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# Effect of Flupirtine on Cell Death of Human Umbilical Vein Endothelial Cells Induced by Reactive Oxygen Species

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**ABSTRACT.** Flupirtine (KATADOLON®), known as a nonopiate centrally acting analgesic drug, was tested as to its potential to prevent apoptosis of human endothelial cells induced by reactive oxygen species (ROS). It was found that Flupirtine displayed no effect on viability and cell proliferation of human umbilical vein endothelial cells (HUVEC) up to a concentration of 10 μg/mL. Apoptosis, induced by ROS and generated by hypoxanthine/xanthine oxidase (EC 1.1.3.22) (HX/XOD) or t-butyl hydroperoxide, was reduced after preincubation with Flupirtine for 3 hr by 35% and 41%, respectively. The maximal cytoprotective effect against apoptosis was observed at a drug concentration of 1 to 3 μg/mL. Flow cytometric studies revealed that Flupirtine was able to decrease the number of necrotic cells as well as of apoptotic cells. Neither the simultaneous administration of Flupirtine with the apoptosis-inducing agent nor the preincubation of HUVEC with Flupirtine influenced the increase in the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ caused by the production of ROS. BIOCHEM PHARMACOL **56**;12:1615–1624, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** Flupirtine; apoptosis; HUVEC; reactive oxygen species; endothelial cells

One major mechanism to maintain homeostasis of the number of cells in a given multicellular organ of an animal or plant is the control of cell death by apoptosis [1]. A number of disorders caused by an excess of cell death have been described, some prominent examples being virus-induced lymphocyte depletion (AIDS) [2] or cell death in neurodegenerative disorders with Alzheimer's disease [3], prion diseases [4], and Parkinson's disease [5].

Apoptosis can be induced by several factors, e.g. by endogenous signals during morphogenesis or tumor formation [6] or by extracellular physiological activators, damage-related inducers, or therapy-associated agents or toxins [7]. In recent studies, it has been shown that exposure of cultured rat neurons to (1) the envelope protein of HIV-1, gp120 [8, 9], (2) isolated scrapie prion protein [10, 11], and (3) fragments of the β-amyloid peptide [12] induces neuronal death by apoptosis.

Recently, we were able to establish that Flupirtine (KATADOLON®), used clinically as a centrally acting, nonopiate analgesic agent [13, 14], is able to block the apoptotic process in neurons induced by gp120 (HIV-1) [15, 16], prion-peptide [17] as well as β-amyloid peptide [18]. Experiments revealed that the antiapoptotic effect of Flupirtine is due to an up-regulation of the antiapoptotically acting proto-oncogene *bcl-2* and a normalization of the glutathione content [18, 19].

The molecular mechanisms by which scrapie prion protein and β-amyloid peptide induce apoptosis are not well known. The activation of glutamate receptors, especially of the NMDA‖ receptor channel, either directly or indirectly followed by an increase in the $Ca^{2+}$ level in the neuronal cells and subsequent cell death, has been discussed [20]. Moreover, ROS seem to be significant factors in this process [16].

ROS also play an important role in the pathogenesis of diseases related to endothelial cells, e.g. arteriosclerosis, sepsis, cancer, and reperfusion injury, because of their potential for oxidation of membrane lipids, protein decomposition, and DNA breakdown [21, 22]. Furthermore, endothelial cells are involved in the pathogenesis of AIDS (as in Kaposi's sarcoma) [23], of Alzheimer's disease [24],

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‖ *Abbreviations:* $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; ECGF, endothelial cell growth factor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; fura-2-AM, fura-2-acetoxymethylester; fura-red-AM, fura-red-acetoxymethylester; $H_2$DCF-DA, 2′,7′-dichlorofluorescin diacetate; HUVEC, human umbilical vein endothelial cells; HX, hypoxanthine; LDH, lactate dehydrogenase; MTT, (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue); NMDA, *N*-methyl-D-asparatate; ROS, reactive oxygen species; t-BOOH, t-butyl hydroperoxide; and XOD, xanthine oxidase (EC 1.1.3.22).

and perhaps of selected neurodegenerative diseases [25]. For these reasons, we wanted to know if Flupirtine, in addition to its neuroprotective activity, could also effect apoptosis induced in an endothelial cell model system. HUVEC were used for the studies reported here. HUVEC are known to undergo apoptosis after exposure to ROS [26], to high glucose concentrations [27], to tumor necrosis factor [28], and after endothelial growth factor deprivation [29]. To generate ROS, the HX/XOD system (EC 1.1.3.22) [16, 30] as well as t-BOOH/$Cu^{2+}$ [31] have been applied.

In the present study, we show that ROS-induced endothelial cell death, mainly of apoptotic origin, is prevented by Flupirtine. Furthermore, we present evidence that Flupirtine does not prevent ROS-elevated intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ and does not act directly as a scavenger of ROS.

## MATERIALS AND METHODS

### *Materials*

FBS, fungizone, L-glutamine, and HEPES were obtained from GIBCO BRL and Medium 199 was purchased from PAA Laboratories. Kanamycin monosulfate, t-BOOH, heparin, histamine, collagenase A (EC 3.4.24.3), trypsin (EC 3.4.21.4), proteinase K (EC 3.4.23.6) and RNAse A (EC 3.1.27.5) were purchased from Sigma, and fura-2-AM, fura-red-AM and $H_2$DCF-DA from Molecular Probes. MTT (M2128), collagenase A, and ECGF were obtained from Boehringer, and the annexin-V assay apoptest was purchased from Nexins. XOD (EC 1.1.3.22) was purchased from Merck. Flupirtine maleate [2-amino-3-ethoxycarbonylamino-6-(4-fluoro-benzylamino)-pyridine maleate] ($M_r$: 420.41) was obtained from Asta-Medica Corp.

### *Cell Culture*

HUVEC were isolated by collagenase digestion from human umbilical cord veins as described by Jaffe *et al.* [32]. Briefly, cells were detached by incubation for 10 min at 37° with 10–20 mL of Hank's solution, containing 0.1 mg/mL of collagenase A and 1 μM $CaCl_2$, and then collected by centrifugation at 100 *g* for 10 min and resuspended in Medium 199, supplemented with 20% FBS, 2 mM L-glutamine, 15 mM HEPES, 25 μg/mL of ECGF, 100 μg/mL of kanamycin monosulfate, 0.25 μg/mL of fungizone, and 100 units/mL of heparin. Cells were cultured in 25 $cm^2$ tissue culture grade flasks at 37° in a humidified atmosphere of air/$CO_2$ (19:1). The medium was changed the next day and subsequently every other day.

Having reached confluency, cells were subcultured after detachment with 0.02% EDTA in Hank's solution with a split ratio of 1:4. HUVEC from at least three individuals were pooled and used for experiments when confluent at passage 4–5; they were seeded into 24-well plates. The effect of Flupirtine on proliferating cells was analysed using cultures 24 hr after plating. In one series of experiments, ECGF was omitted from the culture medium for 12 hr prior to analysis.

### *Determination of DNA Fragmentation*

For these experiments, HUVEC were dissociated from the culture plates with trypsin/EDTA. The single cell suspension was washed twice with phosphate-balanced saline/10% FBS, and then the extent of apoptotic DNA fragmentation was determined as described [33]. Briefly, HUVEC ($1 \times 10^6$ cells) were centrifuged at 200 *g* for 7 min at 4°. The resulting pellets were separated into intact nuclei and free DNA fragments after cell lysis in TET buffer [10 mM Tris–HCl (pH 7.2), 1 mM EDTA, and 0.2% Triton X-100] by centrifugation at 13,000 *g* for 10 min at 4°. DNA was isolated from both fractions after digestion with proteinase K (100 μg/mL, 10 min, 37°; 4 hr) and RNAse A (50 μg/mL) by precipitation with iso-propanol-70% ethanol at 4°. Precipitates were spun down, decanted, air dried for 30 min, and then resuspended in TE buffer [10 mM Tris–HCl (pH 7.2), and 1 mM EDTA, 48 hr, 37°]. Finally, the suspension was subjected to agarose gel electrophoresis and DNA was visualised by ethidium bromide staining.

### *Cell Viability*

The viability of cells was determined with the MTT colourimetric assay system [34], followed by evaluation with an ELISA reader (BioRad 3550).

### *Flow Cytometric Analysis*

Flow cytometric measurements were performed on a Becton and Dickinson FACScan cytometer with an argon laser tuned to 488 nm. Forward and side scatter light as well as green and red fluorescence intensities were recorded. The data were analysed using LYSYS II software. HUVEC were gated for analysis by their forward and side-scatter light properties.

To detect apoptotic cells, a commercially available test kit ("apoptest") was used according to the protocol given by the manufacturer. Briefly, 445 μL of cell suspension ($2 \times 10^5$ cells/mL), 5 μL of annexin-V-FITC conjugate solution, and 50 μL of propidium iodide solution (100 μg/mL) were incubated for 10 min on ice. Then the cells were analysed without any further washing steps. Due to a breakdown of cellular energy metabolism during apoptosis, phosphatidylserine—a specific cytoskeleton ligand—appeared on the cell surface. This translocation allowed the annexin-V-FITC conjugate to bind. Apoptotic cells exhibited a strong binding of annexin-V-FITC and a weak propidium iodide fluorescence (630 nm), whereas secondary necrotic cells were found as a double positive population.

### *Induction of Apoptosis in HUVEC*

ROS were generated using HX and XOD [16, 30]. If not mentioned otherwise, 20 mU/mL of XOD and 30 μM HX were used for induction of apoptosis. After 10 hr, less than 10% of the cells were viable under these conditions. Usually, ca. 70% of the HUVEC undergo apoptosis after 6 hr. The level of 70% is sufficient for studies in which a given compound is tested for potential inhibition and/or stimulation of apoptotic cell death.

As a second inducer of apoptosis, t-BOOH (100 μM) was applied in combination with $CuSO_4$ at a concentration of 200 nM [31].

### *Loading of Cells with Fura-2-AM or Fura-Red-AM*

Basically, the procedure described by Jacob was applied [35]. After dissociation of HUVEC with trypsin/EDTA, the single cell suspension was washed twice with Dulbecco's modified Eagle's medium with 20% FBS and the fluorescent dye was added to a final concentration of 4 μM. The cells were incubated for 30 min at room temperature, washed twice with buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, and 1% BSA) and resuspended in the same buffer, supplemented with only 0.1% BSA. After an additional incubation period of 60 min, which is required for hydrolysis of the fura-2 ester, the determinations of $[Ca^{2+}]_i$ were performed within the following 3 hr. The concentration of free intracellular fura-2 acid was determined by addition of $MnCl_2$ (1 mM) in the presence of 10 μM ionomycin, which causes quenching of the free acid [36].

### *Measurement of $[Ca^{2+}]_i$*

After the different incubation periods indicated, 2 mL aliquots of the cell suspension were transferred to a continuously stirred quartz cuvette (maintained at 22°) in an LS-50 spectrophotometer (Perkin-Elmer). The changes in $[Ca^{2+}]_i$ were determined by recording the ratio of the fluorescence intensities between the excitation wavelengths of 340 nm and 380 nm [36]. Flupirtine by itself is a fluorescent compound within an UV-excitation range of 300 to 400 nm, showing a bright green fluorescence which interferes with the fura-2 emission wavelengths chosen for the determination of $[Ca^{2+}]_i$. Maximum excitation of Flupirtine was found to be 330 nm. Because the fluorescence intensities at 340 and 380 nm were different—the emission at 340 nm was brighter than the intensity at 380 nm—addition of Flupirtine led to an apparent increase in $[Ca^{2+}]_i$ by modifying the 340/380 nm fura-2 emission ratio. For example, the ratio 340/380 nm increased to approximately 5% of the peak height of the histamine (20 μM) response after addition of Flupirtine (3 μg/mL). Therefore, the background level of the respective value for the 340/380 nm ratio caused by Flupirtine was subtracted for the described measurements. Furthermore, the experiments were repeated with fura-red, a visible light calcium indicator. The ratios between the excitation wavelengths of 420 and 488 nm were recorded on a Sigma ZWS dual-wavelength spectrophotometer (Eppendorf) [37]. For emission, a cut-off filter with 50% transmission at 590 nm was used.

### *Determination of LDH Leakage Rate*

Cells were cultivated in 24-well plates. After the indicated periods of incubation, the supernatant was removed from the cultures, centrifuged (5 min, 1000 *g*), and aliquots of the supernatants were used for the determination of extracellular LDH activity. For the measurement of this activity, monolayer cell cultures were treated with 3 mL of 0.9% NaCl, (supplemented with 0.1% Triton and 0.1 mM of dithiothreitol), pooled with the pellet from the supernatant of the first centrifugation step, and sonicated. LDH activity was measured as described [38]. The leakage rate was calculated as follows: leakage rate = LDH activity (extracellular)/LDH activity (intracellular plus extracellular) × 100%.

### *Measurement of the Direct Scavenger Effect of Flupirtine on ROS Production by XOD*

The reaction mixture to determine XOD activity consisted of 10 μM $H_2$DCF-DA, 100 μM HX, and Flupirtine (0–10 μg/mL) in a final volume of 2 mL of PBS. The reactions were performed at 25° and were started by adding 40 mU of XOD. The increase in fluorescence intensity at an excitation wavelength of 505 nm was measured in a Sigma ZWS dual-wavelength spectrophotometer (Eppendorf). For emission, a KV-550 cut-off filter (Schott) was used. The initial steepness (0–3 min after addition of XOD) of the obtained curves was taken as a measure of enzyme activity.

### *Determination of Protein Concentration*

Protein content was determined by the method of Bradford [39] using the Protein-Assay-Kit I (Bio-Rad).

### *Statistical Analysis*

For the statistical evaluation the Student's *t*-test was applied [40].

## RESULTS

### *Effect of Flupirtine on Viability of HUVEC*

We first determined the effect of Flupirtine on the viability of confluent HUVEC. As summarised in Table 1, no significant change in cell number in a concentration range of 0.1 to 10 μg/mL of Flupirtine was observed during an incubation period of 24 hr. The amount of protein did not vary between treated and untreated cultures. The MTT assay system was applied as a further test to show that the compound did not affect cell viability. Setting the viability

**TABLE 1. Effect of different concentrations of Flupirtine on viability of confluent HUVEC**

| Flupirtine (μg/mL) | Cell number/well (% of control) | μg protein/well | Viability (% of control) |
|---|---|---|---|
| 0 | 100.0 ± 13.3 | 14.2 ± 1.2 | 100 ± 6.0 |
| 0.1 | 84.4 ± 13.1 | 14.1 ± 1.1 | 97 ± 8.8 |
| 0.3 | 109.1 ± 10.3 | 13.1 ± 0.8 | 104 ± 7.1 |
| 1.0 | 97.4 ± 15.6 | 13.4 ± 1.0 | 103 ± 1.2 |
| 3.0 | 92.1 ± 18.4 | 13.5 ± 1.4 | 96 ± 9.3 |
| 5.0 | 87.9 ± 13.6 | 14.3 ± 1.0 | 98 ± 7.3 |
| 10.0 | 91.0 ± 11.2 | 14.6 ± 1.1 | 102 ± 9.6 |

Nonproliferating, confluent HUVEC were incubated in the absence or presence of different concentrations of Flupirtine for 24 hr. Subsequently, the following parameters were determined: cell number [in the absence of Flupirtine (controls), a density of $1.3 \times 10^5$ cells/cm$^2$ was measured], protein content, and viability, as determined by the MTT assay system (OD: 0.74). The values determined in the absence of the drug were set to 100%. The means and the SD are given; $N = 5$.

of untreated cultures to 100%, Flupirtine caused no change at concentrations between 1 to 10 μg/mL (Table 1).

We next studied the influence of the drug on the proliferation rate of HUVEC. Flupirtine displayed no significant effect on the growth of HUVEC at a concentration of 3 μg/mL. The numbers of untreated and treated cells were determined after incubation periods of 24, 48, and 72 hr (Table 2).

### *Induction of Apoptosis by ROS and Its Prevention by Flupirtine*

ROS were generated both by the HX/XOD and t-BOOH/$Cu^{2+}$ systems. XOD (20 mU/mL) in the presence of 30 μM HX or a concentration of 100 μM t-BOOH in the presence of 200 nM $Cu^{2+}$ were sufficient to cause the characteristic DNA fragmentation pattern [1]. Multiples of ≈180 base pairs were resolved by agarose gel electrophoresis (Fig. 1, lane c [HX/XOD] and lane e [t-BOOH]). Preincubation of HUVEC with 3 μg/mL of Flupirtine for 3 hr abolished DNA fragmentation in ROS-treated cells (Fig. 1, lanes b and f). The positive control (omission of ECGF from the culture medium) also caused apoptotic fragmentation of DNA (lane d).

The protective effect of Flupirtine was further investigated by a quantitative analysis using the MTT assay system. Incubation of the cells with either HX/XOD or t-BOOH/$Cu^{2+}$ caused a reduction in cell viability to 25.2% and 53.5%, respectively (Table 3). Preincubation for 3 hr with Flupirtine (1 to 10 μg/mL) caused a significant increase in cell viability. The highest protective effect was seen at concentrations of 1 and 3 μg/mL in assays with both generators of ROS.

**TABLE 2. Effect of Flupirtine (3 μg/mL) on proliferation of HUVEC**

| Cultivation period after splitting (hr) | Cell number (% of control) | Cell number in the presence of Flupirtine (% of control) |
|---|---|---|
| 0 | 100 ± 2.9 | 100 ± 3.8 |
| 24 | 123 ± 3.5 | 120 ± 6.1 |
| 48 | 155 ± 2.6 | 157 ± 2.1 |
| 72 | 199 ± 4.1 | 202 ± 3.2 |

HUVEC were incubated with or without Flupirtine for up to 72 hr, and the proliferation rate was determined by counting the number of cells. The cell count 24 hr after cell splitting (at $t = 0$ hr) was set to 100%. The means and the SD are given; $N = 10$.

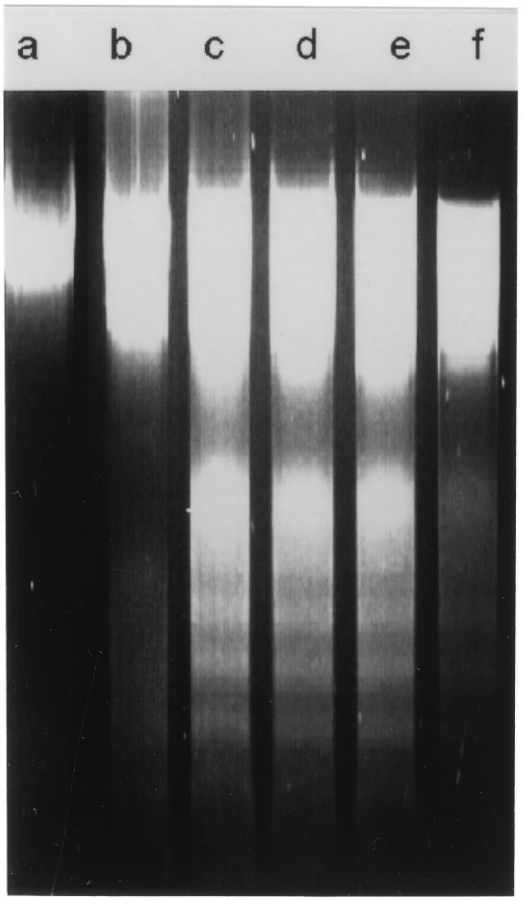


**FIG. 1. Effect of Flupirtine on ROS-induced apoptosis in HUVEC. The cells were (pre)incubated for 3 hr in the absence or presence of 3 μg/mL of Flupirtine. Apoptosis was induced by the addition of ROS, as described under Materials and Methods. After an incubation period of 6 hr, the cells were lysed and DNA was analysed by agarose gel electrophoresis. Cells remained either untreated (lane a) or were incubated as follows: addition of HX/XOD plus Flupirtine (lane b), HX/XOD alone (lane c), t-BOOH/$Cu^{2+}$ alone (lane e), or t-BOOH/$Cu^{2+}$ plus Flupirtine (lane f). Lane d shows the DNA fragmentation pattern, extracted from HUVEC cultivated for 12 hr in the absence of ECGF.**

In order to study whether or not the cytoprotective effect of the drug depends on the duration of preincubation, HUVEC were pretreated with the drug for 0 (simultaneous application of ROS generators and Flupirtine) to 30 hr. In the absence of Flupirtine, HX/XOD reduced cell viability to 26.3 ± 2.9% and t-BOOH/$Cu^{2+}$ to 54.6 ± 5.7%. Simultaneous application of Flupirtine with HX/XOD caused a small increase in cell viability ($P < 0.01$), while the effect on t-BOOH/$Cu^{2+}$-induced reduction was not significant

**TABLE 3. Effect of different concentrations of Flupirtine on viability of cells after induction of apoptosis by ROS**

| Flupirtine concentration (μg/mL) | Viability; treatment: HX/XOD (% of control) | Viability; treatment: t-BOOH/$Cu^{2+}$ (% of control) |
|---|---|---|
| 0 | 25.2 ± 2.7 | 53.5 ± 5.3 |
| 1 | 41.3 ± 4.5† | 69.7 ± 6.6† |
| 3 | 51.5 ± 6.5† | 72.4 ± 7.1† |
| 10 | 37.5 ± 7.3* | 61.3 ± 6.9* |

Preceding the addition of ROS—treatment with 30 μM HX/20 mU/mL XOD or 100 μM t-BOOH/200 nM $Cu^{2+}$—cells were preincubated with Flupirtine for 3 hr. After an incubation period of 6 hr, the viability was determined by the MTT assay system. The values determined for untreated HUVEC were set to 100%. Means and SD are given; $N = 5$.

* $P < 0.0001$.

† $P < 0.001$ (Student's *t*-test versus assay without Flupirtine).

under the same incubation conditions (Fig. 2). However, a preincubation with Flupirtine for 3 hr significantly ($P < 0.0001$) increased cell viability in both ROS-producing systems to 51.3 ± 3.1% [HX/XOD] and 72.9 ± 3.8% [t-BOOH/$Cu^{2+}$], respectively. Maximal protective effects were seen after preincubation periods of 3 to 10 hr, whereas a longer preincubation was less effective (Fig. 2).

In a separate series of experiments, the LDH leakage rate was determined as a second parameter for cell viability. Flupirtine (3 μg/mL) did not affect the LDH leakage rate. In the absence of the compound, the rate measured after an incubation period of 9 hr was 18.7% ± 1.9% in the absence and 21.5% ± 2.4% in the presence of the drug. When HUVEC were treated with the ROS-producing systems HX/XOD or t-BOOH/$Cu^{2+}$ and analysed after 6 hr for LDH leakage, the rate was found to be 86.2 ± 7.3% in the HX/XOD system and 91.3 ± 8.5% with t-BOOH/$Cu^{2+}$. However, Flupirtine displayed a cytoprotective effect in

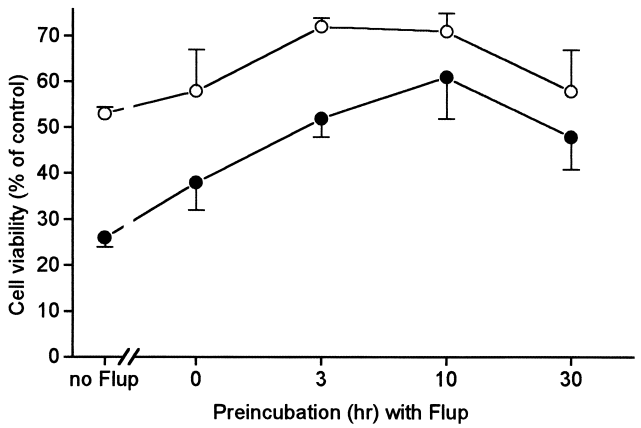


**FIG. 2. Cytoprotective effect of Flupirtine on ROS-induced reduction in cell viability following varying preincubation periods (0–30 hr) with Flupirtine (3 μg/mL). Cell viability of untreated HUVEC, as measured by the MTT assay, was set to 100%. The values for the two ROS generation systems, HX/XOD [●] and t-BOOH/$Cu^{2+}$ [○], were obtained after 6 hr of further incubation. The degree of cell viability in the absence of the drug is included as control. At time 0, the drug was added simultaneously with ROS.**

**TABLE 4. Effect of different concentrations of Flupirtine on ROS production by HX/XOD**

| Flupirtine concentration (μg/mL) | Steepness Relative fluorescence units/3 min |
|---|---|
| 0 | 0.74 ± 0.06 |
| 1 | 0.73 ± 0.06 |
| 2 | 0.69 ± 0.05 |
| 3 | 0.65 ± 0.08 |
| 5 | 0.73 ± 0.11 |
| 10 | 0.80 ± 0.14 |

The reaction was started by addition of 20 mU/mL XOD to 2 mL reaction mixture (10 μM $H_2$DCF-DA, 100 μM HX in PBS) in the absence or presence of Flupirtine (1, 2, 3, 5, and 10 μg/mL). Fluorescence was monitored over a period of 10 min and the initial steepness of the reaction curves (0–3 min after addition of XOD) was estimated as a measure of the velocity of ROS generation. Means and SD of four independent experiments are given.

experiments when HUVEC were pretreated for 3 hr with 3 μg/mL of Flupirtine and subsequently with the ROS-producing systems HX/XOD or t-BOOH/$Cu^{2+}$. When the LDH leakage rate was determined after 6 hr, Flupirtine caused a significant reduction ($P < 0.0001$) in the rates to 62.1 ± 5.8% (HX/XOD) and 65.2 ± 6.1% (t-BOOH/$Cu^{2+}$), respectively. These data support the cytoprotective effect of Flupirtine seen in the analysis with the MTT system.

Flupirtine itself has no scavenger effect on the production of ROS as tested by measuring the fluorescence increase due to the oxidation of $H_2$DCF-DA by HX/XOD. The initial steepness of the reaction curves was taken as a measure of the velocity of ROS production (0–3 min after addition of XOD). Flupirtine had no significant inhibitory effect on ROS production by HX/XOD under the applied conditions (Table 4).

### *Determination of the Effect of Flupirtine on the Fraction of Viable, Apoptotic, and Necrotic Cell Populations during Apoptosis*

In the present study, flow cytometrical analysis, applying the "annexin-V assay apoptest", was performed to differentiate between viable, apoptotic, and necrotic cells. As shown in Fig. 3a, the proportion of the different populations in the untreated controls was as follows: viable cells 63%, apoptotic cells 19%, and necrotic cells 16%. When the cells were treated with HX/XOD, only 8% of the cells remained viable, while 65% were found to be in the necrotic and 19% in the apoptotic state (Fig. 3b). Preincubation (3 hr) with Flupirtine increased the number of viable cells markedly to 31%, whereas the amounts of apoptotic (17%) and necrotic cells (48%) decreased (Fig. 3c).

The distribution of the different cell populations was analysed by the dual-colour immunofluorescence technique with subsequent quantification by flow cytometry, with the major emphasis that of elucidating the effect of Flupirtine on HX/XOD- and t-BOOH/$Cu^{2+}$-mediated cell death dur-

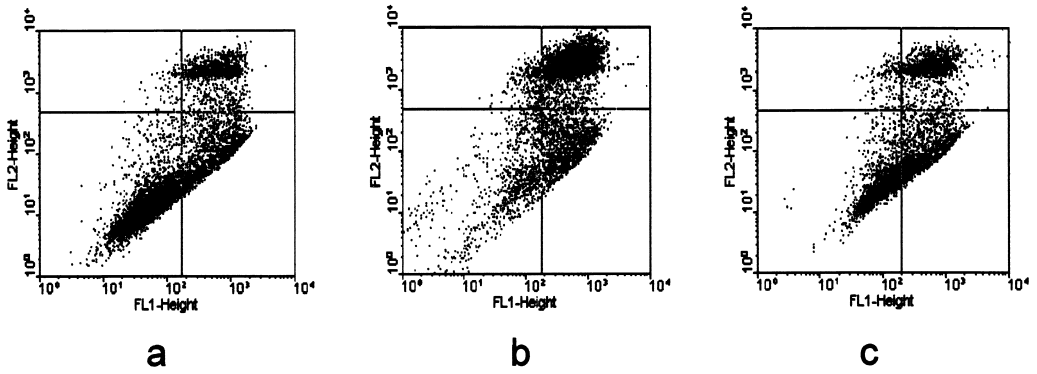


**FIG. 3. Flow cytometrical analysis of untreated control cells (a), cells incubated for 6 hr with HX/XOD (b), or cells preincubated with Flupirtine (3 μg/mL) (c). The preincubation period was 3 hr and incubation time was 6 hr. The abscissa shows the green fluorescence caused by the annexin-V-FITC conjugate, indicating apoptotic cells, and the ordinate denotes the red fluorescence of propidium iodide staining for necrotic cells. The lower left quadrant includes the viable cells, the lower right the apoptotic cells, and the upper right the necrotic cells.**

ing preincubation periods of 0 (administration of the drug together with the ROS-generating system) to 30 hr. In the absence of any inducer, the proportion of viable cells remained unchanged, irrespective of the treatment schedule with Flupirtine, the distribution being 61% of viable cells, 18% of apoptotic cells, and 19% of necrotic cells (Fig. 4a). When HUVEC were treated with HX/XOD, the proportion of viable cells dropped to 11%, while the necrotic cell population increased to 64%. Approximately 19% of the cells were apoptotic (Fig. 4b). This distribution was significantly changed ($P < 0.0001$) if the cells were pretreated with Flupirtine for at least 3 hr prior to the addition of the inducer HX/XOD. Under these conditions, the fraction of viable cells increased to 38%, while that of the necrotic cells decreased to 44%. A preincubation period beyond 10 hr with the compound had a weaker effect. The simultaneous administration of Flupirtine with the inducer did not significantly change the distribution of the cell population.

In a parallel series, apoptosis of HUVEC was induced by t-BOOH/$Cu^{2+}$. Even under these conditions, the reduction in the ROS-induced proportion of viable cells from 61% to 27% and a concurrent increase in necrotic cells from 19% to 52% only occurred if the HUVEC were pretreated with the compound for at least 3 hr (Fig. 4c). At this point, the fraction of viable cells increased to 42%, and that of necrotic cells decreased to 38%. Again, a longer preincubation—exceeding 10 hr—did not have a stronger effect, whereas a simultaneous application (Flupirtine together with the inducer) did not show any effect.

The relatively high number of necrotic cells can be explained by the known phenomenon that cell populations undergoing apoptosis exhibit features of secondary necrosis when apoptosis proceeds to its final stage, if analysed by flow cytometry. Thus, in order to determine whether the effect of ROS on the viability of HUVEC was really based on a controlled active process (i.e. apoptosis) or on the mere influence of oxidative membrane disruption, the cells were preincubated with cycloheximide, a known blocker of protein synthesis [41]. As can be seen in Table 5, the presence of cycloheximide nearly completely abolished the ROS-induced decrease in viability for all parameters investigated. The percentage of apoptotic HUVEC in the annexin-V/propidium iodide assay was lowered by cycloheximide from 16 to 11%, whereas the double positive population of the dead cells decreased from 67% to 18% in the presence of the inhibitor. These results were confirmed by estimation of cell viability (MTT test) of ROS-treated cells in the presence or absence of cycloheximide. Formazan production of the cells recovered from 29% to 91%. In addition to the typical DNA fragmentation, these results show that the cell death caused by ROS in HUVEC is a controlled apoptotic process.

### *Alteration of the Level of $[Ca^{2+}]_i$ by ROS in the Presence or Absence of Flupirtine*

To clarify whether or not the intracellular $Ca^{2+}$ level of HUVEC changes in response to the ROS-generating systems HX/XOD and t-BOOH/$Cu^{2+}$, and whether it might initiate the apoptotic process or not, the $[Ca^{2+}]_i$ level was determined. In contrast to the transient histamine signal which rose to a maximum within 30 sec, HX/XOD caused a steady increase in the fluorescence ratio between 340/380 nm within 5 min. Thereafter, $[Ca^{2+}]_i$ remained at this level (Fig. 5A). The results reveal that the two inducers caused an increase in $[Ca^{2+}]_i$ of 18% [HX/XOD] or 15% [t-BOOH/$Cu^{2+}$] of the maximal response to 20 μM of histamine (Fig. 5B).

Simultaneous addition of Flupirtine together with the HX/XOD treatment did not affect the peak height or the duration of the changes in $[Ca^{2+}]_i$ (Fig. 5B, columns a and b). While in the absence of Flupirtine the increase in $[Ca^{2+}]_i$ was 18 ± 3% (with respect to the histamine response), the $[Ca^{2+}]_i$ level in HUVEC coincubated with the drug and HX/XOD was 17 ± 6%. After preincubation of HUVEC with Flupirtine for 3 or 6 hr, the increase in

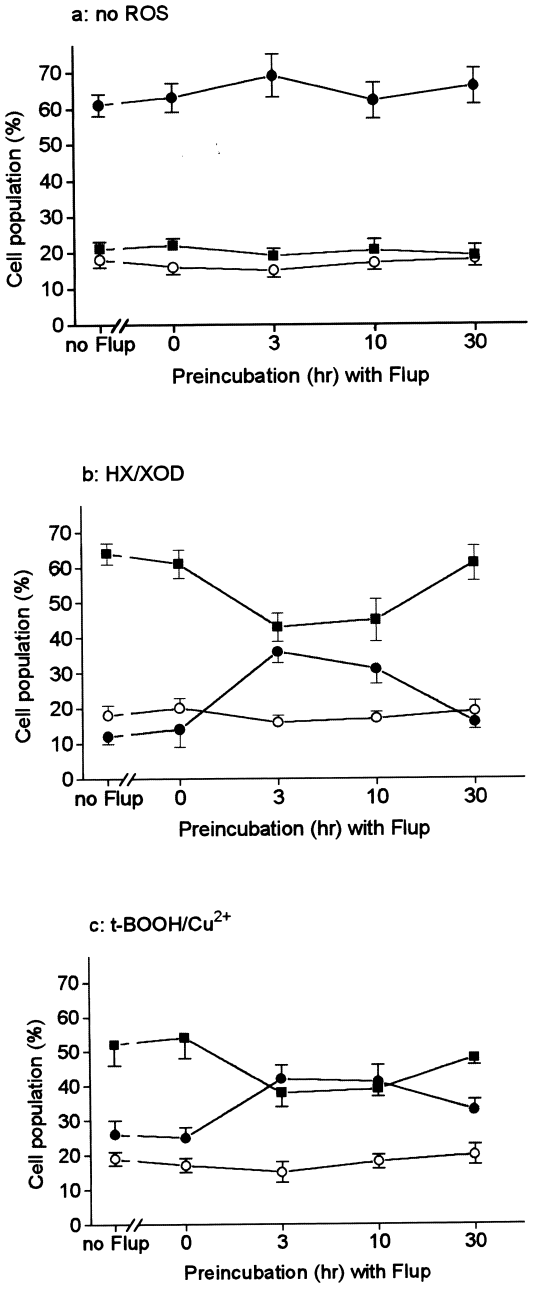


**FIG. 4. Effect of Flupirtine on the distribution of the following populations of HUVEC: viable [●], apoptotic [○], and necrotic cells [■]. HUVEC remained either untreated with respect to a ROS-generating system (a), or were treated for 6 hr with HX/XOD (b) or t-BOOH/$Cu^{2+}$ (c). The preincubation time was 0 (simultaneous addition of Flupirtine and the inducer) to 30 hr. The percentage of the respective cell population in the absence of the drug is included (no drug). Means and SD are given; N = 10.**

$[Ca^{2+}]_i$ was only slightly, but not significantly smaller (Fig. 5B, columns c and d).

To be able to definitely exclude possible influences of Flupirtine on the method used for the determination of the $[Ca^{2+}]_i$ level, experiments were repeated with the visible light excitable calcium indicator fura-red. In this wavelength range (420 nm to 490 nm), Flupirtine exhibited no considerable fluorescence emission. The results obtained with fura-red confirmed the above-described effects obtained with the fura-2 indicator. Again, it was found that Flupirtine displayed no effect on the ROS-mediated change in $[Ca^{2+}]_i$ (not shown).

## DISCUSSION

ROS are known inducers of apoptosis in neurons [42] as well as other somatic cells, e.g. endothelial cells [29], and are considered to be a main basis for neurodegenerative disorders such as β-amyloid peptide-mediated Alzheimer's disease [43] or prion-mediated cell injury [44]. In the present study, two ROS generators, the HX/XOD and t-BOOH/$Cu^{2+}$ systems, were used to induce apoptosis in HUVEC. The extent of apoptosis in HUVEC was measured both by flow cytometry and by DNA fragmentation. Flupirtine significantly reduced the number of dead cells if the drug was given 3 hr before induction of apoptosis. This protective effect on endothelial cells was not as strong as it was on neuronal cells [16, 20]. Based on earlier reports, e.g. on human neutrophils [45], which show that progression through an early phase of apoptosis results in a "secondary necrosis", we assume that most of the cells found in the population of necrotic cells by flow cytometry are in fact late apoptotic cells. This assumption is supported by the finding that after treatment of HUVEC with ROS in the presence of cycloheximide, a known inhibitor of protein synthesis, the number of viable cells was increased enormously. Control cells analysed by flow cytometry showed a viability of only 72%. The relatively low number of propidium-iodide negative cells can be explained by the fact that the cells were detached and washed repeatedly before measurement. Due to the stress during this procedure, HUVEC may exhibit a loss in plasma membrane integrity, leading to higher percentages of damaged cells than found with adherent HUVEC used in the other cell death assays (MTT test; DNA fragmentation assay).

It is known that $[Ca^{2+}]_i$ in HUVEC increases in response to histamine [35]. In the present report, we confirm that these cells also respond to ROS with an increase in $[Ca^{2+}]_i$, as shown by Vischer *et al.* [46]. The response is approximately 5-fold less than that with histamine. The elevation in $[Ca^{2+}]_i$ could not be reduced by Flupirtine, irrespective of the schedule of its application. This is to be seen in contrast to neuronal cells, where Flupirtine is able to inhibit the glutamate-induced increase in $[Ca^{2+}]_i$ [47]. As the NMDA receptor is not present in endothelial cells, we conclude that Flupirtine, besides its influence on NMDA receptor and the decrease in $[Ca^{2+}]_i$, has an additional effect of protection against induced apoptosis.

It was reported previously that apoptosis of endothelial cells could be prevented by increasing the intracellular level of glutathione [48]. As further modulators of apoptosis in endothelial cells, the Fas-Fas ligand system [49] as well as *bcl-2* [50] have been implicated. Using neuronal cells as model system, Flupirtine was shown to prevent induction of

**TABLE 5. Effect of cycloheximide on cell death induced by ROS on HUVEC**

| | Viability as measured by: | | | |
|---|---|---|---|---|
| | Flow cytometry | | | MTT test |
| | % vital | % apoptotic | % necrotic | Viability (% of control) |
| Control | 72 ± 6 | 12 ± 2 | 14 ± 4 | 100 |
| + HX/XOD | 13 ± 3 | 16 ± 4 | 67 ± 7 | 29.3 ± 2.9 |
| + Flu, HX/XOD | 44 ± 3 | 13 ± 3 | 37 ± 5 | 56.7 ± 7.7 |
| + CH, HX/XOD | 66 ± 4 | 11 ± 4 | 18 ± 6 | 91.6 ± 8.1 |

Cells were preincubated with 3 μg/mL Flupirtine (Flu) for 3 hr or with cycloheximide (CH) (1 μM, 30 min) before addition of 30 μM HX and 20 mU/mL XOD to the cells. After an incubation period of 6 hr, the viability was determined by flow cytometry experiments and by the MTT assay system. Means and SD are given; $n = 5$.

apoptosis by normalising the intracellular glutathione level [18, 19]. These experiments revealed that the β-amyloid peptide-mediated decrease in intracellular glutathione could be totally prevented in the presence of the drug. The possible effect of the drug on the Fas-Fas ligand system has not yet been studied. Future experiments will have to be performed to clarify the mode of action by which the drug protects endothelial cells against the apoptotic process.

Interestingly, the cytoprotective effect of Flupirtine on HUVEC is biphasic, a similar effect having been seen earlier using neuronal and lymphoidal cell systems *in vitro* [16]. Optimal protection was observed at concentrations between 1 and 3 μg/mL which were also measured *in vivo*, both in plasma and in cerebrospinal fluid, after oral administration of Flupirtine at the therapeutic daily dose of 600 mg (3 × 200 mg). Under these conditions, the peak concentration ($c_{max}$) of 2.4 μg/mL is seen in the plasma after 90 min [14] and the level remains at 1 μg/mL in plasma for approximately 12 hr [51]. As shown here, a drug concentration of 1–3 μg/mL was found to protect HUVEC against inducer-mediated apoptotic cell death *in vitro*. Higher concentrations of Flupirtine than 3 μg/mL showed no higher cell death prevention for HUVEC. On the contrary, the cytoprotective effect slightly diminished. The concentration dependence found seems to be typical for Flupirtine, as has been demonstrated for other cell types earlier [16].

Studies showing that Flupirtine acts antiapoptotically *in vitro* [15, 17, 18] as well as *in vivo* [52, 53] reveal that the drug displays a neuroprotective activity. Thus, Flupirtine seems to be a promising drug in the treatment of neurodegenerative disorders, e.g. Alzheimer's disease and prion diseases. However, recently published data show that the prion protein, gp120, or β-amyloid peptide toxins do not act apoptotically solely on neurons *in vitro*, but also affect other cell types, e.g. glial cells [44] or endothelial cells [24]. Hence, the results from our study reveal a protective mechanism of Flupirtine in addition to the known effects of the drug on NMDA receptors in neuronal cells. As ROS are also involved in the pathogenesis of neurodegenerative disorders, our data on the viability of endothelial cells *in vitro* might also contribute to the potential therapeutic

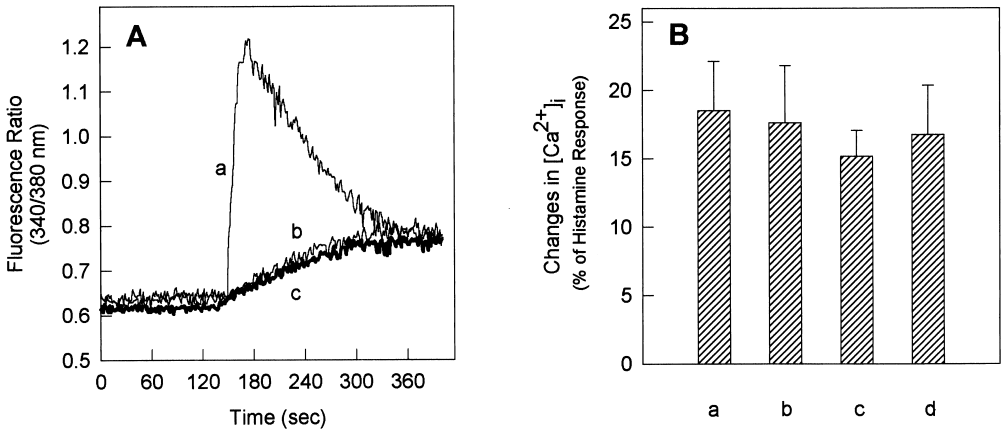


**FIG. 5. (A) Changes in the fluorescence intensity ratio between 340 nm/380 nm in fura-2-loaded HUVEC after treatment with (a) 20 μM of histamine, (b) 30 μM HX/20 mU of XOD, and (c) 30 μM of HX/20 mU of XOD and preincubation with Flupirtine (10 μg/mL). (B) Comparison of the maximal changes in $[Ca^{2+}]_i$ in HUVEC caused by HX/XOD in the presence or absence of Flupirtine. Addition of HX/XOD in the absence of Flupirtine (a). Flupirtine was added to the cells either simultaneously with HX/XOD, at a concentration of 3 μg/mL (b), or 3 and 6 hr prior to the addition of the inducer (preincubation) (c and d). The $[Ca^{2+}]_i$ level after addition of ROS is given as a percentage fraction of the maximal response of the cells to histamine (20 μM) treatment. Means and the SD are given; $N = 10$.**

intervention and to an improvement in the therapeutic strategies in the treatment of dementia in AIDS, Alzheimer's disease, and Prion diseases.

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