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DIRECT EVIDENCE FOR THE INVOLVEMENT OF FREE RADICALS IN ISCHEMIC INSULT TO THE INTESTINE

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Ischemia of rat intestine was induced in vivo by occlusion of the superior mesenteric artery (SMA) for 15 min. Sodium salicylate, 100 mg/kg, given IP, 30 min prior to the ischemic event served as a specific trap for hydroxyl radicals and provided *direct evidence* for the involvement of free radicals during the ischemic insult. Portions of the bowel were sequentially isolated and removed. The hydroxylation products, dihydroxybenzoic acid (DHBA) derivatives were isolated, identified and qunatified by HPLC coupled with electrochemical detection (ECD). The level of 2,5-DHBA (Mean ± SE, ng/g tissue) in the preischemic bowel ($N = 21$) was 241.8 ± 10.0. It rose significantly to 313.3 ± 15.5 in the ischemic specimen ($p = 0.0129$) and remained unchanged in the reperfusion period (322.8 ± 15.5). The histological examination correlated well with these levels: mild villi damage in the ischemic period with no further damage in the reperfusion period.

KEY WORDS: Free radicals, intestinal ischemia

INTRODUCTION

The causative role of oxygen derived free radicals in the pathogenesis of injury associated with ischemia and reperfusion of many tissues has been the subject of intense research in the last decade.[1] The supporting data for these theories are mainly indirect. *Direct* evidence for the presence of free radicals in an ischemia-reperfusion system and the causative relationship between free radicals and the damage is still lacking. A newly developed methodology is now available, where a specific hydroxyl radical trap is used, and it produces stable hydroxylation products that can be specifically identified and quantified with high sensitivity using high pressure liquid chromatography (HPLC) coupled with electro-chemical detection (ECD).[2,3]

The aim of this study was to provide *direct evidence* for the involvement of the hydroxyl radical in in vivo ischemia-reperfusion processes. A correlation between the free radical level and the level of histological changes caused by this process has been made. This correlation could serve to assess the causative role of free radicals in ischemic injury and may serve as a basis for the evaluation of new treatment modalities of this grave disorder.

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MATERIALS AND METHODS

The rat model

Studies were carried out in male rats weighing 70–90 g. Animals were housed in cages supplied with routine animal feed and water. Antibiotics were not used. The rats were anesthetized with ether. Rectal temperature was monitored and maintained at 37°C. Ischemia was induced by occluding the superior mesenteric artery with a microvascular "bulldog" clamp for 15 minutes. Portions of the bowel were sequentially isolated with clamps and removed for further analysis. These bowel samples were taken prior to the application of the microvascular clamp to the SMA ('preischemic sample'), 2 minutes prior to the termination of the ischemic period ('ischemic sample') and 10 minutes after the termination of the ischemic period ('reperfusion sample'). Sodium salicylate served as a specific trap for hydroxyl radicals (salicylic acid, sodium salt, Gold Label, Aldrich).[2,3] It was injected in a saline solution, intraperitoneally, 100 mg/kg, 30 minutes prior to the ischemic event. Dihydroxy benzoic derivatives *(DHBA)* are thus formed by hydroxylation of salicylate and are specifically identified and quantitated by *high performance liquid chromatography (HPLC)* coupled with *electro chemical detection (ECD)*.[2,3]

Statistical analysis of the results

Repeated Measures Analysis of Variance was used for the evaluation of the results.

RESULTS

Ischemic time of 15 minutes produced damage to the tips of the intestinal villi with epithelial denudation and some inflammatory reaction (Figure 1). This histological picture was evident already prior to the termination of the 15 minutes ischemic event. There was no progression of the histological damage during the reperfusion phase. A typical chromatogram of the HPLC-ECD analysis of bowel specimen is shown in Figure 2. The specific peak of 2,5-dihydroxy benzoic acid (2,5-DHBA) is easily resolved, identified and quantified. The level of 2,5-DHBA in the bowel specimens taken at various times prior to the ischemic insult, during ischemia, and in the reperfusion phase are depicted in Table I. Mean ($\pm$SE) of 2,5-DHBA prior to the ischemia was 241.8 + 10.0 ng/g tissue. The mean 2,5-DHBA level *increased significantly* during the ischemic period to a mean level of 313.3 $\pm$ 15.5 ng/g tissue. Analysis of Contrasts showed that the difference between the preischemic and ischemic levels was significant ($F = 7.46$, $DF = 1,20$ $P = 0.0129$). No further change in the mean 2,5-DHBA level was recorded in the reperfusion period. The mean level, 10 minutes following declamping of the SMA was 322.8 $\pm$ 15.5 ng/g tissue.

DISCUSSION

The present study provides for the first time a *direct* evidence for the involvement of hydroxyl radicals in the ischemia-reperfusion injury of the intestine. The high rate of the intestinal metabolism produced a relatively high basal level of DHBA. Neverthe-

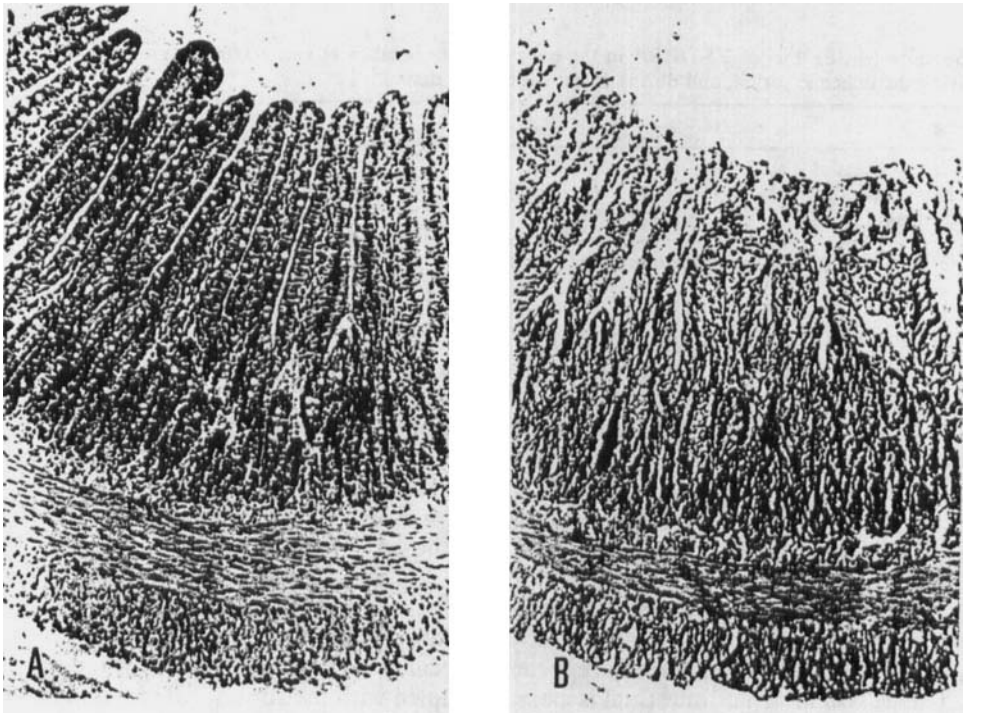

FIGURE 1. A - Non-ischemic bowel with normal architecture. B - Histology of the bowel following 15' of ischemia. Epithelial denudation of the tips of the villi with some inflammation is evident. (H & E stain; magnification X 160).

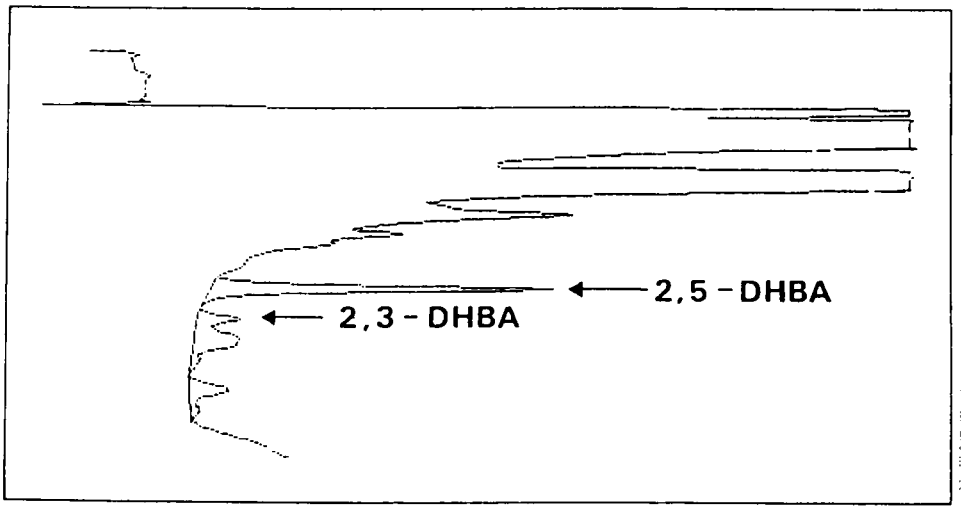

FIGURE 2. A typical HPLC-ECD chromatogram of 10' reperfused bowel. The specific peaks of 2,5-dihydroxy benzoic acid (2,5-DHBA) can be seen.

TABLE I

The mean (± SE) level of 2,5-DHBA in the bowel specimens taken at various times: prior to ischemia, during the ischemic period, and during reperfusion (ng/g tissue)

Time	N	2,5-DHBA
Prior to ischemia	21	241.8 ± 10.0
Ischemic period	21	313.3 ± 15.5*
Reprefusion period	21	322.8 ± 15.5*

*P = 0.0129 (compared to the level prior to ischemia)

less, this level increased significantly during the ischemic phase (P 0.0129). A similar basal level of DHBA has recently been found in the gerbil brain.[4] There too, the basal level was attributed to enzymatic hydroxylations of salicylate.[5] This application of HPLC-ECD is a newly developed methodology which is highly sensitive, with a detection limit of less than 1 ng DHBA. Nevertheless, the use of this methodology in biological samples is gaining credence and further refinements are currently under investigation. The model used in this study involves total arterial occlusion for a rather short period of time. This period was chosen to produce persistent but mild injury to the intestinal villi. This animal model enables sequential sampling of the intestine while the experimental conditions remain undisturbed. Thus, it offers an *internal control level* of DHBA, that further reinforces the validity of the results. The 2,5 DHBA serves as a bona fide reporter molecule for the flux of hydroxyl radicals.

This *in vivo* model of intestinal ischemia, coupled with the sophisticated methodology of free radicals determination may also serve as a useful tool to examine novel treatment modalities for ischemic bowel disorders.

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