Effect of exercise on biological pathways in Apc<sup>Min</sup>/+ mouse intestinal polyps

Kristen A. Baltgalvis,1,3 Franklin G. Berger,2,3 Maria Marjorette O. Peña,2,3 J. Mark Davis,1 and James A. Carson1,3

1Division of Applied Physiology, Exercise Science Department, 2Department of Biological Sciences, and 3Center for Colon Cancer Research, University of South Carolina, Columbia, South Carolina

Submitted 10 September 2007; accepted in final form 23 January 2008

Baltgalvis KA, Berger FG, Peña MM, Davis JM, Carson JA. The effect of exercise on biological pathways in Apc<sup>Min</sup>/+ mouse intestinal polyps. J Appl Physiol 104: 1137–1143, 2008. First published January 31, 2008; doi:10.1152/japplphysiol.00955.2007.—Many epidemiological studies have demonstrated that level of exercise is associated with reduced colorectal cancer risk. Treadmill training can decrease Apc<sup>Min</sup>/+ mouse intestinal polyp number and size, but the mechanisms remain unclear. Understanding the molecular changes in the tumor following exercise training may provide insight on the mechanism by which exercise decreases Apc<sup>Min</sup>/+ mouse polyp formation and growth. The purpose of this study was to determine if exercise can modulate Apc<sup>Min</sup>/+ mouse intestinal polyp cellular signaling related to tumor formation and growth. Male Apc<sup>Min</sup>/+ mice were randomly assigned to control (n = 20) or exercise (n = 20) treatment groups. Exercised mice ran on a treadmill at a moderate intensity (18 m/min, 60 min, 6 days/wk, 5% grade) for 9 wk. Polyps from Apc<sup>Min</sup>/+ mice were used to quantify markers of polyp inflammation, apoptosis, and β-catenin signaling. Exercise decreased the number of macrophages in polyps by 35%. Related to apoptosis, exercise decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells by 73% in all polyps. Bax protein expression in polyps was decreased 43% by exercise. β-Catenin phosphorylation was elevated 3.3-fold in polyps from exercised mice. Moderate-intensity exercise training alters cellular pathways in Apc<sup>Min</sup>/+ mouse polyps, and these changes may be related to the exercise-induced reduction in polyp formation and growth.

There is a strong inverse relationship between exercise and colon cancer risk in both men and women (23). Rodent models of colorectal cancer have been used to confirm that exercise can prevent colorectal tumor formation and progression (1, 9, 27, 40, 47). Apc<sup>Min</sup>/+ mice are a widely accepted model used to study interventions that prevent colorectal cancer. These mice have a nonsense mutation at codon 850 in the Apc gene that predisposes them to both small and large intestinal adenomas (31). Regular moderate-intensity exercise can reduce the Apc<sup>Min</sup>/+ mouse intestinal polyp burden (9, 28). Many theorized mechanisms for the protective effect of exercise on colorectal cancer risk include a decreased gastrointestinal transit time, an altered inflammatory state, and a reduction in circulating growth factors (39). However, the biological mechanisms related to exercise-induced colon cancer prevention remain poorly understood. Systemic changes in exercised Apc<sup>Min</sup>/+ mice, such as insulin-like growth factor-1 (IGF-1), leptin, corticosterone, and inflammatory cytokines, have been investigated and correlated with changes in the Apc<sup>Min</sup>/+ mouse polyp burden (7–9, 28). However, direct exercise effects on biological changes within intestinal polyps have not been investigated in the Apc<sup>Min</sup>/+ mouse.

Tumors arise from many different tissue types, but all tumors have similar characteristics. All tumors produce growth factors, escape apoptosis, and have uncontrolled cell division (12). The growth of tumors can also be attributed to chronic inflammation in both human and animal models of colon cancer (18). Inflammatory cells, such as macrophages, are recruited to the tumor and assist in tumor growth, proliferation, and metastasis (10, 25). Tumor-associated macrophages provide tumors with survival factors, such as interleukin-1β, interleukin-6 (22), and cyclooxygenase-2 (COX-2) (16). COX-2 is an inducible enzyme that is responsible for prostaglandin synthesis, and its activity is elevated in colon tumors (44). Inhibition of COX-2 can prevent or cause regression of colorectal polyps in familial adenomatous polyposis (FAP) patients and in Apc<sup>Min</sup>/+ mice (34, 44, 46).

The main consequence of an Apc mutation is the constitutive activation of nuclear localization of β-catenin and activation of genes associated with cell cycle progression (4, 6). Interestingly, intestinal Apc deletion promotes apoptosis, and Apc<sup>Min</sup>/+ mice demonstrate an increase in apoptosis-related proteins compared with wild-type intestinal epithelia (35, 43). The Wnt pathway also interacts with the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is activated in a number of tumor types, including human colon cancer (11, 37, 48), and in Apc<sup>Min</sup>/+ mouse intestinal polyps (20, 30, 35). Growth factors, such as insulin or IGFs, activate Akt. Akt can regulate β-catenin nuclear localization, leading to polyp formation (26, 48). Exercise may prevent Apc<sup>Min</sup>/+ mouse polyp formation by inhibition of the Apc→β-catenin signaling pathway.

Exercise has been shown to decrease both the number and size of polyps in Apc<sup>Min</sup>/+ mice (9, 28). While there has been considerable focus on the ability of exercise to prevent polyp formation, the ability of exercise to block or repress the growth of existing polyps has important health implications and has not been well described in Apc<sup>Min</sup>/+ mice. The loss of Apc leads to nuclear β-catenin localization, which is responsible for adenoma formation. Local growth factors signal through Akt and can also activate this pathway by phosphorylating and inactivating GSK-3β, which also leads to β-catenin activation. Once adenomas are formed, immune cells are often recruited to tumors to fight the immortalized cells. However, tumor-associated macrophages are associated with tumor growth and the secretion of inflammatory cytokines (25). Apoptosis, or programmed cell death, is typically evaded by tumors and leads to uncontrolled tumor growth (12). Exercise can regulate inflammation (49, 51), apoptosis (45), β-catenin (2, 42, 50), and Akt

Address for reprint requests and other correspondence: J. A. Carson, Univ. of South Carolina, Dept. of Exercise Science, Rm. 405A Public Health Research Bldg., 921 Assembly St., Columbia, SC 29208 (e-mail: carsonj@sc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
signaling (2, 5, 19, 42) in other tissues. It is not known whether exercise can regulate these critical pathways in ApcMin/+ mouse intestinal polyps. The primary purpose of the current study was to determine if exercise alters signaling in polyps related to inflammation and growth. We hypothesized that exercise would attenuate macrophage infiltration and COX-2 expression in intestinal polyps from ApcMin/+ mice compared with sedentary mice.

METHODS

Animals. ApcMin/+ male mice were supplied by the Colorectal Cancer Research Center Breeding Core Facility at the University of South Carolina as previously described (28). A subset of animals (control, n = 13; exercised, n = 12) used in this study for immunohistochemical analysis have previously had their polyp counts published (28). All data presented in the present study have not been published previously. Due to polyp counts being performed on formalin-fixed tissue, methanol-fixed tissues required a second group of mice to isolate polyps for protein expression analysis (see Tissue collection). This additional set of control (n = 7) and exercised (n = 8) mice underwent an identical exercise stimulus as the first set of mice, and the exercise treatments were performed consecutively with animals from the same breeding colony at the University of South Carolina as those used in the initial study examining polyp counts. All animal experimentation was approved by the University of South Carolina’s Institutional Animal Care and Use Committee.

Treadmill protocol. Male ApcMin/+ mice [control (n = 20) and exercised (n = 20)] were exercised as previously described (28). This moderate-intensity exercise protocol has previously shown to reduce male ApcMin/+ mouse polyp number. Briefly, 3.5-wk-old male mice ran on the treadmill (18 m/min; 60 min/day; 6 days/wk; 5% grade) for a total of 9 wk. All training took place at the beginning of the dark cycle with the guidance of a red light. Mice were motivated to run by gentle hand prodding. Controls were kept next to the treadmill but remained in their cages during training. All mice were killed at 13 wk of age, at least 36 h after the last bout of exercise. This exercise protocol induces a significant increase in gastrocnemius muscle citrate synthase activity in ApcMin/+ mice (28).

Tissue collection. Mice were given a subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 mL/kg body wt). The small intestines were carefully dissected distally to the stomach and proximal to the cecum. The large intestine was removed from the distal end of the cecum to the anus. Mesentry tissue was removed with tweezers, and the small intestine was cut into four equal sections. All intestinal sections were flushed with PBS, opened longitudinally, and flattened with a cotton swab. The distal end of the ileum was fixed in 10% buffered formalin for 24 h and transferred to 70% ethanol for histochemical analysis on all animals (n = 12–13 per group). With an additional set of animals (n = 7–8 per group), animals were anesthetized and the intestinal tract was dissected similarly, except ileum and flattened with a cotton swab. The distal end of the ileum was fixed with tweezers, and the small intestine was cut into four equal sections. Tissues were fixed in 10% buffered formalin for 24 h and transferred to 70% ethanol for histochemical analysis on all animals (n = 12–13 per group). With an additional set of animals (n = 7–8 per group), animals were anesthetized and the intestinal tract was dissected similarly, except ileum intestinal polyps were dissected from the intestinal tract and frozen in liquid nitrogen for protein analysis.

Western blotting. Briefly, frozen polyps (n = 7–8 animals per treatment group) were homogenized in Mueller buffer, and protein concentration was determined by the Bradford method, as previously described (27). Crude polyp homogenates (10–20 μg) were fractionated on 8–12% polyacrylamide gels. Gels were transferred to PVDF membranes overnight, and Ponceau staining was used to visually confirm the gel transfer and equal loading. Membranes were blocked in 5% Tris-buffered saline with 0.1% Tween 20 (TBST) milk for 1 h at room temperature. Primary antibodies for phosphorylated β-catenin (Ser 33/37, Thr 41), total β-catenin, phosphorylated Akt (Ser 373), total Akt (Cell Signaling), and Bax (Calbiochem) were incubated at dilutions of 1:500 to 1:1,000 overnight at 4°C in 1% TBST milk. Secondary anti-rabbit or anti-mouse IgG-conjugated secondary anti-bodies (Amer sham Biosciences) were incubated with the membranes at 1:2,000 to 1:5,000 dilutions for 1 h in 1% TBST milk. Enhanced chemiluminescence (Amer sham Biosciences) was used to visualize the antibody-antigen interactions and developed by autoradiography (Kodak, Biomax, MR). Digitally scanned blots were analyzed by measuring the integrated optical density (IOD) of each band using digital imaging software (Scion Image, Frederick, MD).

Immunohistochemistry. Formalin-fixed, paraffin-embedded intestinal sections were Swiss-rolled and were cut on a microtome in 4-μm sections (n = 12–13 animals per treatment group). Sections were deparaffinized in xylene and rinsed in 100% ethanol. Peroxidase activity was squelched with 0.6% H2O2 in methanol for 30 min. Antigen retrieval was accomplished with 0.1% bovine trypsin for 30 min at 37°C. Sections were blocked for 30 min in rabbit serum. Slides were incubated 1:100 with β-catenin (BD Transduction Laboratories), F4/80 (Serotec), or COX-2 (Caymen Chemical) for 1 h at 37°C. Slides were rinsed in PBS and incubated 1:200 with anti-rabbit peroxidase-conjugated antibody for 1 h at 37°C. Color detection was visualized with a Vectorstain avidin-biotinylated enzyme complex (ABC) detection kit and 3,3′-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA). β-Catenin-accumulated crypts were counted within polyps or COX-2 or F4/80-positive cells were counted for each polyp on a microscope at a 400× magnification by a technician blinded to the treatments. Polyps with <5 β-catenin-accumulated crypts were considered small polyps, while polyps with ≥5 β-catenin-accumulated crypts were considered large. The number of these small and large polyps was averaged for each animal. For COX-2 and F4/80 analyses, cells were counted for all polyps within an animal and averaged per animal. Data were also examined by polyp size (<1 mm or ≥1 mm) when appropriate.

TUNEL assay. Apoptotic cells were detected using a kit purchased from Chemicon International. Swiss-rolled, formalin-fixed, paraffin-embedded intestinal sections (4 μm) were stained for apoptotic cells according to manufacturer’s instructions. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were counted on a microscope at a 400× magnification by a technician blinded to the treatments. Cells were counted for all polyps within an animal and averaged per animal, as well as stratified by polyp size.

Statistical analysis. Results are reported as means ± SE. All variables were analyzed with independent t-tests. Histological analyses of polyps were analyzed with independent t-tests between control and exercised mice for all polyps and also within each polyp size when appropriate; t-tests were also performed within the cohorts of control mice and exercised mice examining body mass and spleen mass to ensure homogeneity between the two experiments. The level of statistical significance was accepted as P < 0.05.

RESULTS

Animal characteristics. We have previously reported that this moderate-intensity treadmill running protocol decreases intestinal polyp number 29% (28), and a subset of these animals was used for immunohistochemical analyses (n = 12–13 mice per group) in the present study. A second set of control and exercised mice were also used in the present study to analyze protein expression within polyps (control, n = 7; exercised, n = 8). To ensure homogeneity among the control mice and exercised mice between the two experiments, t-tests were performed on body mass and spleen mass (an indirect marker of polyp burden). There were no significant differences in body mass among the control mice (24.3 ± 0.6 vs. 24.8 ± 0.6 g; P = 0.143) or exercised mice (24.3 ± 0.5 vs. 23.1 ± 0.7 g; P = 0.159) between the two experimental groups. Spleen weight also did not differ between control (167 ± 26 mg; P = 0.230) and exercised mice (91 ± 9 vs.
73 ± 7 mg; \( P = 0.169 \)), suggesting genetic homogeneity among the groups. In these additional mice, spleen weight did decrease with exercise training (132 ± 26 vs. 73 ± 7 mg; \( P = 0.03 \)), similar to our previous study. While some exercise training protocols can induce muscle hypertrophy and promote fat loss, this training protocol was not sufficient to alter body weight (24.0 ± 0.5 vs. 22.5 ± 0.6 g; \( P = 0.064 \)), gastrocnemius muscle weight (113 ± 6 vs. 103 ± 6 mg; \( P = 0.267 \)), or epididymal fat pad mass (214 ± 24 vs. 251 ± 19 mg; \( P = 0.234 \)) between control and exercised Apc\(^{Min/+}\) mice.

**Polyp inflammation.** The effect of exercise on tumor-associated macrophage infiltration was identified by F4/80 immunohistochemistry in the distal ileum of Apc\(^{Min/+}\) mice (Fig. 1, A and B). Nine weeks of treadmill training produced a 35% decrease in F4/80-positive cells in polyps (Fig. 1C; \( P = 0.010 \)). When polyps were stratified by size, only large polyps \( ≥ 1 \text{ mm} \) in diameter showed fewer F4/80 cells in exercised polyps (20 ± 2 vs. 13 ± 3; \( P = 0.037 \)).

To determine if exercise alters polyp COX-2 expression, immunohistochemistry was also used to identify COX-2-positive cells within polyps located in the distal ileum (Fig. 2, A and B). There were no differences in the number of COX-2-positive cells within polyps (\( P = 0.985 \)). Total COX-2 protein was also measured in polyps by Western blot (see Fig. 5A). Total COX-2 protein did not change in polyps with exercise training (see Fig. 5B; \( P = 0.835 \)).

**Apoptosis.** TUNEL staining was used to localize apoptotic cells within polyps (Fig. 3, A and B). Exercise significantly reduced TUNEL-positive cells 73% (Fig. 3C; \( P = 0.009 \)). When stratified by size, exercise reduced the incidence of TUNEL-positive cells in small-diameter polyps (<1 mm) by 78% (40 ± 14 vs. 9 ± 3; \( P = 0.011 \)). Bax is a proapoptotic protein whose induction normally coincides with increased apoptosis. The relative concentration of Bax protein was measured in polyps by Western blot analysis (see Fig. 5, A and B). Exercise significantly reduced polyp Bax protein concentration 43% (\( P = 0.003 \)).

**β-Catenin.** β-Catenin expression immunohistochemistry was performed on Swiss-rolled sections of the distal ileum in both control and exercised mice (Fig. 4, A and B). β-Catenin-accumulated crypts (BCACs) are precancerous lesions found in the Apc\(^{Min/+}\) mouse (15), and this similar pattern of β-catenin expression is found in adenomas (21). β-Catenin-positive crypts within polyps were counted for each polyp. Polyps were also classified by having a small number of β-catenin foci (<5 foci) or a large number of foci (≥5). Exercise had no effect on the number of β-catenin-accumulated crypts in polyps with a small number of foci (\( P = 0.275 \)) or a large number of foci (\( P = 0.420 \); Fig. 4C).

Protein extracts from isolated polyps snipped from the small intestine were also examined for total and phosphorylated β-catenin levels (Fig. 5A). Exercise appears to affect β-catenin phosphorylation, rather than total expression levels. Exercise had no effect on polyp β-catenin protein concentration (\( P = 0.142 \)), but exercise induced phosphorylated β-catenin (Ser 33/37 Thr 41) 2.2-fold (\( P = 0.004 \)), and the ratio of phosphorylated to total β-catenin protein 3.3-fold (\( P = 0.007 \); Fig. 5B).

**Growth signaling.** Exercise had no effect on polyp Akt phosphorylation protein levels (1.00 ± 0.11 vs. 0.88 ± 0.10 normalized IOD), total protein levels (1.00 ± 0.06 vs. 1.07 ± 0.275); \( P = 0.010 \).
knowledge, this is the first study to examine the effect of exercise on \(Apc^{Min/+}\) mouse polyp cellular characteristics and protein expression. We report that exercise reduced the number of macrophages in intestinal polyps from \(Apc^{Min/+}\) mice, but

Fig. 2. COX-2-positive cells (arrows) were counted in polyps located in the distal ileum of male \(Apc^{Min/+}\) mice after 9 wk of treadmill training. \(A\): control polyp (×400 magnification). \(B\): exercised polyp (×400 magnification). \(C\): COX-2 data. Data are means ± SE. Data were analyzed with independent \(t\)-tests. Significance was set at \(P < 0.05\).

DISCUSSION

Many studies have examined the effect of exercise on \(Apc^{Min/+}\) mouse intestinal polyp burden (7–9, 28). To our

Fig. 3. Apoptotic cells in polyps following exercise training. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells (arrows) were counted in polyps located in the distal ileum of male \(Apc^{Min/+}\) mice after 9 wk of treadmill training. \(A\): control polyp (×200 magnification). \(B\): exercised polyp (×200 magnification). \(C\): TUNEL data. Data are means ± SE. Data were analyzed with independent \(t\)-tests. Significance was set at \(P < 0.05\). *Different from control.
The number of COX-2-positive cells remained unchanged by exercise. It appears cells other than macrophages are also sources of COX-2 signaling in polyps. Further work can establish if the beneficial effect of exercise on polyp number and growth is independent of COX-2 signaling. We also found that exercise decreased markers of apoptosis and increased β-catenin phosphorylation in polyps. Together, these findings suggest that exercise can influence several signaling pathways related to polyp formation and growth.

One potential exercise-mediated mechanism of colorectal cancer prevention is its anti-inflammatory effect (36, 51). Our lab has previously published that exercise-trained ApcMin/+ mice have a reduction in circulating IL-6 levels, which is associated with a decrease in polyp number and size (28). This decrease in polyp burden was also correlated with a reduction in the crypt depth:villus height ratio, an indirect marker of intestinal inflammation. In other cancer models, daily strenuous exercise can reduce tumor macrophage and neutrophil infiltration, which is associated with delayed tumor growth (53). While macrophages are typically thought to fight tumors,
tumor-associated macrophages secrete inflammatory proteins that contribute to tumor growth and attract more inflammatory cells (16, 22). In the present study, exercise decreased the number of macrophages in polyps, and the effect was more pronounced in polyps. Since these effects were only found in large polyps, these data suggest that the exercise-induced reduction in ApcMin/+ mouse polyp growth is partially attributed to a reduction in the polyp inflammatory state. COX-2-secretory cells in ApcMin/+ polyps are macrophages (16). The role of COX-2 in tumor progression is the secretion of prostaglandins to stimulate growth. Since COX-2-positive cells and protein expression were unaltered in polyps, exercise’s effect on macrophase polyp formation may not be mediated through reduced COX-2 expression. Therefore, exercise has the potential to block polyp growth by decreasing the polyp inflammatory environment, through immune cell recruitment.

Counteracting cell growth and replication is apoptosis. A common characteristic of tumor cells is that they lack the ability to carry out apoptosis (12). Many ApcMin/+ mouse studies that detect a reduction in tumor burden also detect increases in apoptosis with these same treatments (41, 52). In the present study, small polyps from exercised mice had fewer TUNEL-positive cells. This was accompanied by a decrease in polyp Bax protein expression, a proapoptotic protein. Since this running protocol was associated with a decrease in polyp number and size, these findings were unexpected. However, when examining wild-type and ApcMin/+ mouse intestinal gene expression, apoptotic genes are upregulated in adenomas compared with normal epithelia (35). This appears to be a function of Apc since conditional Apc deletion within the intestine or neural crest cells increases apoptosis (13, 43). ApcMin/+ mice are heterozygotes for the Apc gene; loss of heterozygosity within individual cells leads to polyp formation. Since exercised mice have fewer apoptotic cells in their polyps, this may be a reflection of Apc heterozygosity. These data suggest that exercised mice may have more polyp cells that are heterozygous for Apc and, subsequently, less apoptosis. Further examination of this should be verified by loss of heterozygosity assays (LOH) in sedentary and exercised ApcMin/+ mouse intestinal polyps. In addition, exercise reduced apoptosis only in small polyps, but not in large polyps. These data suggest that exercise-induced decreases in ApcMin/+ mouse polyp formation may be mediated through apoptosis. The lack of a change in apoptosis in large polyps suggests that the exercise-induced reduction in ApcMin/+ mouse polyp growth does not appear to be due to an apoptotic mechanism.

The loss of Apc leads to apoptosis and to β-catenin nuclear localization, which turns on proliferation genes, such as c-myc and cyclin D1 (4, 38). β-Catenin expression is prominent in ApcMin/+ mouse intestinal polyps (17, 20, 21, 29), and precancerous lesions also have abundant β-catenin expression (15). Therapeutics that decrease intestinal tumor burden are associated with changes in β-catenin content and/or localization (20). Immunohistochemistry revealed intense nuclear β-catenin staining of the crypts within polyps. However, exercise did not change the number of β-catenin-positive crypts within polyps. However, β-catenin phosphorylation was increased with exercise. Since β-catenin is downstream from Apc, and Apc loss leads to polyp formation, these data would suggest that exercise modulates the Apc→β-catenin pathway. Further work is needed to determine the downstream targets of β-catenin signaling, as well as β-catenin localization, that are modulated within ApcMin/+ mouse intestinal polyps following exercise training.

Circulating factors, such as insulin or IGF-I, can promote tumorigenesis and growth via the PI3K/Akt pathway (11, 14). A reduction in plasma insulin or IGF-I has been hypothesized as a possible exercise-induced mechanism of cancer prevention (3, 24, 32, 33, 39). Akt activity is increased in ApcMin/+ intestinal polyps, and a reduction in polyp burden is associated with inhibition of this signaling pathway (20, 30, 35). In the present study, phosphorylated and total Akt levels were not different in intestinal polyps between sedentary and exercise-trained ApcMin/+ mice. While other proteins can influence Akt activity, these data coincide with previous ApcMin/+ mouse exercise studies that do not detect a reduction in circulating insulin or IGF-I levels, despite a reduction in tumor number (8, 9, 28). In summary, this study demonstrates that exercise can regulate ApcMin/+ mouse intestinal polyp composition. The effects of exercise that reduce the overall tumor burden (size and number) in the ApcMin/+ mouse likely occur via multiple cellular mechanisms, including reduced immune cell infiltration, apoptosis, and β-catenin signaling.

ACKNOWLEDGMENTS

We thank Tia Davis and Valerie Kennedy for technical assistance.

GRANTS

The research described in this report was supported by National Institutes of Health (NIH) Grant P20-RR-017698 from the National Center for Research Resources. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

REFERENCES


