Activation of Ischemia- and Reperfusion-Sensitive Abdominal Visceral C Fiber Afferents
Role of Hydrogen Peroxide and Hydroxyl Radicals
Gregory L. Stahl, Hui-Lin Pan, and John C. Longhurst

Abdominal ischemia and reperfusion evoke reflex excitation of the cardiovascular system and generate reactive oxygen species. We have shown previously that the reactive oxygen species hydrogen peroxide (H$_2$O$_2$) elicits reflex excitation of the cardiovascular system after serosal application to abdominal organs. However, it is not known if ischemia-sensitive afferents respond to reactive oxygen species or if scavengers such as dimethylthiourea (DMTU) inhibit the response of these afferents to ischemia or reperfusion. Therefore, to provide more information on the neurophysiological mechanisms underlying the activation of these afferents, we studied their responses to H$_2$O$_2$ applied to the receptive field during recordings of single-unit activity of ischemia-insensitive or -sensitive abdominal visceral C fiber afferents in anesthetized cats. Additionally, we recorded single-unit activity of ischemia and reperfusion-sensitive afferents before and after treatment with DMTU (10 mg/kg), which scavenges H$_2$O$_2$ and hydroxyl radicals or the iron chelator deferoxamine (DEF, 10 mg/kg), which inhibits hydroxyl radical formation. Application of 44 μmol H$_2$O$_2$ to afferent endings increased the discharge frequency in nine of 11 ischemia-sensitive units, from 0.01±0.01 to 0.67±0.16 impulses per second. In contrast, only one of 10 ischemia-insensitive C fibers responded to H$_2$O$_2$ application. In an additional 13 ischemia-sensitive C fibers, DMTU significantly (p<0.05) attenuated ischemia-induced increases in discharge frequency from 0.42±0.18 to 0.24±0.1 impulses per second (ischemia versus DMTU+ischemia, respectively). In eight additional C fibers, we found that reperfusion after 5 minutes of ischemia was associated with an increase in discharge activity from a baseline activity of 0.02±0.01 to 0.44±0.07 impulses per second. DMTU significantly attenuated the reperfusion-induced increases in discharge frequency from 0.08±0.04 to 0.18±0.06 impulses per second. DEF significantly (p<0.05) attenuated the increased discharge activity from 0.39±0.07 to 0.10±0.04 impulses per second (ischemia versus DEF+ischemia, respectively) in an additional 11 ischemia-sensitive C fibers. In contrast, iron-saturated DEF did not attenuate ischemia- and reperfusion-induced increases in impulse activity. Thus, ischemia-sensitive but not ischemia-insensitive abdominal visceral afferents respond to H$_2$O$_2$. Furthermore, ischemia- and reperfusion-sensitive afferents decreased their impulse activity to a repeated period of ischemia or reperfusion after DMTU or DEF treatment. These data suggest that reactive oxygen species, particularly H$_2$O$_2$ and hydroxyl radicals, activate abdominal visceral C fibers in the cat during brief periods of ischemia and reperfusion. (Circulation Research 1993;72:1266–1275)

KEY WORDS • dimethylthiourea • deferoxamine • oxygen-derived free radicals • ischemia

Stimulation of both mechanosensitive and chemosensitive sensory nerve endings in abdominal visceral organs can activate reflexly the cardiovascular system. In this regard, mesenteric artery occlusion in cats, resulting in ischemia, evokes reflex excitation of the cardiovascular system.

The specific metabolic stimuli responsible for activating ischemia-sensitive visceral afferent nerve endings are not fully known. Ischemia leads to metabolic changes and the production of a number of factors that potentially could stimulate these sensory endings. For instance, our laboratory has provided evidence demonstrating that severe hypoxia, lactic acid, and cyclooxygenase metabolites can activate these afferent endings.

We recently have classified ischemia-sensitive abdominal afferents into three types. One type responded only during ischemia. A second type responded during ischemia and continued to discharge during reperfusion. A third type responded only during reperfusion.

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We have begun to consider potential stimuli, other than prostaglandins or lactic acid, that could activate these afferent endings during ischemia and reperfusion.

Periods of visceral ischemia followed by reperfusion are well known to generate free radicals and other reactive oxygen species. Reactive oxygen species play a substantial role in the pathogenesis of tissue injury, particularly during the period of reperfusion. Reactive oxygen species produced during ischemia and reperfusion include superoxide and hydroxyl radicals, hydrogen peroxide (H₂O₂), and hypochlorous acid.

Although reactive oxygen species are known to be involved in ischemia/reperfusion injury to the gastrointestinal tract, information about their direct action on neuronal function is limited. Colton et al. observed synaptic depression after application of xanthine and xanthine oxidase mixtures to the squid giant synapse and the lobster neuromuscular junction. H₂O₂ was a major contributor to the effects observed. Furthermore, we have recently shown that application of H₂O₂ to abdominal visceral organs induces cardiovascular reflexes manifest by increases in heart rate, myocardial contractility, and arterial blood pressure. H₂O₂-induced reflexes were attenuated by ganglionectomy, dimethylthiourea (DMTU), or deferoxamine (DEF), suggesting that H₂O₂ and the hydroxyl radical are important oxidative mediators of the reflex. Thus, evidence suggests that reactive oxygen species may modulate neuronal function and induce cardiovascular reflexes. However, the discharge activity of ischemia-sensitive afferents to reactive oxygen species and the ability of free radical generation in vivo to activate afferent endings must be investigated before one can conclude that these products are an important mechanism of afferent activation during ischemia or reperfusion. In this study, therefore, we investigated the following hypotheses: 1) Ischemia-sensitive afferents are particularly responsive to H₂O₂. 2) Blockade of reactive oxygen species attenuates the discharge frequency of ischemia- or reperfusion-sensitive afferents.

Materials and Methods

All animal experiments were conducted in accordance with guidelines set by the University of California at Davis and the “Guide for the Care and Use of Laboratory Animals” (US Department of Health and Human Services, publication No. [NIH] 86-23). We studied 50 cats (3.7±0.3 kg) anesthetized with pentobarbital sodium (35 mg/kg i.v.). The trachea was intubated, and respiration was maintained artificially (Harvard pump, model 661, Ealing, South Natick, Mass.). A femoral artery and vein and the right common carotid artery were cannulated for measurement of pressure or administration of fluids and drugs. In addition, the carotid artery catheter was positioned with its tip in the descending thoracic aorta. Arterial blood pressure was measured with strain-gauge transducers (Statham P23ID) connected to the arterial catheters with a stopcock. Arterial blood gases were analyzed frequently with a blood gas analyzer (model ABL 3, Radiometer America, Inc., Westlake, Ohio) and maintained within physiological limits (Po₂, >100 mm Hg; PCO₂, 28–35 mm Hg; pH 7.35–7.45) by adjusting the respirator’s rate, tidal volume, or O₂ concentration or by administering NaHCO₃ (1 M i.v.). Body temperature was maintained with a circulating-water heating pad and heat lamps.

The surgical preparation has been described previously. Briefly, a midline sternotomy was performed, and the third through eleventh ribs and the middle and lower lobes of the right lung were removed. Both phrenic nerves were isolated and cut. The fascia covering the right paravertebral sympathetic chain was removed. The chain was draped over a mirror platform and covered with warm mineral oil.

Small nerve filaments were dissected gently from the chain using an operating microscope (Zeiss, FRG), and the caudal end was placed across a recording electrode. One pole of the recording electrode was grounded with cotton thread to the animal. The recording electrode was attached to a high-impedance probe (Grass Instruments Co., Quincy, Mass.), and the signal was amplified (Grass model P511 preamplifier) and processed through an audioamplifier (Grass AM48B audio monitor) and an oscilloscope (model 549, Tektronix, Beaverton, Ore.) and recorded on an electrostatic recorder (model ES1000B, Gould, Cleveland, Ohio).

An inflatable occlusion cuff was placed around the descending thoracic aorta distal to the tip of the carotid artery catheter. A midline incision was used to expose abdominal organs. The incision was covered with gauze sponges soaked with warm Ringer’s buffer and closed with towel clips.

Receptive fields were located, and conduction velocities were estimated as we have described previously. Briefly, this involved using a cotton-tipped applicator or a fine-tipped glass rod to locate the receptive fields that generally were found to be 1–3 mm in diameter. The mechanical sensitivity of the ending to von Frey hairs was recorded. Subsequently, the conduction time between the receptive field and the recording electrode as well as the conduction distance were measured. Conduction distance was estimated with a thread placed from the receptive field along the supposed afferent pathway through the prevertebral ganglion along the course of the major splanchnic nerve to the sympathetic chain to the recording electrode. C fibers were classified as those with a conduction velocity of <2.5 m/sec. The fibers included in this manuscript had a conduction velocity range of 0.27–2.30 m/sec, and each had a single receptive field that could be located precisely.

Experimental Protocols

Effect of H₂O₂ on afferent discharge activity. This protocol consisted of 10 animals subjected to 5 minutes of abdominal ischemia followed by 3–5 minutes of reperfusion. After identification of an ischemia-sensitive or -insensitive unit, the abdomen was opened, the receptive field of the ending was located, and a conduction time was measured as noted above. H₂O₂ (44 μmol) then was applied to the receptive field, and the afferent’s activity was recorded as we have described previously. This dose of H₂O₂ was chosen since we have demonstrated previously that it causes maximal reflex cardiovascular reflex responses when it is applied to abdominal visceral organs. If the afferent did not respond to H₂O₂, bradykinin (140 pmol) was applied to the receptive field. We have demonstrated previously that this concentration stimulates many C fibers and a high percentage of ischemia-sensitive afferents.
ents were not included in the study if the discharge frequency did not increase above baseline activity after application of H2O2 or bradykinin. Mechanical threshold was estimated by the von Frey technique.18,19 The esthesiometer set used in this technique (Stoelting Co., Chicago, Ill.) consisted of precisely calibrated nylon filaments of equal length.

**Effect of DMTU on discharge activity.** This protocol consisted of 18 animals subjected to 5 minutes of abdominal ischemia followed by 3–5 minutes of reperfusion. After identification of an ischemia- or reperfusion-sensitive unit, DMTU (10 mg/kg i.v.) was administered over a 10-minute period. DMTU was dissolved in 0.9% NaCl and was prepared fresh daily. This dose of DMTU effectively inhibits the reflex cardiovascular effects of 44 μmol H2O2.15 A repeat period of ischemia and reperfusion was begun 30–45 minutes after the first period of ischemia and at least 10–15 minutes after DMTU treatment. We have shown previously that an increase in afferent activity is repeatable if a 30-minute period is maintained between ischemic events.7 If the afferent activity was suppressed completely, the receptive field was manipulated mechanically to establish that the nerve ending was viable.

**Effect of DEF on discharge activity.** This protocol consisted of 11 animals subjected to 5 minutes of abdominal ischemia followed by 3–5 minutes of reperfusion. After identification of an ischemia- or reperfusion-sensitive unit, DEF (10 mg/kg i.v.) was administered over a 10-minute period. DEF was dissolved in 5–8 ml of 0.9% NaCl and was prepared fresh daily. This dose of DEF effectively inhibits the reflex cardiovascular effects of 44 μmol H2O2.15 A repeat period of ischemia and reperfusion was begun 30–45 minutes after the first period of ischemia and at least 10–15 minutes after DEF treatment. If the afferent activity was suppressed completely, the receptive field was manipulated mechanically to establish that the nerve ending was viable.

We studied an additional 11 ischemia- and/or reperfusion-sensitive C fibers in 11 animals before and after the administration of iron-saturated DEF. Iron-saturated DEF was prepared as we have described previously15 by adding 98 mg FeCl3·6H2O to 1 ml DEF (250 mg/ml) for 1 hour at room temperature. After the initial response to ischemia and reperfusion, iron-saturated DEF (10 mg/kg) was infused for 30 minutes. The animal was rechallenged with ischemia and reperfusion 5–10 minutes after completion of the infusion.

**Data Analysis**

As we have described previously,5,7 discharge rates of ischemia-sensitive afferent fibers were averaged over a 60-second preischemic period just preceding the onset of arterial occlusion. During ischemia or reperfusion, the peak 60-second discharge rate was measured. The latency of response to ischemia for each intervention was measured from the time of arterial occlusion or from the initial application of H2O2 to the receptive field until a 10% increase in discharge frequency compared with baseline activity was observed. The onset latency of reperfusion-sensitive afferents was taken from the onset of reperfusion until a 10% increase in discharge activity compared with the control value was observed. If an afferent did not respond to ischemia or reperfusion after treatment with DEF or DMTU, an onset latency equal in length to the maximum observation period was assigned.

The effect of DMTU, DEF, or iron-saturated DEF treatment was compared with repeated-measures analysis of variance and Scheffe’s post hoc test. Latencies were compared with the Wilcoxon test.20 All statistical calculations were performed with commercially available software (Crunch Software Corp., Oakland, Calif.).

Data are expressed as mean±SEM. Values were considered to be significantly different at p<0.05.

**Results**

**Effects of H2O2 on Afferent Activity**

Ischemia-sensitive C fibers. Figure 1 summarizes the effects of ischemia, H2O2, and bradykinin on ischemia-sensitive C fiber discharge activity. Inflation of the aortic occlusion cuff significantly decreased mean femoral arterial pressure from 87±6 to 17±1 mm Hg (p<0.05). We have shown previously that this degree of arterial occlusion is associated with a significant increase in portal venous and mesenteric lymph lactate concentration.6,21 A 5-minute period of ischemia significantly increased the discharge activity of 11 C fibers, with an increase in discharge activity from 0.2±0.11 to 1.25±0.50 impulses per second after an onset latency of 12±11 seconds. The afferent endings were located in the porta hepatitis (n=5), pylorus (n=1), gallbladder (n=3), pancreas (n=1), or duodenum (n=1) and had an average conduction velocity of 0.96±0.13 m/sec. Mechanical threshold (von Frey hair technique) was 3.41±0.2 g. Application of H2O2 (44 μmol) to the receptive field increased discharge activity by 0.81±0.15 impulses per second after a latency of 15±3 seconds in nine of the 11 C fibers. However, two ischemia-sensitive C fibers did not increase their impulse activity to H2O2.
In contrast, these two C fiber endings did increase their discharge activity to bradykinin from 0 impulses per second to 2.27 and 1.10 impulses per second after an onset latency of 19 and 33 seconds, respectively.

**Ischemia-insensitive C fibers.** Figure 2 summarizes the effects of ischemia, H$_2$O$_2$, and bradykinin on ischemia-insensitive C fiber impulse activity. Inflation of the aortic occlusion cuff significantly ($p<0.05$) decreased mean femoral arterial pressure from 87±7 to 14±1 mm Hg. Ischemia significantly increased the discharge activity of the 13 C fibers from 0.02±0.01 to 0.44±0.17 impulses per second, with seven endings increasing their activity only during ischemia and the remaining discharging during ischemia and continuing during reperfusion. The afferent endings were located in the porta hepatitis (n=6), gallbladder (n=1), pancreas (n=1), liver (n=1), mesentery (n=2), or duodenum (n=2) and had an average conduction velocity of 1.05±0.14 m/sec with an onset latency of 139±15 seconds. These 13 C fibers were then subjected to a second period of ischemia and reperfusion after DMTU administration. DMTU treatment, compared with the first ischemic period, did not significantly attenuate the decrease in mean femoral arterial pressure (14±1 versus 14±1 mm Hg) or alter mean aortic pressure (90±6 versus 88±6 mm Hg) or the onset latency (173±18 versus 139±15 seconds). However, DMTU significantly attenuated the increased discharge activity during ischemia in 10 of 13 C fibers (Figure 4). The other three afferents did not demonstrate a decreased impulse activity during ischemia after DMTU treatment; one afferent exhibited no change in activity, and two afferents exhibited increased activity compared with the initial ischemic period.

**Effect of DMTU on Reperfusion-Sensitive C Fibers**

In five reperfusion-sensitive C fibers displaying an average conduction velocity of 1.38±0.29 m/sec, we investigated the afferents’ responses to repeated periods of reperfusion. We observed a similar increase in discharge activity during the second compared with the initial period of reperfusion (Figure 5A). The onset latency after reperfusion also was unchanged (initial reperfusion of 19±6 seconds versus repeat reperfusion of 14±6 seconds). The receptive fields were located in the liver (n=1), mesentery (n=1), and porta hepatitis (n=3).

Figure 6 shows representative tracings of a reperfusion-sensitive C fiber innervating the gallbladder with a conduction velocity of 0.64 m/sec. Reperfusion increased baseline discharge activity from 0.02 to 0.68 impulses per second during reperfusion after an onset latency of 70 seconds (panel A). DMTU (10 mg/kg) sharply decreased this activity during the second reperfusion period to only 0.02 impulses per second (panel B).

Figure 5B summarizes the effect of reperfusion or reperfusion+DMTU treatment on discharge activity of eight reperfusion-sensitive C fibers. Inhalation of the aortic occlusion cuff significantly decreased mean femoral arterial pressure from 83±9 to 16±2 mm Hg ($p<0.05$) during a 5-minute period of ischemia. Reperfusion significantly increased the discharge activity of these eight C fibers (Figure 5B). The afferent endings were located in the porta hepatitis (n=2), gallbladder (n=2), pancreas (n=2), liver (n=1), or duodenum (n=1). The average conduction velocity was 1.00±0.16 m/sec with an onset latency after the onset of reperfusion of 33±10 seconds. Treatment with DMTU, compared with the first ischemic period, did not attenuate the decrease in mean femoral arterial pressure (17±2 versus 16±2 mm Hg) or alter mean aortic pressure (91±7 versus 83±9 mm Hg). However, DMTU significantly attenuated the increased discharge activity.

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**Figure 2. Impulse activity of ischemia-insensitive abdominal C fibers before (open bars) and after (closed bars) ischemia, H$_2$O$_2$ (44 μmol), or bradykinin (140 pmol). Ischemia and H$_2$O$_2$ data are mean±SEM of 10 afferents. Bradykinin data are mean±SEM of nine afferents. Bars and brackets represent mean±SEM.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nerve Activity (Imp/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>2.0 ± 1.0</td>
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Figure 4. Bar graph showing the effect of ischemia on impulse activity of 10 ischemia-sensitive abdominal C fibers before dimethylthiourea (−DMTU) and after DMTU (+DMTU) treatment. Bars and brackets represent mean±SEM. *Significant (p<0.05) increase in impulse activity compared with the respective control value.

during the second period of reperfusion (Figure 5B) and increased the onset latency to 126±27 seconds (p<0.02).

Effect of DEF on Ischemia- and Reperfusion-Sensitive C Fibers

Figure 7 shows representative tracings of an ischemia-sensitive C fiber innervating the portal vein with a conduction velocity of 1.48 m/sec. Ischemia increased baseline discharge activity from 0.03 to 0.45 impulses per second during ischemia after an onset latency of 221 seconds (panel A). DEF (10 mg/kg) decreased the impulse activity during the second ischemic period to 0.27 impulses per second (panel B).

Figure 8A summarizes the effect of ischemia and reperfusion before (−DEF) or after (+DEF) DEF treatment on the discharge activity of 11 ischemia- and/or reperfusion-sensitive C fibers. Inflation of the aortic occlusion cuff significantly decreased mean femoral arterial pressure from 112±8 to 18±2 mm Hg (p<0.05) during a 5-minute period of ischemia. The discharge activity of nine of 11 C fibers increased significantly during ischemia, and two of the afferents increased their impulse activity during reperfusion. The afferent endings were located in the porta hepatis (n=6), gallbladder (n=1), pancreas (n=1), or portal vein (n=3). The average conduction velocity was 1.09±0.12 m/sec, with an onset latency of 201±22 seconds. Treatment with DEF, compared with the first ischemic period, did not attenuate the extent to which mean femoral arterial pressure was decreased during ischemia (18±2 versus 19±2 mm Hg) or alter mean aortic pressure (112±8 versus 113±10 mm Hg). However, DEF significantly attenuated the increased discharge activity during the second period of ischemia or reperfusion (Figure 8A) and increased the onset latency to 362±38 seconds (p<0.05).

Figure 8B summarizes the effect of ischemia and reperfusion before (−DEF) or after (+DEF+iron)
Three important observations were made in this study: First, ischemia-insensitive C fiber endings rarely respond to H2O2, whereas ischemia-sensitive C fiber endings frequently respond to H2O2. Second, DMTU, an iron-saturated free radical scavenger, attenuates the discharge activity of both ischemia- and reperfusion-sensitive C fibers. Third, iron-saturated DEF treatment on the discharge activity of the 11 ischemia- and reperfusion-sensitive C fibers. Inflation of the aortic occlusion cuff significantly decreased mean arterial pressure from 117±7 to 16±1 mm Hg (p<0.05). The discharge activity of seven of the 11 C fibers increased significantly during ischemia, with the remaining afferents increasing their impulse activity during the reperfusion period (Figure 8B). The afferent endings were located in the porta hepatis (n=5), gallbladder (n=3), pancreas (n=2), or portal vein (n=2).

Discussion

Three important observations were made in this study: First, ischemia-insensitive C fiber endings rarely respond to H2O2, whereas ischemia-sensitive C fiber endings frequently respond to H2O2. Second, DMTU, an oxygen-derived free radical scavenger, significantly attenuates the discharge activity of both ischemia- and reperfusion-sensitive C fibers. Third, iron-saturated DEF treatment on the discharge activity of the 11 ischemia- and reperfusion-sensitive C fibers. Inflation of the aortic occlusion cuff significantly decreased mean arterial pressure from 117±7 to 16±1 mm Hg (p<0.05). The discharge activity of seven of the 11 C fibers increased significantly during ischemia, with the remaining afferents increasing their impulse activity during the reperfusion period (Figure 8B). The afferent endings were located in the porta hepatis (n=5), gallbladder (n=3), pancreas (n=2), or portal vein (n=2).

Figure 6. Representative tracings of a reperfusion-sensitive C fiber innervating the gallbladder with a conduction velocity of 0.64 m/sec. Phasic pressure during ischemia represents aortic pressure. Reperfusion increased baseline discharge activity from 0.02 to 0.68 impulses per second during reperfusion after an onset latency of 70 seconds (panel A). Dimethylthiourea (10 mg/kg) sharply decreased this activity during the second reperfusion period from 0.02 to 0.02 impulses per second (panel B). The afferent still responded to mechanical stimulation of its receptive field (panel B, poke).
reperfusion-sensitive abdominal visceral afferents. Third, the iron chelator DEF significantly attenuates the discharge activity of both ischemia- and reperfusion-sensitive abdominal visceral afferents, suggesting that the important oxidative stress metabolite is the hydroxyl radical. Thus, the present study provides information that indicates, for the first time, that endogenous oxygen reactive species, particularly the hydroxyl radical, are an important mechanism of afferent activation during brief periods of mesenteric ischemia and reperfusion.

Abdominal ischemia activates abdominal visceral afferents and invokes reflex excitation of the cardiovascular system. Ischemia-sensitive abdominal afferents are activated by severe hypoxia, lactic acid, and prostaglandins. We have previously shown that cyclooxygenase blockade significantly attenuated the increased impulse activity of ischemia-sensitive afferents. Several laboratories have suggested that oxygen-derived free radicals can be produced in low oxygen tensions or low-flow conditions. However, during a no-flow condition, when delivery of oxygen to the tissue is stopped, a reduction in free radical generation is observed in organs such as the myocardium. In our model, ischemia is induced by occluding the thoracic aorta. However, some collateral channels are present, because femoral arterial pressure decreases to only 15–20 mm Hg. In this condition, we believe that some molecular oxygen is available to generate free radicals, which stimulate sensory endings after the endogenous scavenging mechanisms have been overwhelmed or saturated. Thus, reactive oxygen species are likely generated to activate afferent endings in our model of abdominal ischemia.

Previously we have shown that H$_2$O$_2$ and possibly hydroxyl radicals are capable of inducing reflex excitation of the cardiovascular system. In the present study, DMTU, an oxygen-derived free radical scavenger, attenuated the increased impulse activity of ischemia-sensitive afferents. Thus, we have provided important electrophysiological data that document the importance of reactive oxygen species in contributing to the activation of ischemia-sensitive abdominal visceral afferents.

In the presence of transition metals, particularly iron, H$_2$O$_2$ can form reactive radicals such as hydroxyl radicals by the metal-catalyzed Haber-Weiss reaction or Fenton chemistry. Indeed, such reactive radicals are frequently responsible for H$_2$O$_2$-mediated damage. DMTU is a nonspecific scavenger of reactive oxygen species that is able to react with H$_2$O$_2$ and hydroxyl free radicals. DMTU attenuated the discharge activity of ischemia- and reperfusion-sensitive afferents in the present study. To investigate more thoroughly the oxidative stress factor stimulating the afferent endings, DEF was administered to bind iron and inhibit hydroxyl radical formation. DEF was found to inhibit significantly the increased impulse activity of ischemia- and reperfusion-sensitive afferents. These data suggest that hydroxyl radicals contribute to excitation of ischemia- and reperfusion-sensitive afferent endings. This is consistent with our previous findings demonstrating that H$_2$O$_2$-induced cardiovascular reflexes are mediated, in part, by hydroxyl radicals. Although DEF can act as a scavenger of reactive oxygen species, the failure of iron-saturated DEF to prevent ischemia and reperfusion stimulation of afferent endings suggests that iron bind-
ing is responsible for its actions, since iron-saturated DEF is an equally good radical scavenger.

We have provided electrophysiological evidence that oxygen-derived free radicals are important activators of ischemia- and reperfusion-sensitive abdominal visceral afferents. We do not know the source of these oxidative stress metabolites in this study. However, several potential sources are possible. The feline gastrointestinal tract is rich in xanthine dehydrogenase, which can be converted to xanthine oxidase during periods of abdominal ischemia and reperfusion. Parks et al. have shown that 20% of the total xanthine oxidase/xanthine dehydrogenase activity in the nonischemic cat duodenum is xanthine oxidase. A twofold increase in xanthine oxidase activity is observed within 1 hour of ischemia without reperfusion. Xanthine oxidase, in the presence of hypoxanthine (ATP metabolism) and molecular oxygen, produces free radicals. Additionally, free radicals can be produced by activated neutrophils, direct donation of electrons from the reduced mitochondrial electron transport chain to molecular oxygen, catecholamine oxidation, and cyclooxygenase and lipoxygenase enzymes. Whatever the source of free radicals may be in this study, it can be assumed that the endogenous radical-scavenging mechanisms would have to be saturated or overwhelmed before activation of the afferent ending.

We have previously documented three types of abdominal visceral afferents responding to ischemia and reperfusion. This is the first report to characterize abdominal visceral C fiber reperfusion-sensitive afferents. We demonstrated that DMTU or DEF significantly attenuated the impulse activity in response to a repeated period of reperfusion. Furthermore, we demonstrated that the afferent activity increased in a repeatable fashion if a 30-minute period was maintained between reperfusion events. We also demonstrated that iron-saturated DEF did not attenuate the increased impulse activity during ischemia or reperfusion. Therefore, it is unlikely that the reduction in afferent activity after DMTU or DEF treatment was merely a time-related effect. Thus, we have provided important data confirming the importance of reactive oxygen species in contributing to the activation of reperfusion-sensitive abdominal visceral C fiber afferents.

We have previously classified abdominal visceral afferents into three broad groups: 1) ischemia-sensitive, 2) reperfusion sensitive, and 3) ischemia and reperfusion sensitive. Data presented in this manuscript suggest that the hydroxyl radical can increase the impulse activity of both ischemia- and reperfusion-sensitive afferents. However, if the hydroxyl radical is responsible for increasing afferent activity, then the question arises of why these afferents do not respond during both ischemia and reperfusion. We can only speculate why this is observed. First, perhaps hydroxyl radicals and other substances (e.g., prostaglandins and lactic acid) synergistically activate afferent endings. If this were true, then ischemia-sensitive afferents might not respond during reperfusion because the other synergistic products are “washed” out or diluted during the reperfusion phase. Second, the location of the afferent ending may expose the endings to different microenvironments. For instance, reperfusion-sensitive afferents may be located closer to a set of oxygen and are relatively less ischemic during the ischemia phase. However, during reperfusion, local antioxidant mechanisms may be overwhelmed by a sudden increase in oxygen concentrations, and oxygen reactive species are formed. Third, it is possible that reperfusion-sensitive afferents could respond to longer periods of ischemia (i.e., >5 minutes).

We have demonstrated previously that the DEF or DMTU doses (10 mg/kg) used in this study effectively inhibit 44 μmol of H2O2-induced cardiovascular reflexes of abdominal origin and are substantially less than the doses used in models of myocardial or gastrointestinal ischemia- and reperfusion-induced injury. We also observed that reperfusion-sensitive afferents demonstrate a repeatable increase in discharge activity after a second period of reperfusion and also after the administration of iron-saturated DEF. This is consistent with our previous observations on ischemia-sensitive abdom-
inal visceral afferents. Thus, the decrease in afferent activity in both ischemia- and reperfusion-sensitive afferents after DMTU or DEF treatment is not due to tachyphylaxis but reflects the actions of DMTU or DEF. Furthermore, since low doses of DEF and DMTU attenuated ischemia- and reperfusion-induced increases in impulse activity, we would propose that these actions are a result of the antioxidant influence of these compounds and not due to a nonspecific effect.

Reactive oxygen species are produced after mesenteric ischemia and reperfusion. In particular, Nilsson et al. demonstrated that reactive oxygen species are produced during abdominal ischemia and reperfusion at a rate of 0.3–0.6 μmol·min⁻¹·100 g intestine⁻¹ during the first 30 minutes of reperfusion. Serosal application of 44 μmol H₂O₂ reflexly increases arterial pressure, myocardial contractility, and heart rate and in the present study increased the discharge activity of ischemia-sensitive afferent endings. Given that H₂O₂ readily enters cells and is rapidly metabolized by catalase and glutathione peroxidase enzymes, it is likely that much of the H₂O₂ applied to the serosal surface was diluted or degraded considerably by the time H₂O₂ had diffused into the interstitium and activated the afferent endings. Therefore, the concentration of H₂O₂ arriving at the afferent ending would have been far less than that applied. Thus, the potential exists in vivo for free radical–induced excitation of abdominal visceral afferent endings.

The specific metabolic stimuli responsible for activating ischemia-sensitive visceral afferent nerve endings are not fully known. Ischemia leads to metabolic changes and the production of a number of factors that potentially could stimulate these sensory endings. Our laboratory has provided evidence demonstrating that severe hypoxia, lactic acid, and cyclooxygenase metabolites activate ischemia-sensitive afferent endings. It may be necessary for the various metabolic factors to be acting in concert to stimulate fully the afferent ending during ischemia. Thus, H₂O₂ may increase the response to other afferent-activating substances, and by removing H₂O₂, the other metabolites may not be as effective a stimulus during ischemia. We did not measure lactic acid or prostaglandin formation in the present study and can only speculate on their role.

It is also possible that oxygen-derived free radicals either may modulate or be the initiating stimulus for the production of other afferent-activating substances. Therefore, eliminating the initial mediator (i.e., oxygen-derived free radicals) potentially may decrease the production of other afferent-activating substances. In this regard, Michael et al. have shown that the production of thromboxane B₂ is reduced during myocardial ischemia and reperfusion after treatment with superoxide dismutase and catalase. Thus, reactive oxygen species appear to modulate enzyme systems (i.e., cyclooxygenase). If the products of these modulated enzyme systems normally cause vasoconstriction (e.g., thromboxane A₂) by preventing their production with a scavenger such as DMTU, there may be less ischemia. However, a previous study in our laboratory indicated that the reflex effects caused by H₂O₂ application to abdominal visceral organs are not altered by cyclooxygenase blockade. Thus, we doubt that cyclooxygenase metabolites are important mediators in the H₂O₂ response. However, we cannot rule out a contribution of other metabolic products or alterations (e.g., lactic acid) in mediating this response.

The effect of oxygen-derived free radicals on mammalian sensory neuronal function has not been studied previously. Colton et al. have demonstrated that H₂O₂ decreased synaptic transmission in the squid giant synapse and the lobster neuromuscular junction. Hence, H₂O₂ decreases synaptic transmission in nonmammalian species. In our study, H₂O₂ excited the endings of ischemia-sensitive but not ischemia-insensitive abdominal C fibers. We have shown previously that the effect of H₂O₂ on these sensory nerve endings does not cause damage, since repeated application causes similar reflex responses. Thus, H₂O₂ appears to produce diverse but, in the concentrations used in the present study, non-damaging neuronal effects in mammalian and nonmammalian species.

The specific metabolic stimuli that underlie activation of abdominal visceral afferents have not been fully elucidated and are most likely multifactorial. Our laboratory has previously shown that hypoxemia, bradykinin, and prostaglandins can sensitize afferents to respond to ischemia or directly activate these endings. Recently, we have shown that cyclooxygenase blockade significantly attenuates the increased impulse activity to ischemia. Additionally, we have shown that lactic acid is an effective activator of ischemia-sensitive abdominal visceral afferents. In the present study, ischemia-sensitive, but rarely ischemia-insensitive, afferents responded to H₂O₂. Thus, although prostaglandins, H₂O₂/ hydroxyl radicals, lactic acid, and possibly severe hypoxemia can stimulate or sensitize these endings during ischemia and/or reperfusion, we suspect that still other metabolic factors may play a role in this multifactorial response. For example, we recently have shown that leukotriene B₄ is produced in lymph fluid during mesenteric ischemia. Since leukotriene B₄ is known to sensitize thin-fiber cutaneous afferents, this is one of several other metabolic factors that will require future study.

In conclusion, serosal application of H₂O₂ to the receptive fields of ischemia-sensitive abdominal visceral afferent endings in cats evokes excitation. DMTU, an oxygen-derived free radical scavenger, attenuates the activity of ischemia-sensitive and reperfusion-sensitive abdominal visceral afferents in cats. Furthermore, inhibition of hydroxyl radical formation from the iron chelator DEF attenuates the activity of ischemia- and reperfusion-sensitive abdominal visceral afferents in cats. Thus, these data and previous data from our laboratory demonstrate that reactive oxygen species, particularly H₂O₂ and the hydroxyl radical, can activate abdominal visceral afferents to elicit excitation of the cardiovascular system.

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