Does rofecoxib increase TNF-α levels?

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Abstract

Objective

Rofecoxib (Vioxx), the first COX-2 selective non-steroidal anti-inflammatory drug (NSAID), was recently withdrawn from the market due to the increased risk of acute myocardial infarction. The precise mechanism responsible for this phenomenon still remains unknown. Tumor necrosis factor alpha (TNF-α) is a cytokine, possibly most responsible for mortality in patients with acute myocardial infarction. However, this study was designed to study possible effects of rofecoxib on the level of TNF-α by using MSU crystal induced inflammation in the rat subcutaneous air pouch model.

Methods

Rat subcutaneous air pouches were produced and examinations commenced 6 days later. Control groups received only MSU crystals, or no crystals or drugs. To begin with, rofecoxib (30 mg/kg), indomethacin (20 mg/kg) or diclofenac (3mg/kg) were administered to groups of 5 rats each. Thirty minutes later, MSU crystals were injected into air pouches, except for the negative control group. Twenty-four hours later, the rats were sacrificed for aspiration of fluid and for the dissection of pouch walls to determine leukocyte counts, pouch wall histology, and to assay IL-10 and TNF-α.

Results

Intra-pouch injection of MSU crystals, compared to non-injected pouches, caused an increase in white blood cell count (WBC) (30 ± 44.7 versus 4508 ± 792.3 cells/mm³), in the numbers of pouch wall vessels (vascular index) (4.8 ± 0.3 versus 11.4 ± 1.5 vessels/high-power field) and in TNF-α (56.6 ± 3.4 versus 79.34 ± 20.9ng/mL), but not in interleukin-10 (IL-10) (66.6 ± 63.6versus 61.48 ± 7.1). WBC and vascular index were significantly reduced in all study groups compared to the control group (p < 0.05). Levels of TNF-α in fluids were unexpectedly and significantly (p < 0.05) increased in all cases. The highest level of TNF-α was found in the rofecoxib group. In contrast to TNF-α, IL-10 levels were significantly (p < 0.05) decreased in all three drug groups. Indomethacin tended to suppress inflammation more effectively. However, there was no significant difference between the groups for IL-10 (p > 0.05).

Conclusion

All three NSAIDs exhibited anti-inflammatory activity against MSU crystal induced inflammation. The difference in anti-inflammatory effects of these three non-steroidal drugs is seen not only in the anti-inflammatory effect on MSU induced inflammation but also in the nature of the effects. Rofecoxib tended to increase the TNF-α level. Whether increased TNF-α levels can help explain the side effect of COX-2 specific inhibitors still requires further studies.

Key words

Diclofenac, indomethacin, rofecoxib, TNF-α, IL-10, WBC, thromboembolism.
Introduction
Acute gout is an intense, extremely painful, inflammatory arthritis with relapsing inflammatory attacks resulting from the formation of monosodium urate crystals in the affected joint space. The drugs usually prescribed for these kinds of acute gout attacks are the non-steroidal anti-inflammatory drugs (1-3). Non-selective non-steroidal anti-inflammatory drugs inhibit two closely related enzymes: cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2). Indomethacin and diclofenac are two non-selective non-steroidal anti-inflammatory drugs widely used for acute gout, and their efficacy is based on few studies (4-7). They are associated with significant side effects in the gastrointestinal tract and central nervous system (8, 9).

Rofecoxib (Vioxx), one of the first COX-2 selective non-steroidal anti-inflammatory drugs, was recently withdrawn by Merck Sharp & Dohme. Indeed, both observational studies and randomized clinical trials showed that rofecoxib is associated with a significantly increased risk of acute myocardial infarction in patients receiving either a daily high dosage (> 25 mg/day) or using them for a long period of time (> 18 months). The precise mechanism responsible for this phenomenon still remains unknown (16). Nevertheless, caution is required because of lack of prospective long-term data, and a strict respect for indications and modalities of clinical use of COX-2 NSAIDs is mandatory.

Materials and methods
Crystal Preparation
Synthetic MSU crystals were prepared as described by McCarty and Faires (20). They were washed, dried and sterilized by autoclaving. Then the needle shape and size of crystals were checked with polarization microscopy. The sterile MSU crystals were suspended in a sterile saline solution at a concentration of 5 mg/ml. This suspension was stirred prior to 5ml injection into 6-day-old rat air pouches.

Rat air-pouch model
Throughout the study, accepted principles of laboratory animal care were followed as well as specific national laws. Five rats comprised each group. This was the lowest number to meet statistical requirements. Rat subcutaneous air pouches were produced under ketamine anesthesia on the dorsum of Sprangue-Dawley rats weighing 150-250 mg at the time of experimentation. Twenty-forty milliliters of sterile air was injected subcutaneously through a 0.25-µm microfilter into the backs of the animals to create a pseudosynovial cavity. A second air injection was given on day 3, if needed, to keep the air pouch inflated. The air pouches had been created under ketamine anesthesia.

Preparations of suspensions of rofecoxib, indomethacin and diclofenac
Solutions of 0.5% methylcellulose in water were stirred while adding the appropriate amount of rofecoxib, indomethacin or diclofenac. The drugs were diluted to a final target volume and sonicated until a homogeneous solution formed. The suspension was stored at 2-8 °C.

The animal groups were the positive control group with only MSU crystals; the negative control group with no crystals or drugs; and the three study groups with MSU crystals and rofecoxib (30 mg/kg), indomethacin (20 mg/kg) or diclofenac (3mg/kg). There were five rats in each group on the 6th day after our air pouch formation. Thir-
ty minutes after the dosing of drugs by gavage, the MSU crystals were injected into the air pouches (except for the negative control group) using ketamine anesthesia in all cases. Twenty-four hours later, at the peak of acute inflammation (based on the results from our preliminary studies), the rats were sacrificed. Pouch wall tissue preparations were dissected, fixed in 10% buffered formaline (pH 7.2-7.4), dehydrated in ethanol, and embedded in paraffin. Five-um sections from identical sites on the pouch wall were sectioned and stained with hematoxylin-eosin-saffron (HES). These sections were used for quantitative visible light microscopic analysis. To obtain quantitative measurements, the numbers of vessels (vascular index, VI) were counted at each of 10 different high power areas that contained at least 4 vessels in each case. Leukocyte counts of air-pouch fluid were performed under regular visible light microscopy (21). TNF-\(\alpha\) and IL-10 levels in the rat air pouches were obtained by enzyme immune assay on the pouch fluid samples that had been stored at -70 °C (Mouse Biotrac Assay, Amersham Bioscience, USA).

**Statistical analysis**
The number of vessels, WBC, percentage of phagocytosis, and concentrations of TNF-\(\alpha\) and IL-10 were analyzed for means and standard deviations. Between-group comparisons were performed using the Wilcoxon Signed Ranks Test.

**Results**
As shown in Table I, the intra-pouch injection of MSU crystals caused an increase in WBC (4508 ± 792.3 vs 30 ± 44.7 cells/mm\(^3\)), the numbers of vessels (11.4 ± 1.5 vs 4.8 ± 0.39 vessels/high-power) and TNF-\(\alpha\) concentration (70.34 ± 20.9 vs 50.0 ± 13.4 ng/ml), but not in IL-10 concentration (61.4 ± 7.1 versus 60.5 ± 63.0 ng/ml).

**WBC**
WBCs in the air-pouch fluid significantly decreased in all study groups compared with the control group \(p < 0.05\). The WBC in the air-pouch fluid tended to decrease more in the indomethacin (2168.8 ± 1245.2 cells/mm\(^3\)) than in the diclofenac (3290.0 ± 1207.9 cells/mm\(^3\)) and rofecoxib (3653.4 ± 1848.7 cells/mm\(^3\)) groups. The differences in WBC between the diclofenac and rofecoxib groups were not statistically significant \(p > 0.05\). However, WBCs were significantly lower in the indomethacin group than in both the diclofenac and rofecoxib groups \(p < 0.05\).

**Numbers of pouch-wall vessels (VI)**
All drugs (indomethacin, rofecoxib and diclofenac) included in study groups showed significant suppressive effect on the numbers of vessels compared to the positive control groups (7.4 ± 1.4, 9.7 ± 0.9, 8.9 ± 1.06 respectively, versus 11.4 ± 1.5). The suppressive effect was significantly greater in the indomethacin group than in both the diclofenac and rofecoxib groups \(p < 0.05\).

**IL-10 and TNF-\(\alpha\)**
Levels of TNF-\(\alpha\) in the air-pouch fluids unexpectedly and significantly \(p < 0.05\) increased in all study groups (rofecoxib, indomethacin and diclofenac; 187.08 ± 42.01, 73.88 ± 10.43, and 69.6 ± 30.8, respectively) versus the positive control group (70.34 ± 20.97 ng/ml). The highest level of TNF-\(\alpha\) was found in the rofecoxib group (Fig. 1). In contrast to TNF-\(\alpha\), IL-10 levels significantly \((p < 0.05)\) decreased (34.0 ± 18.34, 46.6 ± 16.65 and 33.4 ± 19.5, respectively, versus 61.48 ± 7.1 ng/ml) in all three drug groups. However, there was no significant difference between the study groups for IL-10 \((p > 0.05)\).

**Discussion and conclusions**
The most prominent result suggested by this study is the anti-inflammatory effect of three NSAIDs given to rats of the study group in regard to air-pouch fluid. The highest and lowest results for both WBC and VI occurred in the case of the same drug. These similarities in WBC and VI thus confirmed the results of each other. However, not only the quantitative results but also the nature of this anti-inflammatory effect shows some unexpected and interesting differences. The doses of NSAIDs in this study were chosen at the highest levels as defined by the producers of these drugs for rats because the anti-inflammatory effects of the drugs could be expected to be equal. The anti-inflammatory responses in air pouches did appreciably differ in regard to indomethacin, diclofenac and rofecoxib, respectively, as measured by both the WBC and the vascular index. It may be considered that significantly correlated results of the WBC and vascular indices, as inflammatory markers in this study, confirmed each other in regard of the anti-inflammatory responses to the drugs. Since cyclo-oxygenase (COX) is the rate-controlling enzyme in prostaglandin (PGE) production, and TNF-\(\alpha\) is a pro-inflammatory cytokine, COX inhibition is expected to suppress not only the inflammation but also TNF synthesis (21). However, this expectation was not observed, and TNF-\(\alpha\)-lev-

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**Table I. Summary of results.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Negative control</th>
<th>Positive control (30 mg/kg)</th>
<th>Rofecoxib (5 mg/kg)</th>
<th>Indomethacin (20 mg/kg)</th>
<th>Diclofenac (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (cells/mm(^3))</td>
<td>30 ± 44.7</td>
<td>4508 ± 792.3</td>
<td>3653.6 ± 1838.7</td>
<td>2168.8 ± 1245.2</td>
<td>32900 ± 1207.9</td>
</tr>
<tr>
<td>Vascular index (VI)</td>
<td>4.8 ± 0.3</td>
<td>11.4 ± 1.5</td>
<td>9.7 ± 0.9</td>
<td>7.4 ± 1.4</td>
<td>8.9 ± 1.0</td>
</tr>
<tr>
<td>TNF-(\alpha) (ng/ml)</td>
<td>50 ± 13.4</td>
<td>70.34 ± 20.9</td>
<td>187.1 ± 42.0</td>
<td>73.88 ± 10.4</td>
<td>69.90 ± 30.8</td>
</tr>
<tr>
<td>IL-10 (ng/ml)</td>
<td>60.6 ± 63.0</td>
<td>61.48 ± 7.1</td>
<td>46.6 ± 16.6</td>
<td>34.0 ± 18.3</td>
<td>33.4 ± 19.5</td>
</tr>
</tbody>
</table>
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Fig. 1. Graphical comparison of the TNF-α levels of the groups. Rofecoxib caused the significantly (p < 0.0005) highest level of TNF-α in air pouch gavage fluid. However, the difference in TNF-α levels between the positive control group, indomethacin group and diclofenac groups was not significant (p > 0.05).

Levels were seen to increase in the rofecoxib, indomethacin and diclofenac groups, respectively. This result provides consistent evidence, as has been shown by Pinheiro et al., indicating that selective COX-2 inhibitors may cause a TNF-α increase, probably through inhibition of prostaglandin production (23). Similarly, another study showed that profound COX inhibition can lead to a compensatory leukotriene increase, and leukotrienes can cause augmented TNF-α synthesis (24). However, the increased level of TNF-α was very significant in the rofecoxib group. Rofecoxib is one of the selective COX-2 inhibitors, in contrast to indomethacin and diclofenac, which are non-selective COX inhibitors.

In a controversial decision, rofecoxib was recently withdrawn from the market due to increased thromboembolic side effects (25). Such side effects were exposed through a very impressive epidemiologic study (26). However, the reason behind these increased ischemic events in patients who used rofecoxib have remained obscure. Our findings suggest that augmented levels of TNF-α using rofecoxib could be the reason for these side effects because experimental studies have suggested that TNF-α may contribute to the deterioration of cardiovascular function through various mechanisms (27, 28). TNF-α has also been shown to exert pro-inflammatory vascular effects (e.g., induction of oxidative stress, endothelial apoptosis, up-regulation of adhesion molecules and chemokines).

Insulin resistance is increasingly recognized as a chronic, low-level, inflammatory state. Hyperinsulinaemia and insulin action were initially proposed as the common preceding factors of hypertension, low-density lipoprotein cholesterol, hypertriglyceridemia, abdominal obesity, and altered glucose tolerance, linking all these abnormalities to the development of coronary heart disease. The similarities of insulin resistance to another inflammatory state, atherosclerosis, have been described only in the last few decades. Atherosclerosis and insulin resistance share similar pathophysiological mechanisms, mainly due to the actions of the two major pro-inflammatory cytokines, TNF-α and IL-6 (29, 30).

Diclofenac and indomethacin have a suppressive effect on both COX-1 and COX-2; however, they did not cause as much of an increase in TNF-α levels as rofecoxib in this study. Therefore, it may be claimed that the selectivity of COX-2 inhibition can alter the nature of the inflammation and cause an increase in the level of TNF-α. If this is true, then it will drive the researchers to find the relation of rofecoxib and TNF-α to cardiovascular side effects. In APPROVe study, a significant increase in relative risk of thrombotic events was apparent after 18 months of the rofecoxib treatment (31). This may create a confusion to explain this side effect with our short term model of acute inflammation. However, Pignatelli P and co-workers demonstrated that TNF-α amplified the platelet response to collagen; this effect was inhibited by TNF-α receptor antagonist (32). This study showed that TNF-α behaves as a trigger of platelet activation. Platelet activation directly increases acute vascular thrombotic events in contrast to other mechanisms suggested for COX-2 inhibitors (inhibitions of PGI etc).

IL-10 was studied to show the effect of MSU crystal induced inflammation on a different cytokine belonging to another cytokine network. This way will not coincide with the proof of the effect on TNF-α. As expected, the levels of IL-10 were about the same in all study groups. Murakami et al. have shown that retrovirally transfected interleukin-10 suppressed monosodium urate monohydrate crystal-induced acute inflammation in murine air pouches (33). MSU induced inflammation also did not induce IL-10 production in the study of Landis et al. (34). These two studies basically confirm our results for IL-10.

The differences between the behavior of these three non-steroidal drugs pertains not only to their anti-inflammatory effect on MSU induced inflammation but also in regard to nature of their effects. Among other things, rofecoxib tended to increase the TNF-α level. Whether or not the increased TNF-α levels in this study can help to explain the side effect of COX-2 specific
inhibitors still needs further investigation. Because, our study has some limitation such as small numbers of the animals in each group. But we do believe that our study points to significant important details in this matter.

References