Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells

HIROTADA AKIHO, PATRICIA BLENNERHASSETT, YIKANG DENG, AND STEPHEN M. COLLINS

Intestinal Diseases Research Program, Department of Medicine, McMaster University, Hamilton, Ontario L8N3Z5, Canada

Received 27 August 2001; accepted in final form 12 October 2001

Akiho, Hirotada, Patricia Blennerhassett, Yikang Deng, and Stephen M. Collins. Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 282: G226–G232, 2002.—T helper 2 (Th2) cytokines interleukin (IL)-4 and IL-13, which activate signal transducer and activator of transcription 6 (STAT6) are expressed in the muscularis externa during nematode infection and are candidate mediators of the associated hypercontractility. To determine the locus of action of these cytokines, we examined the IL-4- and IL-13-induced hypercontractility of the isolated muscle cells from STAT6+/+ and STAT6−/− mice. We compared the results with cells isolated from Trichinella spiralis-infected STAT6+/+ and STAT6−/− mice. Carbachol chloride (Carbachol) induced the contraction of jejunal muscle cells in a concentration-dependent manner maximal contraction (Rmax 26.7 ± 1.9%). Cells from T. spiralis-infected STAT6−/− mice showed the hypertrophy (cell lengths 41.4 ± 0.8 to 89.0 ± 8.7 μm) and hypercontractility (Rmax 35.5 ± 1.9 and 32.4 ± 2.9%, respectively). Incubation of LMPM from STAT6−/− mice with IL-4 did not enhance the contraction. The hypercontractility in T. spiralis-infected mice was attenuated in STAT6−/− mice (P < 0.02). These results indicate both IL-4 and IL-13 induce hypercontractility of muscle cells via the STAT6 pathway, and this is the basis for hypercontractility observed in T. spiralis-infected mice.

INFLAMMATION OF THE PROXIMAL gastrointestinal tract and airways is accompanied by changes in smooth muscle contractility. This contributes to the clinical expression of diseases such as inflammatory bowel disease and asthma. Studies in asthma have identified T helper 2 (Th2) cells and their cytokines in the development of airway hyperreactivity in atopic and intrinsic asthmatics (4, 35). A previous study of tissue from Crohn’s disease patients also revealed hypercontractility of intestinal muscle cells (33), and preliminary data identify the Th2 cytokines interleukin (IL)-4 and IL-13 as putative mediators (2, 3).

To explore mechanisms underlying inflammation-associated hypercontractility in the gut, we have used mice infected with the nematode parasite Trichinella spiralis. Our studies to date show that the hypercontractility of intestinal muscle strips to carbachol chloride (Carbachol) is T cell dependent (31, 32) and is attenuated in mice deficient in signal transducer and activator of transcription 6 (STAT6) (21), again implicating IL-4 and IL-13 as putative mediators.

Exact locus and mechanisms underlying the actions of these cytokines remains unclear. Goldhill et al. (18) reported that IL-4 administration in vivo enhanced the response to cholinergic nerve stimulation in murine small intestinal longitudinal muscle via a leukotriene D4-mediated effect. This, however, does not exclude a direct effect of Th2 cytokines on smooth muscle cells, as has recently been shown for IL-4 and IL-13 on airway smooth muscle cells (22). Thus inflammation-associated increase in contractility of intestinal muscle strips could reflect a direct effect on smooth muscle cells, an indirect action of IL-4 and IL-13 on enteric nerves (18), or an indirect action mediated by another cell type or via a component of the extracellular matrix.

IL-4 exerts its biological effects by binding to the IL-4 receptor α-chain, a component of both the type 1 and type 2 IL-4 receptor (IL-4R) (14, 16, 37). In the type 2 IL-4R, IL-4Rα is paired with IL-13Rα1, which also binds IL-13 (19, 34). Signal transduction may occur by two separate pathways, phosphorylation and activation of STAT6 by janus kinase 1 (JAK1) and JAK3, which, once activated, dimerizes, translocates to the nucleus, and binds to specific promoter regions to regulate gene transcription (24, 25, 36). Our previous work has implicated the STAT6 pathway in the Carbachol-induced hypercontractility of jejunal muscle strips from T. spiralis-infected mice (21).

Thus the purpose of this study was to determine the locus of action of IL-4 and IL-13 defined as their abilities to alter intestinal muscle contractility by perform-
ing studies on single muscle cells freshly isolated from the intestine of control or *Trichinella spiralis*-infected mice, in the presence or absence of the STAT6 pathway. Specifically, we examined whether the IL-4Rα is expressed on these cells, whether IL-4 and IL-13 induce hypercontractility, and whether this is STAT6 mediated.

**MATERIALS AND METHODS**

**Materials.** The following materials were used in this study: collagenase (CLS type I), trypsin inhibitor, BSA, acrolein, lefunomide, protease, and Mayer’s hematoxylin from Sigma (St. Louis, MO); HEPES from BioShop Canada (Burlington, ON, Canada); IL-4 and IL-13 from R&D Systems (Minneapolis, MN); DMEM and antibiotic-antimycotic from Gibco-BRL Life Technologies (Gaithersburg, MD); rabbit anti-human polyclonal antibody anti-CD3, normal swine serum (NSS), biotinylated swine anti-rabbit, streptavidin-peroxidase conjugate, and Faramount aqueous mounting medium from DAKO Diagnostics (Mississauga, ON, Canada); and diaminobenzidine (DAB) from Zymed.

**Mice.** Studies were performed on male C57BL/6 mice with or without *T. spiralis* infection and STAT6−/− mice between 6 and 10 wk of age. STAT6−/− mice on a C57BL/6 background were originally produced by the gene mutation as described by Takeda et al. (27). Breeding pairs of STAT6−/− mice and their wild-type littermates (STAT6+/−) were obtained from the John Curtin School of Medical Research, Australian National University, Canberra, Australia. Mice were kept in filter-isolated cages in groups of four to five in positive-pressure rooms with a constant ambient temperature and a 14:10-h light-dark cycle. All experiments were approved by the Animal Care Committee at McMaster University and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

**Trichinella infection.** Mice were infected by the administration of 0.1 ml of phosphate-buffered saline containing 375 *T. spiralis* larvae by gavage. The larvae were obtained from infected rodents 60–90 days after infection using a modification of the technique described by Castro and Fairbairn (11). The *T. spiralis* culture originated in the Department of Zoology at the University of Toronto, and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice.

**Immunohistochemistry for histological analysis.** Muscle layers from mice infected with *T. spiralis* were fixed in 10% formalin and embedded in paraffin for histological analysis. Sections were cut at a thickness of 3 μm and immunostaining for T-lymphocytes was performed with a rabbit anti-human polyclonal antibody in a technique similar to the three-step immunoperoxidase method (26). Slides were washed with xylene and ethanol, placed in freshly prepared methanol solution for 30 min, transferred to protease solution (protease 125 units in 50 ml TBS pH 7.6 with 50 μl of CaCl2) for 15 min, and washed with water and Tris-buffered saline (TBS). NSS (1%) was dropped on the slides as a protein-blocking agent for 15 min. Slides were incubated with 1:400 CD3 antibody in 1% NSS overnight in a humid chamber. Slides were rinsed in TBS, and incubated with 1:600 biotinylated swine anti-rabbit in 1% NSS for 90 min, rinsed in TBS, incubated with 1:1,200 streptavidin-peroxidase conjugate in NSS for 90 min. Slides were rinsed in TBS, incubated with DAB for 8 min, counterstained with Mayer’s hematoxylin for 10 min, and mounted in glycerin gelatin.

**Preparation of dispersed smooth muscle cells.** Muscle cells were isolated from longitudinal muscle-myenteric plexus (LMMP) of the C57BL/6 mice jejunum by a method similar to that used by Bitar and Makhlouf (7) to prepare smooth muscle cells from the guinea pig stomach. The uninfected mice and the mice infected 8 days previously with *T. spiralis* were killed by cervical dislocation. The jejunum was removed and placed in DMEM with 1% antibiotic-antimycotic. LMMP was peeled carefully from jejunum. The LMMP were preincubated with or without cytokines (10 ng/ml IL-4, 10 ng/ml IL-13) for 16 h in the 5% CO2 incubator. LMMP was incubated for two successive 10-min periods at 31°C in 10 ml of HEPES medium containing the following (in mM): 98.1 NaCl, 3.87 KCl, 2.42 NaH2PO4, 4.86 l-glutamic-acid, 4.86 fumaric acid, 4.86 pyruvate, 11.17 glucose, 1.79 CaCl2, 1.2 MgSO4, 7H2O, and 23.5 HEPES, pH 7.4, containing 10 mg/ml of collagenase, BSA, and trypsin inhibitor. After incubation, the partly digested LMMP was washed with enzyme-free HEPES medium and reincubated in 10 ml of fresh HEPES medium to allow the cells to disperse spontaneously. Cells were then harvested by filtration through a 210-μm polyester mesh.

**Detection of IL-4R in muscle by RT-PCR.** Expression of mRNA of IL-4Rα in dispersed longitudinal single smooth muscle cells with or without *T. spiralis* infection was investigated by a method described previously (30). Total cellular RNA was isolated based on previously described guanidium isothiocyanate method (12). The concentration of RNA was determined by measuring absorbance at 260 nm and its purity was confirmed using the ratio of absorbency at 260: 280 nm. RNA was stored at −70°C until used for RT-PCR. mRNA was then reversed transcribed as described previously to yield cDNA, and the cDNA was amplified by PCR using gene-specific primers. cDNA (0.1 μg in 50 μl aliquots) were then mixed with 20 pmol each of upstream (5′-GAGT GAG TGG AGT CCT AGC ATC-3′) and downstream (5′-GCT GAA GTA ACA GAA CAG GC-3′) primers for IL-4Rα (13). PCR was performed in 50-μl volumes containing 200 μM 2-deoxynucleotide 5′-triphosphate, 1.5 mM MgCl2, and 2.5 units Taq polymerase with corresponding buffer and distilled water. Messages for IL-4Rα were coamplified using the following parameter: denaturation 94°C for 30 s, annealing 55°C for 30 s, and extension at 72°C for 60 s. PCR products (sizable IL-4R (sIL-4R) 241 bp, membrane IL-4R (mIL-4R) 127 bp) were separated by 2.5% agarose gel electrophoresis and then visualized under ultraviolet light after ethidium bromide staining.

**Measurement of contraction and relaxation in dispersed cells.** Dispersed cells were stimulated by the addition of a 0.8 ml aliquot of the cell suspension to 0.1 ml of the test agent and then incubated at room temperature for 30 s, because we previously found that Carbachol induced the maximal contractile response in jejunal longitudinal smooth muscle cells after 30 s of incubation. The reaction was interrupted by the addition of acrolein in a final concentration of 1%. The median cell length of 50 cells on each slide was measured with a microscope using image-splitting micrometry, and the percent decrease from control in the mean cell length was determined.

**Statistics.** Each experiment was performed at least four times and results are presented as means ± SE. Statistical analyses were performed using Student’s t-test for comparison of two means or one-way ANOVA for the comparison of more than two means. A P value <0.05 was considered statistically significant.
RESULTS

Immunohistochemistry revealed the presence of CD3+ cells in the longitudinal muscle layer from mice infected with T. spiralis 8 days previously (Fig. 1a); no lymphocytes were seen in the muscle layers of noninfected mice (data not shown). As shown in Fig. 1b, both mIL-4R and sIL-4R forms of the α-chain of the IL-4R were expressed on single muscle cells isolated from control and T. spiralis-infected mice. At 29 cycles, there was no change in the PCR products for either the mIL-4R (127 bp) or the sIL-4R (241 bp) between control and infected mice (Fig. 1).

We then examined the characteristics of single muscle cells isolated from the longitudinal muscle layer from control and T. spiralis-infected mice. In muscle from control mice, the mean cell length was 42.4 ± 6.2 μm and this increased by 110% to 89.0 ± 8.7 μm in cells from infected mice (P < 0.05). Reduction in mean cell length after stimulation by 10 nM Carbachol was 26.7 ± 1.9% in control cells compared with 37.5 ± 1.3% in cells isolated from infected mice, reflecting a 40% increase in contractility (P < 0.01). In addition, the half-maximal effective dose (ED50) for Carbachol-induced contraction was 1,000-fold less in cells from infected mice (0.3 pM vs. 0.3 nM in infected and control mice, respectively) (Fig. 2).

To determine the effects of the Th2 cytokines IL-4 and IL-13 on muscle contractility, strips of muscle were preincubated with the cytokine before dispersion of the cells and subsequent stimulation by Carbachol. Preincubation with IL-4 or IL-13 did not alter mean cell length; cell length was 43.1 ± 1.7 and 44.5 ± 3.4 μm in IL-4 and IL-13 exposed cells, respectively, compared with 42.4 ± 6.2 μm in control cells. Carbachol-induced contraction of cells exposed to IL-4 was 35.5 ± 1.9%, reflecting a 33% increase in contractility compared with control cells. In addition, the ED50 for Carbachol-induced contraction was ~22-fold less in IL-4-exposed cells compared with control (2.5 pM vs. 0.3 nM) (Fig. 3).

In STAT6 −/− mice, mean cell length was 41.4 ± 0.8 μm and was similar to that of wild-type mice. Carbachol-induced contraction of cells from STAT6 −/− mice was 23.2 ± 1.1% compared with that of 26.7 ± 1.9% observed in wild-type mice. As expected, preincubation with IL-4 did not alter Carbachol-induced contractility with values of 22.4 ± 1.3% and 0.2 nM for maximal contraction (Rmax) and ED50, respectively, being not significantly different from those of wild-type mice. In IL-13 exposed cells from STAT6 −/− mice, Rmax was increased at 27.7 ± 0.8% (P < 0.05) and the ED50 was significantly less than that of control cells at 40 pM (Fig. 4).

![Image](https://example.com/image.png)

**Fig. 2.** Dose response curve of the Carbachol-induced jejunal smooth muscle cell contraction from control mice (●) and T. spiralis-infected mice (○). Values are means ± SE of 4–5 experiments. *Significant difference compared with control cells at the same concentration. CCh, Carbachol.
To evaluate the role of these cytokines in the muscle changes observed in infected mice, we examined the characteristics of single muscle cells isolated from STAT6-deficient mice in the absence or presence of T. spiralis infection. The mean length of cells from uninfected STAT6−/− mice was 41.4 ± 0.8 µm and this value increased significantly by 15% to 47.4 ± 2.1 µm in infected STAT6−/− mice (P < 0.05). However, this infection-induced increment in cell length in STAT6−/− mice was substantially less than that of 110% seen in wild-type mice. In infected STAT6−/− mice, Carbachol-induced contraction was 25.5 ± 1.4 compared with 23.2 ± 1.1% in noninfected STAT6−/− mice. However, the ED50 fell from 0.1 nM in uninfected STAT6−/− mice to 0.8 pM in infected mice (Fig. 5).

DISCUSSION

Results of this study show that cells isolated from the longitudinal muscle layer of T. spiralis-infected mice exhibit a substantially greater degree of shortening on exposure to Carbachol than cells isolated from control mice. This finding indicates that the inflammation-induced hypercontractility is a property of the muscle cells, rather than the syncytium. Thus muscle cells isolated from this model may be used to examine changes in excitation-contraction coupling that underlie this hyperresponsiveness to Carbachol. The presence of CD3+ cells in the muscularis externa, taken...
together with the observation that the increased shortening of isolated cells is attenuated in STAT6-deficient infected mice, is consistent with the hypothesis that Th2 cytokines mediate these effects at the level of the muscle cell.

We also show that intestinal muscle cells from control or infected mice express the IL-4Rαb and are therefore, able to respond to IL-4 and IL-13. Indeed, we show that when these cells are preincubated with IL-4 or IL-13, they exhibit a greater degree of shortening on subsequent exposure to Carbachol. These findings are similar to those observed in T. spiralis-infected mice and provide further support for the hypothesis that IL-4 and IL-13, acting directly on muscle cells via the STAT6 pathways, mediate muscle hypercontractility during nematode infection.

We believe muscle hypercontractility contributes to the increased propulsive forces seen in extrinsically denervated intestinal segments during T. spiralis infection (5). The hypercontractility observed in muscle from the proximal intestine contrasts with the hypocontractility observed in the distal small intestine (23), thus generating an aboral gradient of force-generation by muscle that likely contributes to worm expulsion (29). Because the successful expulsion of nematode parasites is also STAT6 dependent (6, 21, 28), these data provide further support for a linkage between increased contractility of muscle and the successful eviction of the parasite from the gut. This generates the concept that the muscle cell acts as an extension of the mucosal immune system and, under appropriate signaling from lymphocytes, can alter its physiology to contribute to host defence.

Our results indicate there are two components to the increased responsiveness to Carbachol in muscle from T. spiralis-infected mice. There is an increase in the maximum shortening of muscle cell length, as well as a decrease in the ED50 of Carbachol-induced contraction. Our findings suggest that different mechanisms underlie these changes. In muscle cells from Trichinella-infected mice, there is an ~1,000-fold increase in affinity for Carbachol. This was reduced by ~50% in infected STAT6-deficient mice. Preincubation of muscle from control mice with IL-4 or with IL-13 resulted in smaller decreases in ED50. Changes were more marked in cells treated with IL-13 than with IL-4 and were largely STAT6 dependent. These observations suggest that the increased affinity of the muscarinic receptor for Carbachol on murine muscle cells is increased by Th2 cytokines (IL-13 > IL-4) and that these, together with STAT6-independent mechanisms, contribute to the increased responsiveness of these cells to Carbachol during inflammation induced by T. spiralis. Inflammation-induced changes in muscarinic receptors have also been reported in the airways. Fryer et al. (17) reported that in asthma, there is decreased M2 muscarinic receptor expression on cholinergic nerves. These neuronal M2 receptors inhibit acetylcholine release. Thus the reduced expression of these receptors induces bronchoconstriction via increased release of acetylcholine acting on M3 receptors on muscle. Furthermore, Fryer et al. (17) showed that this downregulation of the M2 receptor could also be induced by exposing the tissue to the Th1 cytokine interferon-γ.

Increased shortening of cells induced by Carbachol was 40% in cells isolated from T. spiralis-infected mice and was only 10% in cells from STAT6-deficient-infected mice, indicating that ~75% of the increased contractility is mediated via the STAT6 pathway. IL-4-induced hypercontractility was 33%, and this was completely STAT6 dependent. In contrast, that induced by IL-13 was only 21% and was largely STAT6 independent. These findings are compatible with a model in which the large STAT6-dependent component of muscle hypercontractility is mediated by IL-4, whereas the minor STAT6-independent component is IL-13 mediated. Increased force generation, resulting in a greater shortening of muscle cells, may reflect changes in the contractile protein content of the cells. Blennerhassett et al. (9) reported that T. spiralis-induced inflammation caused a fivefold increase in the amount of α and γ isoforms of smooth muscle actin per cell. These increases in the smooth muscle-specific actins may affect force production and further demonstrate the plasticity of smooth muscle in the inflamed intestine.

In previous studies in the rat, we reported trophic changes in smooth muscle during nematode infection. These changes were a combination of hypertrophy and hyperplasia (10). These findings are reflected, in part, in the present studies where we show that the length of smooth muscle cells isolated from T. spiralis-infected mice is 110% greater than that of cells isolated from control mice. Although resting cell length was unchanged in uninfected STAT6-deficient mice compared with wild-type mice, there was only a 15% increase in cell length after infection in STAT6-deficient mice. This observation indicates that the trophic effect of inflammation is largely STAT6-dependent. This could not be reproduced by preincubating tissue with either IL-4 or IL-13 for 16 h. Although a longer exposure time might be required to induce the degree of hypertrophy seen after 8 days of infection, it is also possible that the hypertrophy could represent the effects of mediators elaborated downstream from the STAT6 dependent generation of the Th2 response in this model. Transforming growth factor-β (TGF-β) is known to exert trophic effects on smooth muscle (8) and is known to be upregulated by IL-4 (15). Furthermore, we have preliminary evidence that TGF-β is expressed in the muscularis externa during T. spiralis infection in the mouse (1).

From the results of this study, we hypothesize that T cells infiltrating the muscularis externa produce IL-4 and IL-13, which act directly on muscle to 1) increase contractility largely through STAT6-dependent mechanisms; 2) increase the affinity of the muscarinic receptor through STAT6 dependent and independent pathways, and 3) to induce hypertrophy of muscle, most likely via the induction of TGF-β. This scenario, in terms of trophic and contractile changes in smooth muscle, is similar to that portrayed in asthma (20) and
further emphasizes similarities between inflammatory conditions of the lung and gut.

This study was supported by a grant from the Canadian Institutes of Health Research (to S. M. Collins), and from a Research Initiative Award (to H. Akiho) from the Canadian Association of Gastroenterology and AstraZeneca.

REFERENCES


