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Original article

Effect of Celecoxib on the Peripheral NO Production

*[1]Parichehr Hassanzadeh

Abstract

Objective(s)
Celecoxib acts through both COX-2-dependent and -independent pathways. According to the paradoxical effect of NO on the inflammatory and nociceptive signal processing, the present study designed to evaluate the probable contribution of NO in the analgesic and anti-inflammatory properties of celecoxib.
Materials and Methods
Different intensities of inflammatory pain were induced by acute and chronic sc administration of 1%, 2.5%, or 5% formalin and spectrophotometrical analysis of the serum nitrite was performed. Then, in the pretreatment process, the effect of celecoxib (10, 20, or 40 mg/kg/ip) was evaluated on the inflammatory pain induced-nitrite. Also, the effect of celecoxib alone (under non-inflammatory condition) was evaluated on the peripheral NO production and the results compared with that of the vehicle.
Results
Formalin-induced inflammatory pain led to an enhancement of the serum nitrite in intensity- and time-dependent manners. Celecoxib (40 mg/kg/ip), except its ineffectiveness on the nitrite level, induced 1.5 hr after 5% formalin, reduced production of formalin-induced nitrite in other cases. Meanwhile, under non-inflammatory condition, 1.5 hr after the administration of celecoxib (40 mg/kg/ip), a considerable elevation of nitrite was observed. Celecoxib 10 or 20 mg/kg/ip did not show a significant effect on either inhibition or stimulation of the peripheral NO.
Conclusion
NO is involved both in the inflammatory and non-inflammatory conditions. It seems that celecoxib exerts a dual effect on the peripheral NO production; it prevents overproduction of NO due to the induction of inflammatory pain, while, it stimulates NO synthesis at the early stage of its action.

Keywords: Celecoxib, Formalin, Inflammatory pain, Nitric oxide

1- Department of Pharmacology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran.
* Corresponding Author: Tel: +98-21-88028350, 0912-1887745; Fax: 88021913; email: Pari_has@yahoo.com

Introduction

Selective cyclooxygenase-2 (COX-2) inhibitors are second generation nonsteroidal anti-inflammatory drugs (NSAIDs) that are used against arthritis and other inflammatory conditions. These newer pain killers have been associated with a lower incidence of stomach irritation than the other NSAIDs (1). Celecoxib as one of the major members of this group; exhibits anti-inflammatory, analgesic, and antipyretic activities and primarily has been used in the treatment of rheumatoid arthritis, osteoarthritis, acute pain, and painful menstruation (2). Inhibition of COX-2 is considered as a main mechanism by which celecoxib exerts its effect against inflammation and its accompanying pain. Celecoxib has also been used as an adjunctive treatment against polyps and tumors which appears to be irrespective of COX-2 inhibition (3, 4). This has encouraged the researchers to study about the other probable mechanism/s of action of celecoxib rather than the inhibition of COX-2; which has been suggested to be responsible for the cardiovascular toxicity of celecoxib (5). In the recent years, the role of nitric oxide (NO), a multifunctional signaling molecule, both in the inflammatory and anti-inflammatory conditions has been suggested. Since its discovery in 1980s, NO has been a target of intensive research and drug development. This ubiquitous signaling molecule elicits a wide variety of biological responses both in the peripheral and central nervous systems and has been known as a mediator and regulator of inflammatory responses and nociceptive signal processing (6). Spinally released NO has been implicated in the production of hyperalgesic states after neural injury or application of a chronic noxious stimulus (7). A number of observations have previously suggested that NO also acts as a pronociceptive and inflammatory mediator in the periphery (8). Meanwhile, the selective antipyretic role of NO and its effects on opioid-induced hypothermia and antinociception have been previously reported (9, 10). There is also evidence suggesting that analgesic action of acetylcholine is mediated in part by NO synthesis (11). In addition, enhanced antinociception by intrathecally administered neostigmine or clonidine is blocked by NOS inhibitors (12, 13). Rocha *et al* have reported that NO exerts a dual effect on the articular inflammatory pain induced by zymosan (14). Thus, according to the dual effect of NO in nociceptive signal processing, and detection of the inducible isoform of nitric oxide synthase (iNOS) and COX-2 in a variety of cells in the inflammatory setting (15, 16), the present study was designed to evaluate the probable contribution of NO in the mechanism of action of celecoxib. For this purpose, the effect of different doses of celecoxib on the peripheral NO production induced by different intensities of inflammatory pain was evaluated. In parallel, the effect of celecoxib alone (i.e., under non-inflammatory condition) on the peripheral NO level was also investigated.

Materials and Methods

Animals

Experiments were performed on adult male Wistar rats weighing 200-250 g from our own animal facilities. Animals were housed at room temperature with an alternating 12 hr light/dark cycle and had free access to food pellets and drinking water. All experimental procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication #85−23, revised in 1985), and were also approved by the local Ethics Committee.

Induction of inflammatory pain

Inflammatory pain was induced by the acute and chronic administration of different concentrations of buffered formalin (Sigma) solution. In the acute administration, 50 µl of 1%, 2.5%, or 5% formalin was injected using a 27 gauge needle and a microsyringe into the plantar surface of both hind paws in three groups of rats (n=7/group). In daily injections within 4 days, another three groups of rats were selected and in order to minimize the probability of fiber destruction, the administrations were performed as follows: different concentrations of formalin were injected bilaterally on day 1; while on day 2,

the dorsal surface of both hind paws were selected for injection. Similar procedure was repeated on days 3 and 4, respectively (n=7/group). Saline treated control groups followed the same procedures. Formalin-evoked behaviors which had previously been studied in our laboratory (17) were not considered in this work.

Evaluation of the peripheral NO production following induction of inflammatory pain

NO production was evaluated by quantitative determination of total nitrite (metabolite of NO) concentration (18). Briefly, 1.5 hr and 24 hr after the acute administration, and 24 hr after the last injection in chronic administration of different concentrations of formalin (hr 120), the serum nitrite level was determined spectrophotometrically at 540 nm by measuring the absorbance of test samples (0.1 ml), after adding 0.1 ml Griess reagent [(sulphanilic acid (1% w/v) and N-(1-naphthyl) ethylenediamide (0.1 w/v) in 5% phosphoric acid] and comparing these values with those from the standard solutions of $NaNO_2$ (1–100 μM).

Effect of celecoxib on the formalin-induced NO

If the serum nitrite level had been significantly elevated in any formalin-treated group; in the next procedure, celecoxib (Searle) 10, 20, or 40 mg/kg (dissolved in 20% DMSO in saline) was intraperitoneally (ip) administered 20 min prior to the formalin injection. Then, the above mentioned procedures were repeated for the measurement of nitrite.

Effect of celecoxib on the peripheral NO production under non-inflammatory condition

Regarding the same mentioned time course; 1.5 hr and 24 hr after the single injection and 24 hr after the last injection in chronic administration of celecoxib 10, 20, or 40 mg/kg/ip within 4 days (hr 120), the serum nitrite level was determined (18).

Statistical analysis

Two-way ANOVA with subsequent Tukey test was used for the data analysis. Data are presented as mean±SEM, and the level of significance set at $P<0.05$.

Results

Effect of formalin-induced inflammatory pain on the peripheral NO production

At 1.5 hr and 24 hr after the acute administration, 1% formalin failed to induce nitrite formation ($P>0.05$), however, 24 hr after the last injection in chronic administration (hr 120), nitrite level was significantly raised (Figure 1, $P<0.001$). Twenty four hr after the acute and chronic administration of 2.5% formalin, a clear increase of nitrite was observed (Figure 1, $P<0.001$), while, 1.5 hr after the acute administration, there was no significant change as compared to the control ($P>0.05$). The acute and chronic administration of 5% formalin induced a remarkable elevation of nitrite at all time intervals in comparison with the control and other groups (Figure 1, $P<0.001$).

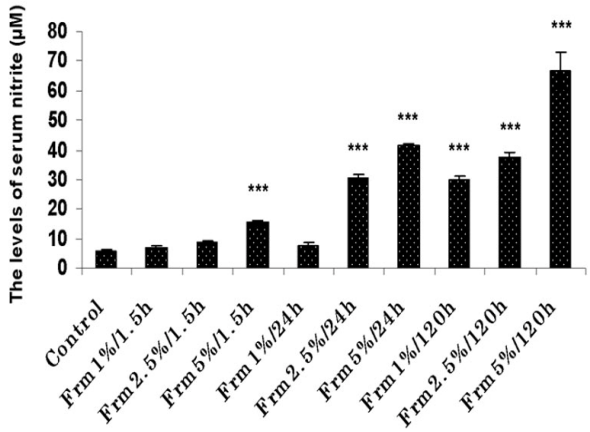

Figure 1. Effect of formalin-induced inflammatory pain on the level of serum nitrite: 24 hr after chronic injections of 1% formalin (120 hr), there was a significant increase of the serum nitrite, however, 1.5 and 24 hr after the acute injection, no obvious change was observed as compared to the control. Twenty four hr after acute and chronic administration of 2.5% formalin, nitrite was elevated, whereas, 1.5 hr after the acute administration, there was no significant change. Acute or chronic injections of 5% formalin led to a significant nitrite raise versus control and other groups. Data represent mean±SEM.

*** $P<0.001$ vs. control.

Frm: Formalin, μM: micromolar, …/1.5 h: 1.5 hr after acute administration of formalin; …/24 h: 24 hr after acute administration of formalin;…/120 h: 24 hr after chronic administration of formalin.

Effect of the pretreatment of celecoxib on the inflammatory pain-evoked NO

Pretreatment with celecoxib 10 or 20 mg/kg/ip did not alter nitrite production induced by the formalin (Figures 2 and 3, $P>0.05$). While, celecoxib 40 mg/kg/ip except its ineffectiveness on the nitrite level induced 1.5 hr after the injection of 5% formalin (Figure 4, $P>0.05$), significantly prevented nitrite elevation in other cases (Figure 4, $P<0.001$, $P<0.01$, $P<0.05$).

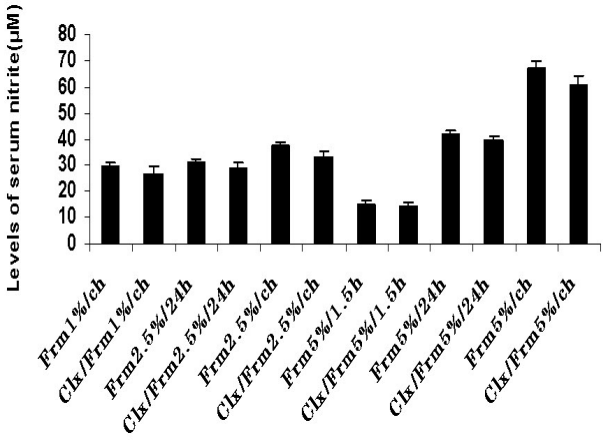

Figure 2. Effect of the pretreatment of celecoxib 10 mg/kg/ip on the inflammatory pain-evoked nitrite: Pretreatment with celecoxib 10 mg/kg/ip did not significantly reduce nitrite levels ($P>0.05$). Data represent mean±SEM.
Clx/…: injection of celecoxib before formalin; ch: chronic injections of formalin;
Frm: Formalin

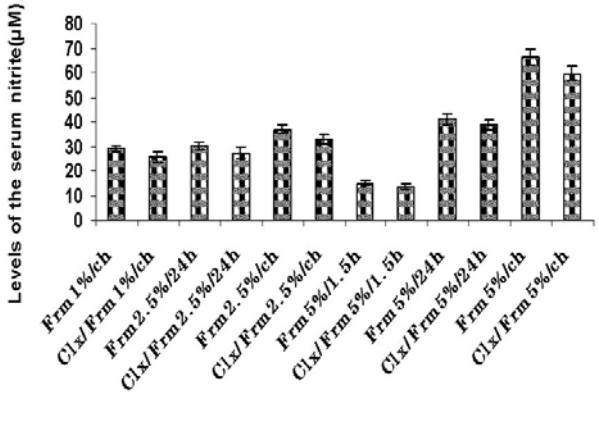

Figure 3. Effect of the pretreatment of celecoxib 20 mg/kg/ip on the inflammatory pain-evoked nitrite: Pretreatment with celecoxib 20 mg/kg/ip did not result to a significant change in the nitrite levels ($P>0.05$). Data represent mean±SEM.
Clx/…: injection of celecoxib before formalin; ch: chronic injections of formalin;
Frm: Formalin

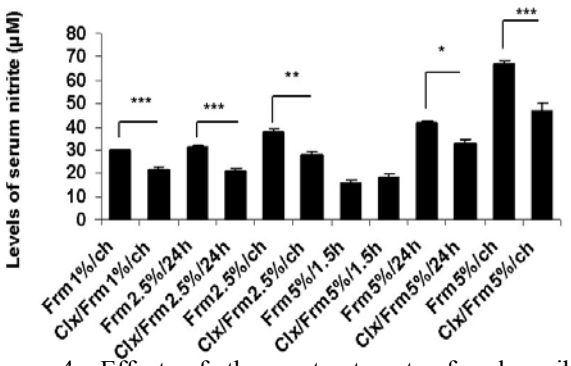

Figure 4. Effect of the pretreatment of celecoxib 40 mg/kg/ip on the inflammatory pain-evoked nitrite: Prophylactic administration of celecoxib 40 mg/kg/ip, except its ineffectiveness on the nitrite level induced 1.5 hr after injection of 5% formalin ($P>0.05$), led to a significant decrease of nitrite in other cases. Data represent mean±SEM.
Clx/…: injection of celecoxib before formalin;
ch: chronic injections of formalin;
Frm: Formalin
*$P<0.05$, ** $P<0.01$, *** $P<0.001$

Effect of celecoxib on the peripheral NO production under the non-inflammatory condition

The acute or chronic administration of celecoxib 10 or 20 mg/kg/ip, did not result to a significant raise of the serum nitrite at all mentioned time intervals (Figures 5 and 6, $P>0.05$). At 1.5 hr after the single injection of celecoxib 40 mg/kg/ip, a significant elevation of the serum nitrite was observed (Figure 7, $P<0.05$). However, 24 hr after the single or chronic injections, no obvious change in the nitrite level was detected, as compared to the control ($P>0.05$).

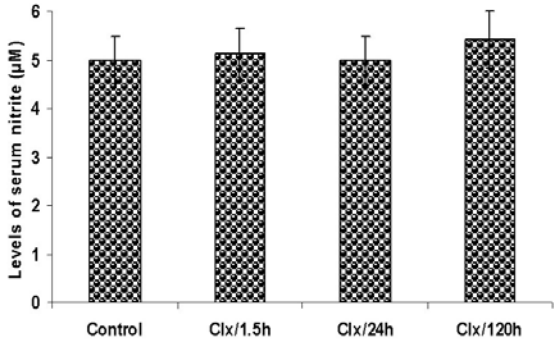

Figure 5. Effect of celecoxib 10 mg/kg/ip on the serum nitrite level: 1.5 hr after single injection and 24 hr after acute and chronic administration of celecoxib 10 mg/kg/ip, there was no significant raise of nitrite versus control ($P>0.05$). Data represent mean±SEM.
Clx/1.5 h: 1.5 hr after single injection of celecoxib, Clx/24 h: 24 hr after single injection of celecoxib, Clx/120 h: 24 hr after chronic administration of celecoxib within 4 days.

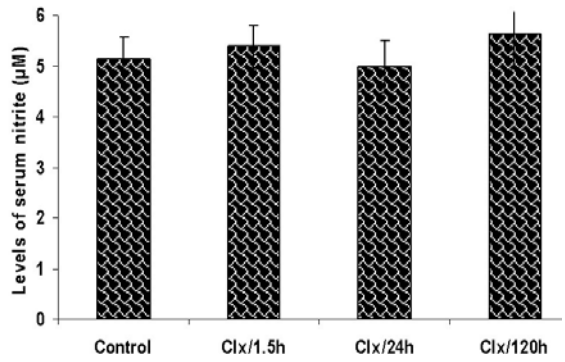

Figure 6. Effect of celecoxib 20 mg/kg/ip on the serum nitrite level: 1.5 hr after single injection and 24 hr after single and repeated injections of celecoxib 20 mg/kg/ip, there was no obvious change in nitrite levels versus control (P>0.05). Data represent mean±SEM.

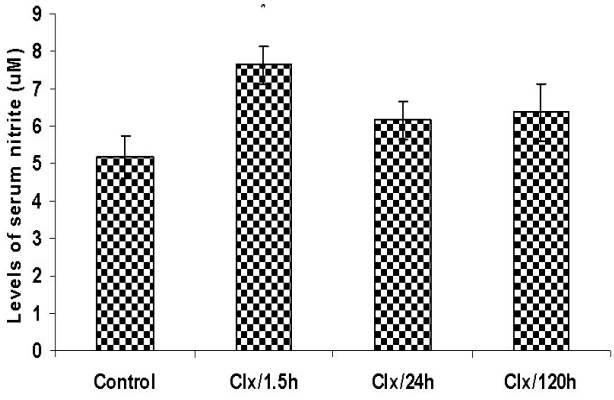

Figure 7. Effect of celecoxib 40 mg/kg/ip on the serum nitrite level: 1.5 hr after single injection of celecoxib 40 mg/kg/ip, a significant elevation of the serum nitrite was observed. However, 24 hr after single or daily injections, there was no significant change as compared to the control. Data represent mean±SEM.
*P<0.05

Discussion

Celecoxib, a widely used NSAID, has been shown to act through both COX-2-dependent and -independent pathways (2, 4). In the present study, regarding the paradoxical effect of NO on inflammatory and nociceptive pathways, and a possible cross talk between NO and COX-2 (19), the hypothesis that NO may be involved in the analgesic and anti-inflammatory properties of celecoxib was evaluated. At the first step, as a model of inflammatory pain, peripheral NO production was assessed following subcutaneous injection of formalin. According to the results, formalin led to an elevation of the serum nitrite in concentration- and time-dependent manners (Figure 1); showing the importance of timing and intensity of the inflammatory pain. Maximum elevation of NO had been induced after the chronic administration of 5% formalin (Figure 1). It seems that exposure to the repeated injections of 5% formalin yields a more intense nociceptive stimulus as compared to 1% and 2.5% formalin and consequently leads to a higher level of the peripheral NO production. Prophylactic administration of celecoxib led to the reduction of inflammatory pain-evoked nitrite in dose- and time-dependent manners (Figures 2-4). This, once again, confirms the role of inflammatory pain in the peripheral NO production, and is in accordance with a number of studies. Ozgocmen *et al* have reported that celecoxib-treated patients show a significant decrease in the nitrite levels (20). Furthermore, Matsuda *et al* have shown that celecoxib inhibits the increase of NO levels in osteoarthritic chondrocytes (21). As known, initiation of PGE_2-induced hyperalgesia is attributable to the actions of COX and NOS and a possible "cross-talk" between iNOS and COX-2 in the inflammatory models has previously been suggested (19). In addition, a number of studies have suggested that COX-2 is located in downstream of iNOS (15, 22). Accordingly, it is supposed that in the inflammatory setting, celecoxib exerts its therapeutic effect via interruption of iNOS activity and therefore, NO-mediated cyclooxygenase activation is prevented. Anti-inflammatory effect of salicylates is also emerged through the inhibition of iNOS (23). However, there are evidences indicating that celecoxib and NS-398 (another COX-2 selective inhibitor) do not affect iNOS activity (4, 24). Meanwhile, it should be mentioned that the nature of the interaction between iNOS and COX-2 varies widely in different cell lines, tissues, and even under different pathophysiological conditions (22). As a whole, it seems that simplistic view on the interaction between COX and NOS is not sufficient to explain the overall inhibition of the inflammatory process by NSAIDs and the role of other inflammatory mediators and

signal transduction pathways should also be considered.

As observed in Figure 4, celecoxib 40 mg/kg/ip failed to prevent nitrite formation 1.5 hr after the injection of 5% formalin. This, at the first glance, appeared to be due to the shortage of time. However, it was better explained when we studied the effect of celecoxib alone (under non-inflammatory condition) on the peripheral NO production; 1.5 hr after the injection of celecoxib 40 mg/kg/ip, spectrophotometrical analysis of the serum nitrite revealed a significant elevation of nitrite as compared to the control (Figure 7). Based on the dual acting anti-inflammatory drugs (25), it is supposed that either inhibition or stimulation of NO synthesis by celecoxib may contribute to its anti-inflammatory and analgesic properties. It is suggested that celecoxib, at least at the early stage of its action, stimulates the basal release of NO which has been shown to play a protective role in many physiopathological conditions (26, 27). Considering the paradoxical effects of NO; it should be mentioned that the cytotoxic, damaging, and cytoprotective properties of NO depend on its relative concentration and the surrounding milieu in which this reactive molecule is produced. In general, under the normal health conditions, NO can act as an anti-inflammatory, but to an already inflamed area, NO can wreak havoc by causing even more inflammation (28). Therefore, efforts should be made to maintain the optimum balance of this double-edged sword; NO.

As shown in Figure 7, 24 hr after the single and chronic administration of celecoxib 40 mg/kg/ip, there was no significant elevation of the serum nitrite. This may be due to the half life of celecoxib (≈11 hr). Thus, despite of the multi-compartmental disposition kinetics of celecoxib and its extensive distribution into the tissues (29), a sufficient concentration of the drug may not be available to stimulate NO synthesis at the mentioned time intervals. Administration of celecoxib 10 or 20 mg/kg/ip did not stimulate NO synthesis (Figures 5 and 6); reminding the importance of dose-response and the validity of data.

As a whole, our data suggest that celecoxib is one of the second generation NSAIDs which potentially possess the advantage to stimulate peripheral NO synthesis. Raising local levels of NO has been shown to lead to the increase of cGMP, vasodilatation, tissue circulation, restoring normal membrane potential, and ultimately pain mitigation (30, 31). Celecoxib has previously been suggested to be unique in causing dilatation of guinea-pig coronary vasculature, relaxation of rat aorta, and exerting a potentiating effect on the NO/cGMP signaling pathway through specific blockade of phosphodiesterase 5 (1, 32). Stimulation of NO synthesis by celecoxib may suggest conservatively a diminished cardiovascular toxicity of this drug, as compared to other COX-2 inhibitors. However, this is still an ongoing discrepancy.

Conclusion

The results of the present study are in accordance with other studies suggesting the involvement of NO in both inflammatory and non-inflammatory pathways. Celecoxib exerts a dual effect on the peripheral NO production; it prevents overproduction of NO due to the induction of inflammatory pain, while, it stimulates NO production at the early stage of its action. In general, the unexpected findings about celecoxib support the notion that celecoxib still possesses an undisclosed molecule-specific property.

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