HIV protease inhibitors activate the unfolded protein response and disrupt lipid metabolism in primary hepatocytes

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First published July 20, 2006; doi:10.1152/ajpgi.00182.2006.—Treatment of human immunodeficiency virus (HIV)-infected patients with HIV protease inhibitors (PIs) has been associated with serious lipid disturbances. However, the incidence and degree of impaired lipid metabolism observed in the clinic vary considerably between individual HIV PIs. Our previous studies demonstrated that HIV PIs differ in their ability to increase the levels of transcriptionally active sterol regulatory element-binding proteins (SREBPs), activate the unfolded protein response (UPR), induce apoptosis, and promote foam cell formation in macrophages. In the present study, we examined the effects of three HIV PIs, including amprenavir, atazanavir, and ritonavir, on the UPR activation and the expression of key genes involved in lipid metabolism in primary rodent hepatocytes. Both atazanavir and ritonavir activated the UPR, induced apoptosis, and increased nuclear SREBP levels, but amprenavir had no significant effect at the same concentrations. In rat primary hepatocytes, cholesterol 7α-hydroxylase (CYP7A1) mRNA levels were significantly decreased by atazanavir (38%) and ritonavir (56%) but increased by amprenavir (90%); 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase mRNA levels were increased by ampranavir (23%) but not by ritonavir and atazanavir; low-density lipoprotein receptor mRNA was increased by atazanavir (20%) but not by ampranavir and ritonavir. Similar results were obtained in mouse primary hepatocytes. Atazanavir and ritonavir also decreased CYP7A1 protein levels and bile acid biosynthesis, while amprenavir had no significant effect. The current results may help provide a better understanding of the cellular mechanisms of HIV PI-induced dyslipidemia and also provide useful information to help predict clinical adverse effects in the development of new HIV PIs.

Although the mechanisms underlying HIV PI-associated dyslipidemia are not fully understood, an increasing body of evidence suggests that multiple cellular mechanisms may be involved and individual HIV PI may have different effects on lipid metabolism (22).

The liver plays a central role in maintaining lipid homeostasis in the body. Under normal physiological conditions, lipid input is equal to lipid output from the body (20). Disruption of either the input or output pathways will result in dysregulation of lipid metabolism. Previous studies have shown that indinavir alters sterol and fatty acid metabolism in primary rat hepatocytes by increasing levels of activated sterol regulatory element-binding proteins (SREBPs), activate the unfolded protein response (UPR), induce apoptosis, and promote foam cell formation in macrophages. In addition, several studies have shown that perturbation of proteasome activities by HIV PIs may also contribute to dyslipidemia (27, 29, 35). Furthermore, den Boer et al. (12) recently reported that ritonavir specifically inhibited postheparin lipoprotein lipase lipase activity, which may decrease plasma triglyceride clearance and ultimately results in hyperlipidemia. Several clinical studies suggest that amprenavir and atazanavir are less likely than other HIV PIs to induce dyslipidemia (1, 17). However, the clinical significance of these observations in terms of decreased cardiovascular risk is still unknown because of limited clinical information. In addition, similar to other HIV PIs, atazanavir also can cause hyperglycemia, insulin resistance, and lipodystrophy, which are independent risk factors for cardiovascular disease (4, 25, 26, 39).

HIV PI-induced endoplasmic reticulum (ER) stress and subsequent activation of the unfolded protein response (UPR) may represent an important cell signaling mechanism of HIV PI-induced metabolic syndromes (28, 44). We have demonstrated that HIV PI ritonavir increases the accumulation of free cholesterol, depletes the ER calcium stores, activates the unfolded protein response (UPR), induces apoptosis, and promotes foam cell formation in macrophages (44). In addition, our recent studies show that all the HIV PIs, except amprenavir, activate the UPR and induce apoptosis to different extents in macrophages (Zhou H, Sirikalaya S, Gurley EC, Pandak WM, and Hylemon PB, unpublished data). However, the effect an individual HIV PI has on activation of the UPR, induction of cell apoptosis, and disruption of the lipid metabolism in primary hepatocytes has not been fully explored.

The objective of the present study was to compare the effects of three different HIV PIs on activation of the UPR, as well as
on lipid metabolism in primary rodent hepatocytes. The results show that both atazanavir and ritonavir activated the UPR and induced apoptosis in hepatocytes, but amprrenavir had no significant effect on UPR activation or lipid metabolism. These three HIV PIs also showed different effects on the expression of key genes involved in lipid metabolism, including CYP7A1, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA-R), and low-density lipoprotein receptor (LDL-R). The current results may help to provide a better understanding of the cellular mechanisms of lipid dysregulation induced by different HIV PIs and also provide useful information for the development of new therapeutic strategies to control HIV PI-associated clinical problems.

**MATERIALS AND METHODS**

**Materials.** Antibodies against C/EBP homologous protein (CHOP), activating transcription factor-4 (ATF-4), X-box-binding protein-1 (XBP-1), lamin B, SREBP-1, and SREBP-2 and horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against β-actin was from Calbiochem (San Diego, CA). Polyclonal antibody against mouse CYP7A1 was a generous gift from Dr. David W. Russell (The University of Texas, Southwestern Medical Center). Bio-Rad protein assay reagent, Criterion XT Precast Gel, HRP-conjugated goat anti-rabbit IgG and Precision Plus Protein KaleidoScope Standards were obtained from Bio-Rad (Hercules, CA). Amprenavir, atazanavir, and ritonavir were generous gifts from GlaxoSmithKline (Barnard Castle, Durham, UK), Bristol-Meyers-Squibb (New Brunswick, NJ), and Abbott Laboratories (Abbott Park, IL), respectively. Biomax MS films were obtained from Eastman Kodak (Rochester, NY). RNAqueous total RNA isolation kit and MAXiscript T7 and ribonuclease protection assay (RPA) II kits were from Ambion (Austin, TX). High-capacity cDNA archive kit and gene expression kits for rat LDL-R, CYP7A1, and HMG-CoA-R were from Applied Biosystems (Foster City, CA). CellTiter 96AQueous One Solution Reagent was from Promega (Madison, WI). All other chemical reagents were from Sigma (St. Louis, MO).

**Isolation and culture of primary hepatocytes.** Primary hepatocytes were isolated from adult male Sprague-Dawley rats (250–300 g) or C57BL/6 mice (20–25 g) using the collagenase-perfusion technique of Bissell and Guzelian (2). Trypan blue exclusion was used to determine cell viability (>90%) before plating monolayers on collagen-coated plates (60 mm). Unless otherwise indicated, cells were isolated and cultured in serum-free Williams’ E medium containing dexamethasone (0.1 μM), insulin (100 nM), penicillin (100 units/ml), and thyroneine (1 μM). Cells were incubated from 12 to 24 h in 5% CO2 environment at 37°C before additions were made to culture medium. Amprenavir, atazanavir, and ritonavir were dissolved in DMSO. HIV PIs were added directly to culture medium (final concentrations 5 to 20 μM) and incubated for 0.5 to 24 h.

**High-performance liquid chromatography assay of the metabolism of HIV PIs.** A HPLC system (System Gold, Beckman Coulter, Montreal, QC, Canada) and a Beckman C18 reverse-phase column (4.6 mm × 25 cm) were used in these experiments. Chromatographic separation was obtained using an analytical C18 column (5 μm) at room temperature under isotonic conditions. The mobile phase was acetonitrile: 20 mM sodium dihydrogenphosphate, pH 6 [60:40 (vol/vol)] and 0.025% triethylamine. The mobile phase was delivered at 1 ml/min. The HIV PI peaks were detected spectrophotometrically at 210 nm.

**Table 1. Sequence information of RPA probes for rat genes**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank No.</th>
<th>Probe Location, bp</th>
<th>Probe Length, bps</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP7A1</td>
<td>7549753</td>
<td>238–439</td>
<td>202</td>
</tr>
<tr>
<td>CYP27A1</td>
<td>30578395</td>
<td>647–998</td>
<td>352</td>
</tr>
<tr>
<td>HMG-CoA-R</td>
<td>296924</td>
<td>31–424</td>
<td>394</td>
</tr>
<tr>
<td>LDL-R</td>
<td>31345479</td>
<td>1023–1424</td>
<td>202</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>203701</td>
<td>129–429</td>
<td>301</td>
</tr>
</tbody>
</table>
internal control. Twenty micrograms of total RNA were used in all RPA assays. After RNase digestion, samples were fractionated on 5% acrylamide/8 M urea gels, and bands were visualized by autoradiography using Kodak Biomax MS film. The density of bands was analyzed using Image J computer software (NIH) (44) and normalized to rat cyclophilin.

Real-time quantitative PCR. Total RNA (5 µg) isolated from mouse primary hepatocytes was used for first-strand cDNA synthesis using a high-capacity cDNA archive kit. The mRNA levels of CYP7A1, HMG-CoA-R, and LDL-R were quantified using the specific gene expression assay kits for mouse CYP7A1, HMG-CoA-R, and LDL-R on an ABI PRISM7700 sequence detection system. The mRNA values for each gene were normalized to internal control β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control was calculated.

Bile acid synthesis by primary hepatocytes. The conversion of [14C]cholesterol into methanol-water-soluble material was determined using a high-capacity cDNA archive kit. The mRNA levels of CYP7A1, HMG-CoA-R, and LDL-R were quantified using quantitative real-time PCR on a roche LightCycler 2.0 system. The mRNA values were normalized to internal control β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control was calculated.

Statistical methods. Student’s t-test was used to analyze the differences between the sets of data. Statistics were performed using GraphPad Pro (GraphPad, San Diego, CA).

RESULTS

Metabolism of HIV PIs in rat primary hepatocytes. Pharmacokinetic studies have shown that most of the HIV PIs have very low systemic bioavailability and are metabolized primarily in the liver by cytochrome P-450 isoforms. Clinical observations indicate that HIV PIs are associated with the development of drug-induced liver injury (38). To optimize the experimental concentrations of HIV PIs in the present study, we first examined the metabolic rates of amprenavir, atazanavir, and ritonavir in primary rat hepatocytes by HPLC. As shown in Fig. 1, the rates of metabolism of tested HIV PIs were similar during the first 6 h of incubation. However, 89% of amprenavir was metabolized after 24 h, whereas larger amounts of atazanavir (45%) and ritonavir (64%) remained after 24 h (Fig. 1 and Fig. 2).

HIV PIs activate the UPR and induce apoptosis in rat primary hepatocytes. Clinical studies have indicated that HIV PIs appear to differ in their ability to cause hyperlipidemia (9, 22). Our previous studies also have shown that most HIV PIs, but not all, were able to activate the UPR and induce apoptosis in mouse macrophages (44). To examine whether HIV PIs have similar effects on the UPR activation in hepatocytes, cells were treated with HIV PIs (30 µM) for various time periods (1 to 24 h), and the expression of CHOP, ATF-4, and XBP-1 was detected by Western blot analysis. As shown in Fig. 3, both atazanavir and ritonavir significantly increased the expression of CHOP, ATF-4, and XBP-1. The expression levels of CHOP, ATF-4, and XBP-1 induced by atazanavir peaked at 2, 6, and 4 h, respectively. The expression levels of CHOP, ATF-4, and XBP-1 induced by ritonavir peaked at 2, 6, and 3 h, respectively. The induction of CHOP, ATF-4, and XBP-1 by atazanavir and ritonavir was concentration dependent (Fig. 4). At 15 µM, the expression of CHOP was increased 49% by atazanavir and 76% by ritonavir; ATF-4 was increased 43% by atazanavir and 63% by ritonavir. The basal level of XBP-1 expression was undetectable but dramatically increased after treatment with atazanavir and ritonavir. However, amprenavir did not significantly affect the expression of CHOP, ATF-4, and XBP-1 even at high concentrations (50 µM). HPLC analysis of amprenavir showed it to be >98% pure and eluted with the same hydrophobicity as reported in the literature (data not shown).

Activation of UPR has been implicated in processes that initiate apoptosis (3, 30). To further examine whether HIV PIs-induced UPR activation is correlated with the apoptosis in rat primary hepatocytes, we treated the cells with 25 µM of HIV PIs for 24 h; morphological changes characteristic of apoptosis were detected with confocal microscopy using Annexin V and propidium iodide staining. As shown in Fig. 5, both atazanavir and ritonavir induced apoptosis, but amprenavir had no significant effect. Our previous studies in macrophages suggest that HIV PIs have different effects on cell viability. In this study, we also compared the cytotoxic effects of atazanavir, atazanavir, and ritonavir on rat primary hepatocytes. As shown in Fig. 6, at 25 µM, the percentages of viable cells after 24 h treatment were 95% for amprenavir, 79% for atazanavir, and 74% for ritonavir, respectively.

Effect of HIV PIs on SREBPs in rat primary hepatocytes. The liver is the major organ responsible for maintaining lipid homeostasis in the body (20). SREBPs are membrane-bound transcription factors that play important roles in the regulation of lipid homeostasis. SREBPs directly activate dozens of genes dedicated to lipid metabolism. In mammalian cells, there are three SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a is highly expressed in cell lines and activates all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides (36). In contrast, SREBP-1c is the predominant isoform expressed in most tissues with especially high levels in the liver, white adipose tissue, skeletal muscle, adrenal gland, and brain. SREBP-1c primarily regulates the transcription of genes required for fatty acid synthesis but not cholesterol synthesis (36). SREBP-2 preferentially regulates cholesterol synthesis. SREBP-1c and SREBP-2 are the predominant isoforms expressed in the liver (19). Previous studies have shown that indinavir activates SREBP-1 and SREBP-2 in both rat primary hepatocytes and in the liver of intact mice (40). We also have shown that both atazanavir and ritonavir activate SREBPs in mouse macrophages (44). In this study, we compared the effects of three HIV PIs, amprenavir, atazanavir, and ritonavir,
on SREBP activation in rat primary hepatocytes. As shown in Fig. 7, after 18 h treatment with 30 μM of atazanavir and ritonavir, the mature forms of SREBP-1 were increased by 48% and 44%, respectively; in contrast, activated SREBP-2 levels were increased by only 13% and 8%, respectively. Amprenavir did not detectably increase either SREBP-1 or SREBP-2 levels. We also observed that activated SREBP levels were not altered by either atazanavir or ritonavir until after 12 h treatment and atazanavir and ritonavir induced SREBPs activation was concentration dependent (data not shown). These results were consistent with our previous studies with indinavir in rat primary hepatocytes (40).

Effect of HIV PIs on mRNA levels of key genes involved in lipid metabolism. Previous studies (40) have shown that indinavir decreases CYP7A1 mRNA levels and increases HMG-CoA-R mRNA levels, but it has no effect on CYP27 and LDL-R mRNA levels in rat primary hepatocytes. Indinavir also significantly decreases CYP7A1 mRNA half-life. In the current study, we examined the effects of three HIV PIs, amprenavir, atazanavir and ritonavir, on the mRNA levels of CYP7A1, HMG-CoA-R, LDL-R, and CYP27 in rat primary hepatocytes by RPA. Cells were treated with various concentrations of HIV PIs (0 to 100 μM) for 12 or 24 h, total RNA was isolated, and the specific mRNA species were quantified by RPA. As shown in Fig. 8A, amprenavir (25 μM) increased CYP7A1 mRNA by 90% and HMG-CoA-R mRNA by 23% after 24 h but had no significant effect on LDL-R and CYP27 mRNA levels. Atazanavir (25 μM) significantly decreased CYP7A1 mRNA by 38% and increased LDL-R mRNA by 20% but did not change the HMG-CoA-R mRNA level after 24 h treatments (Fig. 8B). At the high concentration (100 μM), atazanavir also decreased LDL-R and CYP27 mRNA levels. Ritonavir (25 μM) significantly decreased CYP7A1 mRNA by 56% but had no effect on HMG-CoA-R and CYP27. Ritonavir also decreased LDL-R mRNA at high concentrations (50 and 100 μM) (Fig. 8C).

We further confirmed the effects of HIV PIs on CYP7A1, HMG-CoA-R, and LDL-R mRNA expression in mouse primary hepatocytes using real-time PCR. We isolated mouse primary hepatocytes and treated the cells with individual HIV PI (25 μM) for 24 h; mRNA levels of HMG-CoA-R, CYP7A1, and LDL-R were quantified using real-time PCR. As shown in Fig. 9, amprenavir increased HMG-CoA-R and CYP7A1 mRNA levels by 23% and 53%, respectively, it but had no effect on LDL-R mRNA. Atazanavir decreased CYP7A1 mRNA by 60% and increased LDL-R mRNA by 54%, it but had no effect on HMG-CoA-R. Ritonavir decreased CYP7A1 mRNA by 49% but did not affect HMG-CoA-R and LDL-R mRNA levels. These results are consistent with the RPA results.
Effect of HIV PIs on protein levels of CYP7A1 in rat primary hepatocytes.

To further examine whether HIV PI-induced increase or decrease of mRNA level of CYP7A1 is correlated with the changes of protein expression, we performed the Western blot analysis using a polyclonal antibody against mouse CYP7A1. As shown in Fig. 10, both atazanavir and ritonavir significantly decreased CYP7A1 levels by 28%, but amprenavir had no effect.

Effect of HIV PIs on bile acid synthesis in rat primary hepatocytes.

Bile acid can be synthesized by either the “neutral” or “acidic” pathway in hepatocytes (11). To further determine whether HIV PI-induced downregulation of CYP7A1 expression is correlated with a decrease in bile acid synthesis in rat hepatocytes, the bile acid biosynthesis was estimated by measuring conversion of [14C]cholesterol to water-soluble radioactivity (21) after treatment with HIV PIs for 48 h. As shown in Fig. 11, atazanavir and ritonavir significantly inhibited bile acid synthesis by 47% and 67%, respectively, but amprenavir had no significant effect.

DISCUSSION

Since the introduction of HIV PIs into HAART, the mortality rate of HIV-infected patients has dramatically decreased. However, HAART has changed the clinical profile of HIV infection from a subacute lethal disease to a chronic ambulatory disease (41). Dyslipidemia specifically associated with HIV PIs, which are one of the cornerstones in HAART, has emerged as an important issue in HIV-infected patients. (9, 10, 37). More than 50% of the patients receiving HAART develop lipid abnormalities, including elevated levels of total serum cholesterol, LDL-cholesterol, and triglycerides, which are well-known risk factors for cardiovascular diseases (34). We and others have demonstrated that HIV PIs induced UPR, which may represent an important cell signaling mechanism of HIV PI-induced metabolic syndromes (28, 44).

In the present study, we compared the direct effects of three HIV PIs on activation of the UPR and hepatic lipid metabolism using rodent primary hepatocytes as a model system. Our results demonstrated that these HIV PIs, amprenavir, atazanavir, and ritonavir, had different effects on the UPR activation, cell apoptosis, and lipid metabolisms in primary hepatocytes. Pharmacokinetic studies have shown that all of the HIV PIs are metabolized in the liver by various isoforms of the cyto-
Drug-induced hepatotoxicity has been observed in patients undergoing HAART (38). Although the mechanisms of drug-induced liver injury are poorly identified, it is clear that HIV PIs are a contributing factor, and different HIV PIs may have different effects. The data from the current studies clearly showed that both atazanavir and ritonavir significantly induced apoptosis in rat primary hepatocytes, but amprenavir had no significant effect (Fig. 5 and Fig. 6). Apoptosis is a form of cell death that involves multiple pathways. In addition to a death receptor and mitochondrial pathways, ER stress-induced cellular death also plays an important role in regulating normal cell function (42). Our previous studies have demonstrated that HIV PIs activated the UPR and induced apoptosis, and different HIV PIs varied greatly in their ability to activate the UPR in macrophages (44). Recent studies done by Parker et al. (28) also demonstrated that HIV PIs induced an ER stress response in human HepG2 and TC5 hepatocytes cell lines and mouse 3T3-L1 adipocytes, suggesting that ER stress may contribute to HIV PI-induced lipodystrophy. It also has been shown that ritonavir increases endothelial permeability, decreases levels of tight junction proteins, and increases superoxide anion production. HIV PI-induced endothelial dysfunction represents one of the important mechanisms of vascular lesion formation and also contributes to HIV PI-associated cardiovascular diseases (8). The data presented in Fig. 3 show that atazanavir and ritonavir, but not amprenavir, activated the UPR in primary hepatocytes. Atazanavir- and ritonavir-induced UPR activation was concentration dependent (Fig. 4) and is consistent with our previous results in macrophages (44). Lack of the UPR activation in amprenavir-treated cells (both hepatocytes and macrophages) was not due to the decomposition of the drug. HPLC analysis showed that the purity of amprenavir used in our studies was > 98%, and the HPLC elution profile was the same as reported in the literature.
literature (data not shown). Drug metabolism analysis also indicated that the metabolic rate of these three HIV PIs were similar during the first 6 h of incubation (Fig. 1). Therefore, the different response appears to reflect the intrinsic properties of the individual HIV PI.

Cholesterol and lipid metabolisms are controlled by SREBPs, which are considered to be master regulators of lipid homeostasis (13). Our previous studies showed that indinavir increased the levels of transcriptionally active of SREBP-1 and SREBP-2 in both primary rat hepatocytes and in the liver of intact mice (40). In macrophages, we also found that HIV PIs markedly increased mature forms of nuclear SREBPs, which might contribute to an increase in intracellular lipids and foam cell formation (44). In the current study, we observed that both atazanavir and ritonavir increased the levels of mature SREBPs in hepatocytes (Fig. 7), but amprenavir had little effect. Because the activation of SREBPs by HIV PIs was detected after 12 h treatment and the metabolic rate of amprenavir after 6 h was much faster than that of atazanavir or ritonavir, a lower active drug concentration might contribute to the lower effect of amprenavir on SREBPs in hepatocytes. However,
Hepatocytes. CPY7A1 and CYP27 are the key enzymes in the two major pathways of bile acid biosynthesis: "neutral pathway" and "acidic pathway," respectively (20). Previous studies have shown that indinavir induces free intracellular cholesterol accumulation in rat primary hepatocytes. This is followed by upregulation of HMG-CoA-R and downregulation of CYP7A1 but no effect on CYP27 and LDL-R expression (40). In the present study, we found that HIV PIs had a different effect on CYP7A1 and HMG-CoA-R expression. Atazanavir and ritonavir significantly inhibited CYP7A1 mRNA and protein expression but had no effect on HMG-CoA-R, both in rat and mouse hepatocytes. Our previous studies have shown that indinavir decreased the half-life of CYP7A1 mRNA (40). We also found that both atazanavir and ritonavir decreased CYP7A1 mRNA half-life in rat primary hepatocytes (data not shown). Consistently, bile acid biosynthesis in atazanavir- and ritonavir-treated cells was significantly decreased. Although amprenavir significantly increased CYP7A1 mRNA level, it had no significant effect on CYP7A1 protein levels and rates of bile acid synthesis (Figs. 10 and 11). Clinical observation indicates that atazanavir and amprenavir seem to have less impact on the lipid profile in patients (4, 11, 16, 26). The results from the current study suggest that the increase of LDL-R expression by atazanavir and CYP7A1 expression by amprenavir may contribute, at least partially, to their lower impact on dyslipidemia. However, HIV PIs tested in vitro may have different biological activities in vivo because of the plasma protein-binding properties of these drugs. All of the HIV PIs currently used in clinical trials, except indinavir, are tightly bound to serum proteins. The concentration tested in vitro is much higher than the calculated free plasma concentration. The kinetics between plasma-bound and free HIV PIs in vivo is still not clear. Further in vivo investigations are needed to elucidate the cellular mechanisms of HIV PI-induced dyslipidemia.

In summary, we have demonstrated that individual HIV PIs have different effects on the UPR activation and expression of key genes involved in lipid metabolism in hepatocytes. HIV PI-induced UPR appears to contribute to lipid dysregulation. These in vitro models may be useful in predicting possible clinical adverse effects by HIV PIs on lipid metabolism in vivo.
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REFERENCES


