In a previous experiment about rat in situ intestinal absorption with propranolol (PL) ester derivatives as a model compound, we have found that about 90% of the compounds absorbed in the mesenteric vein was a metabolite hydrolyzed by esterases during a first-pass in the intestine. However, a major intestinal esterase contributing the first-pass metabolism has not been clear. In this study, we performed a rat in situ intestinal absorption experiment with PL ester derivative after a treatment with bis(p-nitrophenyl)-phosphate (BNPP), an specific carboxylesterase inhibitor. The damage against other enzymes according to the treatment with BNPP was evaluated by the activity of aminopeptidase existing in the intestinal brush border membrane. More than 70% of the rat intestinal first-pass metabolism of PL ester derivative was inhibited by the treatment of BNPP under a condition without any damage against aminopeptidase, although about 90% of the hydrolysis of PL ester derivative was inhibited by BNPP in rat intestinal homogenate 9,000 × g supernatant (S9). These data suggest that carboxylesterase contributes the intestinal first-pass metabolism in rat. Also, in vitro inhibition experiment showed that a major esterase involved in hydrolase activity was carboxylesterase in human intestinal S9. A localization of esterase along with the intestinal length was studied in order to demonstrate a contribution of carboxylesterase in the human intestinal hydrolysis. Interestingly, the nearly same esterase activity was observed in the both jejunum and ileum, although P450 activities were higher in the jejunum than in the ileum, as previously reported. Consequently, it was demonstrated that carboxylesterase mainly contributed in the intestinal first-pass hydrolysis in rat and human.