Inhibition of the biosynthesis of prostaglandin E_2 by low dose aspirin: implications for adenocarcinoma metastasis.

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SUPPLEMENTARY DATA

Urinary PGE-M

Urinary PGE-M is an index of systemic PGE_2 production (1, 2). Catabolism of PGE_2 results in a stable end metabolite, 11-α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M) that is excreted in the urine (3-6). Measurement of urine PGE-M is a better measure of systemic PGE_2 than plasma measurements. PGE_2 in plasma is rapidly metabolized in the lungs and consequently may not accurately reflect endogenous PG production (7).

PGE-M concentrations in urine were measured in the laboratory of Dr. Ginger Milne at Vanderbilt University. Urine (1mL) was acidified to pH 3 with HCl and treated with methyloxime HCl, and PGE-M converted to the *O*-methyloxime derivative. The methoximated PGE-M was extracted, applied to a C-18 Sep-Pak (Waters Corp. Milford, MA USA), and eluted with ethyl acetate. An [$^2H_6$]-*O*-methyloxime PGE-M deuterated internal standard was then added. The sample was dried under a stream of dry nitrogen at 37^{o}C and then reconstituted in 75 µl mobile phase A (see below) for LC/MS analysis. LC was performed on a 2.0 x 50 mm, 1.7 µm particle Acquity BEH C18 column (Waters Corporation, Milford, MA, USA). Mobile phase A was 95:4.9:0.1 (v/v/v) 5 mM ammonium acetate:acetonitrile:acetic acid, and mobile phase B was 10.0:89.9:0.1 (v/v/v) 5 mM ammonium acetate:acetonitrile:acetic acid. Samples were separated by a gradient of 85–76% of mobile phase A over 6 min at a flow rate of 200µl/min prior to delivery to a ThermoFinnigan TSQ Quantum Vantage triple quadrupole mass spectrometer. The precursor ion of endogenous-formed PGE-M is m/z 385 and [$^2H_6$]-PGE-M internal standard is m/z 391 with the expected predominant product ions being m/z 336 and m/z 339, respectively. Quantification of a subject's PGE-M was calculated by ratiometric determinations of unlabeled:labeled peak areas corresponding to both precursor and product ions. It should be noted that PGD_2 is metabolized by a mechanism analogous to PGE_2 and metabolites for this eicosanoid are detected using the same m/z transitions as PGE-M. The methoximated metabolites of PGE_2 and PGD_2 are chromatographically separated using this method (Supplemental Fig. S1). The lower limit of detection of PGE-M is in the range of 40 pg, which is approximately 100-fold below levels in normal human urine. Urinary creatinine levels are measured using a test kit from Enzo Life Sciences. The urinary PGE-M levels in each sample are normalized using the urinary creatinine level of the sample and expressed in ng/mg creatinine.

Experimental requirements for comparing aspirin potency between cell lines.

The inhibitory effect of aspirin can be influenced by several factors such as total COX activity, and substrate availability. To minimize the impact of these external factors, all cells were assayed by adding the same concentration of exogenous isotopically labelled arachidonic acid. It is essential that the concentration of substrate is the same in each analysis because the acetylation of the cyclooxygenases by aspirin is inhibited by hydroperoxides. As the initial product of the cyclooxygenases is the hydroperoxide, PGG_2, aspirin's inhibitory effect can be predicted to be inversely related to the substrate concentration (8). All cells were incubated in albumin-free media because albumin enhances aspirin potency (9). Because the IC_{50} values for aspirin previously reported in the literature are dependent on the concentration of substrate , they cannot be extrapolated to other experimental conditions. Cells were pre-incubated with a range of aspirin concentrations for 30 minutes followed by addition of 2 μM AA and the medium was subsequently harvested for GC/NICI/MS analysis of PGE_2 biosynthesis. The results were normalized to controls performed for each experiment where cells were incubated with vehicle only before adding AA (Figure 2B).

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