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In vivo administration of ritonavir worsens intestinal damage caused by cyclooxygease inhibitors

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ABSTRACT

The protease inhibitor ritonavir is part of the highly active anti-retroviral therapy (HAART) successfully used in the treatment of human immunodeficiency virus (HIV)-1 infection. There is evidence that ritonavir alters intestinal permeability and induces damage to the small intestine. Because HIV infected patients taking HAART are at high risk for developing cardiovascular complications, there might be a need for the use of low dose of aspirin (ASA) to prevent ischemic events. Similarly, long term survival exposes HIV infected persons to detrimental interactions of ritonavir with non-steroidal anti-inflammatory drugs (NSAIDs). In the present work we tested whether ritonavir worsens intestinal injury caused by NSAIDs and ASA. C57BL6 mice were treated for 25 days with ritonavir and for a further 5 days with the combination of ritonavir plus ASA or ritonavir plus naproxen. In a second set of experiments C57BL6 mice were cotreated with ritonavir plus misoprostol, a PGE_1 analog. We found that ritonavir administration caused intestinal damage and its co-administration with naproxen or ASA exacerbated the severity of injury and intestinal inflammation, as assessed by measuring haematocrit, MPO, mucosal levels of PGE_2 and mRNA levels of iNOS, MCP-1 and VLA-1. Co-administration of misoprostol protected against intestinal damage induced by naproxen and ritonavir. In conclusion we demonstrated that ritonavir causes intestinal damage and that its association with NSAIDs or ASA worsens the damage caused by COX-inhibitors. Misoprostol rescues from intestinal damage caused by ritonavir. Further studies are need to clarify whether this observation has a clinical readout.

1. Introduction

Gastrointestinal ulcerations and bleedings caused by acetylsalicylic acid (ASA) and traditional non steroidal anti-inflammatory drugs (tNASIDs) are well recognized complication related to the use of these agents occurring at a rate of ≈3% (Abraham et al., 2010; Santucci et al., 1995). Despite the inhibition of gastric acid secretion by proton pump inhibitor (IPP) has been demonstrated effective in reducing the incidence of gastro-duodenal ulcers and gastroduodenal bleeding, this approach has been shown to be poorly effective in protecting against intestinal injury. The basic mode of action of ASA and non selective NSAIDs lies in the inhibition of cyclo-oxygenases (COX), with COX-2 generating prostaglandins at site of inflammation and COX-1 being responsible for generation of prostaglandins involved in protecting the gastrointestinal mucosa (Catella-Lawson et al., 2001; Fiorucci et al., 2007, 2003). Thus, while tNSAIDs are thought to injury the gastrointestinal tract through a COX-1 related mechanism, selective COX-2 inhibitors effectively reduce both gastro-duodenal and intestinal complications, but their use associates with an increased risk to develop cardiovascular ischemic events (Catella-Lawson et al., 2001; Fiorucci et al., 2007, 2003). Because the above limitations, there is still a need for the discovery of safer NSAIDs (Fiorucci et al., 2007, 2003; Fiorucci, 2001) or effective co-treatments that could limit intestinal damage in specific subset of NSAID-taking patients.

Protease inhibitors (PI) as a part of highly active anti-retroviral therapy (HAART) have been used successfully in the treatment of human immunodeficiency virus (HIV)-1 infection. Incorporation of HIV protease inhibitors in the HAART causes profound and sustained suppression of viral replication, significantly reduces the morbidity and mortality, and prolongs the lifespan of patients with HIV infection (Riddle et al., 2001; Moyle and Carr, 2002; Spector, 2003). HAART has changed the clinical profile of HIV infection from a sub-acute lethal

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disease to a chronic ambulatory disease (Clevenbergh et al., 2003). Despite its efficacy in controlling the disease progression, the use of PI therapy associates with an increased risk of development of premature atherosclerosis. An increasing body of evidence suggests that treatment of HIV-infected patients with HIV PIs causes a dyslipidemia which contributes to the development of cardiovascular diseases (Spector, 2003; Clevenbergh et al., 2003; Cohen, 2005). A significant increase in plasma triacylglycerols and total cholesterol concentrations, often associated with abnormal body fat distribution and peripheral insulin resistance (hyperinsulinemia, hyperglycemia and diabetes mellitus), has been detected in HIV PIs-treated patients (Spector, 2003; Clevenbergh et al., 2003; Cohen, 2005; Dubé, 2000) making these subjects putative candidates for the treatment with ASA and/or anti platelet drugs. HIV PI causes gastrointestinal adverse effects including abdominal pain and diarrhea (Wallace and Brann, 2000). Recent studies have shown that the two of most commonly used HIV PIs, ritonavir and lopinavir, trigger apoptosis of intestinal cells by activating endoplasmic reticulum (ER) stress, thus disrupting the intestinal epithelial barrier integrity both in *in vitro* cell cultures and in mice (Wu et al., 2010).

Because tNSAIDs and ASA might be needed in long-term survival HIV infected persons we have designed a study to investigate whether ritonavir exacerbates intestinal injury caused by tNSAIDs and ASA (Zhang et al., 2013; Wallace et al., 2011) and define mechanisms involved in these interactions.

2. Materials and methods

2.1. Materials

Aspirin (ASA), naproxen, ketoprofen, misoprostol and ritonavir were purchased from Sigma. Prostaglandins E1 and E2 (PGE_1 and PGE_2) were purchased from Cayman Chemical.

2.2. Cells

HT29 cells, a human colon adenocarcinoma cell line, were cultured in RPMI-1640 medium containing 10% FCS, 1% glutamine and antibiotics.

2.3. In vitro detection of apoptosis

To test whether ritonavir, TNFα and naproxene drive colon cells to apoptosis HT29 (300.000 cells/wells) were incubated 36 h with medium alone or with the following reagents: ritonavir (15 μM), naproxen (50 μM), TNFα (100 ng/ml), the combination of ritonavir plus naproxen or ritonavir plus TNFα or ritonavir plus naproxene plus TNFα. To test whether ritonavir, TNFα and ketoprofen drive colon cells to apoptosis HT29 (300.000 cells/wells) were incubated 12, 24, 36 and 48 h with medium alone or with the following reagents: ritonavir (15 μM), ketoprofen (50 μM), TNFα (100 ng/ml), the combination of ritonavir plus ketoprofen or ritonavir plus TNFα or ritonavir plus ketoprofen plus TNFα. To investigate the effect of PGE_1 and PGE_2 on Ritonarir induced apoptosis HT29 cells were incubated 36 h with ritonavir (15 μM) alone or in the presence of prostaglandins PGE_1 (1 μM) or PGE_2 (5 μM).

Apoptosis was detected by staining the cells with propidium iodide (PI). Briefly, cell pellets were washed twice in PBS, resuspended in hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate + 0.1% Triton X-100), kept 4–8 h at 4 °C in the dark, and analyzed using a Epics XL flow cytometer (Beckman-Coulter, Miami, FL). The percentage of apoptotic cells was determined by evaluating hypodiploid nuclei after proper gating on DNA content.

2.4. Assessment of caspase 3 and 8 activity

After incubation with appropriate agents HT-29 cells were recovered into lysis buffer (10 mM Tris–HCl (pH 7.3), 25 mM NaCl, 0.25% Triton X-100, 1 mM EDTA). After centrifugation at 13,000 rpm for 30 min at 4 °C, the resulting supernatants were adjusted to 1 mg/ml with lysis buffer and 25 μg total proteins were incubated in 100 μl of caspase buffer (50 mM HEPES (pH 7.2), 100 mM NaCl, 1 mM EDTA (pH 8.0), 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 1 mM DTT) with various fluorogenic substrate peptides (100 μM) including acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl-coumarin) (Ac-DEVD-AFC) for caspase-3 and Ac-Ile-Glu-Thr-Asp-AFC (Ac-IETD-AFC) for caspase-8. Caspase-3 and -8 activities were assayed using a fluorimeter plate reader in kinetic mode with excitation and emission wavelengths of 405 and 519 nm, respectively, continuously measuring release of AFC from substrate peptides (Fiorucci et al., 2003).

2.5. Animal protocols

C57BL6 were from Harlan Nossan (Udine, Italy). Mice were housed under controlled temperatures (22 °C) and photoperiods (12:12-h light/dark cycle), allowed unrestricted access to standard mouse chow and tap water and allowed to acclimate to these conditions for at least 5 days before inclusion in an experiment. Protocols were approved by the University of Perugia Animal Care Committee. The ID for this project is #98/2010-B. The authorization was released to Prof. Stefano Fiorucci, as a principal investigator, on May 19, 2010.

2.6. Intestinal damage, intestinal mucosal prostanoids, haematocrit and MPO

Groups of six mice (not fasted) were randomized and treated as follows: control group: mice received vehicle alone (a solution of 1% methylcellulose); ritonavir group: mice were treated daily for 25 days with ritonavir (50 mg/kg/day/OS); ASA group: mice were treated for five days with ASA (50 mg/kg/day/OS); naproxene group: mice were treated for five days with naproxen (100 mg/kg/OS); ritonavir plus ASA group: mice were administered 25 days with ritonavir (50 mg/kg/day/OS) and for other five days with the combination of ritonavir plus ASA (50 mg/kg/day/OS); ritonavir plus naproxen group: mice were administered 25 days with ritonavir (50 mg/kg/day/OS) and for other five days with the combination of ritonavir plus naproxene (100 mg/kg/OS). In a second set of experiment C57BL6 mice were randomized and treated as follows: control group: mice received vehicle alone (a solution of 1% methylcellulose); ritonavir group: mice were treated for 25 days with ritonavir (50 mg/kg/day/OS); naproxene group: mice were treated for five days with naproxen (100 mg/kg/OS); ritonavir plus naproxene group: mice received 25 days ritonavir (50 mg/kg/day/OS) and for other five days with the combination of ritonavir plus naproxen (100 mg/kg/OS); ritonavir plus misoprostol: mice received 25 days ritonavir (50 mg/kg/day/OS) and for other five days with the combination of misoprostol (100 μg/kg/OS) plus ritonavir. Six hour after the final administration of drugs, the mice were anesthetized with sodium pentobarbital (100 mg/kg) and blood sample was drawn, by intracardiac puncture, for determination of haematocrit (Fiorucci et al., 2003). The small intestine was excised, and the extent of haemorrhagic damage to the small intestine blindly quantified, under a microscope, by measuring the lengths of each lesion in mm and then summing these to obtain a damage score for each mice (Fiorucci et al., 2003). Generation of PGE_2 by gastric and intestinal mucosa was measured according to previously published methods (Fiorucci et al., 2003), using a specific ELISA kit (Cayman Chemical Company, Ann Arbor, MI). Gastric MPO activity was measured using a spectrophotometric assay with tri-methylbenzidine (TMB) as a substrate. Activity is expressed as mU per mg protein.