a powerful antioxidant effect in vivo. This suggests that part of the increased survival is caused by a better protection against plasma lipid and LDL oxidation, formation of oxidation-specific epitopes, and other oxygen radical–mediated processes. Our results with vitamin E are consistent with this assumption. Vitamin E treatment conveyed strong antioxidant protection and reduced mortality. However, it proved far less effective than CCBs, suggesting that the antioxidant effects of these compounds may be responsible for only part of the beneficial effect. On the other hand, vitamin E treatment was not associated with any antihypertensive effect.

### Table 3: Relative Electrophoretic Mobility (REM) in Agarose Gel and Percent Decrease of TNBS Reactivity in LDL Oxidized with X/XO for 18 Hours From Experimental Groups of SPSHR and From Normotensive WKY

<table>
<thead>
<tr>
<th></th>
<th>REM, mm</th>
<th>No. of Experiments</th>
<th>TNBS Reactivity, % Decrease</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY controls</td>
<td>1.8±0.3</td>
<td>n=9</td>
<td>−16.2±3.9</td>
<td>n=8</td>
</tr>
<tr>
<td>SPSHR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>2.1±0.3</td>
<td>n=10</td>
<td>−21.2±4.6</td>
<td>n=8</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>1.5±0.2*</td>
<td>n=10</td>
<td>−11.1±2.5</td>
<td>n=8</td>
</tr>
<tr>
<td>Lacidipine, 1 mg/(kg · d)</td>
<td>1.2±0.2*</td>
<td>n=10</td>
<td>−6.3±1.3</td>
<td>n=8</td>
</tr>
<tr>
<td>Lacidipine, 0.3 mg/(kg · d)</td>
<td>1.3±0.2*</td>
<td>n=9</td>
<td>−6.9±1.4</td>
<td>n=7</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.2±0.3*</td>
<td>n=7</td>
<td>−5.8±1.8</td>
<td>n=6</td>
</tr>
</tbody>
</table>

*P<0.01 vs SPSHR controls by ANOVA followed by t test and Bonferroni’s correction.
†P<0.05 vs SPSHR nifedipine by ANOVA followed by t test and Bonferroni’s correction.

![Figure 3](image-url) Relative agarose gel electrophoretic mobility of oxidized LDL from each SPSHR group. Lane 1, SPSHR, 1 mg/kg per day lacidipine; lane 2, SPSHR, 0.3 mg/kg per day lacidipine; lane 3, SPSHR, nifedipine; lane 4, SPSHR, vitamin E; lane 5, control SPSHR.

![Figure 4](image-url) Presence of native LDL and ox-LDL in sections of the common carotid artery (top) and middle cerebral artery (bottom) from treated and control SPSHR. Serial sections were immunostained with monoclonal antibodies NP153388 (against native rat apolipoprotein B) and MDA2 (against epitopes of ox-LDL), as described in Materials and Methods. Sections containing at least 1 intimal area showing substantial immunostaining were counted as positive, and results were expressed as percentage of all sections of the same artery. *P<0.01 vs the SPSHR control group; #P<0.001 vs the SPSHR control group; **P<0.05 vs the SPSHR control group. Lac indicates lacidipine; Nif, nifedipine; and Vit., vitamin.
hand, given the scarcity of side effects, vitamin E may be the
drug of choice in combination therapy with antihypertensive
drugs such as CCBs.

The doses we used for both CCBs are consistent with those
of other in vivo studies in animal models\cite{12,16,26-30} but clearly
higher than the dosages commonly used in clinical practice.
The daily dose of lacidipine for hypertensive patients is 0.07
mg/kg, \( \approx \) 4- to 14-fold lower than the 2 doses we and others
used in SPSHR, and the maximum daily dose of nifedipine
given to hypertensive patients is 2.0 mg/kg, \( \approx \) 40-fold lower
than what we used. This difference may be related to
differences in bioavailability between rats and humans.\cite{25}
Since 1,4-DHP CCBs are lipophilic, these drugs tend to
concentrate in lipid phases,\cite{43,44} which may enhance their
concentrations in the lipid moiety of LDL and cellular
membranes into arterial wall. However, it is conceivable that
during long-term therapy, a much greater accumulation of
1,4-DHP CCBs in lipids may occur in humans, leading to
consentrations similar to those achieved by much shorter
treatment in rats. Indeed, it has been estimated that 1,4 DHP
CCBs can concentrate \( > 1000 \)-fold in lipid membranes,\cite{43,44}
especially in the brain.\cite{44}

The lysine residues of apolipoprotein B present on LDL are
required for the interaction of LDL with the classic LDL
receptor.\cite{7} As increasing numbers of lysine residues are
derivatized by the oxidation products, LDL recognition by the
macrophage receptors is impaired.\cite{7} In the present study, the
protective effect of 1,4-DHP CCBs was also extended to
apolipoprotein B and thus to lysine residues. Furthermore, the
antioxidant effects of 1,4-DHP CCBs in plasma and LDL ex
vivo are consistent with those observed in vitro.\cite{23,24,45}

The specific role of LDL cholesterol and/or ox-LDL in the
pathogenesis of cerebral atherogenesis and ischemic stroke is
still debated.\cite{9} However, because ox-LDL has multiple athero-
genic effects, including the promotion of cytotoxicity, inflam-
mation, and vascular remodeling,\cite{6-9} a beneficial effect of
interventions reducing oxidation is easily envisaged. Among
others, hypercholesterolemia and ox-LDL impair vascular
relaxation.\cite{7-9} Hence, the antioxidant effect of CCBs may
contribute to their antihypertensive effect.

In addition to the antihypertensive and antioxidant effects,
CCBs also induce suppression of the vascular postcontraction
tone,\cite{28} reduction of cholesterol esterification,\cite{46} relaxation
of intracerebral microarterioles,\cite{47} and the reversal of abnormal
coronary vasomotion in patients with hypercholesterolemia.\cite{48}
They have also been proven effective in preventing athero-
sclerosis in classic experimental models (reviewed in Refer-
ence 49). These effects are supported by the evidence that
long-term nifedipine therapy resulted in a significant slowing
of the appearance of new coronary lesions in patients with
mild to moderate coronary heart disease\cite{49,50} and by studies
that reported beneficial effects of CCBs on stroke (reviewed in
References 11 and 51). Furthermore, in the present study
we showed for the first time that these drugs also reduced the
formation of oxidation-specific epitopes of ox-LDL in the
arterial wall. As in SPSHR, it remains to be established to
what extent this was caused by the inhibition of the oxidation
of LDL or that of oxidation-sensitive processes, such as the
regulation of intracellular gene expression. For example,
lacidipine also inhibits the activation of the transcription
factor nuclear factor-\( \kappa \)B and the expression of adhesion
molecules on endothelial cells induced by ox-LDL.\cite{52} Al-
though atherogenesis of intracerebral vessels occurs later than
coronary atherosclerosis\cite{5} and human atherogenesis of the
abdominal aorta and common carotid arteries,\cite{53} small intimal
lesions occur even in brain arteries of SPSHR (Reference 53
and present observations). The manifold antiatherogenic
properties of 1,4-DHP CCBs could therefore all have con-
tributed to the increased survival.

An abnormal influx of Ca\(^{2+}\) through calcium channels
triggers ischemic neuronal death.\cite{54} CCBs may protect by
influencing this process. However, even at the level of brain
tissue, additional benefits may stem from the antioxidant
effects of CCBs, because during cerebral ischemia large
amounts of reactive oxygen species are generated.\cite{1-5}
For example, after cerebrovascular injury, an increased liberation
of arachidonic acid from membrane phospholipid produces
oxygen radicals, causing brain edema.\cite{55} In addition, endog-
enous scavengers and antioxidants are depleted very soon
after the ischemic insult.\cite{1-5} The reduction of oxidation-
related processes in the arterial wall induced both by CCBs

Figure 5. Presence of oxidation-specific epitopes
of ox-LDL in the common carotid artery. Arterial
sections were prepared as described in Materials
and Methods and immunostained with the avidin-
biotin peroxidase method. Epitopes recognized by
the primary antibody are brown, and the nuclei are
counterstained with hematoxylin. Shown is an
example of a section of carotid artery from the
SPSHR control group (A) and from the SPSHR
group treated with 1 mg/kg per day lacidipine (B)
immunostained with MDA2, a monoclonal antibody
against MDA-lysine, an oxidation-specific epitope.
In A but not in B, a diffuse extracellular presence
of oxidation-specific epitopes can be noted. Stain-
ing patterns obtained with MDA2 were generally
similar in groups treated with CCBs or vitamin E
(magnification \( \times 660 \) in A and B).
and vitamin E may also reduce the formation of foam cells into the intima. To date, preliminary experiments of gene therapy carried with antioxidants may depict a pathophysiological scenario improving intracellular defenses against oxidants generated during ischemia/reperfusion.55

Issues raised against the safety of CCBs in cardiovascular diseases have been recently refuted. A massive meta-analysis57 of 98 randomized controlled clinical trials confirmed the safety of nifedipine in the treatment of hypertension. Furthermore, the prospective defined analysis of data from comparative and noncomparative trials of nifedipine and amlopidine showed that there is no indication of excessive risk of death or cardiovascular events for hypertensive patients treated with CCBs.58 This is consistent with the beneficial effects of CCBs in cerebral ischemia–related syndromes seen in experimental models13–17,43,44 and in humans (reviewed in References 11 and 51). Interestingly, we showed here that prolonged treatment with 1,4-DHP CCBs and in part by vitamin E protected from stroke-related mortality.

In conclusion, the results of the present study demonstrate that 1,4-DHP CCBs inhibit oxidation of LDL and other processes induced by oxygen radicals in vivo. Our data also support the idea that the protective effect of these drugs on cerebral ischemia and stroke may result at least in part from inhibition of oxidative processes. This provides a framework for new experimental and therapeutic approaches.

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References


Calcium-channel blockers such as 1,4-dihydropyridine (1,4-DHP) reduce systolic blood pressure and stroke-related mortality in stroke-prone spontaneously hypertensive rats (SP-SHR). However, the mode of action of these calcium-channel blockers in lowering systolic blood pressure alone has not been clearly linked to the neuroprotective effect in SP-SHR. In this study the authors report on the decrease in plasma lipoperoxide levels and reduced relative electrophoretic mobility and increased trienol-benzenesulfonic acid reactivity of oxidized LDL from lacidipine- or nifedipine-treated SP-SHR. The authors have now shown that the antioxidant-related processes afforded by these calcium-channel blockers may underlie the neuroprotective mechanisms in SP-SHR. These studies are carefully performed using normotensive rats to control for the hypertensive effects of SP-SHR.

The clinical relevance of this work in stroke is not clear. If the oxidative-related process is the main risk factor for strokes in humans with hypertension, one would consider that antioxidants that are known to be without toxic side effects (eg, vitamin E) would be the drugs of choice. Nevertheless, this study points to the importance of LDL oxidation as a risk factor for stroke and that this oxidative process can be targeted by 1,4-DHP calcium-channel blockers and perhaps preferably by antioxidants as well.

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1,4-Dihydropyridine Calcium Channel Blockers Inhibit Plasma and LDL Oxidation and Formation of Oxidation-Specific Epitopes in the Arterial Wall and Prolong Survival in Stroke-Prone Spontaneously Hypertensive Rats
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