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SHORT COMMUNICATION

INDOMETHACIN MODULATES CIRCULATING CYTOKINE RESPONSES TO STRENUOUS EXERCISE IN HUMANS

Shawn G. Rhind,[1,2] Greg A. Gannon,[1,*] Roy J. Shephard[1,2], Pang N. Shek[1,2,3]

Physical stress is associated with circulating cytokinemia. However the mechanisms of cytokine regulation during such stress are not clearly defined. Non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, are widely used in countering the effects of excessive exercise, but their impact on circulating pro- and anti-inflammatory cytokine production in healthy humans also remains unclear. This study investigated the effect of five days of oral indomethacin treatment (75 mg per day) on the serum concentrations of IL-6, IL-10, IL-12, and TNF-α induced by exercising healthy volunteers. The results demonstrate that indomethacin does not alter resting serum cytokine concentrations. Increased circulating levels were noted, however, for all four cytokines with exercise, but with a different time-course. During and after strenuous physical exercise, indomethacin treatment blunted serum IL-6, and augmented TNF-α and IL-10. These findings may have important implications for both host defense and the injuries associated with excessively vigorous exercise.

The production of cytokines by leukocytes and other body cells facilitates intercellular signalling during the activation of innate and specific immunity.[52] These potent effector molecules regulate a wide array of physiological and pathophysiological processes, including the initiation and coordination of immune, acute phase and inflammatory responses, and in consequence they play an important role in human health and disease.[18] Under resting conditions, serum cytokine levels are kept to a very low level by an intricate network of co-stimulatory and feedback loops.[5] However, the concentration and/or activity of many cytokines increases dramatically in response to stressful or pathophysiological conditions.[7,27]

Exercise has been suggested as an appropriate paradigm to study the impact of physical stress on the immune system.[30,32] Strenuous exercise elicits a cascade of cytokine secretion,[24,31,35] the kinetics of which are somewhat analogous to the systemic inflammatory response associated with trauma or sepsis, although exercise generally elicits much lower peak serum concentrations of cytokines.[4,28,39,40] Typically, there is a sequential release of several inflammation-associated cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-12, followed by several anti-inflammatory mediators, including IL-1ra (receptor antagonist), IL-10, and transforming growth factor (TGF)-β.[24,31] The sources and mechanisms of cytokine release remain controversial;[23,36,48] however the increases in serum concentrations during physical stress are likely mediated by a complex interplay of neuroendocrine, metabolic, and inflammatory stimuli.[32,40,45]

The non-steroidal antiinflammatory drug (NSAID) indomethacin, is widely used to treat pain and inflammation.[33] Most of its analgesic and anti-inflammatory actions have been attributed to the inhibition of cyclooxygenases (COX), and the resultant

From the [1]Defence Research & Development Canada–Toronto, Toronto, Ontario M3M 3B9; [2]Faculty of Physical Education & Health, [3]Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, M5S 1A1, Canada
Correspondence to: Pang N. Shek, Ph.D. Operational Medicine Section, DRDC–Toronto (formerly DCIEM), 1133 Sheppard Avenue West, Toronto, Ontario, M3M 3B9, Canada
*Present address: Faculty of Physical Education & Recreation Studies, University of Manitoba, R3N 0V1, Canada
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TABLE 1. Mean resting serum cytokine concentrations in ten healthy adults

Cytokines	Placebo	Indomethacin
IL-6	1.52 ± 0.37	0.96 ± 0.28
IL-10	3.68 ± 0.22	4.05 ± 0.43
IL-12	0.59 ± 0.09	1.03 ± 0.19
TNF-α	0.54 ± 0.11	1.28 ± 0.21

Values represents mean (± SEM) cytokine concentrations (pg/mL) of duplicate determinations.
No significant resting differences were noted between placebo or indomethacin conditions.

reduction in the synthesis of prostaglandins (PG).[56] NSAIDs are also well known immunomodulators[11,53]; for example, indomethacin promotes T cell activation and proliferation[19,50] and augments natural killer (NK) cell cytotoxicity via suppression of PGE_2.[3,37] Recent evidence suggests that NSAIDs affect a wide variety of cellular processes independent of COX inhibition, accounting for at least some of the anti-inflammatory and immunomodulatory properties of these agents.[6,51] A growing number of reports demonstrate that indomethacin and other NSAIDs directly modulate cytokine production both *in vivo* and *ex vivo*,[13,15,22,34,42,44] altering the activity of certain transcription factors including NF-κB.[16] However, there are few data concerning the effects of indomethacin on circulating cytokine responses to acute exercise in healthy persons. Therefore, the purpose of the present investigation was to examine the effect of *in vivo* indomethacin treatment on the serum concentrations of the pro- and anti-inflammatory cytokines IL-6, IL-10, IL-12, and TNF-α in a group of healthy volunteers, during and following a bout of prolonged strenuous exercise.

RESULTS

Low concentrations of IL-6, TNF-α, IL-10 and IL-12 were present in serum samples drawn prior to exercise. However, mean resting cytokine concentrations did not differ significantly between placebo and indomethacin trials (Table 1).

Exercise induced significant increases in the serum concentrations of all four cytokines assayed, although the kinetics differed for each cytokine (Fig. 1). During both placebo and indomethacin trials, modest but significant elevations of TNF-α (P <0.05) and IL-12 (P<0.05) were detected after 1 h of cycling and concentrations peaked at the end of exercise (all P values <0.0001). The highest absolute levels of TNF-α (2.78 $pg \cdot mL^{-1}$) and IL-12 (1.45 $pg \cdot mL^{-1}$) were attained during the indomethacin trials, but because absolute baseline levels were also higher during the indomethacin trials, the largest percentage increases (216% and 100%, for TNF-α and IL-12, respectively) occurred during the placebo trials. Concentrations of both cytokines had returned to their respective pre-exercise values by 2 h following exercise.

Significant increases in IL-6 (all P values <0.0001) and IL-10 (placebo, P<0.05; indomethacin, P<0.0001) were detected only after 2 h of exercise. Concentrations of IL-6 remained significantly above baseline 2-h post-exercise during the placebo trial only, whereas higher IL-10 concentrations persisted for at least 2 h post-exercise under both conditions. The greatest between-trial differences were observed for IL-6 and TNF-α following 2 h of exercise (P<0.05), and for IL-10 2–h post-exercise (P<0.05). TNF-α also showed significant inter-trial effects 2-h post-exercise (P<0.05). There were no significant (P=0.897) inter-trial differences for serum IL-12 concentrations. Values of all cytokines were normal at 24-h (22 h post-exercise) under both placebo and indomethacin conditions.

DISCUSSION

Cytokines participate in a wide array of physiological processes, including the regulation of immune and inflammatory responses.[52] These effector molecules are produced transiently and locally, controlling the amplitude and duration of the response.[5] A variety of experiments have shown that excessive or insufficient production may significantly contribute to the pathophysiology of a range of diseases.[27] Indomethacin and other NSAIDs regulate the production and/or activity of both pro and antiinflammatory cytokines.[9,34,44,54] Although NSAIDs are commonly used to treat inflammatory disorders[33], few studies have evaluated their immunomodulatory effects in healthy adults undergoing strenuous exercise, and previous investigations have failed to substantiate their impact on spontaneous cytokine secretion.[8,14,43] The present study demonstrates that oral administration of indomethacin (75 mg per day for five days) can alter the pattern of exercise-induced cytokinemia.

In accordance with previous reports,[1,10,23,29] we observed that serum concentrations of both pro- and anti-inflammatory cytokines increase with strenuous exercise. Immunoreactive concentrations of the pro-inflammatory cytokines TNF-α and IL-12 more than doubled during the early exercise period, but had returned to resting levels 2-h post-exercise. In contrast, the kinetics of the antiinflammatory cytokines IL-6 and IL-10 were slower, with serum concentrations not increasing significantly until after 2-h of exercise and remaining elevated 2 h post-exercise. The serum

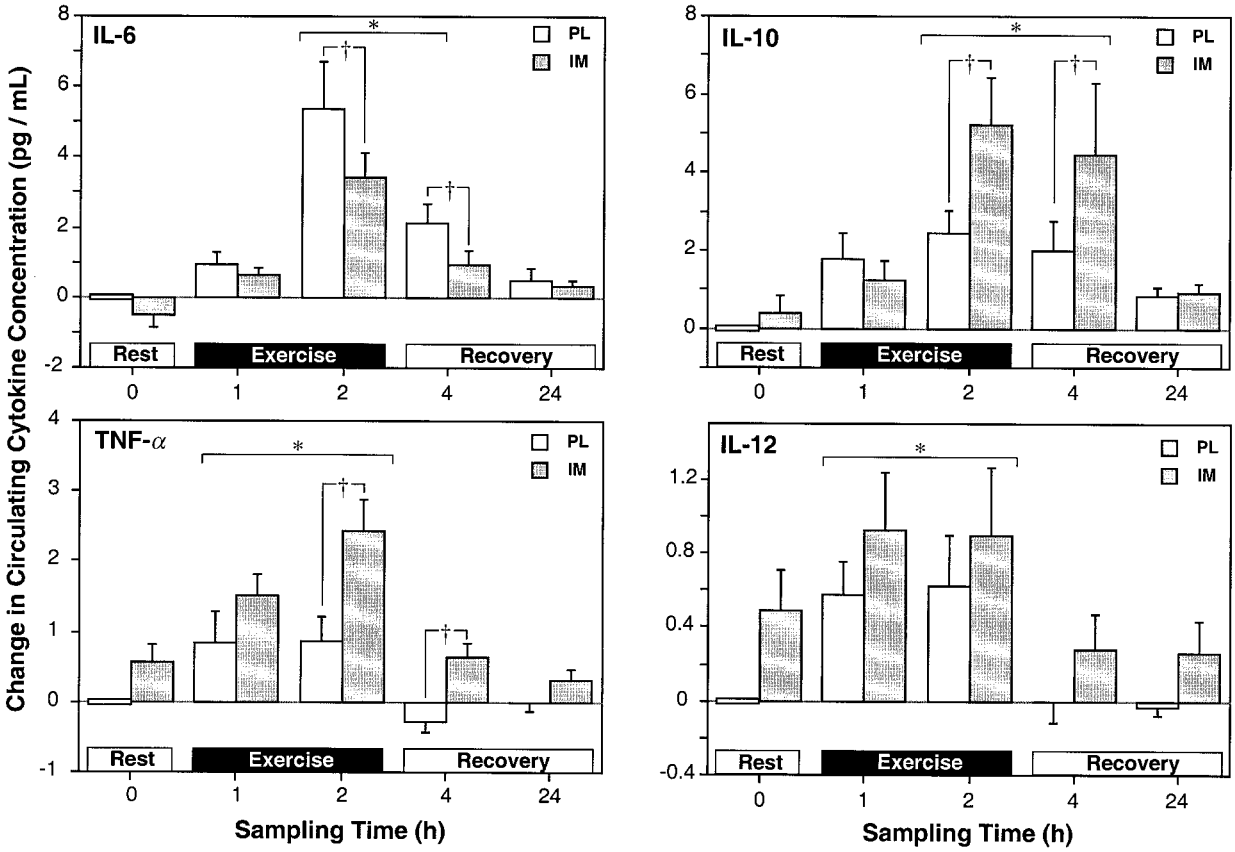

Figure 1. Effect of indomethacin on circulating cytokine concentrations during physical stress.

Mean (± SEM) changes in serum cytokine levels (pg/mL) during placebo (PL; open bars) and indomethacin (IM; filled bars) trials. Asterisks indicate statistically significant ($P<0.05$) differences within a trial as compared to rest and daggers indicate significant ($P<0.05$) between trial differences ($n=10$).

concentrations are consistent with an initial burst of proinflammatory cytokine secretion, followed by the sustained release of antiinflammatory mediators as observed previously in response to strenuous activity [24,26]; the data suggest that IL-6 and IL-10 may serve to counter-regulate the production of proinflammatory cytokines and induction of a systemic inflammatory response following a bout of vigorous physical activity.[31,35]

A key finding of this investigation was that indomethacin treatment differentially modulated spontaneous circulating cytokine responses to exercise. Specifically, we observed that indomethacin augmented peak concentrations of exercise-induced TNF-α (~150%) and IL-10 (~100%), and reduced IL-6 concentrations (40–50%) following 2-h of exercise. In contrast, IL-12 concentrations were not significantly altered within the indomethacin-treated group, despite an apparent trend towards higher IL-12 levels during the indomethacin trial.

The apparently paradoxical observation that indomethacin augments circulating levels of the potent proinflammatory cytokine TNF-α during physical stress is compatible with earlier demonstrations that NSAIDs enhance concentrations of this cytokine both *in vivo* and *in vitro*.[8,17,34,38,42,54] The differences in serum TNF-α concentrations between placebo versus indomethacin trials could be induced at least partially by the direct action of indomethacin on mononuclear cells in vivo. If humans are injected with small doses of LPS and co-treated with NSAIDs, the circulating levels of TNF-α are higher than in controls not receiving NSAIDs.[20,47] Similarly, release of TNF-α by LPS-stimulated, isolated mononuclear cells is potentiated by addition of NSAIDs in vitro.[46] Kinetic evaluation of TNF-α gene expression shows that the stimulatory effect of NSAIDs is related to the persistence of TNF-α synthesis.[8] These findings are consistent with the kinetics of exercise-induced increases in TNF-α and implicate endotoxin as a possible mediator of cytokine induction during exercise.[4,30,39]

There is ample evidence that arachidonic acid metabolites are endogenous regulators of immuno-inflammatory responses, including cytokine production.[3,53] For example, PGE_2 and other cAMP-elevating agents stimulate monocytic IL-6 production, while

strongly inhibiting TNF-α production.[12,17,42] Thus, in addition to the direct potentiating effects of indomethacin on the production of these cytokines, it is possible that indomethacin treatment differentially regulates IL-6 and TNF-α levels indirectly via an inhibition of PGE_2. In support of this idea, we have demonstrated previously that strenuous exercise can significantly enhance circulating PGE_2 concentrations, noting also that this response can be blocked by oral indomethacin treatment.[37] Therefore, it can be speculated that PGE_2 contributes to the regulation of exercise-induced cytokine production. Furthermore, evidence[2] that TNF-α stimulates the release of monocytic PGE_2 is consistent with an autoregulatory loop, in which the early release of TNF-α during exercise induces endogenous inhibitors of further cytokine release (e.g., IL-1ra and IL-10). Thus, under the current experimental conditions, a reduction in exercise-induced PGE_2 synthesis by indomethacin treatment could facilitate TNF-α production, while simultaneously inhibiting IL-6 production.

Moreover, in a form of negative feedback, PGE_2 downregulates the release of TNF-α from various inflammatory cells.[17] PGE_2–mediated downregulation of TNF-α, which occurs at the level of the gene, is dependent upon release of the counterregulatory cytokine IL-10,[49] as evidenced by its efficacy in inhibiting TNF-α production and systemic inflammation.[25] Nevertheless, our observation that indomethacin treatment augmented exercise-induced IL-10 concentrations was somewhat surprising, given that previous investigations have shown the opposite effect of various NSAIDs on circulating IL-10 levels using a variety of experimental models.[21,34,41,44] The mechanism for this pronounced antiinflammatory activity with exercise is not known, but may be directly related to higher circulating TNF-α concentrations, which could elicit IL-10 production.[25]

In contrast to its positive enhancing effect on TNF-α and IL-10 concentrations, our results showed that indomethacin treatment reduced serum concentrations of IL-6 during exercise and eliminated the post-exercise rise in IL-6 concentrations seen during the placebo trial. These findings are compatible with *in vitro* concentrations of IL-6 mRNA in LPS-stimulated monocytes.[55] The action of PGE_2 on monocytes involves stimulation of IL-6 gene transcription. Indomethacin does not affect PGE_2-stimulated IL-6 gene expression; instead, it acts at the level of cellular prostaglandin metabolism, inhibiting COX-catalyzed PGE_2 synthesis and regulation of NF-κB.[51]

In summary, indomethacin did not alter resting serum cytokine concentrations. However, therapeutic doses of indomethacin blunted circulating IL-6 concentrations and augmented concentrations of TNF-α and IL-10 during and after strenuous exercise. Circulating IL-12 levels were unaffected by indomethacin treatment. Altered cytokine responses caused by antiinflammatory therapy may have important implications for both host defense and the injuries associated with excessively vigorous exercise.

TABLE 2. Physical and physiological characteristics of participants

Variable	Mean ± SD
n	10
Age (y)	26.3 ± 5.4 (20–35)
Height (m)	1.78 ± 0.07 (1.65–1.92)
Mass (kg)	79.3 ± 10.3 (69–192)
BMI (kg/m^2)	25.03 ± 3.0 (24.6–30.2)
$\dot{V}O_{s\ peak}$ (mL/kg/min)	44.0 ± 3.5 (38.2–49.5)
HR_{max} (beats/min)	192 ± 7.2 (181–200)

Values are means ± SD (range); BMI = body mass index; HR_{max} = maximal heart rate.

MATERIALS AND METHODS

Study Design

A randomized, double-blind crossover design was used. Subjects completed two experimental cycle ergometer exercise tests separated by an interval of at least two weeks. Drug or placebo was administered for 5-days prior to each experimental session. This design provided a washout period for systemic clearance of drug metabolites.

Participants

Ten healthy, non-smoking men volunteered to participate in the study (see Table 2 for physical and physiological characteristics). Following approval from the Institute's human ethics committee and an appropriate medical examination, the procedures, risks, and benefits of the study were explained, and informed written consent was obtained from each subject. Exclusion criteria included hypersensitivity to NSAIDs and a history of gastrointestinal ulcers. None of the subjects reported taking NSAIDs during the month prior to the investigation. During the study, the participants were required to take only the prescribed NSAID or placebo preparation .

Testing and exercise procedures

Each subject underwent a graded maximal cycle ergometer test to determine their $\dot{V}O_{2\ peak}$. Experimental exercise trials consisted of 2-h of cycle ergometer exercise at a power output eliciting 65% of the individual's $\dot{V}O_{2\ peak}$. Exercise intensity was monitored by direct measurements of oxygen consumption (Sensor Medics metabolic cart), as previously reported.[37]

Indomethacinlplacebo administration

Participants consumed one sustained-release capsule of indomethacin (75 mg INDOCID® SR; Merk Frosst Canada Inc., Mississauga, ON, Canada) or placebo (180 mg of lactose; Novopharm Ltd., Scarborough, ON, Canada) each morning with breakfast, beginning 5 days before each trial. Capsules were packaged such that neither the participants nor investigators knew the nature of the capsules. Compliance was controlled by return of empty capsule containers after the study and by observation of capsule ingestion on scheduled test-days.

Blood sampling and cytokine measurements

Venous blood samples were collected from the antecubital vein, using a 22–gauge intravenous catheter (Insyte®, Becton Dickinson Vascular Access, Sandy, UT, USA), with the subject sitting upright. Specimens were drawn into non-additive sterile glass vacutainers (Becton Dickinson, Franklin lakes, NJ, USA) immediately before (0-h; rest), during (1, 2-h; exercise) and after (2, 24-h) the experimental period.

Serum concentrations of IL-6, IL-10, IL-12 and TNF-α were measured in duplicate, using commercially available sandwich ELISA kits (Quantikine(tm) HS, R&D Systems Inc., Minneapolis, MN; sensitivities: 0.094, 2.0, 0.5 and 0.2 $pg \cdot mL^{-1}$, respectively), as detailed in the manufacturer's guidelines. Optical density, with wavelength correction, was read using an automated microplate photometer (EL340, BIO-TEK Instruments, Winooski, VT, USA).

Statistical analyses

Cytokine concentrations before and after exercise were compared between groups, using a two-way ANOVA with repeated measures over time. When the *F*-ratio showed a significant interaction effect, specific post-hoc analyses determined the source of differences. Significance was accepted at a level of $P<0.05$. Data are expressed as mean ± SE unless otherwise noted.

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