Ionizing radiation induces iNOS-mediated epithelial dysfunction in the absence of an inflammatory response

SAMARA L. FREEMAN AND WALLACE K. MACNAUGHTON
Gastrointestinal Research Group and Department of Physiology and Biophysics,
University of Calgary, Calgary, Alberta, Canada T2N 4N1

Ionizing radiation induces iNOS-mediated epithelial hyporesponsiveness to secretagogues through an unknown mechanism. We investigated the role of the inducible isoform of nitric oxide (NO) synthase (iNOS)-derived NO in radiation-induced hyporesponsiveness. C57BL/6 mice were sham treated or exposed to 10-Gy γ-radiation and were studied 3 days later. Tissues were mounted in Ussing-type diffusion chambers to assess chloride secretion in response to electrical field stimulation (EFS) and forskolin (10 μM). Transport studies were also repeated in iNOS-deficient mice. White blood cell counts were significantly lower in irradiated mice, and there was no inflammatory response as shown by myeloperoxidase activity and histological assessment. iNOS mRNA levels and nitrate/nitrite concentrations were significantly elevated in irradiated colons. iNOS immunoreactivity localized to the epithelium. Colon from irradiated wild-type, but not iNOS-deficient, mice exhibited a significant reduction in the responsiveness of the tissue to EFS and forskolin. The hyporesponsiveness was reversed by L-arginine, 1400W, and dexamethasone treatments. iNOS-derived NO mediates colonic hyporesponsiveness 3 days after irradiation in the mouse in the absence of an inflammatory response.

Radiotherapy; secretion; intestine; electrolyte transport; transgenic mice

EXPOSURE TO IONIZING RADIATION is a mainstay of treatment of abdominopelvic malignancies. However, this treatment is associated with a high incidence of untoward gastrointestinal symptoms. The severity of the manifestations of acute gastrointestinal dysfunction may be sufficient to result in interruption or cessation of radiation treatment, which in turn may prevent a curative outcome for the patient (22). Although acute radiation enteritis is usually self-limiting and can be managed symptomatically, severe bouts of acute enteritis are a predictor of more severe, chronic complications months to years after therapy (21, 22, 24).

Nonspecific DNA damage leads to S-phase interruption and cell death in actively dividing cells, which is a well-established consequence of ionizing radiation and mediates its antineoplastic activity (23). However, the high proliferative activity of cells in the crypt region of the intestinal epithelium renders this region a site of bystander injury after abdominopelvic exposure (21). Although the gastrointestinal side effects of exposure to ionizing radiation are generally ascribed to its effect on epithelial mitotic success and subsequent depletion of cells, more recent evidence indicates that epithelial dysfunction is initiated before significant epithelial cell loss. The primary dysfunction observed is a profound hyporesponsiveness to secretagogues in most species studied. Although this phenomenon has been described by us (8, 18) and others (11, 12), the underlying mechanism is not clear. We had, in earlier studies, described a role for mast cells (18), but more recent evidence suggests that mast cells do not mediate the perpetuation of the epithelial dysfunction (8), and effects on the epithelial cell itself have been suggested (16) but by no means unequivocally proven.

Understanding radiation-induced epithelial hyporesponsiveness to secretagogues is critical because the physiologically regulated secretion of chloride and water prevents transepithelial migration of bacteria, bacterial products, or antigens that could trigger an inappropriate and potentially chronic inflammatory response (29). Hyporesponsiveness to secretagogues is characteristic of inflammatory diseases of the gut and has been demonstrated in animal models (19) and human inflammatory bowel disease (14, 25). The exact mechanism underlying this apparently generalized response to inflammation is unclear, but recent data suggest that nitric oxide (NO) derived from the inducible isoform of NO synthase (iNOS) may be involved. NO is synthesized in numerous cell types, is a known modulator of epithelial function, and has been implicated in models of intestinal inflammation and human disease. In a model of acute colitis in the mouse, there is elevated iNOS expression and activity, as well as epithelial hyporesponsiveness to secretagogues (19). This hyporesponsiveness was partially reversed by pretreatment with a selective iNOS inhibitor. Similar observations have been made in a model of postinflammation dysfunction in a rat model (1). An earlier study showed that after exposure to ionizing radiation, NO is elevated in mouse intestine by an L-arginine-dependent mechanism (27). More recently, we have shown (17) that iNOS levels in the rat intestine were elevated as early as 2 h after radiation treatment.
From the preceding findings we have formulated the hypothesis that radiation-induced epithelial dysfunction, which may predispose the gut to chronic complications, is mediated by iNOS-derived NO. Here we describe the expression, activity, and function of iNOS-derived NO in colons from wild-type and iNOS-deficient mice exposed to therapeutically relevant doses of ionizing radiation. The studies demonstrate a central role for NO in radiation-induced dysfunction.

METHODS

Animals. Male C57BL/6 mice (Charles River; Montreal, QC, Canada), 4–6 wk of age, were housed at a constant temperature (22°C) and photoperiod (12 h light, 12 h dark). Mice were given 5–7 days to acclimatize to these conditions before inclusion in an experiment. They were allowed free access to standard lab chow and tap water. All procedures involving animals were approved by the University of Calgary Animal Care Committee, and experiments were conducted in accordance with the regulations established by the Canadian Council on Animal Care.

Irradiation. Conscious mice were physically restrained in plastic tubes during radiation. Mice were exposed to 10-Gy parallel-opposed field total body radiation from a 60Co therapeutic irradiator at a dose rate of 1.25 Gy/min (Theratron 780-C, Atomic Energy of Canada, Kanata, ON, Canada) at the Tom Baker Cancer Centre (Calgary, AB, Canada). Intestinal mucosal responses to irradiation are dose dependent (11), and this dose corresponds to that which we (18) and others (11) have used previously. Sham-treated mice were placed in the treatment field but were not exposed to the radioactive source. Mice were studied 3 days after irradiation or sham treatment.

White blood cell counts. Aliquots (50 µl) of whole blood were collected from anesthetized mice by cardiac puncture before tissue collection. The blood sample was added to 440 µl of 3% acetic acid and 10 µl of 1% crystal violet. White blood cells were counted in a hemocytometer. A 1-mm2 counting area was used. Cells falling on the center line at the top and right-hand sides were included in the count, whereas the cells on the center line at the bottom and left-hand sides were excluded. Cell counts were expressed as the number of white blood cells/ml of whole blood.

Myeloperoxidase activity. The colon was excised, and a whole thickness sample was taken for determination of myeloperoxidase (MPO) activity as previously described (3).

Nitrite and nitrate eluted as separate peaks. Concentrations were measured at 450 nm on a UVmax kinetic microplate reader (Bio-Rad, Hercules, CA), and densitometry was performed using Quantity One version 4.0.1 software (Bio-Rad).

HPLC. Nitrite and nitrate levels were determined by HPLC as previously described (20). Brieﬂy, the colon was excised and washed and incubated in 1 ml of PBS. The samples were incubated for 10 min at 37°C and then homogenized and centrifuged at 12,000 g for 10 min. The supernatant was taken for HPLC. The supernatant was centrifuged at 14,000 g for 2 min to remove any particulate matter. The samples were deproteinized by ultrafiltration through nitrocellulose filters (Centrisart C-4 ultrafiltration tubes, mol wt cutoff: 5,000; Sartorius, Germany) and then diluted in a 1-to-3 ratio with HPLC-grade water. The samples were deproteinized by ultrafiltration through nitrocellulose filters (Centrisart C-4 ultrafiltration tubes, mol wt cutoff: 5,000; Sartorius, Germany) and then diluted in a 1-to-3 ratio with HPLC-grade water. The samples were deproteinized by ultrafiltration through nitrocellulose filters (Centrisart C-4 ultrafiltration tubes, mol wt cutoff: 5,000; Sartorius, Germany) and then diluted in a 1-to-3 ratio with HPLC-grade water.

Electrolyte transport experiments. Mice were killed by cervical dislocation. The colonic tissue was rapidly excised and placed in ice-cold (4°C) Krebs buffer (in mM: 115 NaCl, 2.0 K H2PO4, 2.4 MgCl2, 1.3 CaCl2, 25.0 NaHCO3 and 8.0 KCl) bubbled with carbogen (5% CO2-95% O2). Feces were removed, and the colon was cut along the mesenteric border. The tissue was mounted in Ussing-type diffusion chambers (Navyeye, Sparks, NV) and bathed on both the serosal and mucosal sides with Krebs buffer (pH 7.4, 37°C) containing 10 mM glucose (pH 7.4). Buffers were circulated and oxygenated with a gas lift system using 95% O2-5% CO2. A voltage-damp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL) was used to maintain the potential difference across the tissue at 0 V. The short-circuit current (Isc) required to clamp the tissue was used as a measure of active electrogenic ion transport across the epithelium. Isc was recorded using a digital data acquisition system (MP100, Biopac Systems, Santa Barbara, CA) and analysis software (AqKnowledge, version 3.2.6, Biopac). The tissue conductance (Isc/potential difference) was measured as an indicator of epithelial ionic permeability. Electrical field stimulation (EFS) of jejunal segments in vivo (48) was used to clamp the tissue. Electrical field stimulation (EFS) of jejunal segments in vivo (48) was used to clamp the tissue and depolarize secretomotor neurons in the preparation to cause chloride secretion (5), which was measured as an indicator of epithelial ionic permeability. Electrical field stimulation (EFS) of jejunal segments in vivo (48) was used to clamp the tissue and depolarize secretomotor neurons in the preparation to cause chloride secretion (5), which was measured as an indicator of epithelial ionic permeability.
which the selective iNOS inhibitors L-N^6-(1-iminoethyl)lysine (L-NIL, 3 µM; Ref. 6) or 1400W (5 µM; Ref. 9) were applied serosally. After a 10-min incubation with either L-NIL or 1400W, responses to EFS and forskolin were measured. Histology. For immunohistochemistry, segments of mouse colon were removed and fixed in Zamboni’s fixative overnight. They were then rinsed and stored in PBS with azide and cryoprotected in PBS containing sucrase. Sections (12–14 µm) were cut using a cryostat (Microm) and placed onto poly-d-lysine-coated microscope slides. Sections were washed three times in PBS and were incubated with a rabbit anti-iNOS primary antibody (Transduction Laboratories; Lexington, KY) at 4°C overnight. Sections were then rinsed with PBS and incubated with donkey anti-rabbit CY3 conjugated secondary antibody (Jackson Laboratory, Bar Harbor, ME) for 90 min. They were then washed three times in PBS, cover-slipped, and viewed under epifluorescence using a Zeiss Axioplan microscope. Images were captured and digitized with a charge-coupled device video camera and Northern Exposure imaging software (Carson Vision, Edmonton, AB, Canada).

Drugs. Routine buffer reagents were obtained from BDH (Toronto, ON, Canada) unless indicated otherwise. 1400W was obtained from Alexis Biochemicals (San Diego, CA). Dexamethasone sodium phosphate was obtained from Sabex (Boucherville, QC, Canada). Other chemicals were obtained from Sigma Chemical (Mississauga, ON, Canada).

Statistics. Data are expressed as means ± SE. Statistical analyses were conducted using GraphPad Instat software (version 3.0, GraphPad Software, San Diego, CA). One-way ANOVA with a post hoc Tukey test was used to compare more than two groups. Unpaired Student’s t-test was used to compare two groups. A probability (P) value of <0.05 was considered significant.

RESULTS

Response to irradiation. Sections of colon were assessed histologically to determine whether there were any morphological changes in the colon 3 days after exposure to 10-Gy γ-radiation. At the light microscopic level, there were no differences between the sections taken from the sham-treated animals and those from the irradiated animals (data not shown). Crypt architecture was preserved in all cases, and the epithelium was structurally intact, other than occasional sectioning artifacts observed in both sham-treated and irradiated tissue. There was no obvious inflammatory cell infiltrate into the mucosa of colons from irradiated mice.

MPO was measured as an indicator of granulocyte (primarily neutrophil) influx into the tissue (3). There was no statistical difference between the MPO levels found in the sham (n = 6) and irradiated (n = 7) tissue (Table 1). Furthermore, white blood cell counts were significantly (P < 0.001) lower in irradiated animals compared with sham-treated mice (n = 6 per group) (Table 1).

Expression of iNOS. Expression of iNOS mRNA, as determined by RT-PCR, was induced in colonic segments taken from irradiated mice (Fig. 1). Expression of iNOS in control animals was either absent or was present at very low levels in colonic tissue from sham-treated mice (n = 9). Densitometry showed a significant upregulation of iNOS expression in irradiated colon (n = 7) compared with controls (Fig. 2). Not surprisingly, iNOS expression was not observed in colonic segments from either sham-treated or irradiated iNOS-deficient (iNOS−/−) mice (n = 6 per group; C57BL/6-Nos2tm1Lau, backcross generation N10; Jackson Labs, Bar Harbor, ME; Fig. 1). Optical density of the PCR product of GAPDH, which was run as the housekeep-

![Fig. 1. Inducible nitric oxide (NO) synthase (iNOS) mRNA expression in samples of colon taken 3 days after either sham treatment (Sh) or exposure to 10-Gy γ-radia-tion (Rad) in wild-type (iNOS+/+) vs. iNOS knockout (iNOS−/−) mice. Figure is a representative reverse image of ethidium bromide fluorescence under ultraviolet light. Expression of iNOS was observed in colons from irradiated wild-type mice but not from iNOS−/− mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message was run as housekeeping gene.]

![Fig. 2. Expression and activity of iNOS in colons 3 days after either sham treatment (open bars) or exposure to 10-Gy γ-radiation (filled bars). A: ratio of density of iNOS-to-GAPDH bands after RT-PCR. Significant induction was observed after irradiation. *P < 0.05 vs. sham treatment. B: iNOS activity was determined by measurement of nitrate and nitrite by HPLC. Significantly more nitrate and nitrite were observed in tissues after irradiation. *P < 0.05 vs. sham treatment.](https://example.com/fig2.png)
ing" gene, was not significantly different between sham and irradiated groups (n > 6 per group).

Expression of iNOS protein was assessed immunohistochemically in colons from sham-treated and irradiated mice (Fig. 3). iNOS immunoreactivity (iNOS-IR) was observed in colons from sham-treated mice. In contrast, iNOS-IR was observed in colons from mice killed 3 days after irradiation. The iNOS-IR was confined primarily to the surface epithelium, but in some mice it was observed in cells at the base of the crypts. It was not possible to determine if epithelial cell type (enterocyte, goblet cell, intraepithelial lymphocyte) expressed iNOS.

HPLC was used to measure nitrite and nitrate levels in the tissue to provide an indirect measure of NOS activity. Nitrite and nitrate levels were significantly elevated in the colons of irradiated mice (n = 11) compared with controls (n = 7) (Fig. 2).

Electrolyte transport studies. Colonic segments from sham-treated and irradiated mice were mounted in diffusion chambers and voltage clamped after challenge with EFS and forskolin. Exposure to EFS and forskolin caused a significant increase in $I_{sc}$ (Fig. 4). Reduced responses to both EFS and forskolin were significantly lower in colonic tissues from irradiated mice (sham, n = 8; IR, n = 6) (Fig. 4). Acute inhibition of iNOS activity with the selective iNOS inhibitors L-NIL or 1400W reversed the hyporesponsiveness of the irradiated tissue (Fig. 4). Similarly, pretreatment of mice with dexamethasone, which blocks iNOS expression, reversed the radiation-induced hyporesponsiveness to EFS and forskolin (sham + saline, n = 6; sham + Dex, n = 6; IR + saline, n = 6; IR + Dex, n = 6) (Fig. 5).

Another measure of epithelial function is tissue conductance, which is an estimate of the epithelial permeability. Exposure of mice to radiation resulted in a significant increase in tissue conductance (Fig. 6). This was also reversed by pretreatment of the tissue with either L-NIL or 1400W (n > 6 for all treatment groups).

To further demonstrate that iNOS was involved in the hyporesponsiveness of the colon to irradiation,
these studies were repeated in iNOS−/− mice. There was no effect of radiation on responsiveness of colonic segments from iNOS−/− mice to EFS (sham, n = 4; IR, n = 5) or forskolin (sham, n = 4; IR, n = 4). The iNOS−/− mice responded to EFS and forskolin to the same degree as sham-treated iNOS−/− mice (Fig. 7). Furthermore, iNOS−/− mice exhibited no radiation-induced increase in tissue conductance, in contrast to wild-type mice (sham, n = 4; IR, n = 5) (Fig. 7).

Fig. 6. Tissue conductance in segments of colon mounted in diffusion chambers. Conductance was measured 3 days after either sham treatment (open bars) or exposure to 10-Gy γ-radiation (filled bars). Radiation-induced increase in conductance was reversed by pretreatment of tissue with selective iNOS inhibitors L-NIL and 1400W. W, water (vehicle control). *P < 0.05 vs. sham vehicle control.

DISCUSSION

In this study we have shown that, 3 days after exposure to a single 10-Gy dose of γ-radiation, the mouse colon is hyporesponsive to EFS and forskolin compared with sham-treated controls in vitro. This hyporesponsiveness is caused, as in models of colitis, by iNOS-derived NO. Of particular interest in the present study is the fact that NO-induced epithelial hyporesponsiveness occurs in the absence of an inflammatory response.

We initially hypothesized that the hyporesponsiveness observed after irradiation was caused by the induction of an inflammatory response. Intestinal inflammation, such as that triggered in animal models of inflammatory bowel disease, or in human inflammatory bowel disease, is associated with epithelial hyporesponsiveness.
responsiveness to secretagogues (10, 13, 19). Furthermore, local abdominopelvic irradiation of anesthetized rats was accompanied by a rapid neutrophil influx into the intestinal submucosa and mucosa (4). One of the crucial findings of the present study, however, is the fact that the hyporesponsiveness observed after irradiation is not associated with an inflammatory response. Several pieces of evidence support this. First, there was no inflammatory cell influx observed in histological sections of colon from irradiated mice, at least at 3 days after 10-Gy radiation (data not shown). Our observations at 3 days after irradiation do not preclude granulocyte influx at an earlier time point after irradiation, although it is unlikely that this would occur to any appreciable degree and still be completely resolved in 3 days. Histological assessment of colonic samples after 10-Gy radiation confirmed that, at least at the light microscopic level, the epithelium was structurally intact and the normal crypt architecture was observed. Thus significant cell loss and epithelial denudation does not account for the observed hyporesponsiveness at this time point. Similarly, epithelial patency was observed after irradiation in the rat small intestine (18).

Second, colonic MPO activity, which is a measure of granulocyte infiltration into the tissue, was not different between sham-treated and irradiated mice (Table 1). Our previous studies (18) have shown that in the rat ileum and colon hyporesponsiveness to EFS after irradiation occurs in the absence of an acute inflammatory response as measured by MPO. The difference between our studies and that of Buell and Harding (4), which showed a rapid inflammatory influx into the tissue, is the mode of irradiation used. We have treated animals with whole body irradiation, whereas their studies were in animals receiving local abdominopelvic exposure, which would leave sufficient hematopoietic tissue unaffected to allow for granulocyte repopulation and subsequent recruitment into the injured site. Finally, in our studies, whole body exposure of mice to 10-Gy γ-radiation was associated with a white blood cell count that was significantly depleted at 3 days, suggesting that there was a reduced pool of potential inflammatory cells available for recruitment to sites of injury. Taken together, these results suggest that a radiation-induced inflammatory response was not a contributing factor in the hyporesponsiveness of the tissue to secretagogues in vitro at this time point.

Of note in this study is the observation that radiation-induced epithelial hyporesponsiveness is mediated by iNOS-derived NO, despite the lack of an inflammatory response. Increased expression of iNOS mRNA was associated with increased production of NO, as shown by HPLC measurement of nitrate and nitrite levels in the tissue (Fig. 1). Inhibition of iNOS activity with the iNOS inhibitors L-NIL and 1400W, at doses that have been shown to be selective for iNOS (9, 19), returns the I sc response to EFS and forskolin to levels similar to those observed in the sham-treated mice (Fig. 4). The results further demonstrate an ongoing release of iNOS-derived NO, because the inhibitors were added to the bath only 10 min before challenge with secretagogue. A similar reversal of hyporesponsiveness was observed in mice pretreated with dexamethasone, which prevents induction of iNOS (7). The conclusion that iNOS is involved in the epithelial hyporesponsiveness is further supported by the studies in iNOS-deficient mice, which did not exhibit radiation-induced increase in conductance or hyporesponsiveness to EFS or forskolin. NO has been shown to inhibit secretory responses in rat small intestine in vivo (2, 26). Our previous studies revealed an iNOS dependency of inflammation-induced hyporesponsiveness, both during acute inflammation in mouse colon (19) and 6 wk after a bout of colitis in the rat (1). The present observation that iNOS-derived NO mediated epithelial hyporesponsiveness to secretagogues in the absence of an inflammatory response is important in that it suggests that generation of NO represents a common mechanism underlying the inability of the epithelium to respond to secretagogues in a number of clinical situations.

Our data have indicated an apparent difference in responsiveness of sham-treated wild-type and iNOS-deficient mice. Although responses to EFS were reduced in the colons from iNOS−/− mice, responses to forskolin were not. If the iNOS−/− mice were basally hyporesponsive in the same sense or through the same mechanism that irradiated wild-type mice were hyporesponsive, then we would have expected reduced I sc responses to both EFS and forskolin. Given the difference in responsiveness to EFS and forskolin in the iNOS−/− mice, we suggest that perhaps the iNOS−/− mice exhibit a different degree of enteric neural control of epithelial function. Differences, as yet undescribed, may exist between these strains with respect to enteric nervous system “wiring” and/or function. If this is the case, then our data exemplify the caution that must be exercised in comparing physiological data derived from wild-type versus genetically deficient animals.

The cellular source of iNOS that contributes to radiation-induced hyporesponsiveness to secretagogues is not clear, but the data presented here (Fig. 3) suggest that it is localized to the epithelium. In some mice, epithelial expression was restricted to the surface epithelial cells, whereas in other mice, expression was observed in both surface and crypt cells. We have previously shown (17) that whole body exposure of rats to 10-Gy γ-radiation resulted in induction of iNOS mRNA in ileum and colon as early as 2 h after irradiation. In the rat, however, expression returned to control levels by 24–48 h, whereas in the present study we still have evidence of iNOS induction at 3 days after exposure. We have also found that, contrary to the rat, in which radiation-induced iNOS immunoreactivity is always localized to epithelial cells along the length of the crypt-villus axis, iNOS in irradiated mouse colon is found primarily in the apical epithelial cells. The secretion of chloride and water by the colonic epithelium is thought to be localized to the relatively undifferentiated crypt enterocytes (28). Clearly, in those mice in which iNOS expression is present in the crypt, NO may act on chloride-secreting enterocytes in a paracrine, or even autocrine, manner. Furthermore, it is possible
that NO could still affect crypt function in those mice in which iNOS immunoreactivity was confined to the surface epithelial cells. NO, although a free radical, is relatively unreactive, and this, along with its hydrophobicity, may allow it to have effects several cells away from its site of production. Furthermore, the mucosal microcirculation could provide a means of transport for apically derived NO to the crypt region.

The implications for epithelial hyporesponsiveness are important when one considers the host defense role played by secretion. The secretion of chloride and, hence, water into the crypt lumen prevents or limits the translocation of bacteria, bacterial products, or antigens into the lamina propria, where they may trigger or perpetuate an inflammatory response (29). Change in epithelial barrier function, which also includes increased permeability, is a predictor of and likely contributes to relapses of inflammation in Crohn's disease (30). Given the link between the acute and chronic development of inflammatory conditions in the gut, there is a clear impetus to discover the mechanisms of, and therapeutic interventions for, radiation-induced epithelial hyporesponsiveness to secretagogues.

The mechanism whereby NO reduces epithelial responsiveness to secretagogues after irradiation remains to be determined. Recently, it was shown that NO reduced the expression of the α-subunit of Na⁺-K⁺-ATPase in the MTAL kidney cell line (15). Furthermore, Na⁺-K⁺-ATPase activity is reduced in colonic epithelial cells after irradiation (16). Na⁺-K⁺-ATPase provides the electrochemical gradient that favors chloride flux into the enterocyte, with subsequent secretion through the apical chloride channel. A link among radiation, NO, and Na⁺-K⁺-ATPase has not been described. Further studies will be required to more fully explore the effect of NO on radiation-induced epithelial secretory dysfunction.

The authors thank J ane Wiggins (Tom Baker Cancer Centre, Alberta Cancer Board, Calgary, AB) for calculation of radiation dose and operation of the Theratron irradiator, Dr. Marcello Muscara for measuring nitrogen oxides by HPLC, and Dr. Keith Sharkey for assistance with iNOS immunohistochemistry. This work was supported by a grant (MT-14660) from the Medical Research Council of Canada. W. K. MacNaughton is an Alberta Heritage Foundation for Medical Research Scholar.

Address for reprint requests and other correspondence: W. MacNaughton, Dept. of Physiology and Biophysics, Univ. of Calgary, 3330 Hospital Dr. NW, Calgary, AB, Canada T2N 4N1 (E-mail: wmacnaug@ucalgary.ca).

Received 17 August 1999; accepted in final form 28 October 1999.

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