DEVELOPMENT OF A NOVEL DIRECT BIOMARKER OF ASPIRIN ACTION ON CYCLOOXYGENASE-2

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Several lines of experimental and clinical evidence support the role of cyclooxygenase (COX)-2 in colorectal tumorigenesis (Wang and DuBois, 2010). Thus, selective COX-2 inhibitors (named coxibs) prevent colorectal adenoma recurrence, in patients with sporadic colorectal adenomas (reviewed in Patrignani and Patrono 2016). However, a similar efficacy was demonstrated by the administration of low-dose aspirin which is considered to act by a selective inhibition of COX-1. Aspirin acts by inhibiting the COX activity of COX-1 and COX-2 through an irreversible acetylation at serine 529 and 516, respectively (Loll et al., 1995; Lecomte et al.; 1994). However, whether the drug affects COX-2 activity in colorectal adenomas/adenocarcinomas when administered at low-doses to humans is not completely clarified yet. To address this clinical relevant issue, we aimed to develop a direct marker of aspirin action by assessing the extent of acetylation of COX-2 at serine 516 using a novel strategy, Protein AQUA, enabling absolute protein quantitation by liquid chromatography-mass spectrometry (LC-MS). The method consists of the use of peptides with incorporated stable isotopes as ideal internal standards to mimic native peptides formed by proteolysis (Gerber et al., 2003). First, we characterized the appropriated enzymatic digestion of COX-2 to obtain a peptide containing the residue serine 516, VGAPFSLK, and its acetylated form, VGAPF[S(Ac)]LK. This was obtained through a double digestion of COX-2 using two proteases, trypsin, which cleaves at the C-terminus of lysine (K) and GluC, which cleaves at the C-terminus of glutamic acid (E) residue. Then, the synthesis of these AQUA peptides which incorporated the stable isotopes (13C and 15N) was obtained. A LC-MS/MS method using specific precursor-to-product ion transitions was then developed. This AQUA method was used for quantification of COX-2 expressed in human intestinal epithelial cells and its acetylated form by aspirin. Cultured epithelial cells were lysates and proteins were fractionated by SDS-PAGE gel electrophoresis, and regions of the gel corresponding to the molecular mass range from 60 and 80 kDa (which comprises the molecular mass of COX-2, i.e. 73 kDa) are excised and subjected to in–gel digestion with Trypsin and GluC. Then, the samples were subjected to LC-MS and data were processed by integrating the appropriate peaks in an extracted ion chromatogram for the native and internal standard, followed by calculation of the ratio of peak areas multiplied by the absolute amount of internal standard (e.g., 500 fmol). Using this technique, in human epithelial cells treated with IL-1□□2 ng/ml for 18h at 37°C to induce COX-2 expression, we measured 0.46±0.15 fmol/□g protein (Mean±SD, n=3) of COX-2. In epithelial cells, untreated with IL-1□, COX-2 levels was below the detection limit of the technique (i.e. 0.052 fmol/□g protein). Western blot analysis confirmed the induction of COX-2 in Il-1□ stimulated epithelial cells. The incubation of aspirin (0.1-1000 □M) with human epithelial cells was associated with a concentration-dependent inhibition of PGE2 production (in response to 0.5 □M of arachidonic acid) and an IC50 of 12 (95% CI: 7-23) □M. We assessed the extent of % acetylated COX-2 in epithelial cells incubated with aspirin 50 □M (for 1h at 37°C) associated with inhibition of COX-2 activity by 80%. The % acetylated COX-2 was 67.33±16.26% (n=3).

In conclusion, we have developed a proteomic method to assess the extent of acetylation of COX-2 at serine 516 in epithelial cells. This biomarker can be used to assess the impact of aspirin on COX-2 in cell cultures (in vitro) and human tissues after dosing with aspirin (in vivo). The assessment of this biomarker will allow to clarify the mechanism of action of aspirin as anti-cancer agent.

Gerber et al., PNAS 2003; 100: 6940–5.

Lecomte et al.; JBC 1994; 269: 13207-15.

Loll et al., 1995; Nat Struct Biol 1995;2: 637-43.

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