ROLE OF OXYGEN-FREE RADICALS ON THE MOTILITY OF RAT ILEUM EFFECTS OF XANTHINE PLUS XANTHINE OXIDASE

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Abstract - The major objective of the present study was to characterize the effects of oxidants generated by xanthine (X) plus xanthine oxidase (XO) on isolated rat ileum motility. The effects of three concentrations of X/XO on the basal tone of the rat ileum preparation were studied for 20 minutes. Developed tensions were measured in mg/mg tissues and then expressed as percentage of baseline tension. Also the effects of 2X concentration of X/XO in the presence and absence of superoxide dismutase, catalase, mannitol, histidine, and deferoxamine were evaluated. The results were expressed as mean ± SE. Xanthine plus xanthine oxidase produced relaxation of ileum. Superoxide dismutase (a superoxide anion metabolizer) and catalase (a hydrogen peroxide scavenger) did not protect ileum from effects of X/XO, suggesting that neither superoxide anion nor hydrogen peroxide involve in X/XO-induced relaxation of ileum. The results of this study suggest that hydrogen peroxide formed extracellularly by X/XO may enter the cells and interact with intracellular iron to form a highly reactive oxidant, hydroxyl radical. The finding that two powerful hydroxyl radical scavengers, dimethylthiourea (DMTU) and mannitol offered protection against X/XO-induced relaxation of ileum suggest formation of hydroxyl radical within the cells. Pretreatment with deferoxamine, a potent iron chelator, reduced the relaxation of ileum, indicating that hydroxyl radical plays an important role in mediating the X/XO-induced relaxation of ileum. In addition, the ability of exogenously administered histidine to reduce relaxation suggests that singlet oxygen is another oxygen derivative which is responsible for relaxation of ileum-induced by X/XO.

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Key Words: Isolated ileum muscle, xanthine, xanthine oxidase, hydroxyl radical, singlet oxygen, superoxide dismutase

INTRODUCTION

Several diseases in the gastrointestinal tract are characterized by the formation of oxygen-free radicals, e.g. ischemia and reperfusion (1) and inflammation (2). The main source of oxygen radical formation in the case of ischemia and reperfusion is xanthine oxidase which, combined with accumulation hypoxanthine, leads to substantial superoxide anion ($O^{\circ-}2$) formation upon reperfusion (2).

Hydroxyl radicals (°OH) generated from $O^{\circ-}2$ by Haber-Weiss or Fenton-type reaction have also been implicated as toxic products formed by the xanthine/xanthine oxidase system (3). However, the formation of hydroxyl radical from superoxide anion and hydrogen peroxide requires the presence of iron. Superoxide anion and xanthine oxidase reaction have been shown to cause release of iron from ferritin (4,5).

Singlet oxygen ($^1O_2$) is another oxygen derivative which has also been suggested as a toxic agent that can be generated from $O^{\circ-}2$ in the xanthine/xanthine oxidase system (6).

The most vulnerable region of the gastrointestinal wall in ischemia/reperfusion and inflammation is mucosa (1). However, changes in the intestinal motility have been reported as a consequence of inflammation (7), which implies that oxygen free radicals can disturb the smooth muscle function.

According to the above studies, the aim of this study was to establish which oxygen free radicals are generated by the X/XO system by using different antioxidants, and to establish whether these oxygen free radicals produce changes in intestinal motility. Therefore, the effects of X/XO on ileum smooth muscle in the presence or absence of superoxide dismutase, catalase, dimethylthiourea and mannitol, and histidine were evaluated. Also to find whether intracellular iron plays a role in X/XO-induced changes in ileum motility, the effect of X/XO in the presence and absence of deferoxamine, an iron chelator, was evaluated.

MATERIALS AND METHODS

Measurement of tension and functional experiments on isolated rat ileum:

The experiments were conducted on isolated rat ileum suspended in 10 ml organ bath containing Krebs-Ringer solution. Male Wistar rats (200-250 g) were anesthetized with a intra-peritoneal injection of pentobarbital sodium (60 mg/kg). The abdomen was opened, a length of ileum was removed and placed in a dish containing cold Krebs-Ringer solution (20). The mesentery was trimmed away and 1.5-2.0 cm strips were cut from the length of ileum. After rinsing the contents of strips, one end of the strip was tied to a stainless steel wire stirred up at the 10 ml organ bath filled with Krebs-Ringer solution, while the other end was attached to a Grass FT-03 force displacement transducer attached to a Grass polygraph recorder for recording responses (contraction or relaxation) of muscle strips. The solution in the bath was maintained at 37°C and constantly bubbled with a mixture of 95% O_2 and 5% CO_2. All samples were allowed to equilibrate for 60 minutes at a resting tension of 1.0 g and were washed at 15 minutes intervals. Acetylcholine (0.5 mg/ml), which produces a sub maximal contraction of ileum, was used in all experiments to assess the viability of the preparations. The response of the preparations to acetylcholine was recorded for 5 minutes and the preparations were washed at 5 minute intervals until the tension reached the resting level. In the functional experiments, X/XO and its effects on the rat ileum were monitored for a total period of 20 minutes. After this treatment the preparations were washed every 5 minutes until the tension reached the resting level. Subsequently, acetylcholine was added to the organ bath and response was recorded for 5 minutes. In another experiment, antioxidants were added 20 minutes prior to the addition of X/XO with the exception of SOD and catalase which were added 3 minutes prior to addition of X/XO. Developed tensions were measured in mg/mg tissues and then expressed as percentage of baseline tension. All solutions made in Krebs-Ringer buffer solution, and 0.1-0.5 ml of solutions were added to the bath to elicit responses.

Generation of oxygen-free radicals:

Xanthine (X) and xanthine oxidase (XO) were used to generate $O°2$ (8). Superoxide anion is produced by the following reaction :

$$\text{Xanthine} + 2O2 \xrightarrow{\text{XO}} 2O°2 + 2H+ + \text{uric acid}$$
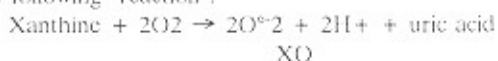
The three concentrations of X and XO used in these studies were termed 1X, 2X, and 4X as follows (8):

(1X)-xanthine (10 mM) and xanthine oxidase (0.025U/ml);

(2X)-xanthine (20 mM) and xanthine oxidase (0.05U/ml);

(4X)-xanthine (40 mM) and xanthine oxidase (0.10U/ml).

Solutions of xanthine were made in Krebs-Ringer solution and 0.1 to 0.4 ml of the solution was added in the organ bath to elicit responses. Xanthine oxidase was obtained from the Sigma Chemical Company as a suspension from milk (0.1 U/mg protein) which was added directly into the bath containing the ileum segments.

Protocol

Six group of animals were used for this study. Six animals were examined for each groups.

Group I.

The effects of three concentrations (1, 2,and 4X) of X/XO on the basal tone of the ileum preparation were studied for 20 minutes.

Group II:

The effects of 2X concentration of X/XO were investigated in the presence and absence of SOD. SOD (100 U/ml) was added to the organ bath containing the ileum tissues 3 minutes prior to the addition of X/XO. The effects were monitored for 20 minutes.

Group III:

To assess the role of hydrogen peroxide in the response induced by X/XO, catalase was used. Catalase (500 U/ml) was added to the bath 3 minutes before addition of X/XO. The effects were monitored for 20 minutes.

Group IV:

The effects of 2X concentration of X/XO in the presence and absence of various concentrations of dimethylthiourea and mannitol on the basal tone were evaluated for 20 minutes. These scavengers were added to the bath 20 minutes before addition of X/XO.

Group V:

To evaluate the role of iron in the X/XO-induced relaxation, the iron chelator deferoxamine was used. The effects of 2X concentration of X/XO in the presence and absence of deferoxamine (50 and 100 mM) were investigated for a period of 20 minutes. Deferoxamine was added to the bath 20 minutes before addition of X/XO.

Group VI:

The effects of X/XO in the presence and absence of histidine were examined for 20 minutes. Histidine (100 and 200 mM) was added to the organ bath 20 minutes prior to addition of X/XO
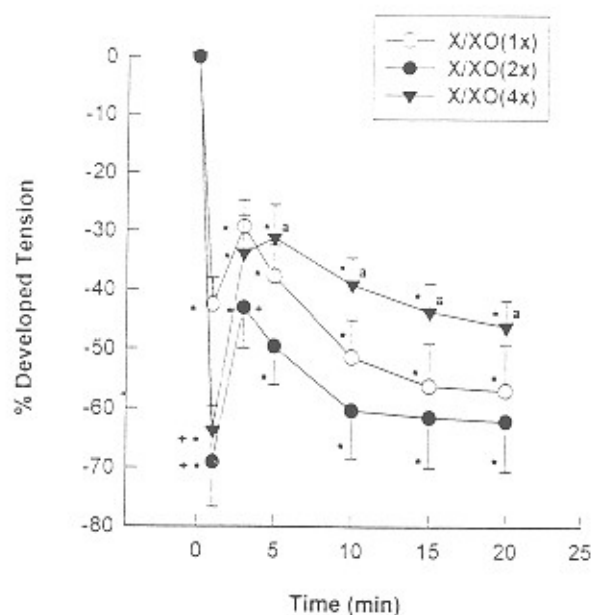
Fig. 1. Effects of various concentrations of xanthine plus xanthine oxidase (1x, 2x, and 4x) on basal tone of ileum preparations.

Results are expressed as mean ± SE

* P < 0.05, comparison of values at different times with respect to values at "0" time within groups.

+ P < 0.05, 1x vs 2x or 4x.

a P < 0.05, 2x vs 4x

STATISTICAL ANALYSIS

The results were expressed as mean ± standard error (±S.E.). The data were analyzed by two-way analysis of variance (ANOVA) using repeated measures followed by the test of least significant difference (LSD) (9). For comparison within the groups, one-way ANOVA was used. A P value of <0.05 was considered significant.

RESULTS

Effects of X/XO on Ileum Preparations

The summary of results of the three concentrations of X/XO are shown in Figure 1. Xanthine plus xanthine oxidase produced relaxation of ileum preparations. The response of tissue to exogenous addition of X/XO was a biphasic, with an initial relaxation occurring at approximately 1 minute followed by a second relaxation maximum at 20 minutes. Relaxation was concentration-dependent up to 2X concentration of X/XO; the relaxation with 4X was smaller than with the 2X concentration. The resting tension returned to baseline after repeated washing, and the time required for ileum strips to fully return to baseline was roughly 15-30 minutes post exposure to X/XO.
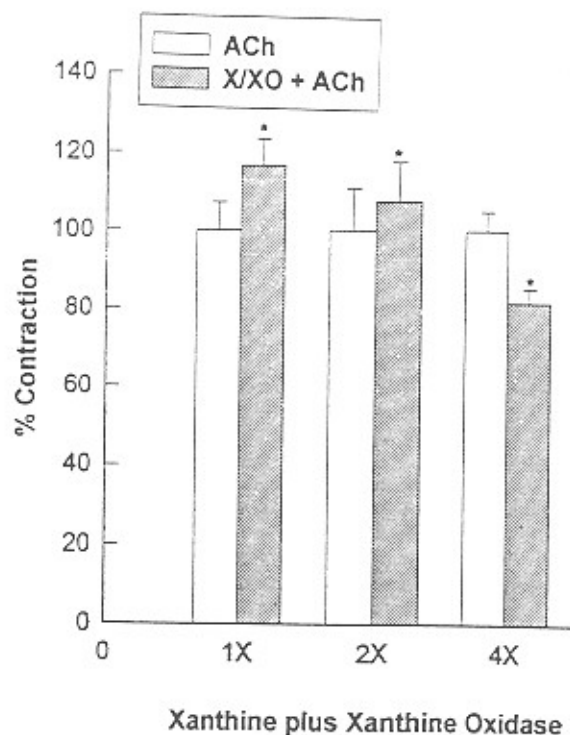

Fig. 2. Effects of acetylcholine (Ach, 0.5 μg/ml) in absence or presence of three concentrations of xanthine plus xanthine oxidase (X/XO) on basal tension of ileum preparation.

Results are expressed as % change from maximal Ach response taken as 100%.

* P < 0.05, comparison of values after exposure to various concentrations of X/XO with respect to values before exposure to X/XO within groups.

Experiments were also conducted to determine whether the responses to acetylcholine (Ach) were affected by the presence of X/XO. The contractile response to Ach (0.5 mg/ml) was tested before and after treatment of tissues with X/XO. The results of ACh response in absence and presence of three concentrations of X/XO are summarized in Figure 2. The responses to Ach following X/XO exposure were greater for 1X, and 2X; and smaller in magnitude for 4X than the initial ACh contraction.

Effect of superoxide dismutase on X/XO-induced relaxation of ileum :

The pretreatment with SOD did not prevent the relaxing effect of X/XO (Figure 3).

Effect of catalase on X/XO-induced relaxation of ileum

The tension developed in the presence of catalase was not significantly different from that developed in its absence (Figure 3)

Effect of superoxide dismutase plus catalase on X/XO-induced relaxation

There was no significant difference in X/XO-induced relaxation of ileum in the absence or presence of SOD+ CAT (Figure 3).
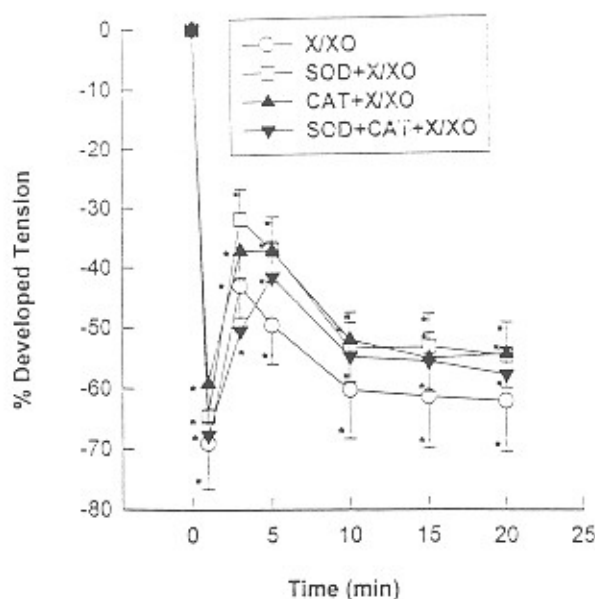

Fig. 3. Effects of 2X concentration of xanthine plus xanthine oxidase (X/XO) in absence or presence of 100 U/ml superoxide dismutase (SOD), 500 U/ml catalase (CAT), and SOD (100 U/ml) + CAT (500 U/ml) on ileum preparations.

Results are expressed as mean ± SE

* $P < 0.05$, comparison of values at different times with respect to values at "0" time within groups.

Effect of dimethylthiourea on X/XO-induced relaxation of ileum

High concentrations of DMTU were used because the site of hydroxyl radical generation was not clear, and hydroxyl radical had high reactivity, reacting with molecules within a 14 Ao ranges in a period of less than 10-6 seconds. Therefore, for an °OH scavenger to be effective, it must be present in concentrations so that it will comprise a significant proportion of the total molecules. The effects of 2X concentration of X/XO in the absence and presence of 1.6 mg/ml DMTU are summarized in Figure 4. The results showed that pretreatment with DMTU reduced the initial and second maximum relaxation. Also in the presence of DMTU, the timeframe of peak tension was similar to controls (X/XO). DMTU alone produced small relaxation which was maximal at 15 minutes after which remained constant during 40 minutes of treatment.
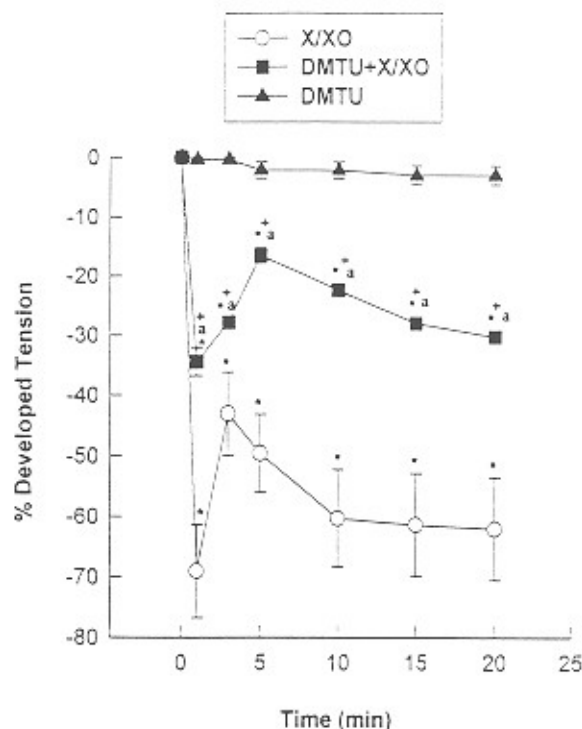

Fig. 4. Effects 2X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of dimethylthiourea (DMTU) on ileum preparations.

Results are expressed as mean ± SE

* $P < 0.05$, comparison of values at different times with respect to values at "0" time within groups.

+ $P < 0.05$, X/XO vs DMTU (1.6 mg/ml) + X/XO

a $P < 0.05$, DMTU vs DMTU + X/XO

Effect of mannitol on X/XO-induced relaxation of ileum

To further ascertain the role of hydroxyl radical in X/XO-induced relaxation of ileum, another scavenger of hydroxyl radical, mannitol, was studied. Figure 5 summarizes the effects of 2X concentration of X/XO on rat ileum in the absence and presence of mannitol (80mM).

Mannitol by itself produced small contraction after 5 minutes which remained constant during 40 minutes of treatment. There was a significant difference between control (X/XO) and mannitol treated groups (MO +X/XO). These results suggest that the relaxation is mediated through the hydroxyl radical.
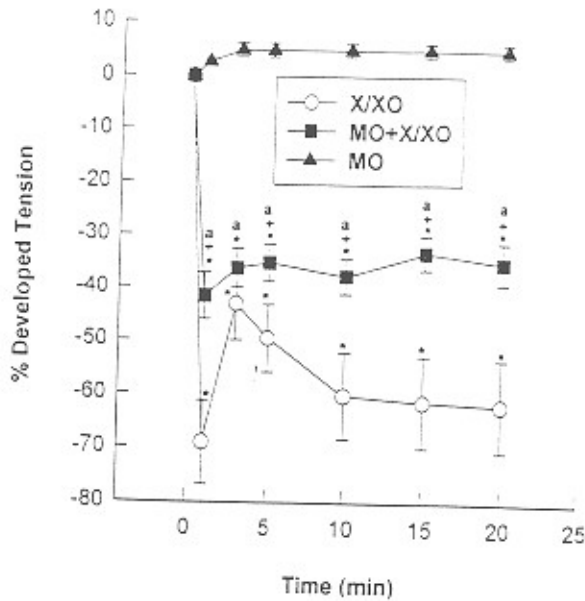
Fig. 5. Effects of 2X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of mannitol (MO) on ileum preparations.

Results are expressed as mean ± SE

* P < 0.05, comparison of values at different times with respect to values at "0" time within groups.

+ P < 0.05, X/XO vs MO (80 mM) + X/XO

a P < 0.05, MO vs MO + X/XO

Effect of deferoxamine on X/XO-induced relaxation of ileum

To assess whether intracellular iron plays a role in relaxation-induced by X/XO, the iron chelator deferoxamine was used. The effects of 2X concentration of X/XO in the absence and presence of deferoxamine are summarized in Figure 6.

Deferoxamine significantly reduced the relaxation induced by X/XO, and this effect was concentration-dependent. Deferoxamine alone produced small contraction of tissues during the experiments. The response of tissues to ACh before and after treatment of tissues with deferoxamine were not significantly different, which meant that deferoxamine at these concentrations did not have damaging effect on ileum. In the presence of deferoxamine (50 mM) ileum tension was not significantly different from control groups (X/XO) in the first 10 minutes, however, the tension was significantly different at 15 and 20 minutes. Pretreatment with deferoxamine (100 mM) reduced the relaxation at all times significantly. This finding suggests that hydroxyl radical is involved in the relaxation induced by X/XO.
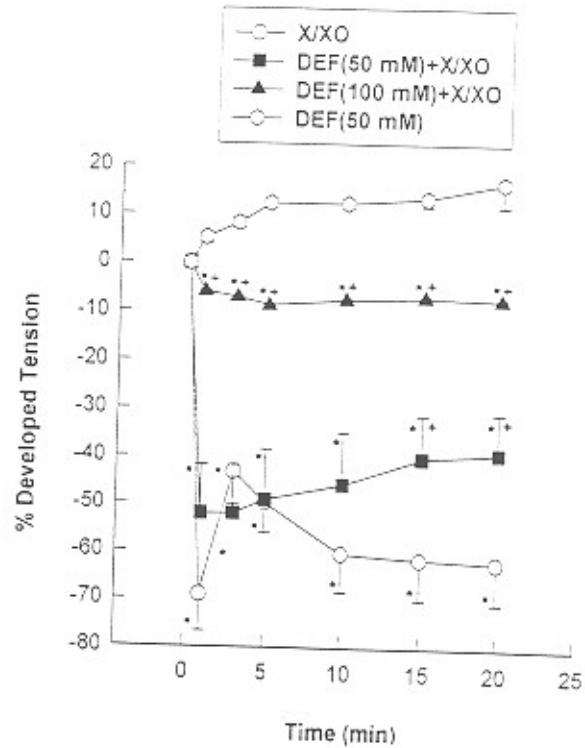

Fig. 6. Effects of 2X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of deferoxamine (DEF) on ileum preparations.

Results are expressed as mean ± SE

* P < 0.05, comparison of values at different times with respect to values at "0" time within groups.

+ P < 0.05, X/XO vs DEF (50 mM) + X/XO or DEF (100 mM) + X/XO

Effect of histidine on X/XO-induced relaxation of ileum

The effects of xanthine plus xanthine oxidase in the absence and presence of two concentrations of histidine on ileum preparations are summarized in Figure 7.

Xanthine plus xanthine oxidase produced relaxation of ileum as expected. Histidine alone did not have damaging effect on tissue, because the response of tissue to ACh before and after treatment with histidine was not significantly different. Pretreatment with histidine significantly reduced the relaxation-induced by X/XO, and this reduction was concentration-dependent. Pretreatment with 100 and 200mM histidine reduced the initial and second maximum relaxation. These results suggest that $^1O_2$ also plays a role in X/XO-induced relaxation
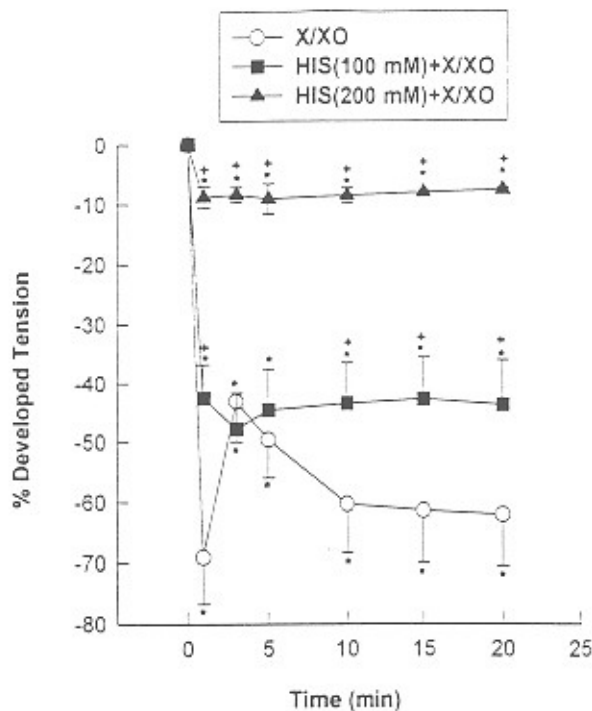
Fig. 7. Effects of 2X concentrations of xanthine plus xanthine oxidase (X/XO) in absence or presence of two concentrations of histidine (HIS) on ileum preparations.

Results are expressed as mean ± SE

* P < 0.05, comparison of values at different times with respect to values at "0" time within groups.

+ P < 0.05, X/XO vs HIS (100 mM) + X/XO or HIS (200 mM) + X/XO

DISCUSSION

Xanthine and xanthine oxidase have been used by other investigators to generate reactive oxygen metabolites (ROMs) (8,12).

The concentrations of X/XO used in this investigation were formulated based on the concentrations used by other investigators (8,12). The results show that 1X, 2X and 4X concentrations of X/XO produced relaxation of ileum. A concentration of 2X xanthine plus xanthine oxidase resulted in maximal relaxant effect. Concentration of 4X elicited a response that was smaller than 2X concentration. One would have expected greater relaxation with 4X concentration of X/XO. This unexpected finding could be due to several reasons. Firstly, the higher concentrations of X/XO through the production of high amounts of oxygen free radicals may inhibit the cyclooxygenase enzyme with the loss of production of prostaglandins, and hence reduced relaxation. Secondly, it is possible

that there is destruction of xanthine oxidase activity by oxygen free radicals (OFRs).

A smaller response with 4X concentration of X/XO could also be due to high levels of OFR production, which may damage the luminal epithelium or the underlying ileum smooth muscle. The cytotoxic effects of reactive oxygen metabolites are well documented (13,14,15). Xanthine/xanthine oxidase have been shown to have direct cytotoxic effects on cultured gastric mucosal cells (16). Xanthine oxidase itself has been shown to alter myofibrillar sulfhydryl content and ATPase activity in vitro (17). Therefore, higher concentration of X/XO may be altering the contractile apparatus of the ileum smooth muscle, resulting in a smaller response.

It may also be that the non-enzymatic sources of antioxidants (Vit-E, Vit-C and β-carotene) are depleted in the presence of high concentrations of OFRs generated by 4X concentration of X/XO. So, increased cytotoxic effects on smooth muscle could be due to decreased antioxidant reserve. Oxidants have been shown to inactivate antioxidant defense systems. For example, H_2O_2 or a combination of $O^{\circ-}2$ and H_2O_2, can inactivate CuZn-SOD (18). In addition, $O^{\circ-}2$ can convert catalase to inactive derivatives (19). Therefore, such changes may decrease the response produced by 4X concentration of X/XO.

To investigate the toxic effect of X/XO, the influence of the X/XO on ACh-induced contraction of rat ileum was studied. Acetylcholine-induced contraction was greater in 1X and 2X concentrations of X/XO-treated groups than in control groups (without X/XO). There is evidence that suggests the enhancement of contractile activity of smooth muscle which is chronically exposed to H_2O_2 in vivo (20). They reported that hyper responsiveness of the airways to ACh may be related to epithelial dysfunction. Therefore, the increased sensitivity of ileum smooth muscle to ACh could be due to damage of the epithelium.

Although 1X and 2X concentrations of X/XO increased the sensitivity of ileum to Ach, 4x concentration of X/XO reduced muscle responsiveness to Ach. It is possible that the biologic activity of Ach could be destroyed by oxidation via 4x concentration of X/XO might damage the cell membrane and alter the coupling of the muscarinic receptor to its effector system. Similar findings were reported by some investigators who found that pretreatment of guinea pig ileum with oxidants reduced the subsequent contractile response to carbachol (21).

Another explanation for reduced contractile responses to Ach with 4x concentration of X/XO may involve intracellular damage by oxidants as proposed by Goldhaber and co-workers (22). They investigated the effects of exogenous free radicals on electromechanical
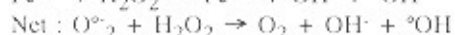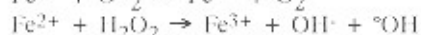
function and metabolism in isolated rabbit and guinea pig ventricle, and found that free radicals may contribute to electrophysiologic abnormalities and contractile dysfunction by inhibiting glycolysis and oxidative phosphorylation.

The results showed that X/XO induced relaxation of ileum does not appear to be mediated through superoxide anion since SOD which metabolizes $O^{\bullet-}_2$ to H_2O_2 could not abolish the relaxation.

The protective effect of SOD against oxidant injury has been questioned by some investigators because they could not find SOD to be protective (23). Yagoda and co-workers (23) have been shown that some commerical preparations of SOD were contaminated with endotoxin. Another possibility of SOD were contaminated with endotoxin. Another possibility exists that the inability of superoxide dismutase to reduce the X/XO - induced relaxation may be result from a failure of its endocytosis, which is required before it can protect against H_2O_2 - induced injury (24).

The lack of effect of SOD to prevent the relaxant effect of X/XO could also be due to the fact that superoxide dismutase metabolizes $O^{\bullet-}_2$ to H_2O_2 (25). The amount of H_2O_2 produced under these circumstances may be sufficient to induce relaxation of ileum. In this investigation, however, X/XO-induced relaxation does not appear to be medicated through hydrogen peroxide since catalase could not reduce the relaxation of ileum. One possibility is that generation of H_2O_2 exceeded the catalytic capacity of catalase. Another possibility is that H_2O_2 crosses the cell membrane and produces hydroxyl radicals which may be responsible for relaxation of ileum.

The results showed that neither $O^{\bullet-}_2$ nor H_2O_2 is responsible for relaxation of ileum. However, if there is insufficient enzymatic protection, these two species, can interact via the Haber-Weiss reaction to generate highly reactive intermediates, the most important of these being the hydroxyl radical (26). Superoxide anion does not directly react with H_2O_2 but rather donates an electron to a transition metal which then reduces H_2O_2 to form $^\circ OH$ as following:

$$Fe^{3+} + O^{\bullet-}_2 \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^\circ OH$$
$$Net : O^{\bullet-}_2 + H_2O_2 \rightarrow O_2 + OH^- + {}^\circ OH$$

Therefore, it is possible that hydroxyl radicals are generated. The results suggest that X/XO-induced relaxation of ileum is mediated through hydroxyl radical, since two powerful hydroxyl radical scavengers, dimethylthiourea and mannitol, reduced the relaxation of ileum.

However, the formation of hydroxyl radical from superoxide and hydrogen peroxide requires the presence of transition metal ions, the most significant of which are iron ions (14). The results of this study indicate that pretreatment with the iron chelator

deferoxamine provided significant protection against X/XO-induced relaxation. This finding indicates that iron is involved in X/XO-mediated relaxation of ileum as a result of its participation in the Haber-Weiss reaction.

A role for intracellular iron and intracellular formation of the hydroxyl radicals has been implicated in some studies (27,28,29). Repine and co-workers (27) showed that with increasing the concentration of intracellular iron of Staphylococcus aureus, their susceptibility to killing by H_2O_2 enhanced. Similarly, Kvietys and co-workers (29) reported that deferoxamine protected the endothelial cells from the cytotoxic effects of the hypoxanthine/xanthine oxidase reaction mixture or H_2O_2. Furthermore, Gannon and co-workers (28). Demonstrated that pretreatment of the endothelial cells with deferoxamine, an iron chelator, prevented neutrophil-mediated injury.

Protection by deferoxamine has also been shown in several studies. Deferoxamine at appropriate concentration afforded protection against X/XO-induced intestinal injury (15). Deferoxamine has also been shown to be protective against ischemia/reperfusion -induced gastric ulceration (30).

There are some concerns regarding the use of deferoxamine, because deferoxamine, besides of its iron-chelating effect it has the capacity to react with superoxide to form a relatively stable nitroxide; however, the reaction of deferoxamine with $O^{\circ-}2$ is very slow (31). Deferoxamine has also hydroxylradicals scavenging property. However, to form $^\circ OH$, which then might react with deferoxamine, iron in the ferrous oxidation state is needed. Deferoxamine strongly promotes the oxidation of the ferrous ion, thus maintaining iron in the ferric state and inhibiting its reaction with H2O2.

The results of this study indicate that in the presence of histidine, a singlet oxygen ($^1O_2$)scavenger, X/XO-induced relaxation was significantly reduced. The protective effect of histidine was concentration-dependent and was comparable to classical free radical scavengers such as mannitol.These results suggest that singlet oxygen may be another oxygen-derived metbolite (in addition to $^\circ OH$ radical) involved in X/XO-induced relaxation.

Singlet oxygen can be generated from superoxide anion in the X/XO system (6). Its high reactivity can damage lipids and constituents of biological membranes, and can inactivate enzymes, cause DNA damage and oxidation of mitochondrial components (32,33).

In the literature, there is no study on the effects of singlet oxygen on ileum; however, some investigators studied the effects of $^1O_2$ in other tissues (32,34). Singlet oxygen has been shown to produce a negative inotropic effect on isolated papillary muscle (32,34).

Singlet oxygen derived from activated neutrophils has also been implicated in causing myocardial and peripheral vascular depression(32).

In summary, results indicate that oxygen derived metabolites generated by X/XO produce a relaxation of rat ileum. The relaxant effect of X/XO was blocked by DMTU, mannitol; and histidine, suggesting that the relaxation is mediated through hydroxyl radical and singlet oxygen generated by X/XO. Pretreatment with deferoxamine, a potent iron chelator, reduced the relaxation of ileum, indicating that iron plays an important role in mediating the X/XO-induced relaxation of ileum. The partial protection offered by the antioxidants used in this study indicate some possibilities. It may be that the effect is not mediated by the hydroxyl radical, but by an intermediate, higher oxidation state of iron (Fe^{4+}) or ferryl species formed by the Fenton reaction (35).This ferryl species is a potent hydroxyl-like oxidant and could readily lead to cytotoxicity. Another rpossibility is that hydroxyl radical has been formed at places inaccessible to scavengers, where iron can promote the generation of hydroxyl radical. Thecompartmentalization of the defense system relative to the site of oxidant attack may also be an important tissue, where even efficient antioxidants are powerless to defend the cell, if the oxidant is generated at a site where it can interact with its target before being degraded.

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