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Sensitive, Selective, and Irreversible Inhibition of Cyclooxygenase-2 Activity by Copper

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Cyclooxygenase (COX) is a proinflammatory enzyme that catalyzes the rate-limiting reaction to produce many important prostaglandins from arachidonic acid.[1] COX has two isoforms. COX-1 is the constitutive type, widely expressed in various tissues, that serves the basal production of prostaglandins. COX-2 is the inducible type that is upregulated by inflammatory mediators and growth factors such as interleukin-1 and tumor growth factor β. COX produces prostaglandin H_2 (PGH_2) by two consecutive catalytic activities. Arachidonic acid is converted to prostaglandin G_2 (PGG_2) by the authentic "cyclooxygenase" activity at first, and then PGG_2 is reduced to PGH_2 by the peroxidase activity of the enzyme. PGH_2 is further converted to prostaglandin E_2 (PGE_2), a prostaglandin that has physiological functions such as platelet aggregation.[1]

COX has a heme at the active site and the redox state of the heme is crucial for cyclooxygenase and peroxidase activities of the enzyme.[2] However, COX is subject to self-inactivation, possibly because of the oxidation of a tyrosine residue at the heme active site of the enzyme.[3] Therefore, COX is liable to lose its activity by oxidative modification of the enzyme.

COX-2 expression is reported to increase in inflammatory diseases such as rheumatoid arthritis (RA),[4,5] and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS)[6] and Alzheimer disease (AD)[7] where the enzyme oxidatively crosslinks with β-amyloid.[8] Therefore, nonsteroidal anti-inflammatory drugs (NSAIDs), typical inhibitors of COX-2, may have beneficial effects on these diseases.[9–11] There is evidence that aberrant metabolism of Cu^{2+}, a redox-active biometal that can cause the oxidation of proteins, may also play a pathogenic role in these conditions.[12–15]

Herein, we report that COX-2 is specifically inactivated by the physiological concentration of Cu^{2+} ions, and discuss its relevance in a clinical situation.

Table 1 shows the effects of various biometals on the production of PGE_2 by COX-2 activity. We measured PGE_2 production to reflect COX-2 activity, as PGE_2 is the major end-product of the partially-purified enzyme,[17] and can be sensitively quantified by EIA. We did not find any production of PGE_2 from the sample without COX-2 (data not shown). At 500 nM, only Cu^{2+} inhibited PGE_2 production significantly ($P < 0.01$, t test) by ≈ 50%. The ligand for Cu^{2+} (3 μM glycine) itself caused no inhibitory effect on the enzyme (data not shown). The background levels of Cu^{2+} in our buffers is routinely measured at less than 20 nM,[18] which was considered negligible compared with the concentration of Cu^{2+} added in this study.

Table 1. Inhibition of PGE_2 production by various biometals. [a]

Metal ion	PGE_2 production [μg L^{-1}]
(–)	215.9 ± 16.7
Cu^{2+}	119.0 ± 8.9[b]
Fe^{3+}	202.4 ± 26.5
Zn^{2+}	198.2 ± 16.8
Co^{2+}	182.0 ± 24.9
Ni^{2+}	236.5 ± 3.2
Mg^{2+}	182.4 ± 47.4

[a] Ovine COX-2 (140 nM) was incubated with a metal (500 nM) for 1 h at 37 °C. The data indicate the average ± SD of triplicate results. [b] Significantly decreased compared to COX-2 alone sample ($P < 0.01$, t test).

Cu^{2+} inhibited COX-2 activity in a concentration-dependent manner (Figure 1). The IC_{50} for Cu^{2+} was ≈ 500 nM, which is a stoichiometric ratio of 3.5:1 (Cu^{2+}:enzyme). On the other hand, in the presence of 10 μM EDTA, the inhibitory effect of Cu^{2+} at concentrations up to 2000 nM was abolished (Figure 1). The amount of PGE_2 produced in the presence of EDTA without Cu^{2+} was less than that in the sample lacking EDTA. This might be explained by EDTA chelating some Fe on the heme from the active site of COX-2, so decreasing the activity of the enzyme.

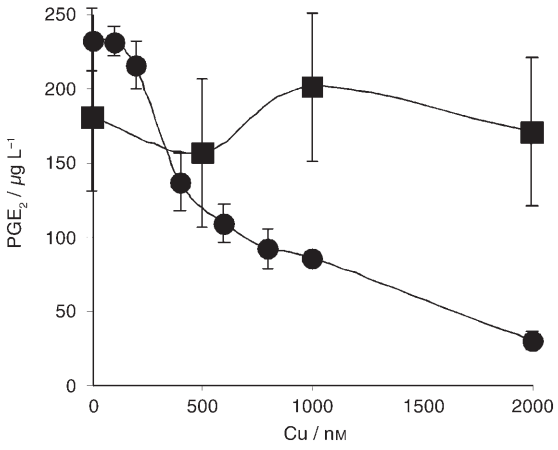

Figure 1. Cu inhibition of COX-2 activity is concentration-dependent. Ovine COX-2 (140 nM) was incubated with various concentrations of Cu with (squares) or without (circles) 10 μM EDTA for 1 h at 37 °C. The data indicate the average ± SD of triplicate results.

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We next examined the effect of Cu^{2+} on the inhibition of COX-2 activity by indomethacin, a nonspecific inhibitor of COX (Figure 2). At 100 nM, Cu^{2+} itself did not inhibit PGE_2 production, consistent with our results in Figure 1. However, the same concentration of Cu^{2+} (100 nM) significantly potentiated the inhibition of PGE_2 production by low concentrations of indomethacin (5–10 nM, $P < 0.05$, t test).

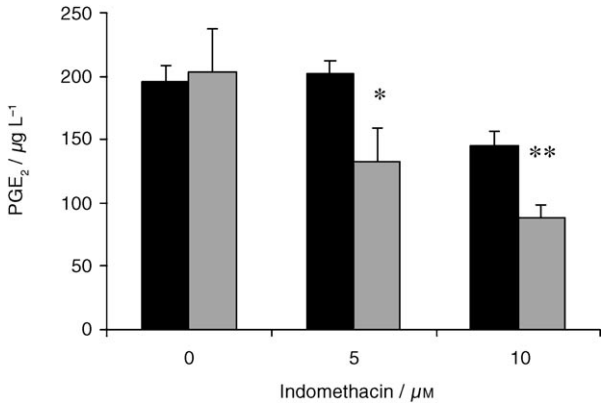

Figure 2. Synergistic effect of Cu on the inhibition of COX-2 activity by indomethacin. Ovine COX-2 (140 nM) was incubated with (gray bars) or without (black bars) 100 nM Cu at various concentrations of indomethacin for 1 h at 37 °C. The data indicate the average ±SD from triplicate results. * $P < 0.05$, ** $P < 0.01$, t test.

To determine whether the inactivation of COX-2 by Cu^{2+} is a reversible process, we investigated the effect of EDTA on COX-2 activity after the inactivation by Cu (Figure 3). Cu^{2+} (1 μM) inhibited COX-2 activity compared to the samples without Cu ($P < 0.001$, t test), consistent with the earlier data (Figure 1). Whereas the inhibition is prevented by co-incubation with EDTA (10 μM) (Figure 1), when EDTA (1–10 μM) was added after the incubation with Cu, it did not restore the inhibition of COX-2 activity, indicating that Cu^{2+} -mediated inhibition was irreversible.

We have shown that Cu^{2+} inhibits COX-2 activity specifically among biometals. This inhibition may be relevant to the regulation of COX-2 activity in vivo, because the concentrations of Cu necessary to inactivate COX-2 ($IC_{50} \approx 500$ nM) are much lower than in human serum (15–30 μM)[19] and the human brain (40–90 μM).[20] By way of comparison, Cu^{2+} also inhibits the activity of SOD1 (20 nM) with an IC_{50} of 2.35 μM.[21] Although there is almost no free Cu^{2+} in the cell,[22] the total levels of Cu rise with age[23,24] and may exist in a pool that could exchange with COX-2.

Although the mechanism by which Cu^{2+} inactivates COX-2 is unclear, the irreversible change of the activity (Figure 3) implies that Cu^{2+} causes the oxidative modification at the active site of the enzyme possibly by hydrogen peroxide or hydroxyl radicals. As dopamine is present, it is possible that the Cu^{2+} is reduced to Cu^{+}, which may be the species responsible for the chemical attack. Oxidative modification of a tyrosine residue in COX is associated with the self-inactivation of the enzyme.[3] It is not likely that the inhibitory effects of Cu^{2+} on COX-2 activity is merely caused by direct dopamine oxidation, as Fe^{3+}, an even more potent means of oxidizing dopamine,[25] did not affect COX-2 activity (Table 1). Also, when EDTA is added after one hour to COX-2 that has been incubated with Cu^{2+} the enzyme maintains considerable activity (Figure 3); at this point all the Cu^{2+} has been chelated by the excess EDTA, so the residual activity indicates that the dopamine substrate has not been exhausted through being oxidized by Cu^{2+}.

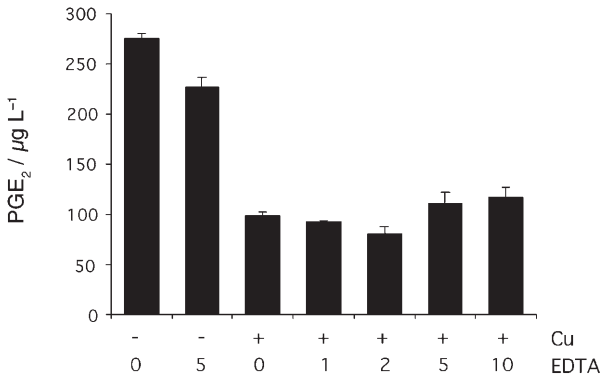

Figure 3. Inhibition of COX-2 activity by Cu is irreversible. Ovine COX-2 (140 nM) was incubated with or without 1 μM Cu for 1 h at 37 °C. After that incubation, various concentrations of EDTA (μM) were added to the sample and incubated for 30 min at 37 °C. The data indicate the average ±SD from triplicate results.

We also observed a synergistic effect of Cu^{2+} on the inhibition of COX-2 activity by indomethacin (Figure 2). Cu^{2+} at a subtoxic concentration might make the enzyme liable to inactivation by indomethacin, or Cu^{2+} might directly bind to indomethacin and reinforce the pharmacological effect of the agent. Cu^{2+} is known to form a complex with NSAIDs including indomethacin[26] and to potentiate their anti-inflammatory activities.[27–30] The structural basis for this awaits determination but the possibility that the pharmacological action of NSAIDs is potentiated by tissue Cu^{2+} may be important in treatment strategies of inflammatory conditions.

The inhibition of COX-2 activity by Cu may be a basis for the physiological and pathological roles of Cu in aging, RA, and AD. COX-2 activity is known to increase by aging in the rat brain.[31] Cu content also increases with age, especially in the brain.[21,23,24,32] This could serve a protective function to suppress the unwarranted COX-2 activity. On the other hand, Cu levels are decreased in erythrocytes and mononuclear cells in patients with RA,[12,13] and in the hippocampus of AD brains,[14,15] although COX-2 expression is induced in affected regions of these diseases.[4,5,7] Therefore, an imbalance between intracellular Cu^{2+} level and COX-2 expression may occur in these diseases. In fact, intramuscular administration of Cu^{2+} complexes has been reported to induce symptom remission in RA patients.[33] Cu^{2+}-thiocomplexes also have been described to have anti-inflammatory and anti-arthritic effects in rats[34] and "copper bracelets" have been described to be of therapeutic value in arthritis sufferers.[35] Our current studies demonstrate that small (nanomolar) elevations of Cu^{2+} could serve to inhibit COX-2 activity. These data may help illuminate the com-

plex interplay between Cu^{2+} and COX-2 activity in conditions such as RA, AD, and ALS.

Experimental Section

Standard metal solutions were obtained from the National Institute of Standards and Technology for Cu^{2+}, Zn^{2+}, and Fe^{3+} (10 mg mL^{-1} in 10% HNO_3), and from Plasma Chemical Corporation for the other metals (1 mg mL^{-1} in 2% HNO_3). Stock solutions of metal ions were prepared at 10 mM with 60 mM ligand (histidine for Zn^{2+}, citrate for Fe^{3+}, and glycine for the other metals, all from Sigma, St. Louis, MO) in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS, Sigma). pH was adjusted to 7.4 with 1 N sodium hydroxide. Indomethacin (Sigma) was dissolved in ethanol at the concentration of 10 mM. Every reagent was diluted with PBS from stock solutions.

The inhibition assay of COX-2 activity was performed according to the method of Mattammal et al., 1995.[16] Purified ovine COX-2 (Cayman Chemical, Ann Arbor, MI) was incubated at 140 nM with metals, EDTA, or indomethacin in a total volume of 100 μL for 1 h at 37 °C, in the presence of 100 μM dopamine (Sigma) as a reducing co-substrate. To examine the effect of EDTA after the inactivation of COX-2, EDTA was added after 1 h incubation of COX-2 with Cu^{2+} and incubated for 30 min. After this incubation, the enzymatic reaction was initiated by adding 5 μL of 20 μM hematin (Sigma) and 5 μL of 2 mM arachidonic acid (Acros Organics, Geel, Belgium), and reacted for 3 min at 37 °C. The reaction was stopped by adding 100 μL cold ethanol. The production of PGE_2 was quantified by STAT-Prostaglandin E_2 EIA Kit (Cayman Chemical) according to the manufacturer's instructions.

Data were expressed as mean ± SD. Statistical differences were determined by t test. $P < 0.05$ was considered to be significant.

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