Regional differences in the response of feline esophageal smooth muscle to stretch and cholinergic stimulation

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Received 12 March 2001; accepted in final form 8 August 2001

Regional differences in the response of feline esophageal smooth muscle to stretch and cholinergic stimulation. Am J Physiol Gastrointest Liver Physiol 281: G1460–G1467, 2001.—There are no objective differences in neural elements that explain regional differences in neural influences along the smooth muscle (SM) esophageal body (EB). Regional differences in muscle properties are present in the lower esophageal sphincter (LES). This study examines whether regional differences in SM properties exist along the EB and are reflected in length-tension relationships and responses to cholinergic excitation. Circular SM strips from feline EB at 1 cm (EB1) and 3 cm (EB3) above LES and from clasp and sling muscle bundles of LES were assessed in normal and calcium-free solutions with and without bethanechol stimulation. Neural inhibition was assessed by electrical field stimulation (EFS). EB3 developed significantly higher tension in response to stretch and to bethanechol than did EB1. The relaxation response to EFS in bethanechol-precontracted strips was less in EB3 than in EB1. In LES, clasp developed higher resting tension than sling but less active tension in response to bethanechol. EFS-induced relaxations of sling and clasp tissues precontracted by bethanechol were not different. In calcium-free solution, length-tension differences between EB3 and EB1 persisted, but those of LES clasp and sling were abolished. Therefore, regional myogenic differences exist in feline EB circular SM as well as in LES and may contribute to the nature of esophageal contraction.

Differences in the cholinergic responses of smooth muscle from clasp and sling regions of the lower esophageal sphincter (LES) and between LES and esophageal body (EB) smooth muscle are known to exist. Studies of the clasp and sling fibers from cat and human LES (20, 21) have demonstrated that the clasp has greater tension but less cholinergic responsiveness than the sling. Furthermore, in response to cholinergic stimulation, there are differences between the LES and the EB in the predominant calcium source used for contraction (3). Therefore, the present studies were performed to 1) assess differences of the active (response to bethanechol) and passive properties of cat esophageal smooth muscle isolated from proximal and distal regions of the EB and 2) compare these differences in the EB with those seen in the clasp and sling regions of the LES.

MATERIALS AND METHODS

Tissue removal. The experimental protocol was approved by the University Health Network Animal Care Committee. Adult cats of either sex weighing between 2.5 and 5.0 kg were anesthetized with ketamine hydrochloride (0.15 ml/kg im; Bimeda-MTC, Cambridge, Ontario, Canada) and euthanized with pentobarbital sodium (0.5 ml/kg iv; Bimeda-MTC). A midline incision was made, and the chest was opened. A length of esophagus from 10 cm above the LES to 4 cm below it was removed and immediately placed in Krebs solution equilibrated with 5% CO2-95% O2 and maintained at pH 7.4 ± 0.05.

Muscle strip preparation. The specimen was freed of surrounding fascia and stretched to its in situ length. The esophagus was then opened up along the greater curvature,
and the mucosa was removed by sharp dissection. The circular and clasp fibers of the LES were readily visible (21). Muscle strips measuring 2 mm in width and 10 mm in length were obtained from the circular smooth muscle esophageus and from the circular (medial side) and sling fibers of the LES. Care was taken to ensure that the orientation of the muscle fiber bundles ran parallel to the long axis of the muscle strips. A silk thread was tied to each end, and the strips were transferred to 25-mL organ baths containing Krebs solution bubbled with 5% CO2-95% O2 at 37°C and maintained at pH 7.4 ± 0.05. One end of the strip was fixed to an electrode holder and the other end to an isometric force transducer (model FT-03; Grass Instruments, Quincy, MA) coupled to a chart recorder (model 79E; Grass Instruments).

The force transducer was supported on a rack-and-pinion clamp (Harvard Apparatus) that facilitated accurate length adjustment of the muscle strips. Transmural electrical field stimulation (EFS) was delivered by a Grass stimulator (SP-9) through platinum wire electrodes placed on either side of the tissue strips. EFS consisted of 0.5-ms square-wave pulses in a 5-s train at 10 Hz and a strength of 50 V. Tissue strips were hung loosely with no tension being applied to them in the organ baths for a 1-h equilibration period before the studies commenced.

Length-tension relationships. To compare the length-tension relationships of muscle strips from different regions, each strip was gently stretched to initial length (L0), which was first determined with a micrometer as the length (in mm) at which a rapid stretch caused a small transient deflection of the recorder pen (~50 mg of tension). At L0, any slack in the strip or silk ties was eliminated and further stretch began to produce tension in response to stretch. The muscle strip was then allowed to equilibrate at L0 for 30 min, during which time the strips could develop some tension, which was called spontaneous tension. Muscle strips were then slowly stretched sequentially and tested at increments of 25% of their L0 to a maximum of 250% L0 according to the protocols below.

Stretch-induced and total tension. Initial stretch of 25% L0 resulted in the development of tension in response to stretch, which was allowed to stabilize for 15 min, at which time the amplitude was recorded. Bethanechol (10−5 M) was then added to the chamber and was allowed to act on the strips for 15 min. The tension at this time was defined as the total tension. EFS was then carried out in the bethanechol- contracted muscle strips to determine the maximal relaxation response in these precontracted muscles. Bethanechol was then washed out from the recording chamber with normal Krebs for 15 min, during which time baseline tension was reestablished at the given level of stretch before the strips were stretched to the next length and the protocol cycle was repeated.

To assess the passive mechanical properties of the tissue, a separate set of length-tension experiments was carried out in a calcium-free Krebs solution. The protocol was similar to that described above, the only difference being the substitution of normal Krebs with calcium-free Krebs solution.

A separate set of muscle strip experiments was carried out to examine the neurogenic component of the spontaneous length-tension relations in the EB and LES. In these experiments, length-tension relationships of the muscle strips were carried out in the presence of TTX (10−6 M). The parameter assessed was tension developed in response to stretch.

At the end of all experiments, the strips were blotted on filter paper and the wet weight was determined. Tension was then normalized to the wet tissue weight and expressed in milliNewtons per milligram.

Composition of solutions. The Krebs solution had the following composition (in mM): 115 NaCl, 4.6 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 22 NaHCO3, 2 CaCl2, and 11 glucose. The calcium-free medium contained (in mM): 115 NaCl, 4.6 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 22 NaHCO3, 11 glucose, and 0.1 EGTA.

Drugs. All drugs were obtained from Sigma Chemical (St. Louis, MO). Bethanechol and TTX were prepared in double-distilled H2O and added directly to the organ bath chamber from stock solution of 100× dilution.

Statistical analysis. At L0, any tension generated during the 30-min equilibration period in the absence of stretch or bethanechol was defined as spontaneous tension. The parameters measured at each muscle strip length were 1) tension developed in response to stretch, which was called stretch tension, and 2) maximum total tension, the total tension developed at each level of stretch after bethanechol (10−5 M) challenge. Active tension at each stretch length was calculated as maximum total tension in response to bethanechol minus tension in response to stretch. Maximal relaxation of tonically contracted muscle in response to EFS is expressed as a percentage. Statistical comparisons were made with two-tailed paired Student’s t-test (using Instat Graph Pad software). Data are presented as means ± SE, and n is the number of cats. P < 0.05 was considered significant.

RESULTS

EB. A dose-response curve was performed for bethanechol (3–100 μM) on muscle strips from the EB prestretched to 130% L0. The region of the EB 3 cm above the LES (EB3) consistently demonstrated a greater contractile response to bethanechol stimulation than the region 1 cm above the LES (EB1; Fig. 1; n = 6). From this curve, 10−5 M bethanechol was chosen because it resulted in near-maximal contraction in all strips studied. Subsequent studies were then performed on strips from both EB3 and EB1.

Muscle strips from all the regions examined showed an increase in tension as their length was increased.

Fig. 1. Comparison of concentration-dependent increase in tension in response to bethanechol in the esophageal body (EB). Note esophageal circular smooth muscle from 3 cm above (EB3) the lower esophageal sphincter (LES) demonstrated a greater contractile response to bethanechol stimulation than at 1 cm above the LES (EB1). aP < 0.05; n = 6.
Neither circular muscle from EB3 or EB1 produced any measurable spontaneous tension at L0 (Table 1). Circular muscle from EB3 developed significantly higher tension in response to stretch than muscle from EB1 (Fig. 2A; n = 9), and the first increase in tension occurred at less stretch in EB3 (150% L0) than in EB1 (175% L0). Moreover, the magnitude of the tension in response to stretch was greater in EB3 than either sling or clasp muscle strips (Table 1). To examine the role of neural innervation, separate length-tension curves were determined in the presence of the sodium channel blocker TTX. TTX (10−6 M) blocked EFS contractions in all muscle strips studied. However, in the presence of TTX, there were no significant changes in the length-tension relationships of EB3 and EB1 in response to stretch (Fig. 2B), the differences between the two regions persisting.

In response to cholinergic stimulation, regional differences within the EB circular muscle were again observed in the length-tension experiments. Circular muscle from EB3 (34.47 ± 7.6 mN/mg) produced significantly greater maximum total tension in response to bethanechol (10−5 M) than the circular muscle from EB1 (18.18 ± 3.2 mN/mg; *P < 0.05 for EB3 vs. EB1; Fig. 3). Although the maximum active tension in response to bethanechol was similar at EB1 and EB3, the maximum active tensions occurred at different lengths: 125% L0 for EB3 and 150% L0 for EB1 (Table 1 and Fig. 3). This latter difference is a reflection of the cholinergic response in EB3 being most prominent between L0 and 150% L0, whereas the response in EB1 was evident at all lengths of stretch studied.

Length-tension relationships were also determined in calcium-free Krebs solution in separate sets of strips. Similar to experiments in normal Krebs solution, EB3 developed significantly greater tension than EB1 in response to stretch (Fig. 4A). In calcium-free Krebs solution, bethanechol did not cause contraction, nor was any change observed in the length-tension relationships in calcium-free Krebs solution (n = 5). Unlike the control studies, in which spontaneous tension and tension in response to stretch were greater in the clasp up to 175% L0, no significant differences in the length-tension curves were observed between the sling and clasp muscle strips under conditions of calcium-free Krebs (Fig. 8A). The two curves were virtually identical and similar to the sling curve in normal Krebs solution. Bethanechol stimulation produced no contraction of either sling or clasp muscle at any stretch in the calcium-free Krebs environment, the two length-tension curves again being virtually identical (Fig. 8B) and similar to the sling curve in normal Krebs solution.

Maximal relaxation induced by EFS was assessed after bethanechol addition to the muscle strip chambers. Circular muscle from EB3 tends to relax less in response to EFS than the muscle from EB1 (at 150% L0, 34% vs. 63%; *P < 0.05; Fig. 5A).

**LES.** Both sling and clasp muscles exhibited spontaneous tension development at L0 (Table 1). Clasp muscle developed higher tension than sling muscle with stretch up to 175% L0 (Fig. 6A; n = 9), but beyond this stretch the two muscles were similar (Table 1). As in the control conditions, in the presence of TTX clasp muscle developed higher stretch tension than sling muscle with stretch up to 175% L0 (Fig. 6B; n = 5). Moreover, neither spontaneous tension nor tension developed in response to stretch was affected by addition of TTX to the baths (i.e., control length-tension curve vs. TTX-treated length-tension curve) at any of the lengths studied.

In response to bethanechol (10−5 M), maximum total tension generated by sling and clasp tissue at each stretch did not show any significant difference (Fig. 7). However, in response to bethanechol, the active tension (maximum total tension minus tension in response to stretch at each length) was more than two times greater in the sling muscle than the clasp muscle (Table 1 and Fig. 7).

Length-tension studies were also performed in calcium-free Krebs solution (n = 5). Unlike the control studies, in which spontaneous tension and tension in response to stretch were greater in the clasp up to 175% L0, no significant differences in the length-tension curves were observed between the sling and clasp muscle strips under conditions of calcium-free Krebs (Fig. 8A). The two curves were virtually identical and similar to the sling curve in normal Krebs solution. Bethanechol stimulation produced no contraction of either sling or clasp muscle at any stretch in the calcium-free Krebs environment, the two length-tension curves again being virtually identical (Fig. 8B) and similar to the sling curve in normal Krebs solution.

| Table 1. Summary of the effect of stretch and cholinergic stimulation on tension development in esophageal body and lower esophageal sphincter |
|-----------------|-----------------|-----------------|
| Tension, mN/mg  | EB              | EB1             | Clasp           | Sling           |
| Spontaneous     | 0.05 ± 0.02     | 0.05 ± 0.01     | 1.99 ± 0.2      | 0.68 ± 0.6†     |
| Stretch induced |                 |                 |                 |                 |
| 150% L0         | 5.62 ± 1.59*    | 1.06 ± 0.22     | 6.47 ± 0.47     | 1.97 ± 0.45†    |
| 250% L0         | 35.34 ± 7.0*    | 14.51 ± 3.8     | 16.86 ± 1.0     | 19.29 ± 1.19    |
| Total           | 9.69 ± 2.06*    | 6.4 ± 0.7       | 10.34 ± 0.87    | 11.29 ± 1.53    |
| 150% L0         | 34.47 ± 7.6*    | 20.63 ± 4.9     | 18.66 ± 1.81    | 21.8 ± 1.42     |
| Active          | 4.06 ± 1.02     | 4.94 ± 1.07     | 3.06 ± 1.10     | 9.29 ± 1.54†    |
| ActiveMax       | 6.24 ± 1.30     | 4.94 ± 1.07     | 4.06 ± 1.10     | 9.29 ± 1.54†    |
| Length activeMax, % L0 | 125  | 150  | 150  | 150  |

Values are means ± SE. EB, esophageal body; EB1 and EB3, tissue from 1 and 3 cm above the LES, respectively; LES, lower esophageal sphincter. Spontaneous tension is the tension at the initial length of the muscle strip (L0). Stretch-induced tension and total tension are all from stretch lengths of 150% L0 and 250% L0. The length at which maximal active tension (activeMax) occurred is listed in the table above. Pairwise comparisons were made between regions by Student’s t-test (n = 9). *Significantly different from corresponding value for EB1. †Significantly different from corresponding value for clasp muscle.
AfterbethanecholcontractionintheLES,therewas
no significant difference in relaxation induced by EFS
between sling and clasp muscle strips (Fig. 5B).

DISCUSSION

This study in the cat is the first study to examine
length-tension relationships in different regions of the
circular smooth muscle of the EB. Previous studies of
length-tension relationships of the EB have examined
the distal 3–8 cm in the human (20, 29, 30) and distal
0.5–2 cm in the cat (4, 21). However, these studies did
not assess regional differences by differentiating be-
tween the proximal and distal portions of the smooth
muscle esophagus but rather pooled all of the results.
Similarly, in the region of the LES, only one study in
the human assessed the sling and clasp fibers sepa-
rately (20), and studies in the cat used rings or large
segments of the LES that included both sling and clasp
fibers (4, 21). We found that circular smooth muscle
from EB3 develops greater tension with stretch, and
this difference remains in calcium-free Krebs solution;
2) is functionally more responsive to cholinergic exci-
tation; and 3) is less responsive to inhibitory innerva-
tion than the more distal (EB1) smooth muscle. We
also found that in the isolated regions of smooth muscle
from the LES 1) clasp develops greater spontaneous
tension and tension with stretch than sling, 2) sling
muscle demonstrates a much greater response to cho-
inergic excitation, and 3) regional differences in sponta-
nous and stretch-induced tension are abolished in
calcium-free Krebs solution. The studies reported in
this paper support the hypothesis that regional myo-
genic differences are present in the EB as well as in the
LES and are reflected in the length-tension character-
istics and in the cholinergic sensitivity of the muscle.
In the EB, a study of only two sites does not determine
whether these regional differences represent a gradi-
ent along the esophagus or two or more discrete re-
gions. The previously demonstrated gradient in neural
responsiveness may favor the former interpretation.

EB. Comparison of the length-tension relationships
between EB1 and EB3 and between the esophageal
and LES clasp and sling regions demonstrates a num-
ber of differences that likely relate to the physical
properties of the contractile and noncontractile elements in the different muscles and, potentially, to functional differences.

Tottrup et al. (30) have provided a proposed model for esophageal smooth muscle based on the Hill-Maxwell model that includes the parallel elastic component (PEC), contractile element, and series elastic component. In muscle strips of the human EB, there is little or no spontaneous active tone (29) and the passive length-tension relationships are the same in normal Krebs and in calcium-free medium. Our findings in the cat are similar, and furthermore the differences between EB1 and EB3 persisted in calcium-free medium. That is, the passive length-tension relationship in the EB is determined almost exclusively by the elements in the PEC that are independent of the active contractile machinery. Our findings were not altered by neural blockade with TTX, indicating that the true muscle response was assessed. Previous studies have also demonstrated that tension was not affected by addition of TTX in either the LES or EB (20, 29). However, those studies did not differentiate between sling and clasp fibers in the LES or between different regions along the EB. Nevertheless, in the human in vivo, there appears to be an active component to resting tone of the EB, because amyl nitrate inhalation can reduce the tone (11, 15). Presumably this is neurally mediated.

The differences therefore in the length-tension relationship between EB1 and EB3 must reside primarily in those elements unrelated to active contraction. Differences in muscle fiber arrangement within the EB may be present but have not as yet been assessed in esophageal muscle. Connective tissue such as proteoglycans, glycoproteins, elastins, and collagen are found in visceral smooth muscle and can affect the ability of smooth muscle to develop tension with stretch. Collagen concentration and the number and types of collagen cross-links contribute to the passive components of the length-tension curves. Previous studies examining collagen composition in the longitudinal and circular layers of the EB and LES revealed no differences between muscle types (29). More recently, connective tissue composition in the lamina propria and submucosa of the opossum smooth muscle esophagus found that the connective tissues of the two regions are similar with regard to fiber orientation, but the lamina propria contains relatively more collagen III (small fibril) and the submucosa contains relatively more collagen I (large fibril) (14). However, these studies did not compare collagen content along the EB at different

Fig. 4. Comparison of length-tension relationship in the EB with calcium-free Krebs solution. A: circular muscle from EB3 developed significantly higher tension with stretch than muscle from EB1. B: in calcium-free Krebs, bethanechol stimulation did not effect the length-tension relationship in the EB. (*P < 0.05, n = 5).

Fig. 5. Maximum relaxation to electrical field stimulation (EFS) of bethanechol-induced contraction in EB (A) and LES (B). A: circular muscle from EB3 tends to relax less in response to EFS than the muscle from EB1 (at L1.5 34% relaxation vs. 63% relaxation; *P < 0.05; n = 9). B: muscle strips from the sling and clasp regions did not show a significant difference of relaxation in response to EFS.
lengths or between clasp and sling fibers. It is possible that there exists a greater amount of collagen and greater number of collagen cross-links in EB3 than EB1 to account for the greater tension in response to stretch.

In addition to the differences in the passive length-tension relationships between EB1 and EB3, cholinergic stimulation with bethanechol demonstrated significant differences in the active contractile responses of the two regions. Both the maximum total and active tension in response to bethanechol were greater in EB3 muscle. The shape of the active component of the length-tension curve showed increased tension with increasing length reaching a maximum, followed by a decline with further stretch. This optimum tension is presumably reached at a level of optimum overlap between the sliding filaments. The maximum active tension occurred at less stretch in EB3 and rapidly declined with increasing stretch, whereas that in EB1 not only occurred at a greater length but persisted as stretch was increased. This is in contrast to maximum total tension, which was consistently greater at EB3 than EB1 at all lengths studied. These findings suggest that the contractile proteins and/or the mechanisms that couple excitation to contraction may be different in the two regions. Differences in these proteins in phasic and tonic smooth muscles of the opossum EB and LES, respectively (28), and in the myosin phosphorylation of these muscles in the cat esophagus (31) have been demonstrated, but this type of analysis has not been applied to regional differences in the EB.

Therefore, at EB3, the tension that developed with stretch beyond 175% L0 was in large part determined by the passive components in the PEC, whereas at EB1, active contractile elements were contributing to at least 225% L0. That is, distally the PEC is more compliant and the active component is more responsive at greater stretch, but the active component produces less overall tension than that proximally at EB3. From a functional point of view, this combination of differences could be seen as advantageous to distal propulsion if a less compliant proximal region with more forceful contraction led into a more compliant region where active muscle tone could be more readily modu-

![Fig. 6. Comparison of length-tension relationship in LES. A: clasp muscle developed significantly higher spontaneous tension and tension in response to stretch up to 175% L0 (\(P < 0.05; n = 9\)). B: in the presence of TTX (10^{-6} M), regional differences in tension development in response to stretch persisted (\(P < 0.05; n = 5\)).](http://ajpgi.physiology.org/)

![Fig. 7. Comparison of length-tension relationship and effect of bethanechol on sling (A) and clasp (B) muscles. In response to bethanechol (10^{-6} M), total tension generated by sling and clasp did not show any difference. Maximum active tension (maximum total tension minus tension in response to stretch) of sling was greater than clasp muscle (\(P < 0.05; n = 9\)).](http://ajpgi.physiology.org/)
lated to accommodate receptive relaxation. Of interest in this regard, the contracted distal circular muscle from EB1 tended to relax more in response to EFS than the proximal muscle from EB3. Although the relaxation was due to release of inhibitory neurotransmitter from nerves, there is no definite objective evidence that the inhibitory neural elements are different in number relative to the smooth muscle content or in release of neurotransmitter along the EB to explain an increasing functional effect distally along the esophagus (16, 18, 19). Is it possible that, in addition to myogenic differences in responsiveness to cholinergic excitation, there are also regional myogenic differences in the response to the inhibitory neurotransmitter nitric oxide? If so, this difference may impact on the timing of the contraction and therefore peristaltic velocity because delay of the contraction is related to action of the inhibitory neurotransmitter (1, 6, 17, 32, 33).

The functional gradient in the nitregeric inhibitory influence (increasing distally) and in the cholinergic excitatory influence (decreasing distally) along the esophagus appears to have importance in regulating the timing of the peristaltic contraction, the former delaying the contraction and therefore peristaltic velocity because delay of the contraction is related to action of the inhibitory neurotransmitter (1, 6, 17, 32, 33).

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Fig. 8. Comparison of length-tension relationship in the LES with calcium-free Krebs solution. A: in calcium-free Krebs, the length-tension relationship of sling and clasp are identical. B: bethanechol had no effect on LES length-tension curves between sling and clasp strips in calcium-free Krebs ($P > 0.05; n = 5$).

LES. Studies of the isolated LES clasp and sling muscles further defined the length-tension relationships in these muscles and the differences in their responsiveness to cholinergic stimulation, generally confirming the previous findings in the isolated muscles in the human (20) and in the more intact combined tissues in the cat (21). The clasp muscle has much more spontaneous tone than the sling but is much less responsive to cholinergic stimulation. Nevertheless, although the total tension at each stretch with cholinergic stimulation was slightly greater in the sling, this difference was not significant, suggesting that the spontaneous tone in the clasp leaves limited extra room for further contraction.

The increase in tone in the clasp is evident with stretch up to 175% $L_0$, but at stretch of 200% $L_0$ and greater, the two muscles are similar, indicating that the noncontractile PEC elements are at this stage dominant and are very similar in the two muscles. The latter similarity is further evident in the length-tension relationships in calcium-free medium in which the active component is now gone from the clasp muscle and the two curves are identical in the presence or absence of bethanechol. We did not explore to what extent the loss of clasp tone related to an intracellular or extracellular source of calcium (3, 9). However, as with the differences seen in the EB smooth muscle, further investigation is necessary to establish the mechanisms responsible for the differences.

These additional findings have interesting functional implications in vivo. Because the LES pressure profile shows a higher pressure in the left lateral-posterior aspect in both the human (22, 25, 27) and the cat (21), this aspect being most sensitive to atropine, it is likely that the sling, in addition to the clasp, is an integral and important physical (13) and physiological contributor to the LES. For this reason, Schneider et al. (25) have raised the importance of regional LES differences in patients with achalasia, and similar at-
tention has been paid to the two muscle regions and their potential role in the pathogenesis of gastroesophageal reflux disease (27).

In conclusion, the different mechanical properties of the EB smooth muscle from these two regions suggest that regional differences likely contribute to esophageal biomechanics and may play an important role in esophageal peristalsis. How these regional differences contribute to the peristaltic contraction (i.e., contractile characteristics, including contraction amplitude, duration, and latency to onset) have yet to be determined. Our studies indicate that the differences in cholinergic responsiveness would impact at least on the contractile properties such as contraction amplitude, but we did not assess any role in determining latency of the contraction. Similarly, the LES regional differences are open to investigation in health and disease.

We thank Junzhi Ji for expert technical assistance.

This work was supported by the Canadian Institute of Health Research.

REFERENCES


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